PHOTOAFFINITY LABELING THE NUCLEOTIDE SITES OF THE SARCOPLASMIC RETICULUM Ca^{2+}-ATPase

Thesis presented for the degree of
Doctor of Philosophy of the
University of Cape Town

by

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PUBLICATIONS

The following manuscripts have arisen in part from work carried out and presented in this thesis:

Champeil, P., Riollet, S., Orlowski, S., Guillain, F., Seebregts, C.J. and McIntosh, D.B. (1988) J.Biol.Chem. **263**, 12288-12294

ATP Regulation of Sarcoplasmic Reticulum Ca^{2+}-ATPase. Metal-free ATP and 8-Bromo-ATP bind with high affinity to the catalytic site of phosphorylated ATPase and accelerate dephosphorylation.

Seebregts, C.J. and McIntosh, D.B. (1989) J.Biol.Chem. **264**, 2043-2052

2'3'-o-(2,4,6-trinitrophenyl)-8-azido-adenosine mono-, di-, and triphosphates as Photoaffinity Probes of the Ca^{2+}-ATPase of Sarcoplasmic Reticulum. Regulatory/Superfluorescent Nucleotides Label the Catalytic Site with High Efficiency.

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ABSTRACT

We have synthesized a new class of photoaffinity analogs, 2',3'-o-(2,4,6-trinitrophenyl)-8-azido-ATP, -ADP and -AMP (TNP-$8N_3$ATP, -ADP and -AMP), and their radiolabeled derivatives, and characterized their interaction with the sarcoplasmic reticulum Ca^{2+}-ATPase. The TNP-$8N_3$-nucleotides were synthesized from ATP in three steps involving bromination in the 8-position of the adenine ring followed by displacement with an azido group and then trinitrophenylation of the resulting $8N_3$-nucleotide with TNBS. Inclusion of the oxidizing agent, DTNB, in the final reaction was found to be necessary to prevent reduction of the azido group by the released sulfite anion and also elevated the yield of trinitrophenylation to about 80%. Purity was determined spectrophotometrically, as well as by anion exchange TLC and reversed phase HPLC. In the dark, the compounds were found to display most of the features of the parent TNP-nucleotides and interacted with the Ca^{2+}-ATPase in a similar way. When activated by illumination, the probes were specifically incorporated into SR vesicles with high efficiency at alkaline pH. The site of labeling was identified as being on the A_1 tryptic fragment.

High affinity binding of all three analogs to SR vesicles was determined by three different methods. Direct binding to intact vesicles was measured by incubation with TNP-$8N_3$-[8-^{14}C]-nucleotides under equilibrium conditions followed by filtration. High affinity binding (k_D = 0.1, 0.2 and 0.2 µM for the mono-, di- and triphosphate, respectively) to the extent of about 4 nmoles/mg protein was followed by a nonsaturable increase at higher nucleotide concentrations and probably represents nonspecific binding to the membrane. Specific binding to the catalytic site was measured by following the inhibition by TNP-$8N_3$-nucleotides of active site crosslinking by glutaraldehyde on polyacrylamide gels (k_D = 0.04, 0.3 and 0.4 µM for the mono-, di-

and triphosphate, respectively). Finally, we utilized the fluorescent properties of the TNP group to measure binding to two types of nucleotide site. Binding of TNP-8N_3-nucleotides to SR vesicles in the absence of ATP and/or Ca^{2+} and presence of EDTA resulted in a small increase in fluorescence, which saturated as the concentration was increased. Inclusion of Mg^{2+}, under the same conditions, resulted in additional binding which was not seen with TNP-ATP. Again, this probably represents partitioning of the more hydrophobic, chelated TNP-8N_3-nucleotides into the SR membrane. In the presence of catalytic amounts of both ATP and Ca^{2+}, the TNP-8N_3-nucleotides undergo a large increase in fluorescence, which also saturates as the fluorophore concentration is raised.

TNP-ATP has been found to bind with high affinity (in the micromolar range)to a regulatory site and to accelerate enzyme activity similarly to that observed with higher concentrations of ATP. Consistently, ATPase activity was stimulated 1.0, 1.4, and 1.5 fold respectively by micromolar concentrations of TNP-8N_3 AMP, TNP-8N_3ADP, and TNP-8N_3ATP. At higher concentrations, TNP-8N_3-nucleotides inhibited ATPase activity, suggesting competition at the catalytic site. The large increase in fluorescence seen upon binding of TNP-8N_3-nucleotides under conditions producing acceleration of activity suggested that the high fluorescent site is a regulatory site.

Irradiation of SR vesicles with TNP-8N_3-nucleotides under equilibrium conditions resulted in covalent incorporation of the nucleotide to the SR vesicles. The incorporation of TNP-8N_3-nucleotides was pH dependent, increasing at alkaline pH and exhibiting a pK of 7.4. Inhibition of ATPase activity as a result of covalent incorporation showed the same pH dependence. Thus while labeling at pH 6.0 had little effect on ATPase activity, labeling at pH 9.0 resulted in an inhibition of ATPase activity stoichiometric with levels of incorporation. TNP-8N_3-nucleotide was covalently incorporated both under conditions of

equilibrium binding to the catalytic site, as well as to the fluorescent/regulatory site under turnover conditions in the presence of ATP. The pH dependence of incorporation was the same irrespective of labeling conditions, consistent with labeling a single locus. Higher levels of labeling were obtained, however, when illumination was carried out under turnover conditions suggesting either a higher affinity for this site or a more efficient labeling process, perhaps as a result of an increase in hydrophobicity of the site. At optimal concentrations of TNP-8N_3-nucleotide and ATP, specific covalent incorporation to the turning over enzyme was obtained to the extent of 80% per irradiation period. An increase in labeling is also obtained by reirradiating labeled vesicles in the presence of fresh TNP-8N_3-nucleotide. The covalently incorporated TNP-8N_3-nucleotide, both under turnover and nonturnover conditions, inhibited active site crosslinking with glutaraldehyde suggesting that both catalytic and regulatory nucleotides are located at the catalytic site.

The fluorescence properties of the covalently incorporated TNP-8N_3-nucleotide were measured following removal of the free nucleotide with ion exchange resin. A large fluorescence increase was observed when SR vesicles covalently labeled with TNP-8N_3-nucleotide were incubated with AcP + Ca^{2+} or with P_i + Me_2SO, but not with ATP + Ca^{2+}. Possibly, ATP is sterically prevented from entering the nucleotide site by the covalently incorporated TNP-8N_3-nucleotide, while AcP and P_i are still able to induce phosphorylation. A similar phenomenon is seen after modification of the enzyme with FITC and so the two probes are probably situated close together. The fluorescence enhancement upon induction of turnover was the same irrespective of whether the TNP-8N_3-nucleotide was incorporated into the low fluorescence site under nonturnover conditions, or to the high fluorescent site under turnover conditions. These latter conditions were identical to those in which binding of TNP-8N_3ATP and TNP-8N_3

ADP stimulated ATPase activity. The crosslinking results again indicated that covalent incorporation of nucleotide during enzyme turnover is more efficient than under nonturnover conditions.

The covalently incorporated TNP-8N_3-nucleotide was localized on the A_1 tryptic fragment both under turnover and nonturnover conditions and thus this part of the molecule must form a fold of the nucleotide binding site in addition to the B fragment. Preliminary peptide mapping results indicated a single peptide carrying most of the radioactive label. Significantly, both the nonturnover and turnover labeled peptides eluted in the same place during reversed phase HPLC and is consistent with a model in which the catalytic, regulatory and high fluorescence sites all share the same locus. The pH dependence of labeling indicates that an ionizable group with pK of about 7.4 is involved and is preferentially labeled when ionized. The most likely candidate is a sulfhydryl group with pK of 7.5, but another amino acid like Tyr (pK = 10) may be the site of labeling as the pK is dependent on the protein environment.

The probes described here may be useful in labeling a number of other ATP binding enzymes particularly those, including myosin, (Na^+ + K^+)-ATPase and F_1-ATPase, which interact with TNP-ATP. The fluorescent properties, high affinity binding, and potential for nucleotide site discrimination displayed by the TNP-8N_3-nucleotides with the Ca^{2+}-ATPase, are favorable properties in a photoaffinity probe.

ABBREVIATIONS

aATP	arylazido ATP
AcP	acetyl phosphate
AP-PL	adenosine phosphopyridoxal
AP_2-PL	adenosine diphosphopyridoxal
AP_3-PL	adenosine triphosphopyridoxal
AP_4-PL	adenosine tetraphosphopyridoxal
Bz_2ATP	3'(2')-o-(4-Benzoyl)benzoyl ATP
CHES	[2-(cyclohexylamino-ethanesulfonic acid)]
DMF	dimethylformamide
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
EF-Tu	_E.coli_ elongation factor
EGTA	ethylene glycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EPR	electron paramagnetic resonance
E-P	phosphoenzyme
E_1-P	ADP-sensitive Ca^{2+}-ATPase phosphoenzyme
E_2-P	ADP-insensitive Ca^{2+}-ATPase phosphoenzyme
F_1	the F_1 subunit of the ATP synthase from mitochondria (MF_1), chloroplasts (EF_1) and _E.Coli_ (EF_1)
FITC	fluorescein 5' isothiocyanate
5'-FSBA	5'-(p-fluorosulfonyl)benzoyl adenosine
HEPPS	(N-2-hydroxyethyl-piperazine-N'-3-propanesulfonic acid)
HMM	'heavy' meromyosin
HPLC	high performance liquid chromatography
IAA	iodoacetamide
IAEDANS	5-(2-iodoacetamidoethyl)aminonaphthalene-1-sulfonate
MES	[2-(N-morpholino)-ethanesulfonic acid]
Me_2SO	dimethylsulfoxide
MOPS	3-(N-morpholino)-propane-sulfonic acid

NAP_3-ATP	arylazido-ß-alanine ATP
NAP_4-ATP	arylazido-4-aminobutyric ATP
NAP_6-ATP	arylazido-6-aminocaproic ATP
$8N_3$ATP/ADP	8-azido-ATP/-ADP
$2N_3$ATP/ADP	2-azido-ATP/-ADP
NEM	N-ethyl maleimide
P_i	inorganic phosphate
PITC	phenylisothiocyanate
PLP	pyridoxal phosphate
PMSF	phenylmethylsulfonylfluoride
SDS PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
TEA	triethylamine
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNP-ATP/ADP/AMP	2',3'-o-(2,4,6-trinitrophenyl)-adenosine 5'-triphosphate/diphosphate/monophosphate
TNP-$8N_3$ATP/ADP/AMP	2',3'-o-(2,4,6-trinitrophenyl)-8-azido-adenosine 5'-triphosphate/diphosphate/monophosphate
TRIS	tris(hydroxymethyl)-methylamine

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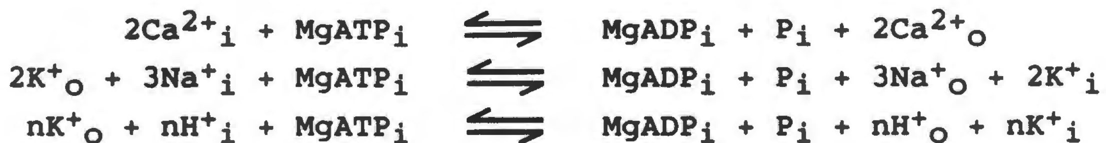
1 INTRODUCTION

1.1 General Properties of Ion Pumps

Ion pumps or cation-linked ATPases are one of three categories of enzymes catalyzing reactions involving phosphate monoesters (Knowles, 1980). This category they share with the kinases and mutases which transfer γ-phosphate groups to molecules other than water, the final acceptor of the phosphatase catalyzed γ-phosphate cleavage.

Ion transport systems can be divided into four categories (Tanford, 1983; Pedersen and Carafoli, 1987a, b): a) ATP driven pumps with phosphoryl enzyme intermediates, or P-type ATPases; b) multisubunit ATP-linked proton transport systems without phosphoryl protein intermediates or F-type ATPases; c) light driven proton pumps (Bacteriorhodopsin) and d) obligatory exchange transport systems. Another group, called the V-type ATPases has been identified, and includes the H^+-ATPases associated with membrane bound organelles other than the mitochondrion, endoplasmic reticulum or SR. Recently, a further group distinct from those above has been identified, comprising the proton translocating ATP synthases from archaebacteria (Danon and Stoeckenius, 1974; Denda et al., 1988).

The P-type or aspartophosphoryl cation motive ATPases, sometimes referred to as E_1E_2 ATPases, catalyze reactions of the type illustrated below (Kyte, 1981).



$$2Ca^{2+}_i + MgATP_i \rightleftharpoons MgADP_i + P_i + 2Ca^{2+}_o$$
$$2K^+_o + 3Na^+_i + MgATP_i \rightleftharpoons MgADP_i + P_i + 3Na^+_o + 2K^+_i$$
$$nK^+_o + nH^+_i + MgATP_i \rightleftharpoons MgADP_i + P_i + nH^+_o + nK^+_i$$

They include calcium pumps (of which the Ca^{2+}-ATPase of SR is an example), sodium pumps, proton pumps and bacterial potassium pumps, and all contain an aspartic acid residue, which is phosphorylated during the catalytic cycle (Degani and Boyer, 1973; Post and Kume, 1973; Nishigaki et al., 1974; Dame and Scarborough, 1981). In addition, they are all inhibited by

vanadate (Macara, 1980), and have a reaction cycle characterized by two major conformational states, E_1 and E_2. K^+ appears to have a common role in the P-type ATPases (Green et al., 1988). It acts as a counterion in the sodium and proton pumps and has a similar function in the bacterial pump where it is required for dephosphorylation. K^+ also activates dephosphorylation in the Ca^{2+} and fungal H^+ pumps.

The F-type ATPases are large multisubunit complexes that predominantly function in ATP synthesis coupled to proton flux down an electrochemical gradient (Senior and Wise, 1983; Amzel and Pedersen, 1983; Vignais and Satre, 1984; Pedersen and Carafoli, 1987a, b; McCarty and Hammes, 1987). The enzymes found in bacteria, chloroplasts and mitochondria consist of a hydrophilic F_1 section, containing the nucleotide binding sites, and an ionophoric F_o part responsible for cation flux through the membrane. The major differences between individual species lies in the F_o section, while the F_1 sections are very similar. The F_1 section is made up of five different subunits usually in a stoichiometric ratio of $\alpha_3\beta_3\gamma\delta\epsilon$ (at least for MF_1 and CF_1). Considerable sequence homology exists among the α-, β- and γ-subunits of all species examined with β- subunit showing as much as 70% homology between species (Walker et al., 1982, 1985). α- and β- subunits themselves show significant sequence homology. The F_1 moeity appears to contain six nucleotide binding sites located on α- and β- subunits only (Cross and Nalin, 1982; Gromet-Elhanan and Khananshvili, 1984; Khananshvili and Gromet-Elhanan, 1985). The F-type ATPases do not form phosphoprotein intermediates, but do contain tightly bound nucleotides, which may play an analogous role. They are also inhibited by a number of agents, although the pattern of inhibition may vary between enzymes from different sources. Except for a small region round the phosphorylation site, no sequence homology with the P-type ATPases exists (Modyanov et al., 1985; Ernster et al., 1986).

The V-type ATPases are probably the largest group of ATPases, and they comprise those enzymes associated with membrane bound organelles, other than the mitochondrion and the

endoplasmic or sarcoplasmic reticulums (Sze, 1985; Bowman and Bowman, 1986; Mellman et al., 1986; Rudnick, 1986; Pedersen and Carafoli, 1987a, b). They all translocate protons, do not form phosphoryl enzyme intermediates, and are composed of multiple subunits. There are two major polypeptide components of 60-65 kDa and 70-89 kDa as well as a number of smaller peptides. The V-type ATPases have a particular pattern of inhibitor sensitivity. Many V-type ATPases appear to participate in the uptake of biogenic amines or amino acids and they have also been implicated in receptor recycling. It appears likely that there are still a number of physiological roles in which they will be found to play a part. There is, at present, no sequence information available for any V-type ATPase.

The more recently characterized archaebacterial H^+-ATPases cross-react immunologically with the F-type ATPases and also resemble them in function, but they differ in subunit composition, sensitivity to ATPase inhibitors and pH optima (Inatomi, 1986; Hochstein et al., 1987; Nanba and Mukohata, 1987; Lubben et al., 1987; Mukohata et al., 1987). The particular inhibitor sensitivities found in the archaebacterial H^+-ATPases are similar to those found in the V-type ATPases (Danon and Stoeckenius, 1974; Mukohata and Kaji, 1981; Kristjansson et al., 1986; Mukohata and Yoshida, 1987a; Lubben and Schafer, 1987; Konishi et al., 1987). The primary structure of the H^+-ATPase from Sulfolobus acidocaldarius has recently been determined and found to bear weak homology to both the α and β subunits of EF_1-ATPase (Danon et al., 1988), although a polyclonal antibody raised against the ATP synthase of Halobacterium halobium cross-reacts with much higher affinity to the anion sensitive ATPase of red beet than to either CF_1-ATPase or the Ca^{2+}-ATPase (Mukohata et al., 1987) .

Despite the differences between classes, there have been attempts to synthesize the observed similarities into a general model for active ion pumping (Hammes, 1982; Scarborough, 1984; Eisenberg and Hill, 1985; Pedersen and Carafoli, 1987b). This kind of model is generally restricted to F- and P-type ATPases for which there is extensive mechanistic information. In terms

of one such model (Pedersen and Carafoli, 1987b), the enzyme is postulated to have a hydrophobic active site which excludes H_2O and makes phosphate transfer an energetically favorable event. The main thermodynamic barrier involves the binding of reactants, which is facilitated by interaction between residues on the protein and on the reactant molecules. A similarity is thus seen between the way in which both types overcome a similar problem.

1.1.1 P-type ATPase structure

Structurally, the P-type ATPases are all large (α) polypeptides of MW 70-140 kDa. The $(Na^+ + K^+)$-ATPase has, in addition, a closely associated (ß) polypeptide of MW 53 kDa and as yet unknown function. The amino acid sequences of a number of P-type ATPases have been determined (Hesse et al., 1984; Kawakami et al., 1985; MacLennan et al., 1985; Shull et al., 1985; Addison, 1986; Brandl et al., 1986; Hager et al., 1986; Kawakami et al., 1986; Ovchinnikov et al., 1986; Serrano et al., 1986; Shull and Lingrel, 1986; Solioz et al., 1987). Extensive sequence homology (> 80%) is found in ATPases sharing the same ion specificity while pumps of different ion specificity show only about 20-30% homology, concentrated in about nine to twelve homologous regions scattered throughout the molecule (Serrano et al, 1986; Green et al., 1988; Serrano, 1988), although a higher degree of homology exists between the $(Na^+ + K^+)$-ATPases and the $(H^+ + K^+)$-ATPases of animal cells (Serrano et al., 1986).

Ten regions of extensive homology have been identified and are shown in Fig. 1.1 (Serrano et al., 1986; Serrano, 1988). Of the ten shown, five (b, c, f, h, and i) are the most highly conserved. Region f contains the aspartic acid residue phosphorylated during catalysis, while regions g and i appear to be located in the nucleotide binding site as they contain the residues affinity labeled by FITC and 5'-FSBA, respectively. Region e is the only conserved region predicted to span the membrane. An evolutionary tree relating all known P-type

```
                           a                                        b
                •  -     -•                    - • --  ••-- -  •  -• •  -
Ca    108 QERNAENAIEALKEYEPEMGKVYR 131    140 IKAKDIVPGDIVEIAVGDKVPADIRL 165
Na-K  148 QEAKSSKIMESFKNMVPQQALVIR 171    178 INAEEVVVGDLVEVKGGDRIPADLRI 203
H-K   159 QEFKSTNIIASFKNLVPQQATVIR 182    189 INADQLVVGDLVEMKGGDRVPADIRI 214
H     161 QEFQAGSIVDELKKTLANTAVVIR 184    191 IPANEVVPGDILQLEDGTVIPTDGRI 216
K      86 AEGRSKAQANSLKGVKKTAFARKL 109    121 VPADQLRKGDIVLVEAGDIIPCDGEV 146

                   c                             d
           -•-• -••••    -                --             • - -
Ca    175 VDQSILTGESVSVIK 189    209 FSGTNIAAGKAMGVVVATGVNTEIGK 234
Na-K  211 VDNSSLTGESEPQTR 225    242 FFSTNCVEGTARGIVVYTGDRTVMGR 267
H-K   222 VDNSSLTGESEPQTR 236    253 FFSTMCLEGTAQGLVVSTGDRTIIGR 278
H     225 IDQSAITGESLAVDK 239    246 FSSSTVKRGEGFMVVTATGDNTFVGR 271
K     153 VDESAITGESAPVIR 167    177 TGSTRILSDWLVIECSVNPGETFLDR 202

                      e                                         f
           -  --- -  -•  -  --            -   - -    --•- -    -  •••••-•
Ca    296 FKIAVALAVAAIPEGLPAVIT 316    326 MAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTN 359
Na-K  319 VIFLIGIIVANVPEGLLATVT 339    349 MARKNCLVKNLEAVETLGSTSTICSDKTGTLTQN 382
H-K   330 MVFFMAIVVAYVPEGLLATVT 350    360 LASKNCVVKNLEAVETLGSTSVICSDKTGTLTQN 393
H     323 LRYTLGITIIGVPVGLPAVVT 343    353 LAKKQAIVQKLSAIESLAGVEILCSDKTGTLTKN 386
K     252 VTVLVALLVCLIPTTIGGLLS 272    282 MLGANVIATSGRAVEAAGDVDVLLLDKTGTITLG 315
                                                                        ↑

           g                                    h
          ••-           - • -  •  -    --  •  •--  •-•••    •      •-
Ca    513 VKGAP 517    592 FVGCVGMLDPPRIEVASSVKLCRQAGIRVIMITGDNKGTAVAICRRIGI 640
Na-K  505 MKGAP 509    583 FVGLISMIDPPRAAVPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGI 631
H-K   516 MKGAP 520    594 FAGLVSMIDPPRATVPDAVLKCRTAGIRVIMVTGDHPITAKAIAASVGI 642
H     473 VKGAP 477    526 ILGVMPCMDPPRDDTAQTVSEARHLGLRVKMLTGDAVGIAKETCRQLGL 574
K     394 RKGSV 398    439 VLGVIALKDIVKGGIKEAFAQLRKMGIKTVMITGDNRLTAAAIAAEAGV 487

                                i
          -•   •  •  --   •     - •-•••• ••-••• •- --•-
Ca    675 FARVEPSHKSKIVEFLQSFDEITAMTGDGVNDAPALKKAEIGIAM 719
NA-K  688 FARTSPQQKLIIVEGCQRQGAIVAVTGDGVNDSPALKKADIGVAM 732
H-K   699 FARTSPQQKLVIVESCQRLGAIVAVTGDGVNDSPALKKADIGVAM 743
H     607 FAEVFPQHKYRVVEILQNRGYLVAMTGDGVNDAPSLKKADTGIAV 651
K     491 LAEATPEAKLALIRQYQAEGRLVAMTGDGTNDAPALAQADVAVAM 535

                        j
          ---  -- •----      - --  -  -- -
Ca    721 SGTAVAKTASEMVLADDNFSTIVAAVEEGRAI 752
Na-K  735 AGSDVSKQAADMILLDDNFASIVTGVEEGRLI 766
H-K   746 AGSDAAKNAADMILLDDNFASIVTGVEQGRLI 777
H     653 GATDAARSAADIVFLAPGLSAIIDALKTSRQI 684
K     537 SGTQAAKEAGNMVDLDSNPTKLIEVVHIGKQM 568
```

Figure 1.1 <u>Conserved Sequences in P-type ATPases</u>

The 10 areas of homology between 5 P-type ATPases, published by Serrano (1988). The enzymes compared are the slow twitch SR Ca^{2+}-ATPase (MacLennan <u>et al</u>., 1985), the (Na^+ + K^+)-ATPase from sheep kidney (Shull <u>et al</u>., 1985), the (H^+ + K^+)-ATPase from <u>S.cerevisiae</u> (Serrano <u>et al</u>., 1986) and the K^+-ATPase from <u>E.coli</u> (Hesse <u>et al</u>., 1984). Asterisks represent conserved residues and dashes conservative replacements. The aspartic acid residue phosphorylated during catalysis is marked with an arrow.

ATPases to the (Na^+ + K^+)-ATPase is shown in Fig.1.2 (Jorgensen, 1988).

The linear sequence and location of predicted transmembrane regions and structural domains in a number of P-type ATPases is shown in Fig. 1.3. Variations in the number and location of transmembrane regions, depending on the predictive method used, have been reported, but the structure shown here probably represents a good average assuming a common structure. The organization of the structural domains is very similar between groups although the bacterial enzymes are shorter as they lack the last two transmembrane regions as well as a portion of the N-terminus. Both the location of the trypsin sensitive sites and the introns shown in Fig. 1.3 may delineate structural domains.

The N-terminus region is the most variable, but nevertheless is predicted to have an α-helical structure in all ATPases. It is separated by the first two closely spaced transmembrane regions from a hydrophilic region made up of about 130-150 amino acids and containing the first four regions of homology. This region is predicted to be mainly organized into a ß-sheet structure and is bounded by the next two closely spaced transmembrane regions, three and four, with a highly conserved region occurring in four. The next region contains a large hydrophilic region made up of about 350-450 amino acids and containing the other five regions of homology as well as the phosphorylation site and the postulated nucleotide binding site. This region is predicted to consist mainly of an alternating α/ß type structure. In both the Ca^{2+}-ATPase and the (Na^+ + K^+)-ATPase these two hydrophilic domains, which contain most of the conserved stretches, are separated by a trypsin sensitive site. Following this region, there are another four transmembrane regions separated by short hydrophilic stretches and which are separated, in the (Na^+ + K^+)-ATPase by the five known introns, again suggesting a separation of domains.

A number of studies have indicated a similarity in the secondary and tertiary structures of the P-type ATPases. CD spectroscopy has demonstrated that a number of P-type ATPases have a similar secondary structure composition (Hennessey and

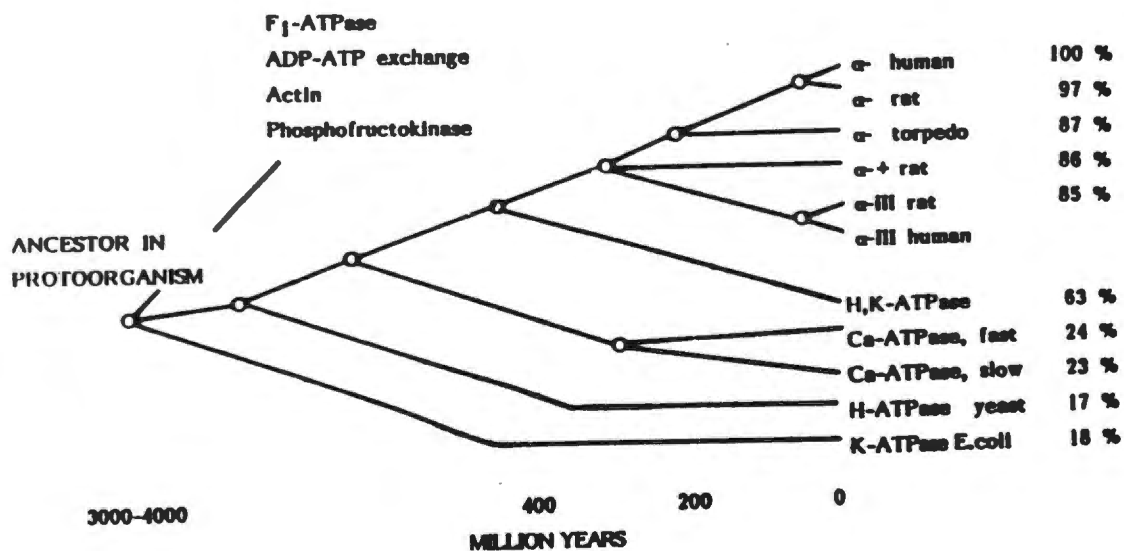


Figure 1.2 <u>Evolutionary tree for the P-type ATPases</u>

An evolutionary tree postulated for the known P-type ATPases assuming divergence from a common ancestor, and relating all species to the α-subunit of the human (Na^+ + K^+)-ATPase (Jorgensen <u>et al</u>., 1988).

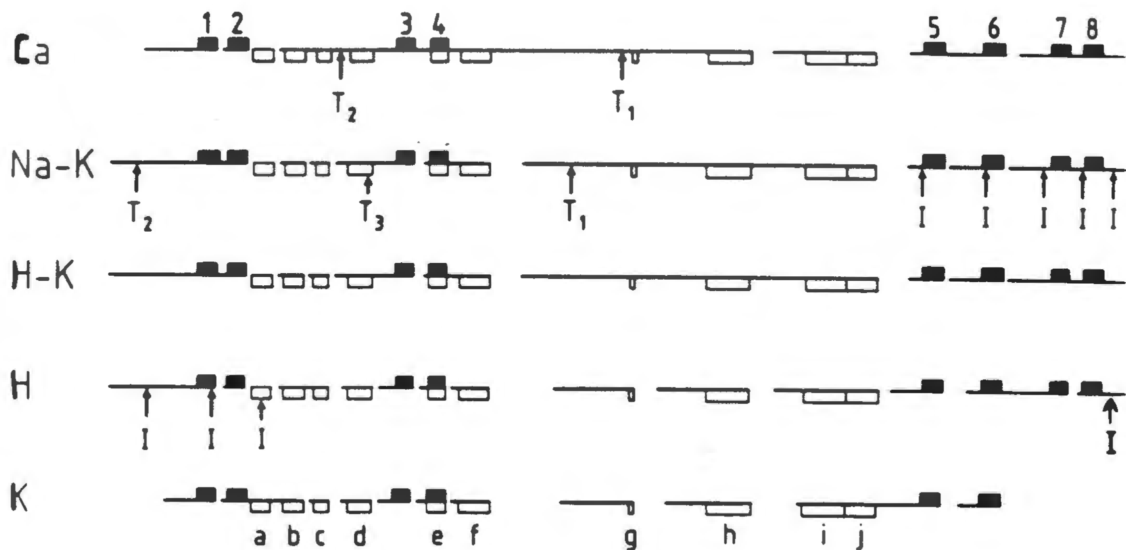


Figure 1.3 <u>Linear sequence alignment of P-type ATPases</u>

Comparison of the organization of the linear sequences of the 5 P-type ATPases compared in Fig.1.1 published by Serrano (1988). The enzymes compared are the same as in Fig.1.1, shaded boxes 1-8 are predicted transmembrane regions and open boxes a-j are the 10 homologous regions. Trypsin sensitive sites (T) and the position of known introns (I) are also shown.

Scarborough, 1988), while Raman spectroscopy has confirmed the assignment of secondary structure units in the $(Na^+ + K^+)$-ATPase based on hydrophobicity plots, and has shown unambiguously that the transmembrane regions are made up of α-helices (Ovchinnikov et al., 1988). Similar models for the tertiary structure organization of a number of P-type ATPases have been postulated (MacLennan et al., 1985; Shull et al., 1985; Addison, 1986; Brandl et al., 1986; Hager et al., 1986; Serrano et al., 1986; Solioz et al., 1987; Ovchinnikov et al., 1988).

Two regions of homology have been identified between P-type ATPases and other ATPases. The first is an observed homology around the phosphorylation site of the P-type ATPases and a region of the F_1-ATPases (Fig.1.4) (Modyanov et al., 1985; Ernster et al., 1986). In the F_1-ATPases the phosphorylated Asp residue is replaced by a Thr and adjacent to this is a Lys residue which, in the F_1-ATPases, is labeled by $8N_3ATP$ (Hollemans et al., 1983). Homology has also been detected between the region around the site of labeling of the Ca^{2+}-ATPase with AP_3-PL (Yamamoto et al., 1988) and rat embryonic skeletal muscle myosin heavy chain (Strehler et al., 1986).

1.1.2. Structure of nucleotide binding sites

First indications of a common nucleotide binding domain followed comparisons of the three-dimensional structure of the NAD binding site of a number of dehydrogenases as well as the FMN binding site of flavodoxin (Chandrasekhar et al., 1973; Buehner et al., 1973; Rao and Rossmann, 1973; Rossmann et al., 1974; Ohlsson et al., 1974). This recurring super-secondary structure, or 'Rossmann fold', consists of three parallel strands of pleated sheet connected by helical regions (Rao and Rossmann, 1973). In the dinucleotide site, two such folds lie adjacent to one another with an approximately two-fold symmetry, and connected by a helical, or less well defined, region (Rao and Rossmann, 1973). The structure of the dehydrogenase NAD binding site is shown in Fig. 1.5. The sequence ßA, αB, ßB, αC, ßC forms the adenine nucleotide binding site while the nicotinamide

P-type ATPases	
	*
K-ATPase	LLDKTGTITLGQ
Na/K-ATPase	SDKTGTLTQNR
Ca-ATPase	CSDKTGTLTTNQ

H-ATPases (β)	
	+
E. coli	TSTKTGSITSVQ
Bovine mitochn.	TTTKKGSITSVQ
Maize chloropl.	TSTKKGSITSVQ
Spinach chloropl.	TSTKEGSITSVQ
R. blastica	TSTKAGSITSVQ

H-ATPases (α)	
E. coli	VKGKTGSLTALP
R. blastica	GDFGAGSLTALP
Tobacco chloropl.	SSLGEGSMTALP

Figure 1.4 Homology between the phosphorylation site of P-type ATPases and a region in the F-type ATPases

Comparison of the amino acid sequence in a region around the phosphorylation site of a number of P-type ATPases with a homologous region in the F-type ATPases, showing the site of phosphorylation in the case of the former (*) and the site of labeling with $8N_3$-ATP in the case of the latter (+) (Modyanov et al., 1985; Ernster et al., 1986).

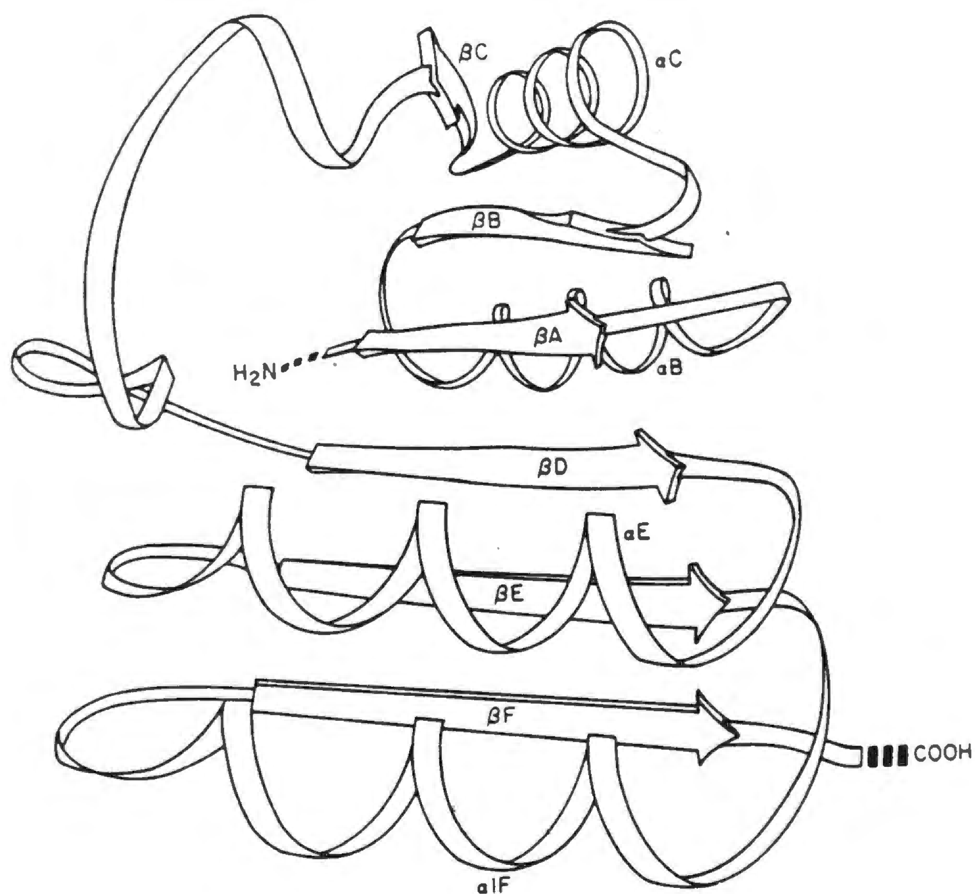


Figure 1.5 Structure of the dinucleotide binding site

Schematic of the coenzyme binding domain in dehydrogenases (Ohlsson et al., 1984). α-helices are represented by coils and strands of ß-sheet by arrows pointing in the direction of the C-terminus.

binding site occurs in the adjacent ßαßαß region (Rossmann et al., 1974).

The basic ADP binding unit of ß-sheet and α-helices, is a recurring motif which has been found to exist in a number of globular proteins (Sternberg and Thornton, 1976; Wierenga et al., 1985). Recognition of this has allowed the refinement of the normal secondary structure prediction which is usually < 60% efficient (Kabsch and Sander, 1983), to greater than 70% (Taylor and Thornton, 1983, 1984). In addition, a 'fingerprint' has been described (Wierenga et al., 1986), following identification of the essential structural features of several amino acid side chains of the ßαß fold (Wierenga et al., 1985). The structure of a typical ßαß fold is shown in Fig. 1.6, with the eleven essential elements (Wierenga et al., 1986). The first requirement is for a basic or hydrophilic amino acid at the first position of the ß-strand (open triangle). The hydrophobic core between the ß-strands and the α-helix is formed by six hydrophobic residues (open squares). The capability for making a sharp turn between the first ß-strand and the α-helix is given by two Gly residues (closed circles), while the third (middle) Gly allows for the close approach of the pyrophosphate group to the N-terminus of the α-helix. Lastly, a good H-bond is made between the acidic amino acid side chain (divided open circle) and the 2'-OH of the ribose moeity. Some variability exists at the first position (divided open triangle) as well as at the positions of the hydrophobic residues, so the three Gly residues and the acidic residue are defined as an essential 'core' region (Wierenga et al., 1986).

An evolutionary tree has been proposed tracing the incorporation and evolution of the mononucleotide binding structure in various dehydrogenases (Buehner et al., 1973), assuming divergence from a common structure more than 3.2 X 10^9 years ago (Rossman et al., 1974; Sternberg and Thornton, 1976). Similar structures have been reported to exist in a number of kinases (Bryant et al., 1974; Schulz and Schirmer, 1974; Schulz et al., 1974; Sachsenheimer and Schulz, 1977; Evans and Hudson, 1979; Watson et al., 1982; Yuasa et al., 1983). The largest

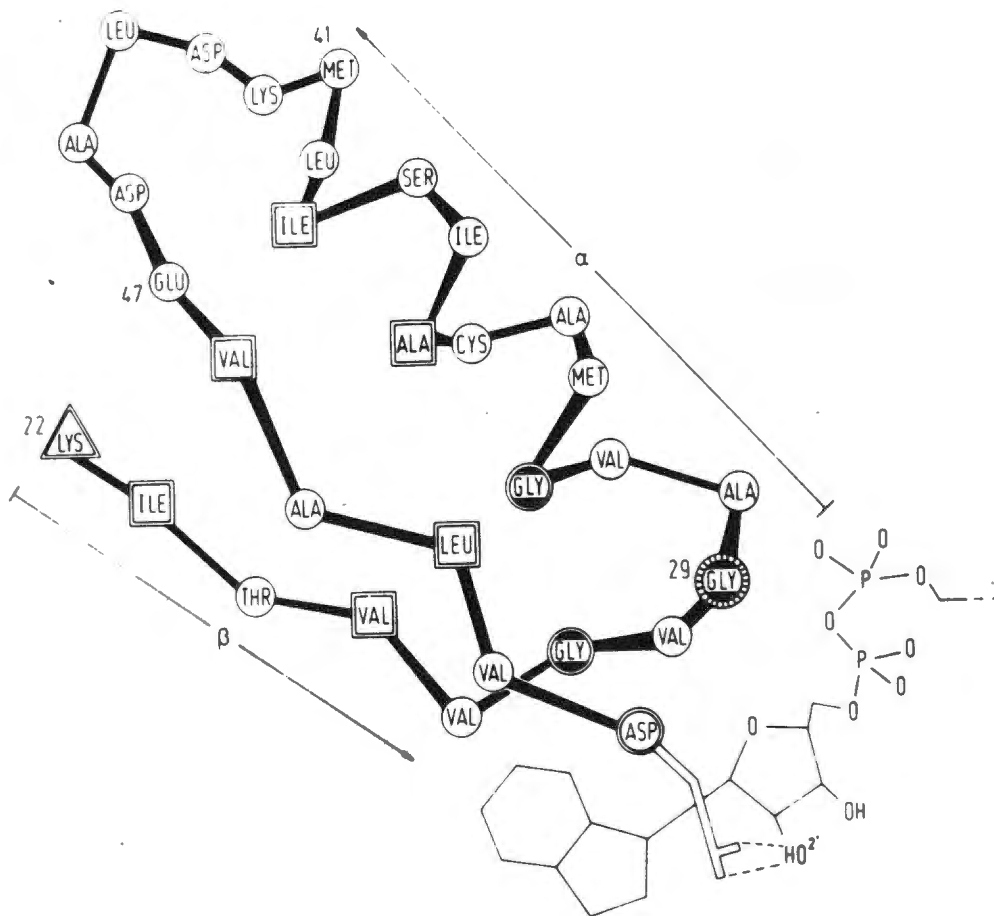


Figure 1.6 <u>Structure of the ßαß fold</u>

Schematic of the ADP-binding ßαß fold of dogfish lactate dehydrogenase showing conserved and core residues. (△ ,basic or hydrophilic; □, small and hydrophobic) (Wierenga, 1986). The 11 'fingerprint' residues are framed by double lines.

body of structural information is available for adenylate kinase and the structure of the nucleotide site in this enzyme has been used as a model for ATP site structure in a number of other enzymes, including the sarcoplasmic reticulum Ca^{2+}-ATPase (Green et al., 1988), and is therefore considered in some detail.

1.2.1.1 ADENYLATE KINASE

Adenylate kinase has been purified from many sources and the X-ray structure of the porcine enzyme has been reported in fine detail (Schulz and Schirmer, 1974; Sachsenheimer and Schulz, 1977). The MgATP binding site of this enzyme has been well characterized both by X-ray diffraction (Schulz et al., 1974; Pai et al., 1977) and NMR (Hamada et al., 1979; Smith and Mildvan, 1982; Fry et al., 1985). The structure of the two crystal forms of the enzyme as well as the position of bound nucleotide is shown in Fig. 1.7 (Fry et al., 1986). The positions of the MgATP binding site from X-ray diffraction and NMR analysis differ (Smith and Mildvan, 1982), and at present, the more recent postulate appears correct. These authors place MgATP in close contact with five strands of parallel ß-sheet structure and with one of the interconnecting helices (Fry et al., 1986) similar to that seen in the dehydrogenases (Rossmann et al., 1974). Thus, although there is little sequence homology between adenylate kinase and the dehydrogenases, there seems to be a distinct similarity in three-dimensional structure in the region of the nucleotide binding site (Fry et al., 1986). In this model (Fry et al., 1985, 1986), the 2 widely conserved consensus sequences described below (Walker et al., 1982) are placed in the MgATP site as is Lys-21, the homologous region of which, in the F_1-ATPases, has been shown to interact with the γ-phosphate of ATP (Parsonage et al., 1988). These findings,in addition to the use of salicylate (Fry et al., 1985) to locate the ATP site in the earlier study, seem to confirm the positioning shown in Fig.1.7 (Fry et al., 1986). The earlier model, however, appears more consistent with recent affinity labeling results (Tagaya et al., 1987), although a conformational

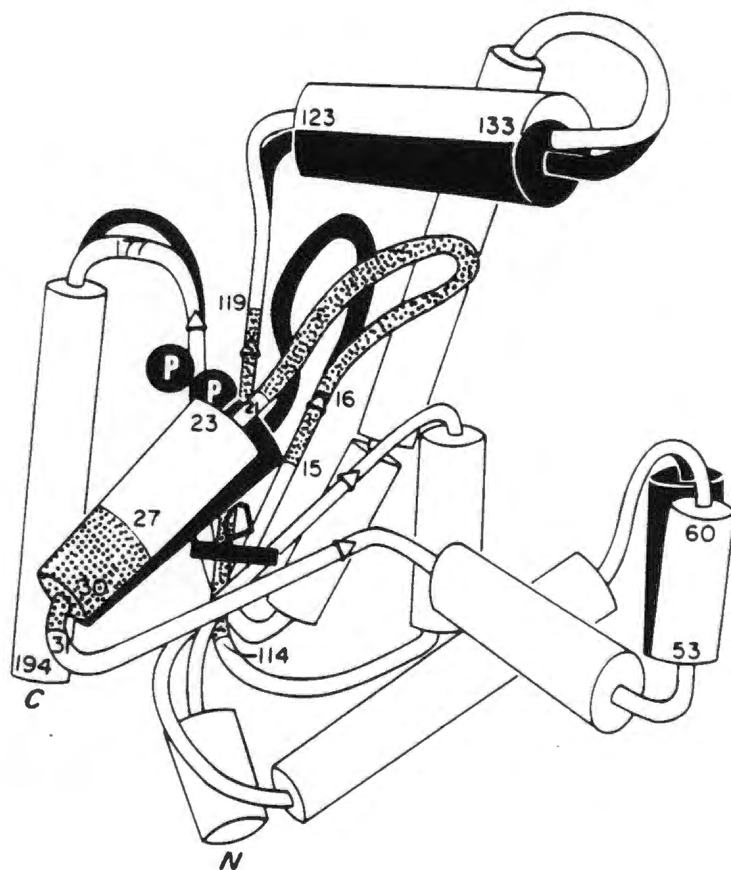


Figure 1.7 <u>Structure of adenylate kinase</u>

The 2 crystal forms of adenylate kinase are shown (A, solid; B, open), with the postulated position of the bound metal-ATP (Fry <u>et al</u>., 1986). α-helises are depicted by cylinders and strands of ß-sheet by arrows pointing from N- to C-terminus. The A conformation changes to the B following the binding of ATP. The 3 homologous segments are shown by stipling in the B conformation.

change on binding nucleotide could also account for these results.

Two highly conserved regions, A and B, have been identified following comparison of the primary structures of adenylate kinase with those of phosphofructokinase, myosin, and F_1 α and ß subunits (Walker et al., 1982). The A sequences have a number of conserved features but most notably the conserved sequence:

G-X-X-X-X-G-K(T)-X-X-X-X-X-X-I/V

which is usually followed by a basic amino acid. The B sequences have the following conserved sequence:

R/K-X-X-X-G-X-X-X-L-Hy-Hy-Hy-Hy-D

where Hy stands for a hydrophobic residue. In adenylate kinase, the A sequence corresponds to the flexible Gly-rich loop structure with the basic amino acid (K) following the loop forming a favorable interaction with the phosphate group on the bound nucleotide, in addition to the extra positive effect of the α-helix dipole (Hol et al., 1978). Sequence B forms a hydrophobic ß-sheet structure at the back of the nucleotide binding pocket. A third region has been identified in adenylate kinase that is conserved in a number of nucleotide binding proteins (Fry et al., 1986) following detailed NMR measurements of the interaction of ß, γ-bidentate Cr^{3+}ATP and MgATP with rabbit muscle adenylate kinase as well as with a synthetic peptide containing the nucleotide binding site (Fry et al., 1985).

The three regions of homology which have been identified in a large number of nucleotide binding proteins, although they are not always all present in a particular structure, have been summarized by Fry et al. (1986). The 3 regions are stippled in the structure shown in Fig. 1.7.

Segment 1, corresponding to sequence A (Walker et al., 1982), is a glycine-rich flexible loop that is terminated by a cationic residue, Lys or Arg (Lys-21 in adenylate kinase) and probably interacts with the γ-phosphoryl group of MgATP. Although previously thought to be diagnostic of nucleotide binding sites, segment 1 may have a more basic role as seen by it's presence in the biotin subunit of transcarboxylase, which

does not interact with nucleotides, as well as at a site, far removed from the AMP binding site, in glycogen phosphorylase (McLaughlin et al., 1984). The function of this loop most likely involves it's ability to undergo a conformational change. Three possible roles for a conformational change at segment 1 have been suggested (Fry et al., 1986): (i) control of accessibility to the substrate binding site, (ii) modification of binding site affinities, (iii) relocation of catalytic groups toward the reaction center of the bound substrate. In adenylate kinase, segment 1 is located in the middle of a cleft and thus it may control accessibility to the substrate binding site by alternately blocking and opening the cleft. Secondly, a conformational change affecting segment 1 could alter the affinity of the binding site for MgATP and MgADP by a change in orientation of the terminal cationic residue (Lys-21). Thirdly, a conformational change bringing segment 1 close to the triphosphate chain of MgATP could facilitate the phosphoryl transfer reaction by proximity to the hydroxyl group of Ser-19 and the amide NH protons of the protein backbone.

Segment 2, corresponding to Sequence B from Walker et al. (1982), is mainly an α-helix consisting of two lysines separated by three residues, two of which are hydrophobic and a third that is variable, even among adenylate kinases from different species. In adenylate kinase the hydrophobic residues form part of the pocket in which the adenine-ribose moeity of MgATP is located. The first lysine residue, Lys-27, can be positioned such that it's NH_3^+ nitrogen is 5.7 ± 2.0 Å from the β- and γ-phosphorus atoms of MgATP and may interact with them. Affinity labeling studies of the cyclic nucleotide-dependent and src protein kinases with FSBA have shown that in these enzymes the Lys residue homologous to Lys-27 is near the γ-phosphate of bound ATP (Kamps et al., 1984).

Segment 3 is a hydrophobic strand of parallel β-pleated sheet terminated by an Asp residue that flanks the triphosphate chain of MgATP, including the reaction center. It probably serves to exclude H_2O and minimize hydrolysis. The end ASP-119 in adenylate kinase may accept a hydrogen bond from a H_2O ligand

of Mg^{2+} on MgATP or alternatively, by directly coordinating Mg^{2+}, it may facilitate the migration of Mg^{2+} from ß, γ-coordination in MgATP to α, ß-coordination in MgADP. An analogous role has been proposed for ASP-372 in phosphoglycerate kinase (Watson et al., 1982) and possibly also for phosphofructokinase (Evans et al., 1981), and ras P21 (Gibbs et al., 1984; Shih et al., 1982).

1.1.2.2 OTHER KINASES

The structure of a number of other kinases in the region of the nucleotide binding site have been determined, two of which are shown schematically in Fig.1.8 (Serrano, 1988). It is evident that the folding of the protein and interaction with ATP is different both for phosphoglycerate kinase and for phosphofructokinase, although both contain the A and B consensus sequences (Walker et al., 1982). In phosphoglycerate kinase, the two consensus sequences do not appear to be directly involved in binding the nucleotide (Bryant et al., 1974; Banks et al., 1979; Watson et al., 1982). Instead the hydrophobic pocket accommodating the adenine ring is formed by two loops while the ribose moeity H-bonds to two acidic residues (Banks et al., 1979; Watson et al., 1982). The α and ß phosphates interact with two Lys groups and are located at the N-terminus of an α-helix which, in this case, however, does not form part of the hydrophobic pocket.

In the case of phosphofructokinase, the nucleotide site differs from both adenylate kinase and from phosphoglycerate kinase (Evans and Hudson, 1979; Kolb et al., 1980; Evans et al., 1981). Here the hydrophobic pocket is made up of an α-helix and a loop. Again, the adenine and ribose moeities make H-bonding interactions with amino acid residues through the chelated Mg^{2+} group (Kolb et al., 1980; Evans et al., 1981).

In hexokinase, it has also been shown that the ATP binding site again is different (Shoham and Steitz, 1980; Steitz et al., 1981). Here the adenine group is located in a shallow slit adjacent to an α-helix with no ß-sheet type structure and the phosphates are not located at the end of an α-helix (Steitz et

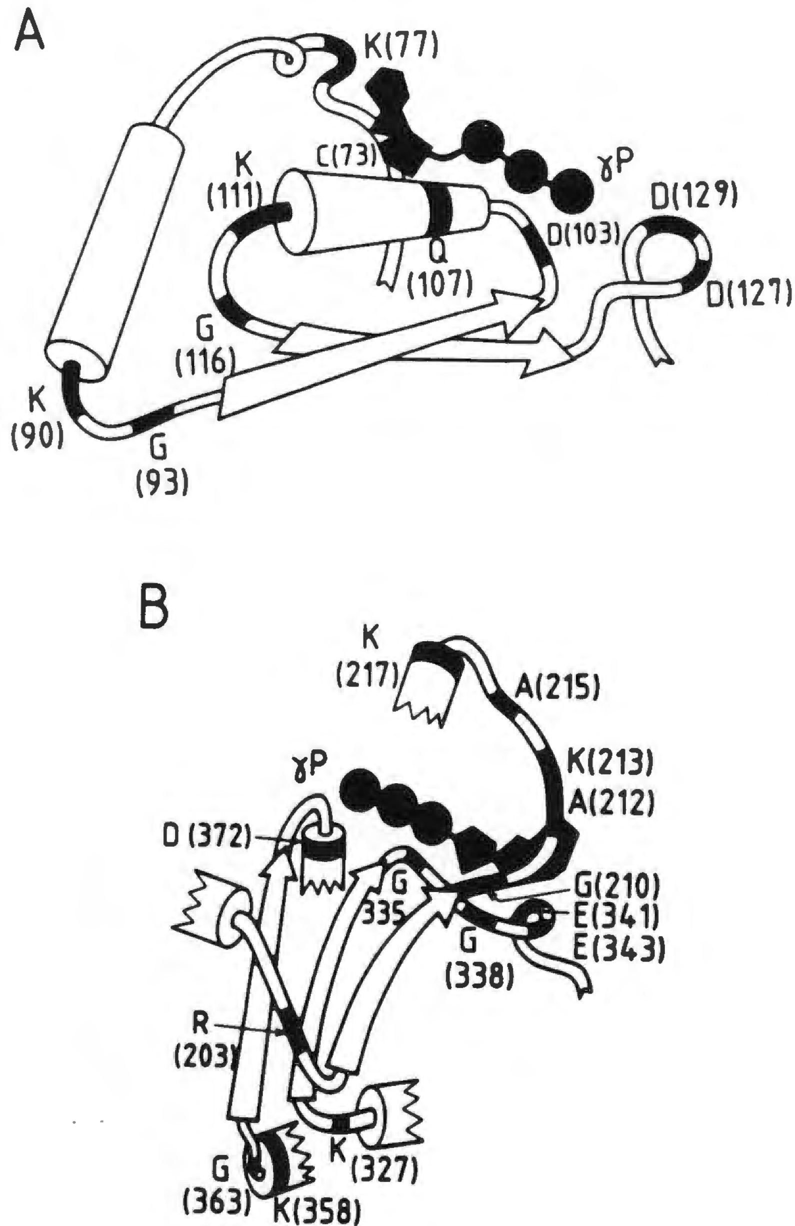


Figure 1.8 <u>Nucleotide binding site of phosphoglycerate kinase and phosphofructokinase</u>

The protein folding in the region of the nucleotide binding site are shown for phosphofructokinase (A) and for phosphoglycerate kinase (B) (Serrano, 1988). α-helises are depicted by cylinders and strands of β-sheet by arrows pointing from N- to C-terminus. Residues implicated in nucleotide binding or conserved in other ATP binding proteins are indicated by dark regions.

al., 1981). Similarly, in pyruvate kinase, a novel interaction with ATP is seen even though an identifiable mononucleotide binding fold exists (Levine et al., 1978). Here the nucleotide lies perpendicular to the axis of a ß-barrel structure (Levine et al., 1978).

1.1.2.3 F_1 NUCLEOTIDE SITE STRUCTURE

It is now well accepted that F_1 from a variety of sources contains six nucleotide binding sites per enzyme unit (Cross and Nalin, 1982; Xue et al., 1987a), and that these are located solely on α and ß subunits (Senior and Wise, 1983; Vignais and Satre, 1984). It is also generally accepted that the 3 catalytic sites reside on the ß subunit (Senior and Wise, 1983) and in terms of the $\alpha_3\beta_3\gamma\delta\epsilon$ subunit stoichiometry of F_1 that the other 3 noncatalytic sites exist either on ß or at an interface between α and ß (Gromet-Elhanan and Khananshvili, 1984; Khananshvili and Gromet-Elhanan, 1985; Kironde and Cross, 1987a, b).

The primary structures of a number of species have been determined and extensive homology is seen between α and ß subunits from different species as well as between α and ß themselves (Walker et al., 1982, 1985; Gorboczi et al., 1988a). Comparison of the primary structures of both α and ß subunits with a number of other proteins including myosin, adenylate kinase and phosphofructokinase have revealed the presence of two consensus sequences A and B (Walker et al., 1982). Both these sequences occur in the nucleotide binding site of adenylate kinase. A corresponds to the Gly-rich flexible loop structure and B to a strand of ß-sheet forming part of the binding pocket. Adjacent to one of the homologous regions is a small stretch showing homology with a region around the phosphorylation site in the P-type ATPases (Modyanov et al., 1985; Ernster et al., 1986; Gorboczi et al., 1988a).

The predicted secondary structure of the F_1-ATPase (Walker et al., 1984; Duncan et al., 1986) shows alternating α-helices and ß-sheets which can be folded into a ßαß structure (Gorboczi

et al., 1988a) and models of the tertiary structure have been predicted in which the nucleotide domain is folded into a structure similar to that found in adenylate kinase (Fig.1.9) (Duncan et al., 1986; Gorboczi et al., 1988a). The predicted fold contains a number of residues which have been implicated in nucleotide binding by chemical labeling studies (Gorboczi et al., 1988a). The same model is thought to apply to the folding of the α subunit (Maggio et al., 1987). It has been postulated that the identified site on the ß-subunit represents the catalytic site (Gorboczi et al., 1988a) while the other postulated site (Gromet-Elhanan and Khananshvili, 1984; Kironde and Cross, 1987) may be elsewhere and less conserved evolutionary (Gorboczi et al., 1987a).

A number of essential residues, including several within the conserved regions, have been specifically mutated (Parsonage et al., 1987a, b, 1988). In particular, Lys-155 was specifically mutated to Gln or Glu (Parsonage et al., 1987a). In the postulated three-dimensional structure of F_1, Lys-155 corresponds to Lys-21 in adenylate kinase, which is located at the end of the Gly-rich flexible loop, and has been postulated to interact with the phosphate groups of ATP (Fry et al., 1985; Dreusicke and Schulz, 1986). Mutation to Glu was found to be less detrimental to binding than was mutation to Gln, which apparently contadicts the interaction hypothesis (Parsonage et al., 1987a, 1988). Both mutations had little effect on catalysis per se and thus cannot be directly involved in phosphoryl transfer reactions (Parsonage et al., 1988). MgADP binding was not affected and so the effect appears specific for the γ-phosphate group of ATP (Parsonage et al., 1988). Another seemingly anomalous finding was that two Tyr residues, both highly conserved and modified by Nbf-Cl and 5'-FSBA respectively, when changed to Phe, had little or no effect on activity (Parsonage et al., 1987a). It has been proposed that the mutations may prevent the deformation in the twist of the central ß-sheet of the nucleotide binding domain which results in conformational signal transduction (Parsonage et al., 1987b). Another apparent discrepancy is that the sites of labeling with nucleotide site directed probes differ from the

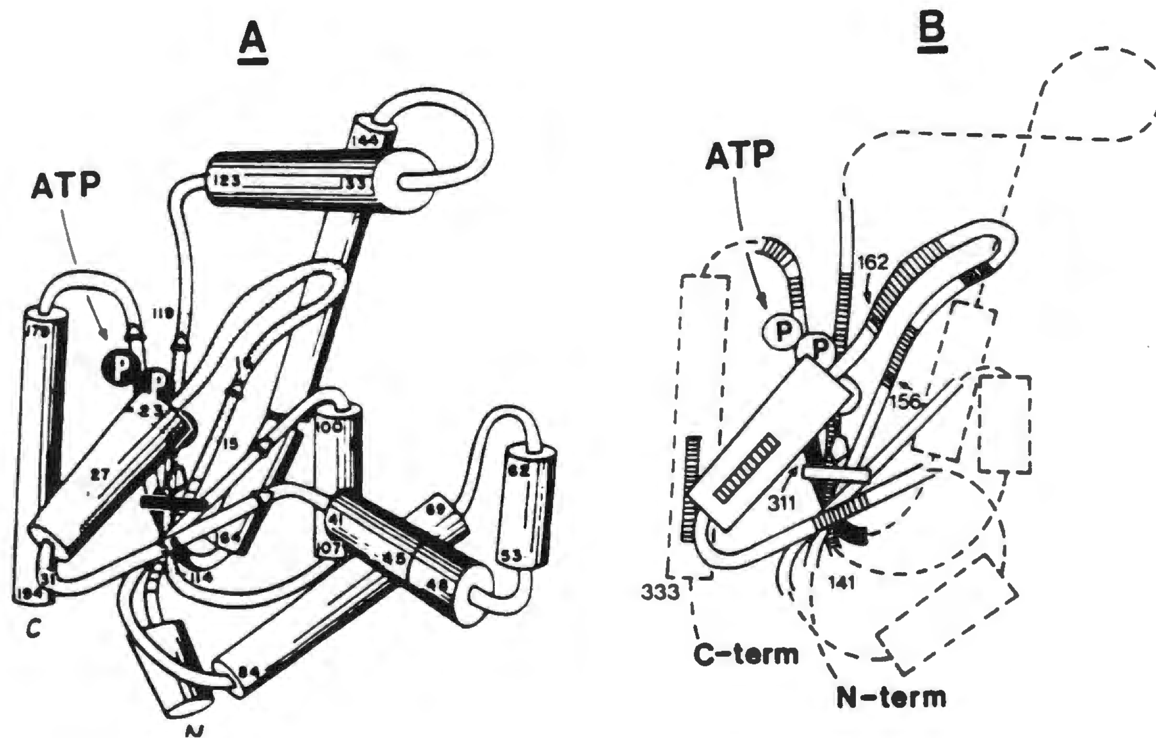


Figure 1.9 Postulated tertiary structure of the F_1-ATPase ß-subunit

The postulated tertiary structure of rat liver F_1 ß-subunit (B) (Gorboczi et al., 1988a) based on the structure of adenylate kinase (A) as determined by X-ray crystallography and NMR spectroscopy (Fry et al., 1985). Shaded areas in (B) represent homologous amino acids.

sites at which mutation results in inhibition of catalytic activity (Gorboczi et al., 1987b).

A fifty amino acid peptide has been synthesized which corresponds to amino acids 141-190, and in the postulated structure corresponds to a strand of the ß-sheet (sequence B) followed by the Gly-rich loop (Sequence A) (Walker et al., 1982) and then an α-helix (Gorboczi et al., 1987b). The peptide complexes with ATP and precipitates. It also induces a seven-fold increase in fluorescence of TNP-ATP similar to the intact enzyme (Grubmeyer and Penefsky, 1981a).

Another well characterized enzyme with a nucleotide site similar to that in adenylate kinase is the elongation factor (EF-Tu) from E.Coli (Jurnak, 1985; la Cour et al., 1985). The guanine nucleotide binds to a region composed of a ß-sheet with flanking α-helices. The phosphates are located at the end of an α-helix joined by a Gly-rich flexible loop which is highly conserved between species and which have the consensus sequences A of Walker et al. (1982) (la Cour et al., 1985).

The A consensus sequence (Walker et al., 1982) has also been identified in the ras oncogenes encoding the p21 transforming proteins of Harvey and Kirsten murine sarcoma virus (Dhar et al., 1982; Tsushida et al., 1982; Gay and Walker, 1983). This homology has been further extended to a number of other oncogene products (Kamps et al., 1984; Gibbs et al., 1984; Sternberg and Taylor, 1984), and a model has been postulated for the GDP binding domain of p21 based on that of EF-Tu (Fig.1.10) (McCormick et al., 1985).

A large number of other nucleotide binding proteins have been identified as containing the A consensus sequence (Moller and Amons, 1985; Argos and Leberman, 1985; Husain et al., 1986) Doolittle et al., 1986; Higgins et al., 1986; Starzyk et al., 1987). A note of caution has, however, been sounded as some proteins, which are obviously unrelated or do not bind nucleotides, have this sequence (Argos and Leberman, 1985; Doolittle et al., 1986; Serrano, 1988).

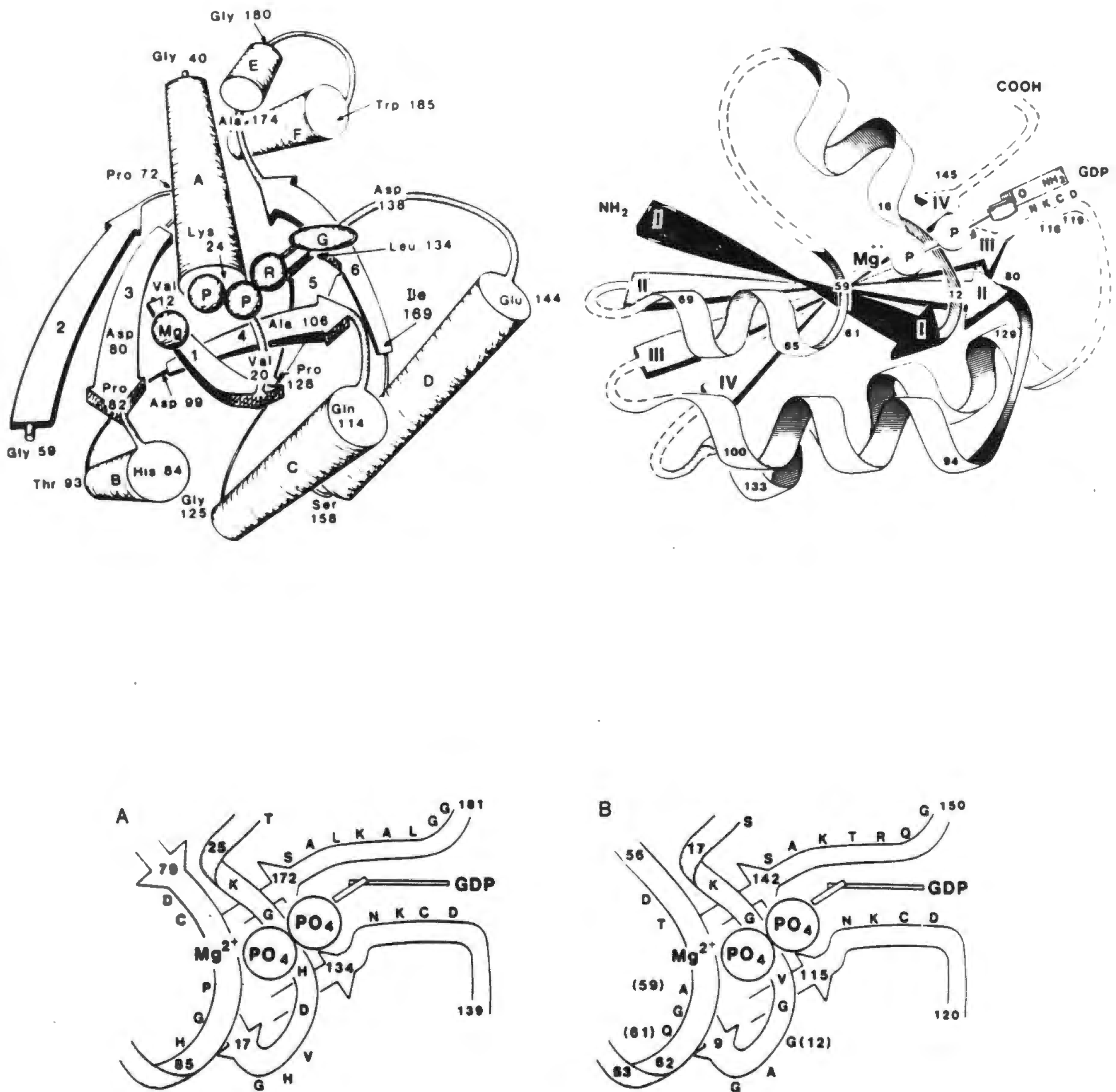


Figure 1.10 <u>Postulated tertiary structures of EF-Tu and p21</u>

A stylized schematic of the tertiary structure of the GDP-binding domain of EF-Tu-GDP determined by X-ray crystallography (top left) (Jurnak, 1985) is compared to a model for the tertiary structure of p21 (top right) (McCormick <u>et al</u>., 1985) in which the broken lines represent regions of sequence homology. Schematic representations of the GDP binding sites of EF-Tu (A) and p21 (B) are shown below (McCormick <u>et al</u>., 1985).

1.2 NUCLEOTIDE PROBES OF ACTIVE SITE STRUCTURE

In general, two different types of reagent have been used to obtain specific labeling of proteins, namely group-directed reagents and affinity labels. The group-directed reagents are directed at particular amino acid side chains although the situation is often complicated by cross-reactivity with other residues (Cohen, 1968). The majority of protein modifications depend either on the nucleophilicity of substituents or on their ability to undergo oxidation (Cohen, 1968). Affinity labeling is differentiated from group-directed labeling by the fact that the specificity of labeling is obtained by the properties of the reagent which cause it to bind preferentially to the active site. This results in an increase in local concentration thereby favoring covalent bond formation at the active site as opposed to the rest of the protein (Wofsy et al., 1962; Singer, 1967). Photoaffinity labeling is a class of affinity labeling where the active-site directed probe is inert until activated by light into a highly reactive species. Numerous reviews are available on the topic of affinity labeling (Wofsy et al., 1962; Singer, 1967; Jakoby and Wilchek, 1977; Benisek et al., 1982; Plapp, 1982; Colman, 1983; Vignais and Lunardi, 1985). In addition to the above, there are a number of reviews that deal specifically with photoaffinity labeling (Knowles, 1972; Bayley and Knowles, 1977; Chowdhry and Westheimer, 1979; Czarnecki et al., 1979; Guillory, 1979; Bayley, 1979; Potter and Hayley, 1983). What follows below is an outline of some of the more important affinity and photoaffinity analogs that have been used to specifically modify nucleotide binding sites.

1.2.1 AFFINITY LABELING

1.2.1.1. Fluorescein 5'-isothiocyanate

Fluorescein 5'-isothiocyanate (FITC) has been extensively used to covalently modify P-type ATPases. The reagent reacts with unprotonated primary amino groups and appears to behave as

an affinity analog of ATP by binding specifically to nucleotide sites (Pick, 1981b).

The interaction of FITC with SR vesicles has been well characterized (Pick and Karlish, 1980; Pick, 1981a, b; Pick and Bassilian, 1981). The probe binds specifically and with high affinity to the Ca^{2+}-ATPase where it undergoes covalent attachment to a lysine residue not essential for activity. Covalent incorporation of the probe, however, inhibits activity, with 100 % inactivation extrapolating to about 7 nmol/mg protein or 1 mol/mol ATPase (Andersen et al., 1982). The site of labeling has been localized to the 45 kDa B fragment postulated to contain the ATP binding site (Pick and Karlish, 1980). Lys-515 has been identified as the amino acid labeled in fast twitch fibers (Mitchinson et al., 1982; Brandl et al., 1986). The incorporation is specifically inhibited by ATP and ADP and results in the inhibition of ATP dependent activity of the pump (Pick and Bassilian, 1981). Phosphorylation of the enzyme by P_i, however, is unaffected, as is AcP induced Ca^{2+} uptake (Pick and Bassilian, 1981). Quenching of the fluorescein fluorescence observed upon binding of Ca^{2+} is thought to report a conformational change (Pick, 1981a).

Extensive sequence homology around the site of labeling of a number of P-type ATPases with FITC is observed (Fig.1.11) especially between different species with the same ion specificity.

1.2.1.2. 5'-p-fluorosulfonylbenzoyl adenosine

Another affinity label which has been extensively used in identifying nucleotide site residues is 5'-p-fluorosulfonylbenzoyl adenosine (5'-FSBA) (Pal et al., 1975). The molecule is arranged in an extended conformation with the reactive sulfonyl fluoride moeity postulated to be located in an analogous position to the γ-phosphate of ATP or to the 5'-position of the ribose moeity adjacent to the nicotinamide group of NAD (Wyatt and Colman, 1977). The sulfonyl fluoride group is a reactive electrophilic reagent that can react covalently with

*

Enzyme	Sequence	Ref.
Ca-ATPase (rabbit) (slow)	MFVKGAPEGVIDRCTHIRV	(a)
Ca-ATPase (rabbit) (fast)	MFVKGAPEGVIDRCNYVRV	(b)
Ca-ATPase (rabbit) (skeletal)	MFVKGAPEGVIDKKNYVR	(c)
Ca-ATPase (dog) (skeletal)	MFVKGAPEGVIDRKNYVR	(d)
Ca-ATPase (lobster) (skeletal)	MFVKGAPEGVLDR	(e)
Ca-ATPase (dog) (cardiac)	MFVKGAPEGVA?GDK	(f)
Ca-ATPase (red cell membr.)	MYSKGASEIILR	(g)
Na/K-ATPase (renal)	LLVMKGAPERILDRCSSILI	(h)
Na/K-ATPase (HeLa cell)	LLVMKGAPERILDRCSSILL	(i)
Na/K-ATPase (Torpedo)	LLVMKGAPERILDRCSTILL	(j)
Na/K-ATPase (ray and shrimp)	LLVMKGAPER	(k)
H/K-ATPase (rat stomach)	LLVMKGAPERVLERCSSILI	(l)
H/K-ATPase (hog gastric)	VLVMKGAPEQLSIR	(m)
H-ATPase (N. crassa)	ITCVKGAPLFVLKTVEEDHP	(n)
H-ATPase (yeast)	VCVKGAPLSALK	(o)
K-ATPase (E. coli)	MIRKGSVDAIRR	(p)

Figure 1.11 <u>Homology about the FITC binding domain of P-type ATPases</u>

The linear sequences around the site labeled with FITC of the known P-type ATPases are collected into 3 groups. The areas of homology are boxed. The references are as follows: a, (Mitchinson <u>et al</u>., 1982; MacLennan <u>et al</u>., 1985); b, (Brandl <u>et al</u>., 1986); c, (Kirley <u>et al</u>., 1985); d, (Kirley <u>et al</u>., 1985); e, (Mitchinson <u>et al</u>., 1982); f, (Kirley <u>etal</u>., 1985); g, (Filoteo <u>et al</u>., 1987); h, (Farley <u>et al</u>., 1984; Kirley <u>et al</u>., 1984; Shull <u>et al</u>., 1985; Ovchinnikov <u>et al</u>., 1986); i, (Kawakami <u>et al</u>., 1986); j, (Kawakami <u>et al</u>., 1985); k, (Ohta <u>et al</u>., 1985); l, (Shull and Lingrel, 1986); m, (Farley and Faller, 1985); n, (Addison, 1986; Hager <u>et al</u>., 1986); o, (Serrano <u>et al</u>., 1986); p, (Hesse <u>et al</u>., 1984); q, (Solioz <u>et al</u>., 1987); r, (Walderhaug <u>et al</u>., 1985).

several amino acids including Tyr, Lys, His, Ser and Cys. A number of nucleotide binding enzymes have been successfully modified with 5'-FSBA including dehydrogenases, kinases and ATPases (for review see Colman, 1983). Related compounds include 3'-FSBA and the fluorescent derivatives 5'-p-fluorosulfonylbenzoyl-1,N^6-ethanoadenosine (5'-FSBεA) and 5'-p-fluorosulfonylbenzoyl-2-aza-1,N^6-ethanoadenosine.

Early work on MF_1 demonstrated specific labeling by 5'-FSBA with most of the incorporation occurring in the α and ß subunits (Esch and Allison, 1978). The site of labeling was identified as a Tyr residue (ß-Tyr-368). Labeling of the α-subunit was found to be nonspecific (Esch and Allison, 1979). In a later study it was found that while modification of Tyr-368 occurs rapidly at alkaline pH, at neutral pH modification of another residue, ß-His-427, occurs at a slower rate (Bullough and Allison, 1986). It was concluded that both these residues are located in a single site to which 5'-FSBA binds.

An enzyme which shows structural homology with F_1 and has been specifically labeled with 5'-FSBA is the <u>rec</u> A protein from <u>E.Coli</u>. The attachment of 5'-FSBA was found to occur at a single site, Tyr-364 (Knight and McEntee, 1985b). This site was identical to that labeled by $8N_3ATP$, a surprising result considering the relative positions of the reactive groups (Knight and McEntee, 1985b). The authors postulated that the enzyme must bind $8N_3ATP$ in the strictly unfavorable <u>anti</u> conformation in order to position the azido group in a similar place to the sulfonyl fluoride group (Knight and McEntee, 1985c).

5'-FSBA has also been successfully used to label the (Na^+ + K^+)-ATPase following an initial report that this enzyme is slowly inactivated by incubation with millimolar concentrations of 5'-FSBA over an extended period in an ATP protectable way (Cooper and Winter, 1980). More recently, two specifically labeled peptides have been purified from the 5'-FSBA modified dog kidney enzyme (Ohta <u>et al</u>., 1986). Both peptides are strongly conserved across species while the homology of one of the peptides (Peptide II) extends to other P-type ATPases. The

homology of a number of ATPases in the region of this latter site of labeling is shown in Fig.1.12.

1.2.1.3. Pyridoxal-phosphate and adenosine polyphosphopyridoxal

Since the discovery that pyridoxal 5'-phosphate (PLP) is an inhibitor of several enzymes, besides acting as a cofactor for others, it has been widely used as a group-directed reagent with particular affinity for sugar-phosphate binding sites (McKinley-McKee and Morris, 1972; Murphy, 1977; Minchiotti et al., 1981; Koga and Cross, 1982; Tagaya and Fukui, 1986). The pyridoxal group is specific for the ε-amino group of lysine with which it forms a reversible Schiff base, and is conveniently monitored by it's absorbance peak at 440 nm. Reduction of the Schiff base by borohydride results in the formation of a stable covalent bond. Extension of the usefulness of the pyridoxal moeity resulted from the synthesis of the adenosine polyphosphopyridoxals in which the pyridoxal group is attached to the terminal phosphate of AMP, ADP, ATP or adenosine 5'-tetraphosphate (AP-PL, AP_2-PL, AP_3-PL, and AP_4-PL, respectively) (Tamura et al., 1986; Tagaya and Fukui, 1986).

PLP has been shown to inactivate a number of enzymes by reaction at an active site (substrate protectable) Lys residue. Some specificity is probably conferred on the probe by the phosphate group (McKinley-McKee and Morris, 1972) and, in addition, active site residues are often either more reactive or occur in an environment more favorable for reaction to occur. A number of residues in addition to the substrate protectable Lys are usually modified under conditions leading to extensive inactivation of the enzyme (McKinley-McKee and Morris, 1972; Koga and Cross, 1982; Tagaya and Fukui, 1986). The SR Ca^{2+}-ATPase, however, is specifically labeled on the A1 tryptic fragment with PLP, at low concentrations of PLP, resulting in up to 40% inactivation of the enzyme (Murphy, 1977). Further modification was found to result in deviation from the line extrapolating to modification of 1 residue. The site of labeling, however, remains to be identified. A similar sort of

Ca-ATPase (rabbit) (slow)	FDEITAMTGDGVDDAPALKKAEIGIAMGSG	(1)
Ca-ATPase (rabbit) (fast)	YDEITAMTGDGVNDAPALKKAEIGIAMGSG	(2)
Na/K-ATPase (sheep kidney)	QGAIVAVTGDGVNDSPALKKADIGVAMGIA	(3)
Na/K-ATPase (yeast)	RGYLVAMTGDGVNDAPSLKKADTGIAVEG	(4)
H/K-ATPase (rat stomach)	QGAIVAVTGDGVNDSPALKKADTGIAVEGA	(5)
K-ATPase (E. coli)	EGRLVAMTGDGTNDAPALA ADVAVAMNSG	(6)
K-ATPase (S. faecalis)	QGKKVIMVGDGINDAPSLARATIGMAIGAG	(7)

Figure 1.12 Homology amongst P-type ATPases around the site of labeling of the ($Na^{\pm}$ + $K^{\pm}$)-ATPase with 5'-FSBA

The linear sequences of a number of ATPases are aligned against the region of the (Na^+ + K^+)-ATPase labeled with 5'-FSBA (Site II) (Ohta et al., 1986). The postulated site of labeling is marked (*). The references are as follows: a, (MacLennan et al., 1985); b, (Brandl et al., 1986); c, (Shull et al., 1985); d, (Serrano et al., 1986); e, (Shull and Lingrel, 1986); f, (Hesse et al., 1984); g, (Solioz et al., 1987).

specificity was seen for rabbit muscle pyruvate kinase (Johnson and Deal, 1970). Specific modification also appears to have been obtained in 6-phosphogluconate dehydrogenase where 2 residues were modified and sequenced out of a number of possible sites (Minchiotti et al., 1981). In view of it's low specificity, however, it does appear that PLP will be of limited use in the identification of amino acids making up the nucleotide binding site (Koga and Cross, 1982).

Possible improvement of the nucleotide site labeling properties of PLP may be obtained by coupling the probe to adenosine, AMP, ADP or ATP (Tamura et al., 1986; Tagaya and Fukui, 1986). Specific nucleotide site labeling was obtained for a number of dehydrogenases and kinases. It was, however, found that modification of MF_1 with AP_2-PL gave a similar result to PLP i.e. Full inactivation extrapolated to a value of 10 mol/mol enzyme (Tamura et al., 1986). A similar result was obtained for EF_1 labeling with AP_2-PL (Noumi et al., 1987). AP_3-PL and AP_4-PL, however, were found to specifically modify EF_1 to the extent of about 1 mol/mol enzyme (Noumi et al., 1987), resulting in complete inhibition of ATP hydrolysis. The modification obtained was completely reversed by ATP and resulted in a two thirds modification of the α subunit and the rest on the ß subunit (Noumi et al., 1987). Contrary to the finding above, Rao et al. (1988) obtained specific labeling of isolated EF_1 α-subunit with AP_2-PL and identified the site of labeling as Lys-201. This residue is located in a region predicted to form part of a nucleotide binding fold based on secondary structure predictions and sequence homology with nucleotide binding domains in proteins of known structure (Maggio et al., 1987), and is strongly conserved in twelve species for which the α-subunit structure is known (Rao et al., 1988). Lys-201 occurs at the COOH terminal end of ß-strand number 2 of the nucleotide binding domain, which is postulated to be the approximate position of the γ-phosphate group of ATP.

Specific labeling of adenylate kinase was obtained with AP_2-PL, with incorporation of 1.0 mol reagent/mol enzyme extrapolating to 100% inactivation (Tagaya et al., 1987).

Protection from labeling was afforded by ADP, ATP and MgATP, but not AMP, identifying the metal-ATP site as the site of modification. Lys-21 was identified as the exclusive site of labeling (Tagaya et al., 1987). The site of labeling here is more consistent with the positioning of the nucleotide site postulated by Pai et al. (1977) based on crystallographic measurements of the substrate soaked enzyme. A more recent postulate based on NMR data of the interaction between ß, γ-bidentate CrATP and the enzyme (Fry et al., 1985) position the nucleotide away from the side chain of Lys-21 (Tagaya et al., 1987). As pointed out by Tagaya et al. (1987), however, the position of Lys-21 at the end of a flexible glycine rich region, may bring it into contact with substrate following a conformational change on ATP binding. In addition, it was noted that there is, as yet, no direct evidence supporting the positioning of the 4-formyl group of adenosine polyphosphopyridoxal exactly as for the γ-phosphate group of ATP, although a number of observations support this conclusion (Tagaya et al., 1987). The Lys residue in the ras oncogene product, P21, analogous to Lys-21 in adenylate kinase has also been labeled with GP_3-PL (guanosine triphosphopyridoxal) (Ohmi et al., unpublished observations).

The glycine rich region containing Lys-21 at the end is a highly conserved region found in a number of ATP binding proteins and is referred to as sequence A (Walker et al., 1982). The homologous region in F_1 α-subunit is Lys-175, although Lys-201 is the residue labeled with AP_2-PL (see above) (Rao et al., 1988). So this probe is positioned in a way that makes the labeling of Lys-201 more likely than Lys-175. The residue in the ß subunit equivalent to Lys-21 is Arg-182. This probably explains the lack of labeling of the F_1 ß-subunit with AP_2-PL (Tamura et al., 1986; Noumi et al., 1987).

Specific modification of the SR Ca^{2+}-ATPase was obtained with AP_3-PL; with 100% inactivation extrapolating to 1 mol/mol of incorporated AP_3-PL (6.7 nmol/mg of SR protein), and protected by ATP and ADP (Yamamoto et al., 1988). A single labeled peptide was purified and the site of labeling identified as Lys-

684. This residue is located on the B tryptic fragment and is clearly different from the Lys residue labeled by Murphy (1977) with PLP which most probably represents another Lys in the active site or a uniquely reactive residue. There is no homologue of the conserved sequence A described above in the Ca^{2+}-ATPase and the amino acid sequence around the AP_3-PL binding site is quite different from that described for adenylate kinase (Tagaya et al., 1987). Similar regions were found in other P-type ATPases, however, as well as in myosin heavy chain (Fig. 1.13). In the $(Na^+ + K^+)$-ATPase the FSBA binding region abutts directly on the region homologous to the AP_3-PL binding region in the Ca^{2+}-ATPase. This region has it's homologue in the Ca^{2+}-ATPase as well.

In summary, PLP shows the characteristics of a classical group-directed reagent although some specificity is conferred on the molecule by the phosphate group. Conversion of the compound into an affinity label by attachment of adenosine makes the compound potentially more specific and hence more useful. The use of the affinity label in mapping studies is also evident, and the usefulness of this approach even as an adjunct to crystallographic information is shown in the work on adenylate kinase. Similarly, although crystallographic data indicates that no Lys residue is present in the active site of hexokinase (Shoham and Steitz, 1980), specific labeling was obtained with AP_2-PL (Tamura et al., 1986) indicating that a conformational change must occur on nucleotide binding bringing a Lys residue into the active site.

1.2.2. Photoaffinity Labeling

Photoaffinity labeling as a subset of the general affinity labeling technique is differentiated by the characteristic that the label is inert until activated by light at which stage a reactive radical is generated which can undergo covalent incorporation. In general, photoactivated radicals are highly reactive, the advantage being that the more reactive is the reagent, generally the less selective it is and the wider the

Ca-ATPase (rabbit)	674 -	CCFARVEPSHKSKIVE	- 689
Na/K-ATPase (sheep)	681 -	IVFARTSPQQKLIIVE	- 696
H/K-ATPase (rat)	697 -	MVFARTSPQQKLVIVE	- 712
H-ATPase (N. crassa)	605 -	DGFAEVFPQHKYRVVE	- 620
K-ATPase (E. coli)	489 -	DFLAEATPEAKLALIR	- 504
myosin HC (rat)	395 -	LLKALKYPRVKVGNEY	- 410

(× marks the K of CCFARVEPSHKSKIVE in the Ca-ATPase sequence)

Figure 1.13 Homology of a number of P-type ATPases about the site of labeling of the Ca^{2+}-ATPase with AP_3-PL

The amino acid sequences of a number of P-type ATPases homologous to the region in the Ca^{2+}-ATPase labeled with AP_3-PL are compared as demonstrated by Yamamoto et al. (1988)

spectrum of amino acid residues with which it can react (Singer, 1967).

The photoaffinity labeling technique has some particular advantages over classical affinity labeling (Benisek et al., 1982). Thus temporal decoupling of the covalent reaction step from the noncovalent binding step allows the experimenter to wait until the system has attained a particular state before allowing labeling to occur (Benisek et al., 1982). Another advantage over the k_{cat} and classical affinity probes is the relatively broad reactivity spectrum of the photogenerated carbenes, nitrenes and ketone excited states towards hydrocarbon and nucleophilic groups. Nitrenes and ketones are generally reactive towards nucleophilic groups (Benisek et al., 1982). The other advantage is the relatively short lifetimes in solution of some photogenerated reactive species. This helps to minimize nonspecific labeling as reagents in solution are usually not in close contact with the protein, making covalent bond formation less likely.

The only chemical species potentially capable of insertion into C-H bonds are carbenes and nitrenes (Fleet et al., 1969), both of which were the active components of the two earliest reports on photoaffinity labeling (Singh et al., 1962; Fleet et al., 1969). While aliphatic diazo or azide compounds are highly reactive chemically and also tend to undergo rearrangement to ketenes on photolysis, arylazides are optimally suited to photoaffinity labeling (Fleet et al, 1969).

The first photoaffinity analog of ATP to be synthesized was $8N_3ATP$ (Haley and Hoffman, 1974) and it was used to label red cell membranes, while $8N_3ADP$ was used to label the adenine nucleotide translocator (Schafer et al., 1976).

1.2.2.1. 8-AZIDO-NUCLEOTIDES

The first enzyme for which extensive use was made of $8N_3ATP$ was the F_1-ATPase. $8N_3ATP$ was found to be a substrate for MF_1 and, following illumination, was specifically incorporated into the ß subunit to the extent of 2 mol/mol ATPase, completely

inhibiting activity (Wagenvoord et al., 1977). A similar result was reported for EF_1 with $8N_3ATP$ except that > 90% inactivation, in this case, extrapolated to 1 mol/mol ATPase and the labeling was located mainly on the α subunit (Verheijen et al., 1978). $8N_3ADP$ was found to label about equally the α and ß subunits of MF_1 in the absence of Mg^{2+}, and mainly the α subunit in the presence of Mg^{2+} (Wagenvoord et al., 1979). Again, extrapolation to 100% inactivation corresponded to 2 mol/mol ATPase, and incorporation was found to have no effect on the levels of tightly bound nucleotides (Wagenvoord et al., 1979). $8N_3ADP$ was found to behave in a similar way with CF_1 (Wagenvoord et al., 1981). Finally, it was found that if MF_1 was irradiated with $8N_3ATP$ in the absence of Mg^{2+}, followed by $8N_3ADP$ in the presence of Mg^{2+}, 4 mol/mol ATPase were labeled and 2 mol/mol tightly bound nucleotides remained (Wagenvoord et al., 1980). This was one of the earliest demonstrations of 6 nucleotide sites on intact F_1. The site of labeling of $8N_3ATP$ on the ß subunit has been localized to two regions, residues 1-12, a site which is not essential for catalysis, and the residues Lys-301, Ile-304, and Tyr-311 (Runswick and Walker, 1983; Hollemans et al., 1983).

$8N_3ATP$ has also been used to label the rec A protein from E.coli (Knight and McEntee, 1985a, c), with a reported efficiency of between 10 and 70% (Kowalczykowski, 1986). In this case, the enzyme was specifically labeled with $8N_3ATP$ and the site of labeling localized to the peptide spanning residues 257-280, but sequencing was prevented at this stage by the presence of a cyclized N-terminal Gln residue (Knight and McEntee, 1985a). After further digestion, the site was identified as Tyr-264 (Knight and McEntee, 1985c), the same residue that is labeled with 5'-FSBA (Knight and McEntee, 1985b). This resulted in the conclusion that $8N_3ATP$ must be bound in the sterically unfavorable anti conformation in order that the azido group is located in a similar position to that expected for the reactive end of 5'-FSBA (Knight and McEntee, 1985c). Attempts to label the Ca^{2+}-ATPase specifically at the active site have been largely unsuccessful and are discussed below.

1.2.2.2. Arylazido modified nucleotides

A family of photoaffinity analogs in which an N-4-azido-2-nitrophenyl group is attached to the 3'-OH group of the nucleotide ribose moeity by aliphatic chains of varying lengths have been described (Jeng and Guillory, 1975; Guillory and Jeng, 1977). Arylazido-ß-alanine ATP (NAP_3-ATP) was found to be hydrolyzed by myosin subfragment 1 (SF_1) analogously to ATP and along with arylazido-4-aminobutyric ATP (NAP_4-ATP) and arylazido-6-aminocaproic ATP (NAP_6-ATP) was specifically photoincorporated into SF_1 (Guillory and Jeng, 1977).

NAP_3-ATP and NAP_4-ATP were also found to be substrates for MF_1-ATPase and to be specifically incorporated into the α and ß subunits (Guillory and Jeng, 1977; Lunardi et al., 1977; Cosson and Guillory, 1979). In addition, both NAP_3-ADP and NAP_4-ADP were found to be competitive inhibitors of MF_1 and to be specifically incorporated into both α and ß subunits (Russell et al., 1976; Lunardi et al., 1977; Weber et al., 1985). A non hydrolysable analog, NAP_4-AMP-PNP has also been specifically incorporated into both α and ß subunits at high concentrations and primarily into the α subunit at low concentrations (Lunardi and Vignais, 1982). Schafer et al. (1983) have synthesized a cross-linking reagent, NAP_3-$8N_3$ADP, and obtained specific cross-linking of both α and ß subunits. An analogous compound, 3'-o-[5-azidonaphthoyl]-ADP has been synthesized and covalently incorporated into the α and ß subunits of MF_1 (Lubben et al., 1984). NAP_4-ADP has been found to be a competitive inhibitor of ADP phosphorylation by CF_1-ATPase although it does not act itself as a substrate (Schafer et al., 1978a). NAP_4-ATP acts as a substrate and NAP_4-ADP as an inhibitor of bacterial F_1 and both are incorporated into α and ß subunits at high concentrations of probe and into the α mainly, at lower concentrations (Lunardi et al., 1981). A similar result was obtained with two analogs with shorter linking chains Lunardi et al., 1981)

NAP_3-ATP has also been found to be a substrate for the (Na^+ + K^+)-ATPase and a competitive inhibitor of ATP hydrolysis (Rempeters and Schoner, 1981; Munson, 1981). In addition the

chromium (III) coordination complex of NAP_3-ATP has been synthesized and found to be a nonhydrolysable competitive inhibitor of the (Na^+ + K^+)-ATPase (Munson, 1981). Following incorporation of the tritiated isotope of $CrNAP_3$-ATP, three labeled peptides were identified (Munson, 1983). NAP_3-ATP has also been used in the Ca^{2+}-ATPase and is dealt with below (Carvalho-Alves et al., 1985; Oliveira et al., 1988).

1.2.2.3. 3'(2')-o-(4-Benzoyl)benzoyl ATP

3'(2')-o-(4-Benzoyl)benzoyl ATP (Bz_2ATP) is a photoaffiniy nucleotide analog that has been used in a number of ATP binding enzymes (Williams and Coleman, 1982; Bar-Zvi et al., 1983; Mahmood and Yount, 1984; Tran and Farley, 1986). The probe contains a photoreactive benzophenone group attached either to the 3' or 2'-OH groups of the nucleotide ribose moeity in an approximate ratio of 60:40, respectively (Mahmood et al, 1987). This group is an α,β unsaturated ketone capable of being excited to a highly reactive di-radical triplet state intermediate by low intensity, long wavelength UV irradiation (Williams and Coleman, 1982). Radical oxygen then abstracts a hydrogen atom from nearby methylene residues present in the protein resulting in a transient caged radical pair which quickly degenerates to form a C-C covalent bond (Williams and Coleman, 1982; Kambouris and Hammes, 1985). The advantages of this probe compared to nitrene evolving species are that it is unreactive towards H_2O and that the carbon-carbon bond formed is more stable than covalent bonds involving nitrogen (Bayley and Knowles, 1977). Another advantage is that the covalent bond formation is a fairly nonspecific process allowing labeling of a wide range of sites (Williams and Coleman, 1982). The disadvantages with this probe are the instability of the benzophenone nucleotide ester bond (Admon and Hammes, 1987) and the location of the reactive part of the molecule may not be conducive for mapping studies.

Bz_2-ATP was found to be a good substrate for the MF_1-ATPase and was covalently incorporated into both α and β subunits (Williams and Coleman, 1982). Bz_2-ADP was found to be a strong

competitor of ADP and ATP in CF_1 although it did not act itself as a substrate for photophosphorylation (Bar-Zvi et al, 1983; Kambouris and Hammes, 1985). Photoincorporation of Bz_2-ATP into the tight ADP site resulted in mainly β subunit labeling while labeling the tight ADP site resulted in only β subunit labeling, and the labeling of a third site resulted in approximately equal labeling of α and β (Bar-Zvi et al., 1983; Kambouris and Hammes, 1985; Admon and Hammes, 1987). The efficiency of incorporation into the tight site following removal of free nucleotide was found to be 70% and there was an indication that α subunit labeling may be nonspecific (Bar-Zvi and Shavit, 1984; Admon and Hammes, 1987). Two labeled peptides were isolated following photoincorporation of Bz_2-ATP into CF_1 corresponding to residues 360-378 and 393-397 (Admon and Hammes, 1987). In the former, specific labeling of two residues, Tyr-362 and Asp-369 was obtained; this peptide is highly conserved in a number of F_1-ATPases and probably forms part of the nucleotide binding site. The latter peptide contained low levels of radioactivity in all residues and may be artifactual. Tyr-362 in beef heart MF_1-ATPase is the residue labeled with 5'-FSBA (Bullough and Allison, 1986).

Bz_2ATP is also hydrolyzed analogously to ATP by myosin subfragment 1 (SF1) and the product, Bz_2ADP, has a single site on SF1 (Mahmood and Yount, 1984). Incubation of SF1 with Bz_2ATP results in photoincorporation into the 20, 23, and 50 kDa peptides but only that in the 50 kDa is specific (Mahmood and Yount, 1984; Mahmood et al., 1987). In order to get specific labeling only it is necessary to trap the Bz_2ATP at the active site with a thiol cross-linker and remove all the free probe before irradiation (Mahmood and Yount, 1984). Two fluorescent derivatives, 2'-deoxy-3'-o-(4-benzoylbenzoyl)- and 3'(2')-o-(4-Benzoylbenzoyl)-1,N^6-ethanoadenosine 5'-diphosphate have also been synthesized and behave in a similar way to Bz_2ADP in SF1 (Cremo and Yount, 1987).

The interaction of Bz_2ATP with a number of P-type ATPases has been reported (Tran and Farley, 1986). It was found that incorporation was not prevented by ATP, but was substantially

reduced by prior labeling with FITC. Bz_2ATP did, however, inhibit high affinity ATP binding. A number of labeled peptides were obtained, none of which was protected either by ATP or FITC. The authors concluded that the labeling is nonspecific. It has also been reported that specific incorporation of Bz_2ATP occurs into the Ca^{2+}-ATPase to the extent of 1.3 nmol/mg of SR protein and that most of the labeling occurs on the A tryptic fragment with some labeling of the B fragment and proteolipid (Cable and Briggs, 1984). This labeling, however, was not completely inhibited by ATP and may be explained by labeling outside the active site (Tran and Farley, 1986).

1.2.2.4. 2-azido-nucleotides

2-azido-adenosine (Schaeffer and Thomas, 1958), as well as it's mono-, di-, and triphosphate derivatives, has been used in a number of systems. They have been shown to act as ADP and AMP analogs in binding to the outer membrane of human platelets and inducing aggregation (Cusack and Born, 1976, 1977; MacFarlane et al, 1982), and were also shown to irreversibly photoinactivate adenosine deaminase (Cusack and Born, 1976). $2N_3AMP$ was also used to specifically label the AMP allosteric site on pig kidney fructose 1,6-bisphosphatase (Riquelme and Czarnecki, 1983). 2-azido-adenosine nucleotides have also been reported to be substrates for pyruvate kinase, phosphofructokinase, adenylate kinase and hexokinase (Czarnecki, 1984). In these cases the binding appeared to be related to whether the nucleotide at the active site is bound in an anti (Ohta et al, 1980) or syn (Danenberg and Cleland, 1975) conformation. $2N_3AMP$ was found to not act as a substrate for adenylate kinase (Czarnecki, 1984) probably as a result of a strict site specificity rather than a nucleotide conformation specificity as 8-Br-AMP is also not a substrate (Lascu et al, 1979). The main area of use, however, has been in the F_1 H^+-ATPases (Czarneki et al, 1982; Dalbon et al, 1985; Wise et al, 1987).

An advantage obtained with 2-azido-nucleotides stems from the fact that they are predicted to adopt an anti conformation

about the N- glycosidic linkage (Czarnecki et al, 1982; Czarnecki, 1984) similar to the natural adenine nucleotides in solution (Ikehara et al, 1972a,b; Davies and Danyluk, 1974). Nucleotides with a bulky substituent in the 8 position tend to adopt a syn conformation as a result of unfavorable steric and electrostatic interactions between the bulky group and the ribofuranose - phosphate system (Ikehara, et al 1972a, b) which, in turn, affects the sugar - phosphate conformation (Fig. 1.14) (Sarma et al, 1974). Both the unsubstituted and 8 - substituted nucleotides adopt the g'g' (gauche' - gauche') conformation (O) about the C(5') - O(5') bond. The conformation about the C(4') - C(5') bond, however, is affected by glycosyl torsion. Thus the preferred conformation in unsubstituted purine nucleotides is anti - g/g (Davies and Danyluk, 1974), while in 8 - substituted derivatives the onset of syn orientation is followed by rotation of C(4') - C(5') into the g/t conformations (Sarma et al, 1974). The ribose moeity of 5'-B-purine nucleotides (whether anti g/g or syn g/t) exists as an equilibrium of S and N conformations with the anti g/g showing a slight bias towards the S conformer (Sarma et al, 1974). The conformations of the 8 - substituted nucleotides, moreover, are sensitive to the degree of protonation of the phosphate group and the base N(1). Thus as the pH of the solution is decreased and the monophosphate dianion is converted into a monoanion bearing a protonated N(1) there is an increase of about 15 - 20% in the gg population possibly as a result of a reduction in repulsive electrostatic interactions (Sarma et al, 1972). It does appear, however, that 8 - substituted nucleotides can be constrained into an anti conformation upon binding to enzymes (Stolarski et al, 1984).

One potential disadvantage with the 2-azido derivatives, however, is their tendency to isomerize in solution to the light insensitive tetrazole forms (MacFarlane et al, 1982). Purines substituted with azides in the 2 and 6 positions isomerize to form tetrazole tautomers (Temple et al, 1966a, b) and their potential as phototaffinity analogs appears to be limited by the predominance of the tetrazole tautomer (Wiegand and Kaleja, 1976). Conditions have, however, been described in which the

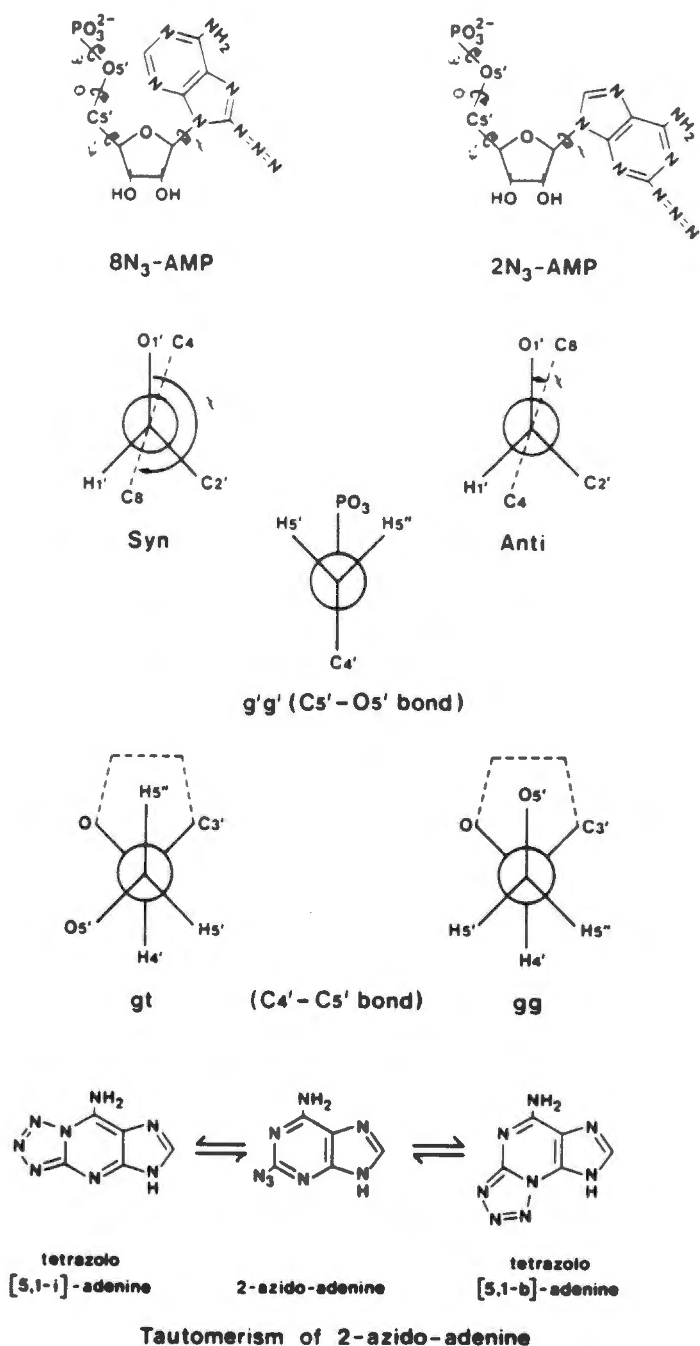


Figure 1.14 Conformations of 2- and 8-substituted nucleotides

Schematic representations of the conformations of nucleotides and their 2- and 8-substituted derivitives (Ikehara et al., 1972a,b; Sarma et al., 1974; Davies and Danyluk, 1974). The conformation about the N-glycosidic bond is shown illustrating the syn and anti conformations.

nucleotide is present predominantly in the azido form at least for the time required to carry out the photoaffinity labeling process (Czarnecki, 1984; Melese et al, 1988). Thus when a methanolic solution of the 2-azido-nucleotide is added to the reaction mixture the predominant species present is the azido form which then undergoes a pH and temperature dependent tautomerism (Czarnecki, 1984). At neutral pH solution approximately 55% of the analog exists in the tetrazole isomer while the azido form is favored at acid pH (Czarnecki, 1984). It has been found, however, that the tetrazole form also binds tightly to MF_1 and CF_1-ATPase and when bound equilibrates to the azido form with a $t_{0.5}$ at 25 ^{o}C of about 41 min (Boulay et al, 1985; Melese et al, 1988). Thus exposure for 50 min resulted in almost complete photoincorporation of the available probe (Melese et al, 1988).

Both $2N_3ATP$ and $2N_3ADP$ have been used extensively in studies into the nature of the nucleotide sites on the F_1-ATPases from mitochondria (MF_1) and chloroplasts (CF_1) and recently a report has appeared on E.Coli F_1 (EF_1). $2N_3ADP$ was initially used to study the tight binding of nucleotides to thylakoid membranes (Czarnecki et al, 1982; van Dongen et al, 1986) on account of the strict specificity these sites exhibit towards their nucleotide substrates evidenced by the failure of 8-azido-nucleotides to displace tightly bound nucleotides (Schafer et al, 1978a; Wagenvoord et al, 1980, 1981). It was found, however, that $2N_3ADP$ inhibits tight binding of ADP and, in addition, binds itself to membrane bound CF_1 with half maximal binding occurring at about 1 µM $2N_3ADP$ which is inhibited by the presence of ADP (Czarnecki et al, 1982, 1983). In addition, $2N_3ADP$ is phosphorylated to $2N_3ATP$ by membrane bound CF_1 and this $2N_3ATP$ binds tightly to the ß subunit (Abbott et al, 1984). $2N_3ADP$ could be successfully photoincorporated into CF_1 and, under all conditions, the site of labeling was identified as the ß subunit (Czarnecki et al, 1982,1983; Abbott et al, 1984) and the tight binding sites for ADP and ATP were located on identical portions of the ß subunit polypeptide (Abbott et al, 1984). $2N_3ADP$ has also been used to study nucleotide binding sites in intact and

inside out mitochondria (Dalbon et al, 1985). It was found to bind with high affinity to the ADP/ATP carrier but not to be transported across the membrane (Dalbon et al, 1985). It has previously been shown that the ADP/ATP carrier has a stricter specificity towards the adenine ring and ribose moeity of ATP and less so for ATPases (Vignais and Lunardi, 1985). Photoaffinity labeling of inside out submitochondrial particles with $2N_3ADP$ resulted in specific photoincorporation into the ATP/ADP carrier as well as the ß subunit of the F_1-ATPase (Dalbon et al, 1985). Further evidence was obtained from the finding that $2N_3ADP$ could be phosphorylated by membrane bound CF_1-ATPase (Czarnecki et al, 1983; Abbott et al, 1984).

$2N_3ATP$ is readily hydrolyzed by both CF_1 (Melese and Boyer, 1985) and MF_1 (van Dongen et al, 1986) and from the kinetics with respect to MF_1, it was concluded that $2N_3$-nucleotides are better substrates than their 8-azido counterparts and are similar to the native nucleotides (van Dongen et al, 1986). The higher K_m and V_{max} values measured for $2N_3ATP$ compared with ATP may be due to the proportion of nucleotide present as the terazole form, as the azido isomer has been found to be a better substrate than the tetrazole (Czarnecki, 1984; van Dongen et al., 1986). Initial reports on photoincorporation of $2N_3ADP/ATP$ into CF_1 showed that $2N_3ATP$ could be photoincorporated into 30% of ß subunits probably representing 1 labeled subunit/F_1 and leave the enzyme quite active (Melese and Boyer, 1985). The enzyme, furthermore, was able to bind ATP tightly and then release it indicating the retention of catalytic cooperativity between at least two sites (Melese and Boyer, 1985; Czarnecki et al, 1985).

In the mitochondrial system it was found that 3 mol/mol of $2N_3ADP$ could be bound to intact F_1, in addition to the 3 mol/mol of endogenous nucleotide, confirming the number of binding site on MF_1 (Boulay et al, 1985). Following photoincorporation of the bound nucleotide, a linear relationship was obtained between % inactivation and photoincorporation extrapolating to a value of 2 mol/mol F_1 for > 90% inactivation (Boulay et al, 1985). It has been suggested that this value may only be 1 if only catalytic sites are labeled (Kironde and Cross, 1986) and

extrapolation to a value of 0.92 mol label/ mol F_1 at full inactivation has been reported (Czarnecki et al, 1985; van Dongen et al, 1986; Lunardi et al, 1987). In CF_1, it has been found that with either catalytic or predominantly noncatalytic site modification, activity loss approximately parallels the extent of modification of a single ß subunit (Melese et al, 1988), although some 5 - 20% of activity may remain after labeling under these conditions. When both catalytic and noncatalytic sites are labeled about equally, however, to a total of 1 mol/mol F_1 some 40 - 50% of activity is retained (Melese et al, 1988). It has, however, also been reported that following extensive incubation, the $2N_3ADP$ could be exchanged into the tight nucleotide sites and that the patterns of photolabeling obtained after enriching either the 'loose' or the 'tight' sites were different (Boulay et al, 1985). Under the former condition only the ß subunit was labeled whilst under the latter both ß (90%) and α (10%) were labeled concluding that MF_1 has three 'loose' sites located exclusively on the ß subunit and three 'tight' sites located at the junction between the α and ß subunits (Boulay et al, 1985; van Dongen and Berden, 1987). A model summarizing the photoaffinity labeling data with both $8N_3AXP$ and $2N_3AXP$ and MF_1 and the postulated interrelationships between the nucleotide binding sites has been reported by van Dongen and Berden (1987) (Fig. 1.15).

$2N_3ADP$ was also used to provide the first demonstration that CF_1 is, in fact, similar to MF_1 and EF_1 in having six nucleotide sites per functional unit (Xue et al, 1987a). It was found that CF_1 can bind more than 3 mole nucleotide per enzyme unit, and specific double incorporation of ß subunits was demonstrated (Xue et al, 1987a). $2N_3ADP$ only is bound at catalytic sites while both $2N_3ADP$ and $2N_3ATP$ are bound at noncatalytic sites (Xue et al, 1987a). Similar to CF_1 (Boulay et al, 1985), it was found that under conditions of catalytic and noncatalytic site labeling in MF_1, different tryptic peptides are labeled (Xue et al, 1987a).

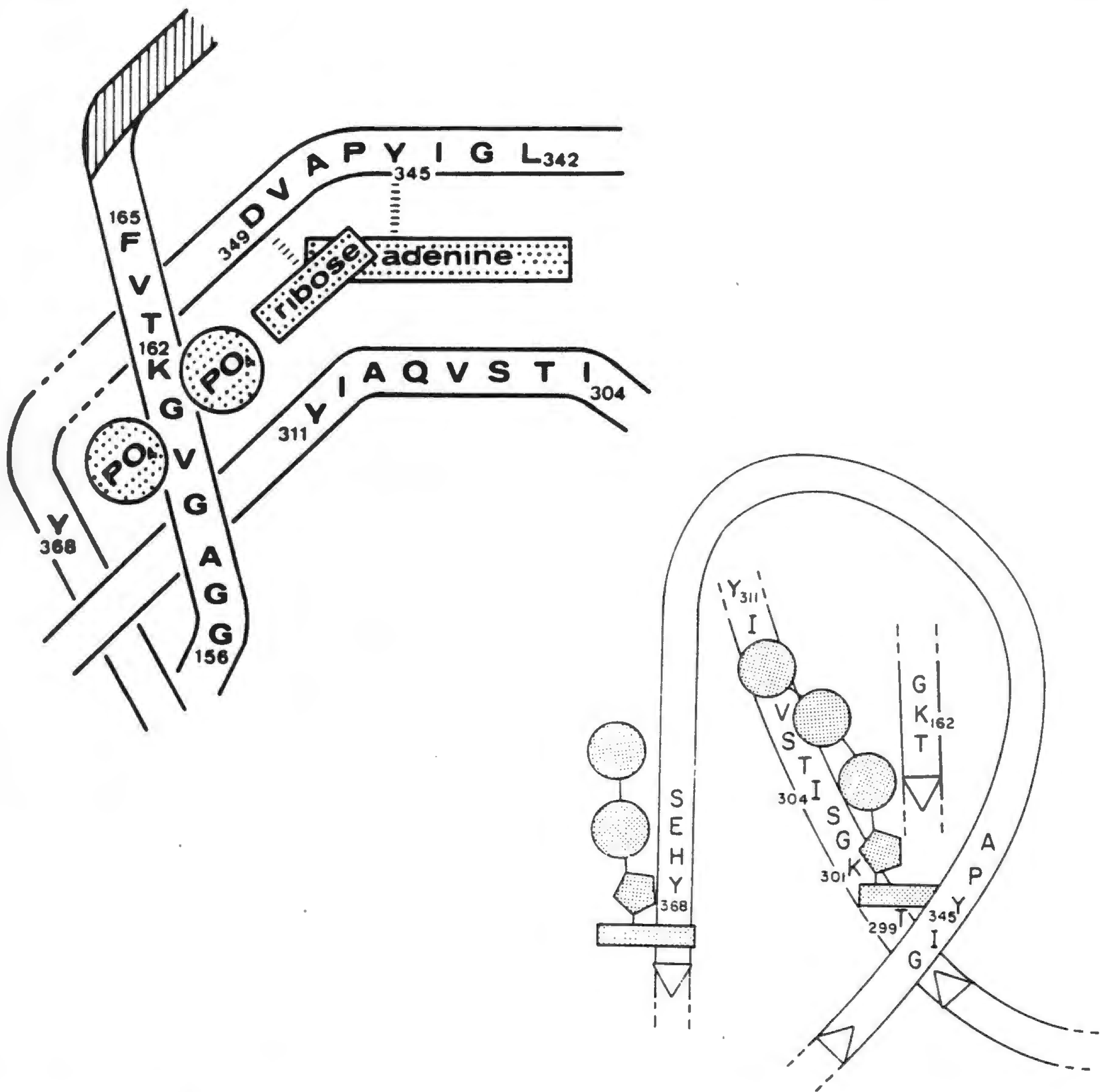


Figure 1.15 Models of the interaction of azido-nucleotides with the nucleotide site/s of MF_1

Two proposed models are shown for the interaction of nucleotides with the ß-subunit of beef heart MF_1. The diagram on the left shows the proposed folding pattern around the site of labeling with both $8N_3ATP$ and $2N_3ATP$ assuming that the 2 nucleotides bind in the same way and that the different regions labeled (304-311 and 342-349, respectively) arises on account of the different orientation of the azido group (Garin _et al_., 1986). The diagram on the right shows the proposed sites of binding of ATP and of ADP (Cross _et al_., 1987).

The site of labeling with $2N_3ADP$ has been determined for MF_1, CF_1 and EF_1. In MF_1 the site of labeling was identified as peptide CB9 and the following amino acids appeared to be modified Leu-342, Ile-344, Tyr-345 and Pro-346 (Garin et al, 1986). It was noted that Tyr-345 was more substantially labeled than the others and it was postulated that the Pro-346 labeling may be an artifact of carry-over from Tyr-345 labeling during the sequencing process (Garin et al, 1986). Cross et al (1987) have also identified the sites of photoincorporation both under conditions of catalytic and noncatalytic site labeling. In the case of noncatalytic site labeling Tyr-345 was also identified (Garin et al, 1986) while Tyr-368 was identified as the site of catalytic site labeling (Cross et al, 1987). They also concluded that either one third of the catalytic or two thirds of the noncatalytic sites had to be modified to obtain near complete inhibition of catalytic activity (Cross et al, 1987). This explains the results of Boulay et al (1985) where 100% inactivation was extrapolated to a value of 2 mole $2N_3$ ADP/mol F_1 while van Dongen et al (1986) and Lunardi et al (1987) obtained a value of 1 mol/mol. After labeling with both $8N_3ADP$ and $2N_3ADP$ it was furthermore shown that catalytic sites in isolated F_1 can be in a tight site (van Dongen and Berden, 1987). In addition to the sites identified above, another site of labeling (Gly-72 - Arg-83) has been identified following labeling under extensive conditions resulting in nearly 4 mol/mol F_1 (Lunardi et al, 1987). The labeling of α subunit previously identified by this laboratory (Boulay et al, 1985) was still seen (Lunardi et al, 1987) and has been confirmed in EF_1 (Wise et al, 1987). The site of noncatalytic site labeling (Leu-342 - Pro-346) (Garin et al,1986) is highly conserved in E.Coli F_1 and maize and spinach chloroplast F_1 (Walker et al, 1984) as well as in ß subunits of human F_1 (Ohta and Kagawa, 1986). $8N_3ATP$ labels the sequence Lys-301 - Tyr-311 (Hollemans et al, 1983) and it has been postulated that labeling by $8N_3ATP$ occurs only via the small proportion of molecules present in the anti conformation as part of the equilibrium about the N-glycosidic bond (Garin et al, 1986). Thus it is seen that $8N_3ATP$ (anti conformation) binds to

the same site as $2N_3ATP$ but labels a different section of the primary sequence (Garin _et al_, 1986). Tyr-368 is also labeled by FSBA (Esch and Allison, 1978).

Evidence was also presented for CF_1 that under conditions of catalytic and noncatalytic site labeling different tryptic peptides were labeled (Xue _et al_, 1987a). These studies were extended and the sites of catalytic and noncatalytic site labeling identified as Tyr-362 and Tyr-385 respectively (Xue _et al_, 1987b). Tyr-362 is also derivatized by 2',3'-o-(4-benzoyl)benzoyl-ADP (Admon and Hammes, 1987). Analogously to MF_1 (Lunardi _et al_, 1987) and EF_1 (Wise _et al_, 1987) the possibility was raised that some of the sites could be at α/β subunit interfaces (Xue _et al_, 1987b).

A similar situation was found for EF_1 and the sites of labeling with $2N_3ADP$ were identified as Tyr-354 (noncatalytic site) and Tyr-331 (catalytic site) (Wise _et al_, 1987), with between 5 and 10% of the labeling located on the α subunit (Wise _et al_, 1987). Two models of protein folding in the region of the nucleotide binding site based on results obtained with the azido nucleotides are shown in Fig. 1.15.

In summary, the $2N_3$nucleotides have provided evidence that the catalytic nucleotides of CF_1, MF_1, and EF_1 comprise portions of the polypeptide around Tyr-362, Tyr-368, and Tyr-331, respectively, and the noncatalytic sites around Tyr-385, Tyr-345, and Tyr-354, respectively, both on the β subunit. In addition to tyrosyl residues, Leu, Ile, and Pro residues have been labeled, indicating that although Tyr may be a preferred target, other, non-nucleophilic residues may also be targets for covalent reaction. The labeling of several residues in a single binding pocket suggests that the nucleotide is not stationary with respect to the protein. In addition, the residue with the majority of the label may not necessarily be the closest, but simply the most reactive of those in the vicinity of the azido group. The 8-azido and 2-azido derivatives provide complementary information on the structure of the nucleotide binding sites allowing a detailed map of the residues around the adenine ring to be constructed.

1.2.3 TNP-NUCLEOTIDES

The TNP-nucleotides are not structural probes in the sense that they have no potential for covalent reaction, but they have been useful on account of their high affinity and fluorescent properties. They have been especially useful in distinguishing different types of nucleotide binding sites of the Ca^{2+}-ATPase (see below) and we chose them, in combination with the azido group, to form the basis for the synthesis of a photoaffinity probe specifically for the Ca^{2+}-ATPase.

Reaction of trinitrobenzenesulfonic acid (TNBS) with the ribose moeity of adenosine results in the formation of the Meissenheimer complex 2',3'-o-(2,4,6-trinitrophenyl)adenosine (Azegami and Iwai, 1964). The corresponding nucleotides, TNP-ATP, TNP-ADP and TNP-AMP (Hiratsuka and Uchida, 1973) as well as TNP-adenosine have been well characterized chemically (Hiratsuka, 1982) and have been utilized in a number of enzyme systems including the myosin ATPase (Hiratsuka and Uchida, 1973), brain pyridoxal kinase (Churchich and Wu, 1981), eel electroplax and canine renal medulla (Na^{+}+ K^{+})-ATPase (Moczydlowski and Fortes, 1981a and b; Munson, 1981), mitochondrial F_1-ATPase (Grubmeyer and Penefsky, 1981a and b), adenosine deaminase, alkaline phosphatase and adenylate kinase (Hiratsuka, 1982), Ca^{2+}-ATPase (Dupont et al, 1982; Watanabe and Inesi, 1982), aspartokinase (Broglie and Takahashi, 1983), chloroplast F_1-ATPase (Wagner et al, 1986) and DNA polymerase I (Oberfelder and McHenry, 1987). TNP-ATP has been found to not act as a substrate for hog cerebral cortex (Na^{+} + K^{+})-ATPase and firefly luciferase (Hiratsuka, 1982), but this does not mean that these enzymes do not bind TNP-ATP.

The TNP group forms a Meissenheimer complex with the 2' and 3' hydroxyl groups of the ribose moeity at alkaline pH resulting in a characteristic absorbance spectrum in the visible region (Hiratsuka and Uchida, 1973). The chromophoric properties, however, are dependent on pH and a progressive decrease in the visible absorbance spectrum occurs along with protonation of the 2' hydroxyl group, with measured pK of 5.1 (Hiratsuka and Uchida,

1973). The TNP group is also fluorescent and has a single emission peak at 561 nm with the excitation spectrum corresponding roughly to the absorbance spectrum. These properties are largely independent of the phosphorylation status of the molecule, but do depend on the polarity of the environment. As the polarity is decreased, the absorption maxima are blue shifted while the fluorescence emission maxima are blue shifted and accompanied by an increase in quantum yield (Hiratsuka and Uchida, 1973). NMR and IR spectroscopic data have indicated an interaction between the adenine base and TNP group (Hiratsuka, 1975), in particular C8 of the adenine ring. As a result the molecule is considered to exist in free solution in a folded anti conformation with the adenine and TNP rings stacking and sharing the pi electron cloud (Hiratsuka, 1975).

TNP-ATP is hydrolyzed by heavy meromyosin (HMM) with very similar K_m and V_{max} values to ATP (Hiratsuka and Uchida, 1973). Binding of the probe to the catalytic site is accompanied by a red shift in the absorbance spectrum postulated to be due to an increase in the hydrophobicity of the environment surrounding the TNP group. The resultant difference spectrum can be used to titrate the number of binding sites (Hiratsuka and Uchida, 1973). Similarly, the fluorescence emission peak of TNP-ATP is blue shifted and increased upon binding to HMM. Fluorescence titration of the nucleotide binding sites has revealed two binding sites for TNP-ATP on HMM with a Kd of 0.8 µM (Hiratsuka, 1976). The TNP-nucleotide bound to the active site has also been used as an energy transfer acceptor from N-(iodoacetyl)-N'-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) bound to the alkali I light chain (Moss and Trentham, 1983) or to the 'fast reacting' sulfhydryl, SH_1 (Tao and Lamkin, 1981). The two TNP-ATP molecules bound to HMM are not homogeneous as 1 mol/mol can be displaced by low concentrations of ATP or ammonium sulfate precipitation while the other molecule is stable to this treatment (Hiratsuka et al., 1977). The former is thought to be located at the initial burst site while the latter is at the steady state ATPase site and is thought to form a stable complex with an amine group (Hiratsuka et al., 1977). As regards the

nature of the difference spectrum, evidence has been presented (Hiratsuka, 1975) that the difference spectrum is the result of disruption of the adenine-TNP interaction by stacking of the adenine ring on an aromatic amino acid at the active site, most likely tryptophan.

Although not a substrate for the (Na^{+}+ K^{+})-ATPase, TNP-ATP binds with high affinity to both eel electroplax (Moczydlowski and Fortes, 1981a and b) and dog renal medulla (Na^{+}+ K^{+})-ATPase (Munson, 1981). In the eel electroplax (Na^{+}+ K^{+})-ATPase, the increase in fluorescence seen on TNP-ATP binding was used to titrate the TNP-ATP binding sites yielding a value of 1 site per αß protomer (Moczydlowski and Fortes, 1981a). TNP-ATP was also shown to bind homogeneously to both catalytic and regulatory sites and it was thus concluded that the (Na^{+}+ K^{+})-ATPase has only one site for TNP-ATP that interconverts between high and low affinity states during the catalytic cycle (Moczydlowski and Fortes, 1981b).

Both TNP-ATP and TNP-ADP have been found to be potent inhibitors of the mitochondrial F_1 ATPase (Grubmeyer and Penefsky, 1981a and b; Kormer et al, 1982; Schafer, 1982) inhibiting both ATP hydrolysis and synthesis. Binding of the analogs was measured both by difference spectroscopy and fluorescence titration yielding a value of two binding sites per enzyme unit (Grubmeyer and Penefsky, 1981a). TNP-ATP binds the first site with an affinity too high to measure and the second with a Kd of 20 - 80 nM. Both these sites are equally competent at hydrolyzing TNP-ATP and occupancy of the second site accelerates hydrolysis at the first giving rise to a model of catalytic site cooperativity (Grubmeyer and Penefsky, 1981b). Alternatively, it has been shown that binding of TNP-ATP to the catalytic site of F_1 results in a difference absorbance spectrum but not in an increase in fluorescence (Kormer et al, 1982), while binding to the tight nucleotide site results in an increase in fluorescence. TNP-ADP has also been found to be a potent inhibitor of photophosphorylation by thylakoid membranes (Wagner et al, 1986). Equilibrium binding measurement with [^{14}-C]-TNP-ADP yielded a value of about three binding sites. TNP-ADP is

poorly phosphorylated to TNP-ATP, but mimics the behavior of ADP + P_i in stimulating basal electron transport. Both TNP-ATP and TNP-ADP have been incorporated into E.Coli F_1 to the extent of 1 mol/mol on the ß subunit and 1 mol/mol on the α subunit (Rao et al, 1988). The fluorescence enhancement upon incorporation into the ß subunit was about 50 - 33% of that into F_1 which has been interpreted as due to the assembled ß having a tighter and more hydrophobic site (Rao et al, 1988). The fluorescence upon binding to the α subunit was totally quenched and the binding was very tight (Rao et al, 1988). Recently, a fifty amino acid peptide has been synthesized which, on the basis of a postulated homology with adenylate kinase, should be located in the active site of the enzyme (Gorboczi et al., 1988). Further evidence for this hypothesis was obtained following the observation that the peptide induced a seven-fold increase in the fluorescence of added TNP-ATP, similar to intact F_1-ATPase.

TNP-ATP is also an inhibitor of ATP dependent initiation complex formation reaction between the E.Coli DNA polymerase III holoenzyme and primed DNA (Oberfelder and McHenry, 1987). TNP-ATP does not serve as a substrate for the enzyme but the fact that it inhibits all three identified holoenzyme reactions to a similar extent was used to demonstrate that all three functions share a common ATP binding site. TNP-ATP has also been used to probe the threonine promoted conformational changes in the aspartokinase region of aspartokinase homoserine dehydrogenase I (Broglie and Takahashi, 1983). TNP-ATP functions as a substrate for the enzyme with a similar Km to ATP but lower V_{max}. Binding results in a nine fold increase in fluorescence while binding of threonine results in a further 77% increase indicating an increase in rigidity or hydrophobicity of the nucleotide binding site in the inhibited form of the enzyme (Broglie and Takahashi, 1983).

1.2.3.1 TNP-NUCLEOTIDE INTERACTIONS WITH SR

TNP-nucleotides have been used extensively in the SR system following initial reports (Dupont, 1982; Watanabe and Inesi, 1982) that TNP-ATP binds specifically to the Ca^{2+}-ATPase with high affinity to the extent of 6-8 nmol/mg protein (1 mol/mol ATPase). Binding is accompanied by a shift in the visible absorbance spectrum as well as a blue shift and seven fold increase in quantum yield of the fluorescence emission spectrum. The most striking feature, however, is that in the presence of micromolar concentrations of ATP and Ca^{2+} bound TNP-ATP undergoes a very large increase in fluorescence (Watanabe and Inesi, 1982). This led to the postulate that TNP-ATP is bound to a separate regulatory site following dissociation of ADP (Watanabe and Inesi, 1982). The increase in fluorescence was seen as a result of a protein conformational change resulting in an increase in hydrophobicity of the binding site (Dupont and Pougeois, 1983) as a result of H_2O exclusion (Dupont and Pougeois, 1983; Nakamoto and Inesi, 1984).

The large increase in fluorescence of TNP-nucleotides bound under turnover conditions has been correlated with phosphorylation of the enzyme (Bishop et al, 1984; Nakamoto and Inesi, 1984; Davidson and Berman, 1987). Prior labeling with FITC or inclusion of ADP prevents TNP-nucleotide binding (Nakamoto and Inesi, 1984). Kinetic studies identified two binding sites (Bishop et al, 1984). The first coincides with the catalytic site (k_D = 2 - 4 µM) with a slow K_{off}. Following dissociation from this site, a second site was detected to which TNP-ATP binds resulting in the large fluorescence increase. Further characterization has shown that TNP-ATP binds competitively to two sites of equal concentration (3.5 nmol/mg protein) and that the affinity and appearance of these sites are modulated by the presence of Mg^{2+} and H^+ (Dupont et al, 1985). Thus in the absence of Mg^{2+} or at acid pH the sites behave like one homogeneous population of intermediate affinity (k_D = 20 µM). At pH > 7 or in the presence of Mg^{2+}, however, they split into two populations of high (k_D = 2 - 4 µM) and low (k_D > 1 mM)

affinity (Dupont et al, 1985). Correlation of the high affinity site with the regulatory nucleotide site has been obtained following the finding that TNP-ATP binding to the phosphorylated enzyme accelerates Ca^{2+} activated hydrolysis (Dupont et al, 1985).

The effects of the monovalent cations H^+ and K^+ on the turnover dependent fluorescence increase have provided insights into the mechanism involved (Bishop et al, 1984, 1986; Davidson and Berman, 1985; Wakabayashi et al, 1986). The presence of K^+ decreases the fluorescence with a $k_{0.5}$ of 48 and 135 mM in the absence and presence respectively of valinomycin (Davidson and Berman, 1985), while the absence of H^+ has the same effect (Wakabayashi et al, 1986). These conditions have been shown to have no effect on the steady state E-P levels (Davidson and Berman, 1985) and have alternately been described to variations in affinity for TNP-ATP (Bishop et al, 1986) and not to variations in the E-P levels (Bishop et al, 1986), or rather to conversion from E_1-P to E_2-P (Wakabayashi et al, 1986). Further evidence for this latter conclusion has been obtained from results where TNP-ATP fluorescence was increased in conditions leading to stabilization of the ADP insensitive phosphoenzyme (Dupont and Pougeois, 1983; Andersen et al, 1985). It has furthermore been shown that uncoupling of the Ca^{2+}-ATPase results in a decrease in the ATP or P_i induced high fluorescence, without a decrease in binding (Berman, 1986). This has been postulated to be due to partial denaturation of the enzyme in the uncoupled state preventing the "synchronized conformational changes resulting in ordered access or restriction of H_2O" (Berman, 1986).

Direct evidence of TNP-nucleotide binding to the regulatory site has been obtained in a number of ways (Bishop et al, 1987). TNP-AMP has been shown to block secondary activation of ATPase activity without itself stimulating activity, and this site of binding has been shown to be the high fluorescence site. A difference in binding affinity for the dephosphorylated (k_D = 20 nM) and phosphorylated (k_D = 0.4 µM) enzyme has been shown indicating two sites of binding (Bishop et al, 1987). Lastly,

it has been shown that TNP-AMP blocks the ADP dependent rapid burst of E-P decomposition, identifying the TNP-AMP binding site with the ADP site (Bishop et al, 1987). The above phenomena have been shown to occur in the monomeric preparation as well (Bishop et al, 1987). It has also been shown that TNP-ATP undergoes a large increase in fluorescence under turnover conditions in the monomeric preparation demonstrating that the monomer undergoes the ADP sensitive E_1-P to ADP insensitive E_2-P transition (Andersen et al, 1985). Finally, it has been found that low concentrations of TNP-ATP accelerate ATP turnover (Dupont et al., 1985) as well as E-P hydrolysis (Champeil et al., 1988).

Fluorescence energy transfer experiments between TNP-AMP and Pr^{3+} as energy acceptors and IAEDANS as donor have led to a model in which the B tryptic fragment is seen as spanning most of the major access of the cytoplasmic region of the enzyme while the A fragment is abutted to the B fragment together forming the nucleotide binding site (Squier et al, 1987)

In summary, TNP-ATP has proved to be a useful probe. It has been used in a large number of systems where it usually binds with high affinity to the nucleotide binding site. The absorbance and fluorescence properties allow easy monitoring of binding and also act as reporters of nucleotide site characteristics. TNP-nucleotides have also been shown in a number of systems to differentiate between kinetically defined catalytic and regulatory sites.

1.3. STRUCTURE AND FUNCTION OF SR VESICLES

Calcium is widely used in regulation in biological systems and it's concentration in various cellular compartments is strictly controlled. The intracellular calcium concentration is normally maintained several orders of magnitude below that in the extracellular fluid. This is achieved by the presence of energy dependent Ca^{2+} transport proteins located in the cell membrane. Intracellular compartments can be used as calcium sinks and in specialized tissues where rapid delivery and removal of calcium is required these pumps are present in high concentration in the membrane. Such an example is skeletal muscle where the SR is used as a storage site for calcium which is rapidly released into the sarcoplasm causing myofibril contraction. To initiate relaxation, the calcium is then removed from the sarcoplasm by the Ca^{2+}-ATPase which in the process, develops a gradient of calcium across the SR membrane of several orders of magnitude.

The SR is a vesicular structure surrounding the myofibrils and abutts directly on the T-tubules which are plasma membrane invaginations which transmit the wave of depolarization from the muscle end plate to the interior of the muscle cell (Peachey, 1965).

1.3.1. Ultrastructure of SR vesicles

SR vesicle preparations are usually obtained by differential centrifugation of white skeletal muscle homogenates from the back and hind leg muscles of young rabbits (Eletr and Inesi, 1972). Further purification may be obtained by detergent extraction (MacLennan, 1970; Meissner et al., 1973) or by separation into light and heavy fractions by isopycnic density gradient centrifugation (Meissner, 1975; Lau et al., 1977; Volpe et al., 1987). Light and heavy SR vesicles are derived from longitudinal and terminal cisternal regions of the SR respectively, and the differences in bouyant density reflect their different protein contents and protein:lipid ratios. The primary function of the longitudinal region is Ca^{2+} uptake and

consequently the predominant protein species is the Ca^{2+}-ATPase (Meissner, 1975). The terminal cisternae are more involved with Ca^{2+} storage and release and so calsequestrin and lower MW proteins make up a greater percentage of the total protein content (Smith et al., 1985; Somlyo, 1985). Calsequestrin is probably located exclusively in the terminal cisternae (MacLennan and Wong, 1971; Meissner, 1975; Jorgensen et al., 1983; Saito et al., 1984). That part of the terminal cisterna abutting directly on the transverse tubule is known as the junctional SR and it is connected by bridging structures called 'feet' to the T-tubule forming a structure called the triad (Franzini-Armstrong, 1970). The junctional membrane is devoid of Ca^{2+}-ATPase (Jorgensen et al., 1979, 1982). Detergent extraction of the SR vesicle preparation removes proteins loosely associated with the membranes (MacLennan, 1970; Meissner et al., 1973)

Protein makes up 60 - 70% of SR vesicles with the balance being lipid (Bailin and Blumenkrantz, 1987). The lipid component is made up of about 80% phospholipid and the rest neutral lipid, mainly cholesterol. The phospholipids consist of phosphatidylcholine (65 - 73%), phosphatidylethanolamine (12 - 19%), phosphatidylinositol (about 9%), phosphatidylserine (about 2%), sphingomyelin (about 4%) and cardiolipin (0.1 - 0.3%) (Tada et al., 1978.

The M_r 110 kDa Ca^{2+}-ATPase accounts for approximately 70 - 90% of the protein component of SR vesicle preparations (Tada et al., 1978). The Mr 45 - 55 kDa acidic calcium binding protein calsequestrin accounts for about 10 - 20% of the protein (MacLennan and Wong, 1971). The high affinity calcium binding protein (Mr 55 - 65 kDa) is a minor component of skeletal SR and together with 4 other minor proteins (M_r 20 - 38 kDa) and a 12 kDa proteolipid makes up about 10 - 20% of the total protein (Bailin and Blumenkrantz, 1987). Two glycoproteins of 53 kDa (Chiesi and Carafoli, 1982; Leonards and Kutchai, 1985) and 160 kDa (Campbell and MacLennan, 1983) have been identified. Their exact roles are unclear although they may be involved in Ca^{2+}-ATPase regulation (Racker and Eyton, 1975). Additional proteins sometimes found in SR vesicle preparations include myosin,

myokinase, phosphorylase and a high MW protein of M_r about 170 kDa (Ross, 1987). The latter 2 can be removed by further centrifugation at low ionic strength in the presence of glycogen (Ross, 1987).

A number of studies have demonstrated an asymmetric orientation of the Ca^{2+}-ATPase in the SR membrane (MacLennan et al., 1971; Dupont et al., 1973; Thorley-Lawson and Green, 1973; Inesi and Scales, 1974; Stewart and MacLennan, 1974; Jilka et al., 1975; Saito et al., 1978; Taylor et al., 1986). When visualized under the electron microscope, the vesicles are seen to be made up of membranes of 60 Å thickness covered with particles of about 35 Å (MacLennan et al., 1971; Jilka et al., 1975). The 30 Å knobs can be removed by treatment of the membranes with trypsin (Thorley-Lawson and Green, 1973; Inesi and Scales, 1974; Saito et al., 1978). Upon freeze-fracture of the membranes, 90 Å particles are seen projecting from the cytoplasmic half of the bilayer (MacLennan et al., 1971; Inesi and Scales, 1974). This has resulted in the conclusion that the ATPase consists of a hydrophobic portion embedded in the membrane and a hydrophilic portion projecting onto the cytoplasmic side (Stewart and MacLennan, 1974). The Ca^{2+}-ATPase appears to have an overall elongated shape with a length of 110 Å and a width of 75 Å (le Maire et al., 1981). The 3-dimensional structure of the Ca^{2+}-ATPase has been determined to 25 Å resolution by image reconstruction of crystals incubated in $NaVO_4$ (Taylor et al., 1986). The ATPase monomers are crystallized in the form of dimers and appear as pear-shaped molecules with a lobe projecting out along one side parallel to the plane of the membrane (Taylor et al., 1986).

Two-dimensional crystalline arrays of Ca^{2+}-ATPase molecules have been observed by negative staining SR vesicles with uranyl acetate (Dux and Martonosi, 1983a,b,c,d; Dux et al., 1985). Two different crystalline units are seen depending on whether the enzyme is crystallized in the E_1 or E_2 form. Crystallization in the E_2 form is obtained by incubating the vesicles with $NaVO_4$ and is inhibited by treatment with Ca^{2+} and ATP but not with ADP, AMP-PNP or AMP-PCP (Dux and Martonosi, 1983a,c). The crystals

formed in this conformation are arranged in pairs consistent with ATPase dimers (Taylor et al., 1984). Crystallization in the E_1 form is obtained following incubation with Ca^{2+} or lanthanide ions and results in a different pattern to that obtained in the E_2 form (Dux et al., 1985). The rate of vanadate-induced crystallization is increased by an inside positive membrane potential (Dux and Martonosi, 1983d), while Ca^{2+} induced crystallization is increased by an inside negative potential (Dux et al., 1985). These findings have resulted in the postulate that the E_1 to E_2 transition may involve a change in oligomericity of the pump (Dux et al., 1985). More recently, crystallization in a form similar to that obtained in the presence of Ca^{2+} has been obtained in detergent raising the possibility that a preparation suitable for X-ray diffraction studies may be obtained (Dux et al., 1987).

1.3.2. Structure of the Ca^{2+}-ATPase

Initial work on the primary structure of the protein was restricted to the water soluble extra-membranous segments and a number of sequences were obtained (Allen and Green, 1978; Allen, 1980a, b; Allen et al., 1980a, b) leading to the elucidation of about 80% of the primary structure of the protein. The entire sequence was later obtained following sequencing of a cDNA for the Ca^{2+}-ATPase (MacLennan et al., 1985; Brandl et al., 1986).

Limited tryptic digestion has also been used as a tool in structural studies. Initial cleavage occurs at a site labeled T1 resulting in two polypeptides, A and B, of approximately equal molecular weight (Thorley-Lawson and Green, 1973; Inesi and Scales, 1974; Stewart and MacLennan, 1974; Rizzolo et al., 1976). A second cleavage takes place at a site labeled T2, cleaving the A fragment into two fragments, A1 and A2, of MW 33 and 22 kDa, respectively (Thorley-Lawson and Green, 1973). The fragments are located in the linear sequence with the A2 fragment at the N-terminus, the B fragment at the C-terminus and the A1 fragment in between (Allen et al., 1980a). Each fragment has a hydrophobic portion situated in the membrane and so the molecule

remains intact following cleavage at T1 and T2 (Thorley-Lawson and Green, 1973; Stewart and MacLennan, 1974; Inesi and Scales, 1974). Initial cleavage results in a small activation of activity but more extensive digestion results in a decline in activity along with the release of peptides from the vesicles (Stewart and MacLennan, 1974; Saito et al., 1978). The trypsin sensitive sites may delineate structural domains (MacLennan et al., 1985).

The most important development in Ca^{2+}-ATPase structure has been the elucidation of the amino acid sequences of rabbit skeletal muscle Ca^{2+}-ATPase from both fast (type II) (Brandl et al., 1986) and slow (type I) (MacLennan et al., 1985) fibers. The two enzymes are very similar with the fast twitch form having four additional residues, three at the C terminus and one near the T1 cleavage site (Brandl et al., 1986). There are 164 changes in amino acid, 66 of which are conservative (S/T, D/E, K/R, F/Y/W, I/L/V/M). The differences have little effect on the predicted secondary structure (Brandl et al., 1986) (Fig.1.16). Most of the variations are found in α helices and bends while the β strands are highly conserved. The most variability occurs in helices at the amino terminus and in the phosphorylation and nucleotide binding domains. The residues making up the helices in the predicted transmembrane regions, the stalk region and a hypothetical hinge region show less variability. The α-subdomain or hinge region and the stalk segment connecting the fourth transmembrane segment to the phosphorylation domain are the most highly conserved regions. Although there are differences between cardiac and slow skeletal muscle Ca^{2+}-ATPase they have been postulated as resulting from the same gene, perhaps with alternate splicing (Brandl et al., 1986).

It is known from freeze-fracture and other studies that the Ca^{2+}-ATPase is a transmembrane protein and the regions in the sequence located in the membrane were predicted by means of a polarity plot (MacLennan et al., 1985). MacLennan et al. (1985) predicted 10 such stretches which are connected, on the lumenal face by five short loops. Most of the molecule is located on

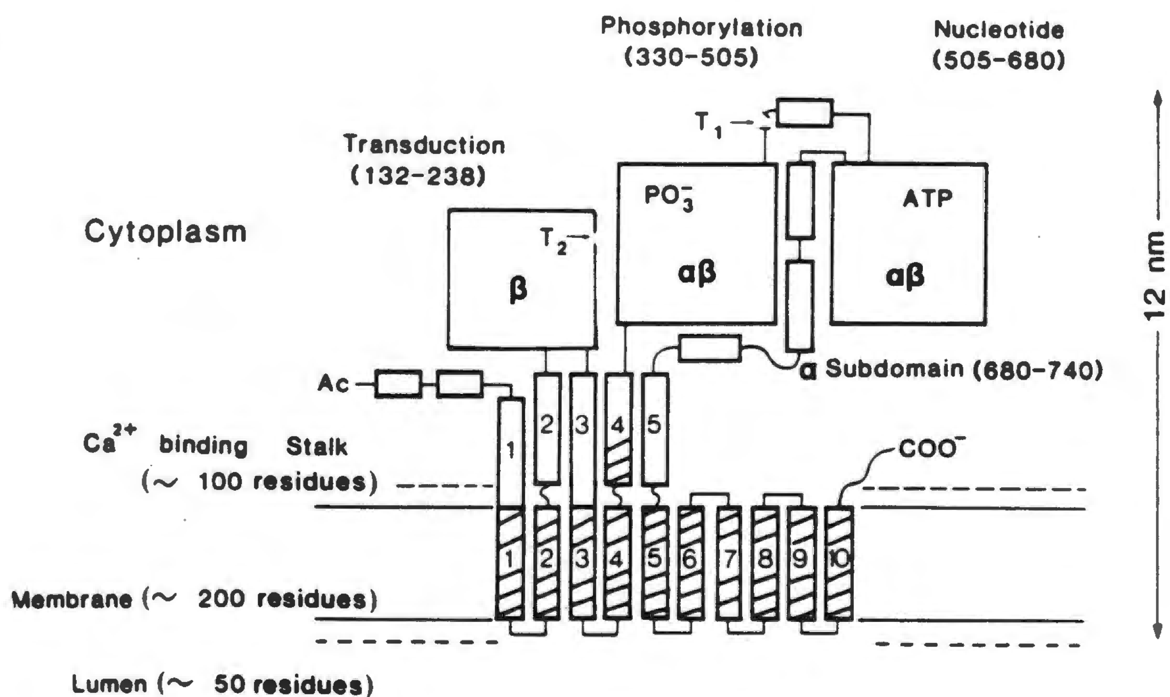


Figure 1.16 Postulated domain folding of the Ca^{2+}-ATPase

A schematic representation of the proposed folding pattern of the Ca^{2+}-ATPase is shown, as well as the proposed division into domains MacLennan et al., 1985; Brandl et al., 1986). The model assumes 10 intramembranous sections. The two trypsin sensitive sites, T_1 and T_2, are shown.

the cytoplasmic side of the membrane where it has been postulated to be arranged into three domains delineated by hydrophobic regions and trypsin sensitive sites (Fig. 1.16). In this model, the hydrophilic domains are connected to the first five transmembrane stretches by five connecting segments arranged into an α-helical net and called the stalk region. The first three stalk segments contain a number of aligned Glu residues which make this region a candidate for the high affinity Ca^{2+} binding site. The first globular domain (residues 132-238) is located between the first and second pairs of transmembrane segments. This region termed the transduction domain consists of seven ß strands which likely form an anti-parallel ß sandwich and contains the T2 cleavage site. The large hydrophilic region between the second and third pairs of transmembrane regions is predicted to be made up of alternating α-helix and ß-strand which would probably fold to form parallel ß-sheets. It is predicted to be arranged into two domains divided by the T1 tryptic site. The N-terminal domain (residues 330-505) contains the phosphorylation site and the C-terminal one (residues 505-680) the site of labeling with FITC and thus the nucleotide binding site. The nucleotide binding domain is connected to the fifth transmembrane segment by a short α-helical segment called the hinge region by analogy to the organization in kinases and dehydrogenases (Bennett and Huber, 1984).

1.3.2.1 SR AFFINITY LABELING WITH THIOL DIRECTED REAGENTS

The Ca^{2+}-ATPase contains twenty-four sulfhydryl residues (Brandl et al., 1986), of which six probably form disulfide linkages tentatively located on the B fragment, while fourteen are probably located on the surface of the protein (Thorley-Lawson and Green, 1977). The highest density of exposed SH groups is found on the A1 fragment, three of which are in the thirty-one residue tryptic peptide containing the phosphorylated aspartic acid residue (Thorley-Lawson and Green, 1977).

Four of these SH residues may be distinguished by their reactivity to NEM (Thorley-Lawson and Green, 1977; Yamada and

Ikemoto, 1978; Kawakita et al., 1980). On incubation with NEM the first SH group to be modified is termed SH_N in the terminology of Kawakita et al (1980), after which, SH_D, SH_N', and SH_F are modified. SH_N modification has no effect on enzyme activity (Yamada and Ikemoto, 1978). Modification of SH_D is accompanied by decreased Ca^{2+} transport as a result of inhibition of E-P decomposition and this effect is markedly accelerated when the labeling is carried out in the presence of Ca^{2+} (Kawakita et al., 1980). Specific modification of SH_D occurs when carried out under mild conditions in the presence of AMP-PNP (Kawakita et al., 1980). The SH_D modified enzyme cannot be phosphorylated from P_i which indicates that the E_2-P conformation is unattainable under these conditions (Davidson and Berman, 1987). Addition of detergent to the SH_D modified enzyme, however, causes a rapid loss of E-P probably by another route (Yamamoto et al., 1984). Further incubation leads to labeling of a 3rd SH residue, SH_N', modification of which has no effect on Ca^{2+} transport (Kawakita et al., 1980). The labeling of SH_F causes inhibition of E-P formation and thus Ca^{2+} transport (Yamada and Ikemoto, 1978). SH_F is not labeled in the presence of AMP-PNP (Kawakita et al., 1980).

SH_D has been located on the A1 tryptic fragment (Saito et al., 1984), while modification of SH_N has led to the modification of 2 residues Cys 344 and Cys 364 to the extent of 0.5 mol/mol ATPase each, with modification of one perhaps inhibiting modification of the other (Saito-Nakatsuka et al., 1987). Labeling of both SH_N and SH_D, resulting in inhibition of ATPase activity, resulted in the same 2 residues being modified. Thus when either is labeled activity is unaffected but when both are modified Ca^{2+} transport is prevented (Kawakita and Yamashita, 1987).

Spin labeled and fluorescent maleimide and iodoacetamide derivatives have been used to obtain information concerning the conformational transitions of the enzyme especially during ligand binding (Yu et al., 1977; Yasuoka-Yabe et al., 1983). Maleimide spin labels have been shown to have a similar selectivity of reactivity towards the Ca^{2+}-ATPase as NEM (Yu et

al., 1977; Yasuoka-Yabe and Kawakita, 1983). One such probe incorporated specifically into the SH groups essential for ATPase activity was found to be located in a highly ordered, less fluid environment than molecules incorporated elsewhere on the enzyme (Yu et al., 1977). It was further shown that this is a result of the tertiary structure of the protein rather than as a result of being buried in a lipid environment (Yu et al., 1977). Another spin labeled probe detected a difference in conformation between ADP binding to E_1-P. The maleimide fluorescent probe ANM has also been shown to have the same selectivity as NEM and the probe has been shown to have a characteristic fluorescence whose intensity is relative to the binding of Ca^{2+} and was ascribed to a conformational change on Ca^{2+} binding to the transport sites (Miki et al., 1981; Yasuoka-Yabe and Kawakita, 1983). Further fluorescent effects were observed on phosphorylating from ATP (Miki et al., 1981) and on binding of AMP-PNP in the presence of Ca^{2+}, irrespective of whether the probe was attached to SH_D or SH_N (Yasuoka-Yabe et al., 1983). The fluorescent probe pyrene maleimide is specifically incorporated into one SH residue under appropriate conditions (Kutenbach and Verjovski-Almeida, 1985). Both the extent and rate of incorporation are increased in the presence of P_i and protected by ATP(Kurtenbach and Verjovski-Almeida, 1985). Evidence was obtained that occupancy of the high affinity Ca^{2+} sites increases the accessibility of medium solvent into hydrophobic domains of the enzyme (Kurtenbach and Verjovski-Almeida, 1985).

Under controlled conditions iodoacetamide spin labels can be incorporated into about 2 SH groups per enzyme without affecting activity (Coan and Inesi, 1977; Laggner et al., 1981). The reaction rate is sensitive to pH and temperature and, in addition when the reaction is carried out in the presence of ATP and Ca^{2+} the rate of labeling is increased and the 'strongly immobilized' signal is enhanced (Champeil et al., 1978). The EPR spectrum has been used to monitor conformational changes induced by ligand binding (Coan and Inesi, 1977; Laggner et al.,1981). Thus, a small but specific broadening of the spectrum occurs on binding

of substrates and P_i (Coan and Inesi, 1977; Laggner et al., 1981). Ca^{2+} greatly increases this effect but reverses the effect of P_i (Coan and Inesi, 1977, 1979). In the absence of substrate, the spin label is incorporated into a homogeneous population of sites while in the presence of Ca^{2+} and substrate the sites labeled appear split into 2 equal populations one of which has a reactivity with the spin label which is increased 10 fold (Coan and Keating, 1982). It has thus been postulated that there are 2 conformations of the substrate binding site present during turnover (Coan and Keating, 1982). It was later found that P_i in the presence of Me_2SO also induces this split in the sites (Coan, 1983). The site of labeling of iodoacetamide and IAEDANS has been shown to be Cys-674 (Suzuki et al., 1987; Yamashita and Kawakita, 1987). The same residue (Cys-674) as well as Cys-670 have been labeled with IAF (iodoacetamido fluorescein) and a model postulated in which the two residues are located at adjacent positions in an α-helical structure allowing both to be labeled with approximately equal probability (Bishop et al., 1988).

1.3.2.2 INTRAMOLECULAR CROSSLINKING OF THE Ca^{2+}-ATPase

It has recently been found that the SR Ca^{2+}-ATPase can be specifically modified with bifunctional aldehyde reagents resulting in intramolecular cross-linking of the ATPase molecule (Ross and McIntosh, 1987a,b). These reagents which react preferentially with lysine groups were found to modify the Ca^{2+}-ATPase resulting in a species with altered hydrodynamic properties when electrophoresed on polyacrylamide gels, exhibiting an increased apparent MW for the cross-linked species of 125 kDa (Ross and McIntosh, 1987a). More extensive treatment results in intermolecular cross-linking to higher MW oligomeric species. Reaction with the reagent glutaraldehyde, under optimal conditions, results in close to 100% modification, which can be completely prevented by the binding of nucleotides or decavanadate, or partially inhibited by prior labeling of the enzyme with FITC or PLP (Ross and McIntosh, 1987a). It has

further been shown that cross-link formation is inhibited upon formation of E_2-P, but not E_1-P, and been postulated that the active site is closed in E_2-P (Ross and McIntosh, 1987b). Inhibition of cross-linking in E_1-P, however is observed in the presence of millimolar concentrations of ATP, demonstrating binding of ATP to a low affinity site on E_1-P (Ross and McIntosh, 1987b). From the pattern of tryptic digestion, it was established that the cross-link is located between the A1 and B tryptic fragments (Ross and McIntosh, 1987a).

1.3.2.3 PHOTOAFFINITY LABELING OF SR VESICLES

At present eight reports have been published reporting the photoaffinity labeling of SR vesicles including $8N_3ATP$ (Briggs et al., 1980; Cable and Briggs, 1984; Campbell and MacLennan, 1983), 3'-o-[3-(2-nitro-4-azidophenyl)-propionyl]-ATP (NAP_3-ATP) (Schoner et al., 1982), 3'-o-(4-benzoyl)benzoic ATP (BzATP) (Cable and Briggs, 1984), 3'-arylazido-ATP (Cable and Briggs, 1984; Carvalho-Alves et al., 1985; Oliveira et al.,1988) and UTP (Ferreira, S.T., and Verjovski-Almeida, S., 1988). The photoaffinity labeling of integral membrane protein components of the SR has also been reported with two arylazido phosphatidylcholine analogs (Volpe et al., 1987)

Contrary to an initial report (Briggs et al., 1980), irradiation of SR vesicles in the presence of $8N_3ATP$ under conditions favoring turnover of the pump, results in little specific photoincorporation (Campbell and MacLennan, 1983; Cable and Briggs, 1984). This could be due to conversion of $8N_3ATP$ to $8N_3ADP$ under these conditions as suggested (Campbell and MacLennan, 1983). Another possibility is that the Ca^{2+}-ATPase has a low affinity for 8- substituted nucleotides reported to adopt a syn conformation (Ikehara et al., 1972; Sarma et al., 1974). This latter possibility is unlikely, however, in view of the finding that 8-BrATP supports Ca^{2+} transport as well as ATP (Champeil et al., 1988) and does $8N_3ATP$ (McIntosh and Seebregts, unpublished observations). Under conditions in which the hydrolysis of $8N_3ATP$ is low, the probe labeled two intrinsic

membrane glycoproteins of MW 53 kDa and 160 kDa (Campbell and MacLennan, 1983).

Photoincorporation of BzATP was obtained to the extent of 1.3 nmol/mg protein under select conditions (Cable and Briggs, 1984). There was a good correlation between BzATP incorporation and inhibition of ATPase activity, and both parameters were antagonized by ATP (Cable and Briggs, 1984). The label was located predominantly on the ATPase A fragment (72 %) but a portion was also found on the B fragment (28 %), and a smaller component on the phospholipid or proteolipid (Cable and Briggs, 1984). In the same study, it was reported that arylazido-ATP is ineffective as a photoaffinity probe, although more recent studies have found arylazido-ATP to be a useful photoaffinity label (Carvalho-Alves et al., 1985; Oliveira et al., 1988).

Arylazido-ATP (aATP) competitively inhibits ATP hydrolysis with a Ki of 10 µM and is itself hydrolyzed by SR vesicles at a slow rate (Carvalho-Alves et al., 1985). The hydrolysis is Ca^{2+} independent and exhibits a biphasic dependence on ATP concentration, with $k_{0.5}$ values of about 10 µM and 300 µM at postulated high and low affinity sites respectively and results in a low level of phosphorylation of about 0.6 nmol/mg protein (Carvalho-Alves et al., 1985; Oliveira et al., 1988). It is postulated that the hydrophobicity of the molecule allows it to mimic P_i + Me_2SO in phosphorylating the enzyme (Oliveira et al., 1988). A similar biphasic dependence on aATP concentration was observed for photoincorporation, which occurred to the extent of about 1 mol/mol ATPase (6 - 9 nmol/mg protein and 1.0 - 1.5 mol/mol ATPase (up to 22 nmol/mg protein) to the high and low affinity sites respectively (Carvalho-Alves et al., 1985). Photoincorporation was inhibited by ATP, P_i, TNP-ATP or Ca^{2+} although the 'kinetic protection' afforded by these compounds did not decrease the photoincorporation by more than 50 % (Carvalho-Alves et al., 1985). Photoaffinity labeling of the high affinity site inhibits the hydrolysis of added free aATP but not ATP while photolabeling the low affinity site irreversibly inhibits ATPase activity (Carvalho-Alves et al., 1985). This is probably due to aATP labeling residues outside the active site;

extensive labeling on non-Ca^{2+}-ATPase components was seen (Carvalho-Alves et al., 1985). More recently, specific photolabeling of the A2 fragment has been obtained by direct illumination of UTP in the nucleotide site (Ferreira and Verjovski-Almeida, 1988). Although low levels of labeling were obtained (0.12 nmol/mg protein), this result has been interpreted as indicating that the postulated transduction domain makes up part of the nucleotide binding site (Ferreira and Verjovski-Almeida, 1988). A surprising result is that the incorporation of UTP is not inhibited by the prior modification of the enzyme with FITC, although phosphorylation by UTP is inhibited. This has been interpreted as meaning that both probes can fit in the nucleotide site (Ferreira and Verjovski-Almeida, 1988).

1.3.3. Ca^{2+} translocation and the Catalytic Cycle

Ca^{2+} ions are transported across the SR membrane coupled to ATP hydrolysis by the Ca^{2+}-ATPase, and the mechanism of action of this enzyme has been the subject of a number of reviews (for example Tada et al., 1978; de Meis and Vianna, 1979; Berman, 1982; Inesi, 1985). The overall reaction catalyzed by the Ca^{2+}-ATPase is given below:

$$2\ Ca^{2+}{}_{in} + MgATP_i \rightleftharpoons MgADP_i + P_i + 2\ Ca^{2+}{}_{out}$$

A reaction cycle based on the E_1, E_2 model postulated for P-type ATPases is often used (Fig. 1.17) (de Meis and Vianna, 1979; Inesi, 1985). The cycle involves some of the intermediates that have been postulated to exist and includes most of the characterized partial reactions. The cycle begins with substrate binding followed by phosphorylation of the enzyme. Thereafter, the two Ca^{2+} ions are rapidly internalized and the enzyme undergoes a slow conformational change leading to Ca^{2+} release inside the vesicles followed by dephosphorylation and completion of the cycle. The cycle is readily reversible and the enzyme can be phosphorylated by P_i and reverse the direction of Ca^{2+} transport.

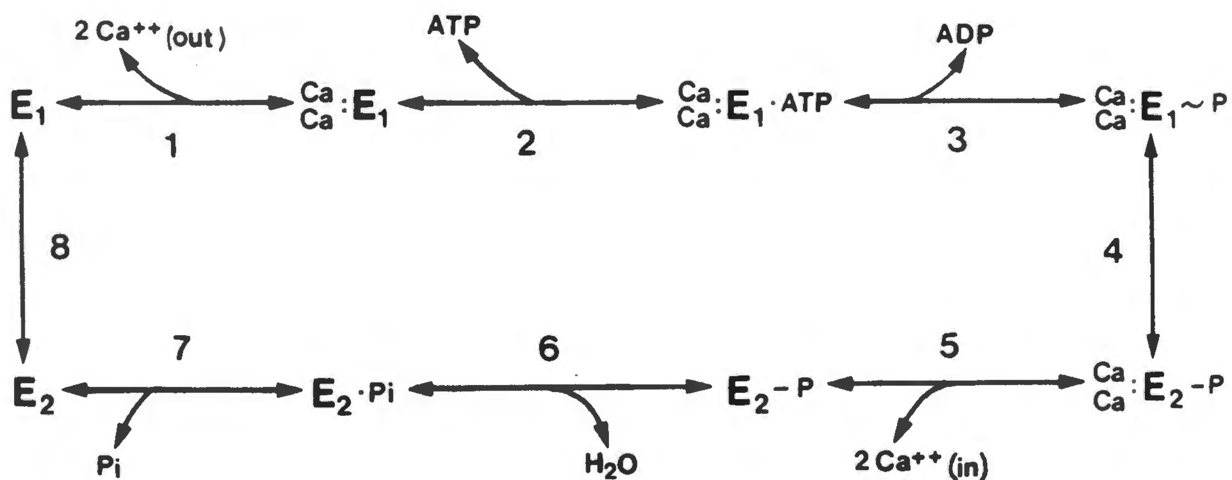


Figure 1.17 <u>Reaction cycle of the Ca^{2+}-ATPase</u>

The commonly used reaction cycle of the Ca^{2+}-ATPase containing most of the principle postulated intermediate species is shown (de Meis and Vianna, 1979).

Jencks and coworkers have postulated that Ca^{2+} transport is governed by a set of rules defining the chemical and vectorial specificity of the enzyme as well as utilization of binding energy to make the reaction proceed at a reasonable rate (Jencks, 1980, 1983; Pickart and Jencks, 1984; Khananshvili and Jencks, 1988). It has, moreover, been found that certain of these rules are incompatible with an E_1E_2 model and been suggested that as some of the postulated intermediates have never been seen, a notation based solely on the chemical composition of the species be adopted (Fig. 1.18) (Stahl and Jencks, 1987). In this model, chemical specificity is obtained by virtue of the fact that the E-P species alone can react with H_2O and P_i while $E._{Ca}$ can react with ADP and ATP ie. Ca^{2+} acts as a chemical switch, defining the chemical reactivity of the enzyme. Vectorial specificity is obtained by allowing E or E.ATP only to interact with the Ca^{2+} on the outside and E-P or E-P.Ca^{2+} to interact with Ca^{2+} on the inside ie. phosphorylation acts as a vectorial switch. Only by obeying these rules, then, is it possible to get tightly coupled Ca^{2+} translocation.

Ca^{2+} BINDING

Ca^{2+} binds with high affinity (K_m = 0.1-0.4 µM) to the outwardly oriented calcium binding sites of the Ca^{2+}-ATPase (Weber et al., 1966; Yamamoto and Tonomura, 1967; Yamamoto et al., 1979; Vianna, 1975; Arav et al., 1983) in a cooperative manner (n_H = 1.8) (Dupont and Leigh, 1978; Inesi et al., 1980; Moller et al., 1980; Arav et al., 1983), with a stoichiometry of 2 Ca^{2+} ions per phosphorylation site (Weber et al., 1966; Inesi et al., 1980). At higher concentrations of Ca^{2+}, binding occurs to the inwardly oriented low affinity calcium binding sites (K_m 0.6 - 1 mM) (Ikemoto, 1974, 1975). Ca^{2+} binding is pH dependent, with an increased affinity occurring at alkaline pH (Meissner, 1973; Verjovski-Almeida and de Meis, 1977; Chiesi and Inesi, 1980) and a model has been proposed in which Ca^{2+} and H^+ compete for the same site (Hill and Inesi, 1982; Inesi and Hill, 1983). Initial studies using changes in tryptophan

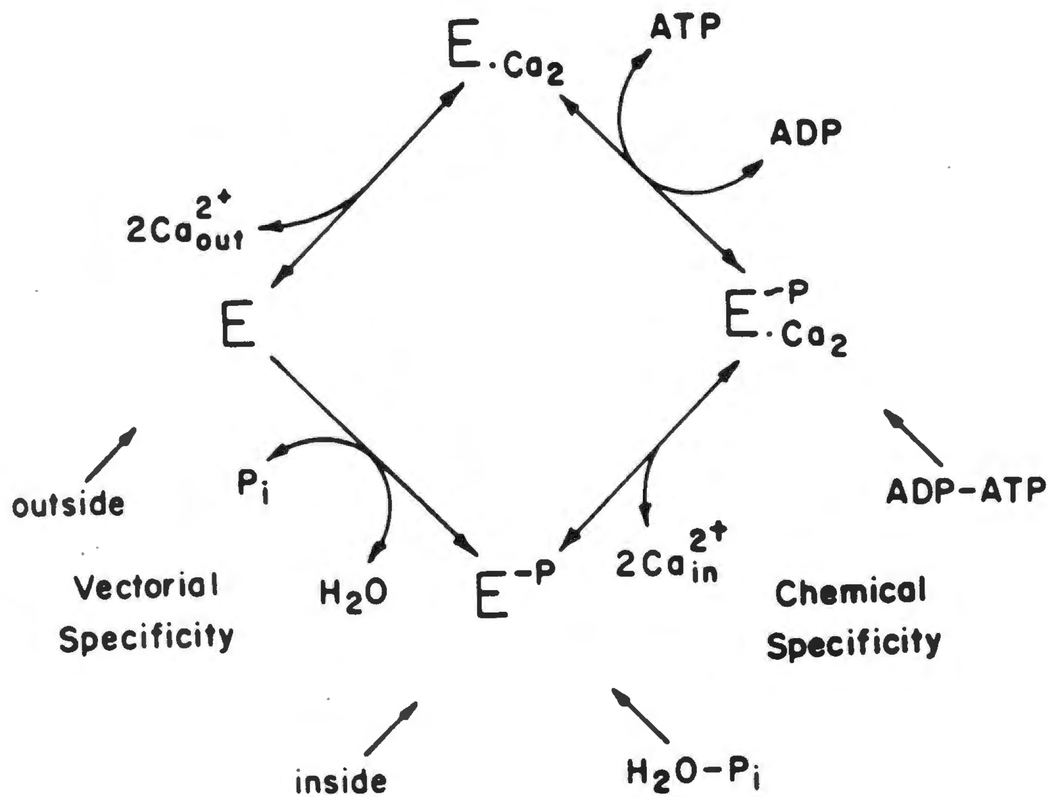
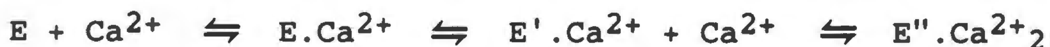


Figure 1.18 Predominant conformational states of the Ca^{2+}-ATPase

A minimal reaction cycle is shown consisting of the 4 main conformations of the Ca^{2+}-ATPase which have been postulated to be required for pumping to occur (Khaanshvili and Jencks, 1988).

fluorescence (Dupont and Leigh, 1978; Dupont, 1982; Champeil _et al_., 1983; Fernandez-Belda _et al_., 1984), EPR spectroscopy (Coan and Inesi, 1977; Inesi _et al_., 1980) and SH group reactivity (Ikemoto _et al_., 1978), demonstrated that Ca^{2+} binding is accompanied by a slow conformational change. The rate of Ca^{2+} binding is accelerated by ATP (Sumida _et al_., 1978; Takisawa and Tonomura, 1978; Scofano _et al_., 1979; Inesi _et al_., 1980; Guillain _et al_., 1981; Pickart and Jencks, 1984; Fernandez-Belda _et al_., 1984), probably by acting on a conformational change associated with binding (Stahl and Jencks, 1984, 1987). A sequential binding mechanism has been postulated in which binding of Ca^{2+} to the first site induces a second site with higher affinity (Dupont, 1982; Champeil _et al_., 1983) according to the following scheme (Inesi _et al_., 1980; Fernandez-Belda _et al_., 1984)



$$E + Ca^{2+} \leftrightharpoons E.Ca^{2+} \leftrightharpoons E'.Ca^{2+} + Ca^{2+} \leftrightharpoons E''.Ca^{2+}{}_2$$

More recent isotope exchange experiments have confirmed these results and shown that both Ca^{2+} binding and release occur sequentially and also that the ion bound first is always released first (Inesi, 1987; Khananshvili and Jencks, 1988). It has been postulated that the Ca^{2+} binding sites are located in the glutamic acid rich stalk region of the Ca^{2+}-ATPase and that rotation of the residues results in Ca^{2+} translocation (MacLennan _et al_., 1985). In an extension of the model, it has been suggested that binding of the first Ca^{2+} draws the subdomains together by electrostatic interactions creating a second site for Ca^{2+} (Tanford (_et al_., 1987). Binding of the second Ca^{2+} causes a conformational change resulting both in occlusion of the 2 Ca^{2+} ions as well as moving of the phosphorylation and nucleotide binding domains together (Tanford _et al_., 1987).

ATP BINDING

Ca^{2+} translocation is obligatorily coupled to the hydrolysis of ATP and pump activity shows a complex non Michaelis-Menten

dependence on substrate concentration (Yamamoto and Tonomura, 1967; Inesi et al., 1967). Direct titration of the nucleotide sites present on SR vesicles has been carried out with ATP in the absence of Ca^{2+} (Dupont, 1977; Moller et al., 1980; Arav et al., 1983) as well as with nonhydrolysable and slowly hydrolysable analogs (Dupont, 1977; Pang and Briggs, 1977). In all cases, one high affinity site was titrated with K_d between 2 and 10 µM. In addition, another site was indicated with K_d for ATP > 0.5 mM (Dupont, 1977). Attempts to titrate postulated low affinity sites has been hindered by difficulties encountered in measuring binding at ATP concentrations above about 0.2 mM (Yates and Duance, 1976). Similar results were obtained with the nonhydrolysable analogs AMP-PNP and AMP-PCP (Dupont, 1977; Pang and Briggs, 1977) and the slowly hydrolysable analog AMPCPP (Dupont, 1977). The high affinity TNP-nucleotides have also been used to titrate the nucleotide binding sites of the Ca^{2+}-ATPase and been found to bind to a total of 1 mol/mol enzyme (Dupont, 1982; Watanabe and Inesi, 1982; Davidson, 1986; Bishop et al., 1987). Saturation of the high affinity sites in the presence of Ca^{2+} results in the formation of an aspartophosphoryl-protein intermediate and catalytic activity (Yamamoto and Tonomura, 1967; Makinose, 1969; Inesi et al., 1970; Bastide et al., 1973; Degani and Boyer, 1973). Evidence has been presented for a conformational change following ATP binding (Coan and Inesi, 1977; Coan et al., 1979; Arav et al., 1983) and further evidence has been presented that a rate limiting conformational change occurs of the $E.Ca^{2+}.ATP$ intermediate prior to phosphoryl transfer (Petithory and Jencks, 1986; Stahl and Jencks, 1987). Higher concentrations of ATP result in a stimulation of activity (Yamamoto and Tonomura, 1967; Inesi et al., 1967; Kanazawa et al., 1971; The and Hasselbach, 1972; de Meis and Fialho de Mello, 1973; Verjovski-Almeida and Inesi, 1979; Taylor and Hattan, 1979; Ariki and Boyer, 1980; Moller et al., 1980; McIntosh and Boyer, 1983) and various models have been postulated to explain this phenomenon (see below).

1.3.3.2 PHOSPHOENZYME FORMATION

Reaction of the enzyme with catalytic concentrations of ATP and Ca^{2+} results in the formation of an ADP-sensitive phosphoenzyme, E_1-P followed by an ADP-insensitive phosphoenzyme, E_2-P (Makinose, 1969; Kanazawa et al., 1971; Shigekawa et al., 1978; Shigekawa and Dougherty, 1978a, b). Evidence has been presented for a rate limiting conformational change of the $E.Ca^{2+}$.ATP intermediate prior to phosphoryl transfer (Petithory and Jencks, 1986; Stahl and Jencks, 1987). There is a rapid burst of E-P formation followed by a decrease to steady-state levels; similarly, P_i production, after an initial lag phase corresponding to enzyme phosphorylation, rapidly increases then settles down to a steady-state rate (Kanazawa et al., 1971; Froehlich and Taylor, 1975, 1976). Occlusion of bound Ca^{2+} ions occurs in parallel with formation of E_1-P (Nakamura and Tonomura, 1982; Takisawa and Makinose, 1983; Wakabayashi and Shigekawa, 1984). Evidence has been presented that an ADP-insensitive E_2-P is formed after E_1-P (Bastide et al., 1973; Shigekawa and Dougherty, 1978b), and that this transformation is very rapid (Froehlich and Heller, 1985; Wang, 1987). Although Ca^{2+} release has been correlated with E-P transformation (Takisawa and Makinose, 1983; Andersen et al., 1985) it has also been suggested that Ca^{2+} remains occluded following phosphoenzyme isomerization, which is a rapid process, and is released following a slow conformational change (Froehlich and Heller, 1985). Alternatively, it has also been suggested that the Ca^{2+} ions are released before formation of the ADP-insensitive E_2-P (Pickart and Jencks, 1982). The discrepancy has arisen largely as a result of the interpretation of the biphasic kinetics observed following ADP induced decomposition of the phosphorylated enzyme. This has been interpreted as due to rapid dephosphorylation of the ADP-sensitive component followed by a slower conversion of the ADP-insensitive to ADP-sensitive E-P (Froehlich and Heller, 1985). The alternative view is that the biphasic kinetics is due to the rapid binding of ADP and phosphoryl transfer, and the slow rate-limiting dissociation of

ATP (Pickart and Jencks, 1982; Fernandez-Belda and Inesi, 1986; Stahl and Jencks, 1987).

Maximal phosphorylation levels with MgATP and Ca^{2+} as substrates, are approximately 4 nmol/mg of SR protein (Froehlich and Taylor, 1975; Verjovski-Almeida et al., 1978), which corresponds to about 0.5 mol/mol ATPase. Higher levels are obtained when P_i and Me_2SO are used, in a Ca^{2+}-depleted medium (de Meis et al., 1982). The fact that less than stoichiometric amounts of E-P are obtained has been ascribed to the presence both of other protein contaminants as well as inactive ATPases (Barrabin et al., 1984). Following HPLC purification values close to 7 nmol of E-P/mg of SR protein have been obtained in the presence of ATP and Ca^{2+} (Barrabin et al., 1984). This data has received another interpretation in which it is postulated that the HPLC purification separates out phosphorylated from nonphosphorylated enzymes, both active, rather than active from inactive (Ferreira and Verjovski-Almeida, 1988). These authors favor a postulate of "half-of-the-sites" reactivity with ATP as substrate and "full sites" phosphorylation with UTP as substrate (Ferreira and Verjovski-Almeida, 1988).

1.3.3.3 CALCIUM PUMP REVERSAL

The catalytic cycle of the Ca^{2+}-ATPase is readily reversible and vesicles preloaded with Ca^{2+} will drive ATP synthesis coupled to Ca^{2+} efflux when incubated in a medium containing ADP, P_i, and EGTA (Hasselbach, 1978; de Meis and Vianna, 1979). The Ca^{2+}-ATPase may be phosphorylated by high concentrations of P_i at acid pH in the presence of Mg^{2+} and absence of Ca^{2+} (Masuda and de Meis, 1973; Kanazawa and Boyer, 1973), and that the E-P so formed can 'drive' ATP synthesis from ADP when a Ca^{2+} jump is performed in the absence of a Ca^{2+} gradient (Knowles and Racker, 1975; Beil et al., 1977; Punzengruber et al., 1978; Kolassa et al., 1979). It was subsequently shown by de Meis et al. (1980) that in the presence of organic solvent phosphorylation of the enzyme by P_i occurred readily independent of pH and temperature. This resulted in the important conclusion that the main

thermodynamic barrier is partitioning of P_i from the aqueous medium into the hydrophobic active site and that once there the reaction occurs spontaneously (de Meis et al., 1980). This result has been confirmed in a study in which it was shown that the binding of $Mg^{2+}.P_i$ occurs slowly followed by a more rapid enzyme phosphorylation (Champeil et al., 1985). The true substrate for the phosphorylation reaction is $Mg^{2+}.P_i$ (Champeil et al., 1985), although higher concentrations result in inhibition probably as a result of the formation of an unreactive $E.Mg^{2+}$ complex (Guillain et al., 1982, 1984).

1.3.3.4 NUCLEOTIDE MODULATION OF ACTIVITY

The activity of the Ca^{2+}-ATPase is modulated by it's substrate ATP in a complex way (Ikemoto, 1982). The situation is complicated by several unanswered questions regarding for example the oligomericity of the pump and the stoichiometry of active sites.

In initial experiments, it was found that at concentrations of ATP higher than that required for saturation of the high affinity catalytic site, acceleration of activity occurred (Weber et al., 1966; Inesi et al., 1967), often seen as downward curvature of Lineweaver-Burke plots (Yamamoto and Tonomura, 1967; Kanazawa et al., 1971; Moller et al., 1980). A number of steady state and pre-steady state kinetic experiments have suggested that this is the result of an effect on phosphoenzyme turnover (Verjovski-Almeida et al., 1978), variously ascribed to acceleration of E-P formation (Kanazawa et al., 1971), E-P hydrolysis (de Meis and de Mello, 1973; Froehlich and Taylor, 1975; McIntosh and Boyer, 1983; Cable et al., 1985; Champeil and Guillain, 1986; Ross and McIntosh, 1987b; Champeil et al., 1988) and P_i release (de Meis and Boyer, 1978). Alternatively, it has also been attributed to acceleration of the E2 to E1 transition (Froehlich and Taylor, 1975; Sumida et al., 1978; Takisawa and Tonomura, 1978; Scofano et al., 1979; Guillain et al., 1981; Stahl and Jencks, 1984), or to an effect brought about as a result of binding at a second nucleotide site

(Froehlich and Taylor, 1975) in a dimeric model (Froehlich and Taylor, 1976; Verjovski-Almeida and Inesi, 1979; Hymel et al., 1984), or on the same enzyme monomer (Taylor and Hattan, 1979), or as a result of cooperativity in a monomeric model in which nucleotide binding induces a slow transition between two active forms of the enzyme (Neet and Green , 1977). As the modulatory effects are reproduced by the nonphosphorylating analogs AMP-PCP (Yates and Duance, 1976; Taylor and Hattan, 1979; Cable et al., 1985) and TNP-ATP (Dupont et al., 1985), they cannot be the result of phosphorylation of the enzyme.

ATP modulation shows 2-3 distinct phases depending on the substrate concentration (Moller et al., 1980; McIntosh and Boyer, 1983; Dupont et al., 1985). At lower concentrations the acceleration has been interpreted in terms of negative cooperativity in a dimeric model (Moller et al., 1980; McIntosh and Boyer, 1983), as it was found to be absent in detergent solubilized preparations (Dean and Tanford, 1978; Moller et al., 1980), and it may be related to an inhibition of E-P hydrolysis observed in this range (McIntosh and Boyer, 1983). At higher concentrations acceleration is again observed and was postulated to be due to ATP binding to the catalytic site after ADP departure (McIntosh and Boyer, 1983). This latter model is seemingly contradicted by the lack of competition by ATP of product induced inhibition of turnover by ADP (Taylor and Hattan, 1979; Coll and Murphy, 1985).

Support for a different family of nucleotide sites was obtained with the ATP analog TNP-ATP (Dupont et al., 1982; Watanabe and Inesi, 1982; Bishop et al., 1984). TNP-ATP was found to bind to one site per ATPase monomer, and half of the bound probe was displaced to a site of higher fluorescence in the presence of catalytic concentrations of ATP and Ca^{2+}. Occupation of the high fluorescence site by TNP-ATP was furthermore correlated with acceleration of turnover (Dupont et al., 1985), while occupation by TNP-AMP inhibited acceleration by millimolar concentrations of ATP (Bishop et al., 1987). The binding of TNP-nucleotides was also found to be inhibited either by prior labeling of the enzyme with FITC or by addition of ADP

to the phosphorylated enzyme-TNP-nucleotide complex (Nakamoto and Inesi, 1984). This has been interpreted as support for the model in which accelerating ATP occupies the phosphorylated catalytic site following ADP departure (Nakamoto and Inesi, 1984). It was further shown that 2 classes of sites can be distinguished based on their affinity for TNP-ATP in the presence of Mg^{2+} or absence of H^+ and interpreted as evidence for a dimeric model (Dupont et al., 1985).

More recently, it has been shown that millimolar concentrations of ATP accelerate phosphoenzyme isomerization following binding to Ca_2E_1-P (Champeil et al., 1986; Champeil and Guillain, 1986; Ross and McIntosh, 1987b). It was furthermore found that this effect is only produced by metal-free ATP than by Mg.ATP (Champeil et al., 1986; Champeil and Guillain, 1986). ATP acceleration of dephosphorylation has also recently been demonstrated in the 10-100 µM range, by rapid filtration, and again it was found that the metal-free form is responsible for the modulatory effect (Champeil et al., 1988).

In summary, it seems that nucleotides are able to bind to the phosphorylated catalytic site following ADP departure (McIntosh and Boyer, 1983), and accelerate the rates of various partial reactions leading to dephosphorylation of the enzyme. The site of acceleration of turnover depends on the step which is rate limiting under the conditions of the reaction. Under turnover conditions, most of the enzyme is found in a phosphorylated form and consequently, acceleration of E_1-P to E_2-P or dephosphorylation (depending on conditions), are probably responsible for the overall acceleration observed (Champeil et al., 1986).

2 EXPERIMENTAL PROCEDURES

2.1 MATERIALS

ATP (disodium salt) was from Sigma. HPLC grade acetonitrile, methanol and isopropanol were from Burdick and Jackson. TEA (Fluka) was distilled from ninhydrin and stored at 4 ^{o}C under nitrogen. DMF (BDH) was dried over molecular sieve 4A (Merck). TFA was Sequanal grade from Pierce. Constant boiling (6 N) HCl, PITC and amino acid standards (H) were from Pierce. The following synthetic peptides, kindly donated by Dr.R. de L. Milton, were used: #AE8 (PSTDCPMEVIK), #AD6 (KSRLPGPSDTPILPQ(OH)) and #AC9 (SQRGKQGGKARA). All other chemicals used were of analytical grade.

[2-^{3}H]-ATP (ammonium salt), [8-^{14}C]-ATP (ammonium salt), [^{32}P]P_i and [6,6'(n)-^{3}H] sucrose were from Amersham.

2.2 HPLC METHODS

Analytical and semi-preparative HPLC of nucleotides, proteins and peptides was carried out on a WATERS reversed phase Radial-Pak C18 (microbondapak or novapak, of particle size 10 µm and 4 µm, respectively) column contained within a Z module radial compression unit. Solvent was delivered by a Spectra-Physics 8700 XR pump and gradient controller with low pressure mixing and ternary gradient capability. Effluent was monitored on a Spectra-Physics 8300 fixed wavelength (254 nm) detector and/or an LKB 2151 variable wavelength detector.

Amino acid analysis and sequencing were carried out on a complete WATERS picotag system in pyrolyzed tubes. The PITC derivatives were analysed on a Picotag (Waters C18) column kept at 45 ^{o}C in a temperature controller and PTH derivatives were analyzed on a Vydac C18 protein and peptide column also maintained at 45 ^{o}C. Solvent was delivered by a model 510 pump and a model M-45 pump with model 680 gradient controller and model 712 WISP autosampler. Effluent was monitored using a model 441 fixed wavelength detector (254 nm) and data collected

on a model 740 data collection module and/or a Tectronix 4054 A desktop computer.

2.3 SYNTHESIS AND PURIFICATION OF TNP-8N_3-NUCLEOTIDES

TNP-8N_3-nucleotides were synthesized in three steps. Firstly ATP was brominated in the 8 position on the adenine ring. In the second step, the Br was replaced with an azido group and finally, the trinitrophenyl group was added to the ribose moeity.

2.3.1 Synthesis and purification of 8-BrATP

Bromination of ATP was carried out according to the method of Ikehara and Uesugi (1969), modified slightly to accomodate a five-fold increase in starting material. ATP (3 g) was dissolved in 200 ml sodium acetate buffer (1 M, pH 4.0). Freshly prepared saturated bromine water, 50 ml, was added to this and the reaction mixture incubated overnight (17 h) at room temperature in the dark. The solution was neutralized by addition of 1.5 ml $NaHSO_3$ solution (37%, Merck) and left to stand for 1 h at room temperature. Solvent was removed in vacuo at 35 ^{o}C with small additions of methanol. The residue was dissolved in 500 ml H_2O and applied to a column (3 X 25 cm) of H_2O washed Dowex 1 x 8 (Cl^- form, 100 - 200 mesh) by gravity. The loaded column was then washed with 1.8 l H_2O at a flow rate of 300 ml/h and then a linear gradient applied from 0.13 M LiCl + 0.001 N HCl to 0.25 M LiCl + 0.001 N HCl (4 l + 4 l) at the same flow rate. The absorbance of the effluent was monitored at 254 nm with an LKB Uvicord detector. At the end of the gradient the column was washed with a further 2 l of the more concentrated eluent to elute the remaining 8-BrATP. Fractions corresponding to the 8-BrATP peak were identified spectrophotometrically ($_{max}$ = 263 nm), pooled and lyophilized to dryness. The lyophilizate was redissolved in methanol:H_2O (1:1, v/v) and the nucleotide precipitated by addition of excess acetone (13.3 ml for every ml methanol:H_2O added). The precipitate was collected on filters, dried and stored in solid form at -20 ^{o}C. Purity was checked by

HPLC on a Radial Pak SAX column eluting with a gradient of 0 - 10% acetonitrile in 0.15 M KH_2PO_4, pH 7.0. Brominated nucleotides elute after their unmodified counterparts under these conditions.

2.3.2 Synthesis and purification of 8-N_3-nucleotides

Displacement of the Br group with an azido group was carried out at high temperature in organic solvent by reaction with triethylammonium azide according to the method of Schafer et al. (1978b). In order to make the nucleotide soluble in the organic solvent it was first converted to the triethylammonium salt. 8-BrATP (0.9 g, Li salt) was loaded onto a column (1.5 x 7 cm) of DEAE-Sephadex A-25 (HCO_3^- form), eluted with 0.5 M TEA.HCO_3^- and then dried in vacuo by coevaporating with small additions of methanol. The nucleotides were dried in vacuo over P_2O_5 for 12 h, and finally dissolved in 30 ml dry DMF.

The synthesis of triethylammonium azide was carried out according to Vogel (1962) in the following way: NaN_3, 3 g, was added to a boiling tube on ice and 12 ml H_2O added slowly with stirring. 10 ml benzene was layered on top and 0.96 ml concentrated H_2SO_4 added dropwise while stirring. The mixture was stirred for 15 min. A portion of the organic layer (6 ml) was removed and added with 1.1 ml redistilled TEA to the DMF containing 8-BrATP in a stoppered 40 ml boiling tube.

The reaction mixture was heated to 75 ^oC in a water bath in the dark overnight (15 h), and then the solvent was removed in vacuo at 50 ^oC. The residue was redissolved in 20 ml H_2O and applied to a column (3 X 60 cm) of H_2O washed DEAE-Sephadex A-25 (HCO_3^- form) in the dark at 5 ^oC. The nucleotides were applied by gravity and the column was washed with 100 ml H_2O. The nucleotides were eluted with a gradient of 0.06 - 0.6 M TEA.HCO_3^- (3 l + 3 l) at a flow rate of 150 ml/h. Fractions (25 ml each) corresponding to $8N_3AMP$, $8N_3ADP$ and $8N_3ATP$ were identified spectrophotometrically, pooled and evaporated to dryness. Last traces of TEA.HCO_3^- were removed by coevaporation with small additions of methanol. Purified $8N_3$-nucleotides were stored in

methanol at -60 ^{o}C. Purity was checked by HPLC on a reversed phase C18 column eluting with acetonitrile in KH_2PO_4 pH 7.0.

2.3.3 Synthesis and purification of TNP-8N_3-nucleotides

Introduction of the trinitrophenyl group was carried out using a modification of the method developed for ATP by Hiratsuka (1982). A solution of 218 mg of Na_2CO_3 and 151 mg of $NaHCO_3$ in 2.4 ml H_2O was added to a suspension of 96 mg of TNBS and 200 mg of DTNB in H_2O. Immediately thereafter, the reaction mixture was added to 100 umoles of lyophilized 8N_3- nucleotide, and incubated overnight in the dark at room temperature with continuous stirring.

Purification was carried out by HPLC in the following way. 1.2 ml amounts were fractionated on a Radial Pak C18 column using a gradient from 0 - 30% acetonitrile in either 20 or 75 mM KH_2PO_4, pH 5.0 at a flow rate of 5 ml/min. The shape of the gradient and phosphate concentration were altered to optimally position the TNP-8N_3-nucleotide peak. TNP-8N_3-nucleotide peaks were identified spectrophotometrically by the presence of absorbance maxima at 281, 408 and 468 nm, pooled and lyophilized to dryness in the dark. When necessary a second purification was carried out under the same conditions. Samples were desalted on the same column with acetonitrile:H_2O (1:1, v/v) as solvent. The nucleotides were judged pure by the following criteria: a single peak on HPLC, a single spot on TLC and a spectrophotometric peak with UV absorption maxima between 280 and 283 nm and visible absorption maxima at 408 and 468 nm. TLC was carried out on polyethyleneimine cellulose TLC plates developed in 2 M formic acid + 0.5 M LiCl.

TNP-ATP was synthesized as described by Hiratsuka (1982).

2.4 SYNTHESIS AND PURIFICATION OF RADIOACTIVE NUCLEOTIDES

2.4.1 Synthesis and purification of [γ-^{32}P]-nucleotides

[γ-^{32}P]-ATP was synthesized by the exchange reaction of Glynn

and Chappel (1964). TNP-[γ-^{32}P]-ATP and TNP-8N_3-[γ-^{32}P]-ATP were synthesized by the same method as modified by Watanabe and Inesi (1982). In the case of the azido derivatives, dithiothreitol was not included in the reaction mixture as it reduces the azido group (Briggs et al., 1980). The [γ-^{32}P]-nucleotides were purified by anion exchange chromatography and assayed for purity on PEI-cellulose TLC plates developed in 2 M formic acid + 0.5 M LiCl. Pure compounds gave a single UV absorbing spot coinciding with a peak of radioactivity seen by proportional gas flow scanning.

2.4.2 Synthesis and purification of [8-^{14}C]- and [2-^{3}H]-nucleotides

TNP-8N_3-[8-^{14}C]-nucleotides and TNP-8N_3-[2-^{3}H]-nucleotides were synthesized from [8-^{14}C]-ATP and [2-^{3}H]-ATP (both ammonium salt, Amersham) using the methods described above for the cold synthesis but adapted for the micro amounts involved. [8-^{14}C]-ATP (ammonium salt, 255 nmoles) in aqueous solution was converted to the sodium salt by passage through a column (0.7 x 9 cm) of Dowex 50W X 2 (100-200 mesh, Na^+ form). This step was necessary as we found that the ammonium ion in the radioactive solution discharged the colour of the reaction mixture on mixing with bromine water. The eluate was lyophilized to dryness, added to 5 umoles of unlabeled ATP (disodium salt), and dissolved in 0.3 ml acetate buffer (1 M, pH 4.0) in a 0.4 ml sealed plastic tube. Freshly prepared, saturated bromine water (0.1 ml) was added and the reaction mixture incubated overnight at room temperature in the dark. The mixture was injected directly on to a Radial Pak C18 HPLC column and fractionated on a gradient of 0 - 5% acetonitrile containing 0.1 M TEA.HCO_3^- (pH 7.0) over 25 min at a flow rate of 4 ml/min. The 8-Br-[8-^{14}C]-ATP peak was collected and lyophilized to dryness. Last traces of TEA.HCO_3^- were removed by coevaporating four times with 0.5 ml methanol and the nucleotides were finally dried over P_2O_5 at 100 mtorr. The azido substitution reaction was carried out by dissolving the 8-Br-[8-^{14}C]-ATP in 0.3 ml dry DMF and adding to this 12 µl

redistilled TEA and 60 µl benzene saturated with hydrazoic acid prepared as described in section 2.3.2. The reaction mixture was incubated in the dark at 75 ^{o}C for 12 h and then evaporated to dryness at 40 ^{o}C under reduced pressure.

The reaction with TNBS was carried out with 0.24 ml of the buffered TNBS and DTNB reaction mixture described in section 2.3.2, and were purified by the same HPLC protocol. Mono-, di-, and triphosphate derivatives were fairly well separated from all other species with one HPLC elution, but each was routinely rechromatographed to obtain the pure compound. Purity was assesed by the same criteria as for the nonradiolabelled nucleotides, but in addition TLC plates were checked by proportional gas flow scanning to ensure that peaks of radioactivity coincided with the positions of the TNP-8N_3-nucleotides. Nucleotides were stored as an aqueous solution at -20 ^{o}C.

TNP-8N_3-[2-^{3}H]-nucleotides were synthesized by the same method. The yield of radioactive nucleotide in this case, however, was less (approx. 1%) as the bromination reaction mixture was found to promote exchange of the tritium label.

2.5 SDS PAGE ON SLAB GELS

SDS PAGE of solubilized SR vesicles was carried out according to the method of Laemmli (1970). The running gel contained 7% acrylamide and was topped by a layer of stacking gel containing 3% acrylamide. The concentration of BIS acrylamide cross-linker used was 4% of the total amount of acrylamide and the SDS concentration was 0.1% throughout. Protein samples, 8-10 µg, were dissolved in an appropriate volume of solubilization buffer (2% SDS, 5 M urea, 0.5% 2-mercaptoethanol and a few grains of bromophenol blue), then loaded onto the gel. Gels were electrophoresed at 5 ^{o}C at a constant current of 20-30 mA for a few hours until the tracking dye was located a few cm from the bottom. The gels were then removed, fixed in 30% methanol + 10% acetic acid for 30 min, and then stained in 0.1% (w/v) Coomassie Blue in 30% methanol + 10% trichloroacetic acid for at least 3 h.

Destaining was carried out by means of a single wash in 30% ethanol + 10% acetic acid followed by clarification in a couple of washes of 7% acetic acid. The gels were then photographed and dried.

2.6 SR VESICLE PREPARATION

SR vesicles were prepared by differential centrifugation of back and hind leg muscle homogenates from fasted rabbits according to the method of Eletr and Inesi (1972). The vesicles were finally resuspended in a medium of 5 mM imidazole, pH 7.4 + 0.3 M sucrose at a concentration of about 10 mg/ml. Aliquots were rapidly frozen in liquid nitrogen and stored at -60 ^{o}C. Protein concentration was determined from the absorbance at 280 nm of an aliquot dissolved in 20 mM MOPS.TRIS, pH 7.2 and 1% SDS using a conversion factor of 0.90 standardized by the method of Lowry et al. (1951) using bovine serum albumin as standard. Protein composition was routinely checked by SDS PAGE (Laemmli, 1970) and the vesicles usually consisted of 80 - 90% Ca^{2+}-ATPase with small amounts of calsequestrin and sometimes phosphorylase and myosin as additional proteins.

2.7 ATPase ASSAY

Hydrolysis of the γ-phosphate group from nucleoside triphosphates catalyzed by SR vesicles was assayed by three different methods: by measurement of radioactive $[^{32}P]P_i$ following extraction of the nucleotide into charcoal (Grubmeyer and Penefsky, 1981a), by coupling ADP production to oxidation of NADH measured spectrophotometrically (Horgan et al., 1972) and lastly by measuring protons released in a pH-stat method (Dupont, 1985).

2.7.1 Charcoal method

The conditions we used were those described by Dupont et al. (1982). This involved incubation of SR vesicles (0.1 mg of SR

protein/ml) in a medium of 20 mM MOPS, pH 7.2 (25 ^{o}C), 100 mM KCl, 5 mM $MgCl_2$, 5 mM oxalate, 1 mM EGTA or 0.1 mM $CaCl_2$, and varying concentrations of TNP-$8N_3$-nucleotides. Hydrolysis was initiated by the addition of [γ-^{32}P]-ATP to a final concentration of 40, 100 or 200 μM and then 0.2 ml aliquots were removed after 10, 20 and 30 s and quenched with 6% (v/v) ice cold perchloric acid. 0.2 ml charcoal suspension in 0.1 N HCl (250 mg/ml) was then added and vortexed. After spinning down the charcoal in a microfuge at room temperature, 0.2 ml of the supernatant containing hydrolysed [^{32}P]P_i was removed and counted in 5 ml Insta-gel.

Hydrolysis of TNP-$8N_3$-[γ-^{32}P]-ATP was measured in a medium of 50 mM MOPS, pH 7.0 (25 ^{o}C), 100 mM KCl, 0.1 mg of SR protein/ml, 4% (w/w) A23187 and either (i) 1.5 mM EDTA, (ii) 5 mM $MgCl_2$ and 0.2 mM EGTA or (iii) 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The reaction was initiated by addition of TNP-$8N_3$-[γ-^{32}P]-ATP (final concentration = 0.2 mM) and the amount of released P_i was determined by the charcoal procedure described above (Dupont _et al_., 1982).

2.7.2 Coupled enzyme method

Final concentrations in the Stock Buffer were: 20 mM MOPS, pH 6.8, (25 ^{o}C), 100 mM KCL, 5 mM $MgCl_2$, 0.5 mM EGTA, 2.5 mM phosphoenolpyruvate, 0.1 mM NADH, 8 units of pyruvate kinase and 8 units of lactate dehydrogenase. To 2.5 ml of Stock Buffer was added 25 µl SR vesicles (0.2 mg/ml) and 4% (w/w) A 23187. Reaction was initiated by addition of ATP to 1 mM and the Mg^{2+} dependent activity determined spectrophotometrically from the change in absorbance at 340 nm. Following this Ca^{2+} was added to 0.5 mM and the total activity recorded. The Ca^{2+} dependent ATPase activity was determined by subtracting the Mg^{2+} dependent activity from the total activity and converting to umoles/min using the extinction coefficient of NADH (6.22 Abs.units.mM^{-1}. cm^{-1}).

2.7.3 pH-stat method

The pH-stat assay was carried out in the same medium used by Dupont et al. (1985). Final concentrations in the reaction mixture were: 1 mM MOPS, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 40 µM X-537A, 100 µM $CaCl_2$, 22 µg of SR protein/ml, 75 µM ATP and 2 - 5 µM TNP-$8N_3$-nucleotide. The Mg^{2+}-dependent activity was recorded by addition of ATP to the Ca^{2+}-depleted medium and measurement of the time dependent addition of aliquots of NaOH solution via an autoburette to maintain the pH at 7.4. Total activity was recorded after addition of Ca^{2+}, and Ca^{2+} dependent activity calculated by subtraction. H^+ release was converted to nmoles P_i hydrolysed using a conversion factor of 0.7 H^+/P_i released.

2.8 BINDING ASSAYS

Binding of TNP-$8N_3$-nucleotides to SR vesicles was measured by a filtration method (Champeil and Guillain, 1986), inhibition of active site cross-linking by glutaraldehyde (Ross and McIntosh, 1987a, b) and fluorescence titration (Dupont et al., 1982; Nakamoto and Inesi, 1984). All manipulations were carried out in the dark, or in dim light.

2.8.1 Filtration assay

Nucleotide binding by the filtration assay was carried out by equilibrating SR vesicles (0.3 mg of protein) with varying concentrations of TNP-$8N_3$-[8-^{14}C]-nucleotides in 50 mM MOPS.TRIS, pH 7.0 at room temperature, 0.1 mM [^{3}H] sucrose and 5 mM $MgCl_2$. At low concentrations of nucleotide, the reaction volume was increased up to 20 ml to ensure an excess of free over bound nucleotide. After mixing, and equilibrating at room temperature for 15 min, the suspension was filtered through two glassfiber filters (Whatman GF/F) under mild vacuum. The filters were removed, shaken overnight separately in 10 ml Insta-gel (Packard Instruments) + 10 µl perchloric acid and counted by liquid scintillation counting. The [^{3}H] count was used to calculate

the wet volume of the filter and the second filter served as a check on this value.

2.8.2 Cross-linking assay

Intramolecular cross-linking by glutaraldehyde was carried out by a modified procedure of that described previously (Ross and McIntosh, 1987b). SR vesicles, 0.1 mg, were added to 20 ml of 50 mM MOPS/TEA, pH 8.0 with either 1 mM EDTA or 1 mM EGTA + 5 mM $MgCl_2$ and equilibrated at room temperature with the required concentration of TNP-8N_3-nucleotide. The large volume ensured that the free/bound nucleotide ratio was high. Cross-linking was initiated by the addition of 0.5 mM glutaraldehyde and quenched after 2.5 min by addition of 25 µl hydrazine. Vesicles were collected on GF/F filters by filtration under reduced pressure and washed once with 50 mM phosphate pH 7.0. Filters were shaken vigorously overnight in 1 ml electrophoresis sample buffer (0.1% (w/v sodium dodecyl sulfate, 0.01% (v/v) Bromophenol Blue, 0.1% (v/v) 2-mercaptoethanol and 0.1 mM sodium phosphate, pH 7.0). Aliquots of 60 µl were then electrophoresed on 7% acrylamide gels (Ross and McIntosh, 1987a, b). The dried gels were scanned and the appropriate peaks integrated.

2.8.3 Fluorescence titration

Fluorescence titration of TNP-8N_3-nucleotide binding to SR vesicles was carried out under nonturnover and turnover conditions. In the case of the former, final concentrations in the cuvettes were 0.2 mg of protein/ml, 50 mM MOPS/TRIS, pH 7.0, either 1 mM EDTA or 20 mM $MgCl_2$ and variable concentrations of TNP-nucleotides. Measurements were made on an Aminco spectrofluorimeter (model SPF-500) with the excitation and emission wavelengths set at 418 and 530 nm respectively. In the experiments involving enzyme turnover, the final concentrations were 0.2 mg of protein/ ml, 20 mM MOPS/TRIS, pH 8.0, 20% (w/v) glycerol, 5 mM $MgCl_2$, 50 µM $CaCl_2$ and variable concentrations of TNP-nucleotides. Turnover was initiated by the addition of 100

μM ATP and 50 μM $CaCl_2$ and then stopped by addition of 1 mM EGTA. The decrease in fluorescence upon addition of EGTA was taken as indicating the extent of binding to the superfluorescent site.

2.9 PHOTOAFFINITY LABELING OF SR VESICLES

TNP-8N_3-nucleotides were irradiated at room temperature in quartz cuvettes positioned 8 cm from a Xenon light source of 150 W and with a front facing reflector positioned behind the cuvette. The light beam was filtered in front and behind by toluene contained in standard (1 cm path length) quartz cuvettes. Irradiation time was usually 3 min. Under these conditions and in the absence of azido nucleotide, inactivation of ATPase activity was less than 3%.

The standard irradiation medium was 20 mM Buffer, 20% (w/v) glycerol, 5 mM $MgCl_2$, 0.2 mg of SR protein/ml and concentrations of TNP-8N_3-[2-^{3}H]-AMP, EGTA, $CaCl_2$ and ATP indicated in the legends to the figures. The Buffers were as follows: pH 6.0-6.5, MES/TMAH; pH 7.0-7.5, MOPS/TMAH; pH 8.0-8.5, HEPPS/TMAH; pH 9.0-9.5, CHES/TMAH. Controls were prepared by irradiating the vesicles in the absence of azido-nucleotide and then adding the latter to the particular concentration of the test samples.

Covalent labeling of SR vesicles was measured using the radiolabeled nucleotide derivatives. The vesicles (0.2 mg of protein/ml) were collected on glass fiber filters (Whatman GF/F) under mild vacuum and the filters were washed extensively with 50 mM phosphate buffer, pH 7.0. Filters were counted in 10 ml Insta-gel. Protein loss was less than 5%.

2.10 ACTIVE SITE CROSS-LINKING OF PHOTOLABELED VESICLES

Active site cross-linking of photolabeled vesicles with glutaraldehyde (Ross and McIntosh, 1987a, b) was carried out by diluting the irradiated samples 100 fold into 50 ml of 50 mM MOPS/TEA, pH 8.0, and 50 μM $CaCl_2$, to lower the concentration of free nucleotide to below the K_d and then reacting with glutaraldehyde (0.146 mM) for 2 h at room temperature. The

reaction was quenched with 5 µl hydrazine and the vesicles collected on GF/F filters and washed with 50 mM phosphate buffer, pH 7.0. The filters were shaken overnight in 1 ml electrophoresis sample buffer (see section 2.8.2) and then 80 µl aliquots were electrophoresed on 7% acrylamide gels. The concentration of stock glutaraldehyde was determined by isolation of the 2,4-dinitrophenyl hydrazone derivative.

2.11 FLUORESCENCE MEASUREMENTS OF PHOTOLABELED VESICLES

Photolabeled vesicles were prepared as above in a medium at pH 8.5. Controls were prepared by adding TNP-8N_3AMP after irradiation. Following irradiation, 0.1 mM ATP was added to the samples, if ATP was not already present, and then passed through a column (0.3 x 1 cm) of Dowex 1 X4 resin, equilibrated with the irradiation medium to remove noncovalently attached nucleotide. Loss of protein was approximately 10 % and the fluorescence of the samples was compared to controls that had also been passed through the columns. The addition of ATP was found to aid removal of noncovalently bound TNP-8N_3AMP. The fluorescence was measured, as above, on the undiluted samples with the additions indicated in the legend to the figure.

2.12 TUBE GEL ELECTROPHORESIS

Location and quantitation of the covalently attached nucleotide on the ATPase and tryptic fragments and subfragments was carried out using a tube gel electrophoresis method with 10% acrylamide (Weber and Osborne, 1969). The gels were either stained with Commassie Blue or cut into 1 mm slices and dissolved by incubation at 70 ^{o}C with 1 ml of 30% hydrogen peroxide in scintillation vials. The samples were counted in 10 ml Insta-gel.

2.13 PEPTIDE MAPPING

2.13.1 Isolation of labeled peptides

Covalently labeled tryptic peptides were isolated essentially as described by Kirley et al. (1984). Photolabeling was carried out in a medium of 25 mM Hepps/TMAH, pH 8.5, 20% glycerol, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 µM TNP-8N_3-[2-^{3}H]-AMP, 0.2 mg/ml SR and either with or without 250 µM ATP. 40 samples of 2.5 ml, both with and without ATP, were irradiated for 3 min and pooled separately. The two batches were then diluted 1:2 with H_2O and centrifuged for 60 min at 50000 rpm. The labeled pellet was resuspended in 10 mM MOPS.TEA, pH 7.2, 1 mM EDTA and 15 mM NaCl, using a Dounce homogeniser. Trypsin was added to a final concentration of 1:20 (w/w, trypsin:SR). The trypsinization reaction was carried out at 37 ^{o}C for 7.5 h then terminated by addition of 5 mM phenylmethylsulfonyl fluoride and the membranes pelleted by centrifugation at 40000 rpm for 40 min. The supernatant was lyophilized to dryness, redissolved in H_2O, and fractionated by HPLC on a Radial Pak C18 column. Three different binary mobile phases were used each of which involved injecting the sample on in buffer A and eluting with a gradient of buffer B. In the first system, buffer A was 0.1% TFA in H_2O and buffer B was 0.1% TFA in 60% acetonitrile. In the second system, buffer A was 5 mM PIC reagent A (tetrabutylammonium phosphate, WATERS) in H_2O and buffer B was 5 mM PIC reagent A in 60% acetonitrile. In the third system buffer A was 0.01 M ammonium acetate, pH 4.0 and buffer B was 80% isopropanol and 20% buffer A. A flow rate of 3 ml/min was used with the first two systems and 2 ml/min with the third system. The particular mobile phase used is indicated in the legend to the figures. The absorbance of the effluent was monitored at 215-240 nm and at 254 nm and peaks or fractions were collected manually and assayed for radioactivity. The radioactive peaks were lyophilized and then rechromatographed using a different mobile phase. The purified peptides were lyophilized to dryness and stored at -20 ^{o}C prior to amino acid analysis and sequencing.

SR vesicles were also labeled with FITC by incubation at 1 mg/ml in 100 mM TRIS.HCl, pH 8.6, 2mM EDTA and 5 µM FITC for 30 min in the dark at room temperature. The reaction was quenched by the addition of 15 µM 2-mercaptoethanol and then the vesicles collected by centrifugation at 40000 rpm for 60 min. The vesicles were then resuspended in 10 mM MOPS/triethanolamine, pH 7.2, homogenized, pelleted again and then trypsinized as described above for the TNP-8N_3AMP labeled peptides.

2.13.2 Amino acid analysis of labeled peptides

Purified peptides were dissolved in H_2O and an aliquot thereof removed into pyrolyzed test-tubes. Amino acid analysis was carried out according to the complete Picotag method (Waters). Samples were dried in vacuo using the Waters workstation and evacuation vessels. Vapour Phase hydrolysis was carried out overnight (17 h) with constant boiling HCl at 75 ^oC followed by removal of the gas, neutralization with TEA and derivatization with PITC. After removal of the unreacted PITC in vacuo, the derivatized amino acids were dissolved in 100 µl sample diluent (Waters) and placed in the WISP autosampler and analyzed by HPLC. The two buffers used for HPLC were, Picotag Eluent A (0.14 M sodium acetate, pH 6.4 + 6% acetonitrile), and Picotag Eluent B (60% acetonitrile in H_2O). Samples were injected onto the column in 100% A and eluted with a gradient of B. Standard amino acids (Pierce amino acid standard H) were incorporated in the general protocol before and after the hydrolysis step in order to measure the lability of the different amino acids to acid hydrolysis.

2.13.3 Amino acid sequencing

Sequence determination of purified peptides and peptide standards was carried out according to the method of Brandt and Frank (1988). Filter holders were made by drawing out Kimble 12 x 75 mm disposable test-tubes over a flame and were fitted with 10 mm diameter discs of Whatman GF/C filter paper. These were

treated with polybrene and then subjected to a blank run of fixing, derivatization and hydrolysis described below. Thereafter a few microlitres of peptide containing solution was applied to the filter and evacuated in a vacuum vessel. Sequential Edman degradation of the peptide was then performed as described (Brandt and Frank, 1988) and the PTC-amino acids washed into pyrolyzed boiling tubes. The PTC-amino acids were then converted to the PTH-amino acids by treating with acid and then analyzed by HPLC as described above. The gradient used was 10 - 50% acetonitrile in 17 mM ammonium acetate pH 4.5. Experimental peaks were identified by comparing both to amino acid standards and to similarly treated synthetic peptide standards.

3 RESULTS

3.1 SYNTHESIS OF TNP $8N_3$ NUCLEOTIDES

3.1.1 Synthesis and purification of 8-Br-ATP

The elution profile of the reaction products following bromination of ATP and chromatography on a column of Dowex 1 X8 anion exchange resin is shown in Fig. 3.1. The absorbance spectrum of the purified compound is compared with that of ATP in the inset. The peak absorbance at 263 nm is characteristic of 8-BrATP. It was necessary to obtain a good separation at this stage to prevent carryover of ATP which, if allowed, could have resulted in contamination of the final prparation with TNP-ATP. Contamination with even a small amount of TNP-ATP would complicate the interpretation of results because of the high affinity of the analog for the Ca^{2+}-ATPase. Contaminating ATP of the nucleotide prepared in this way was judged to be less than 5% by HPLC. The yield of 8-Br-ATP was 50 % from starting ATP and the final preparation contained a certain amount of LiCl, which was removed in the next step.

3.1.2 Synthesis and Purification of 8-N_3-nucleotide

The elution profile of the reaction products following the azido reaction and chromatography on a column of DEAE-Sephadex A-25 is shown in Fig. 3.2. The absorbance spectrum of $8N_3ATP$ is compared with that of 8-BrATP in the inset. A good separation was obtained between $8N_3AMP$, $8N_3ADP$, and $8N_3ATP$. Smaller amounts of 8-BrAMP, 8-BrADP, and 8-BrATP eluted just ahead and were well separated from their respective azido derivatives. They are not seen in the profile as they do not absorb strongly at 280 nm. The purified compounds had absorbance maxima shifted to 281 nm (Fig. 3.2, inset) consistent with the extension of the conjugated adenine ring structure by the azido group (Schafer *et al.*, 1978). The peak at 281 nm was susceptible to light and disappeared after irradiation (see later).

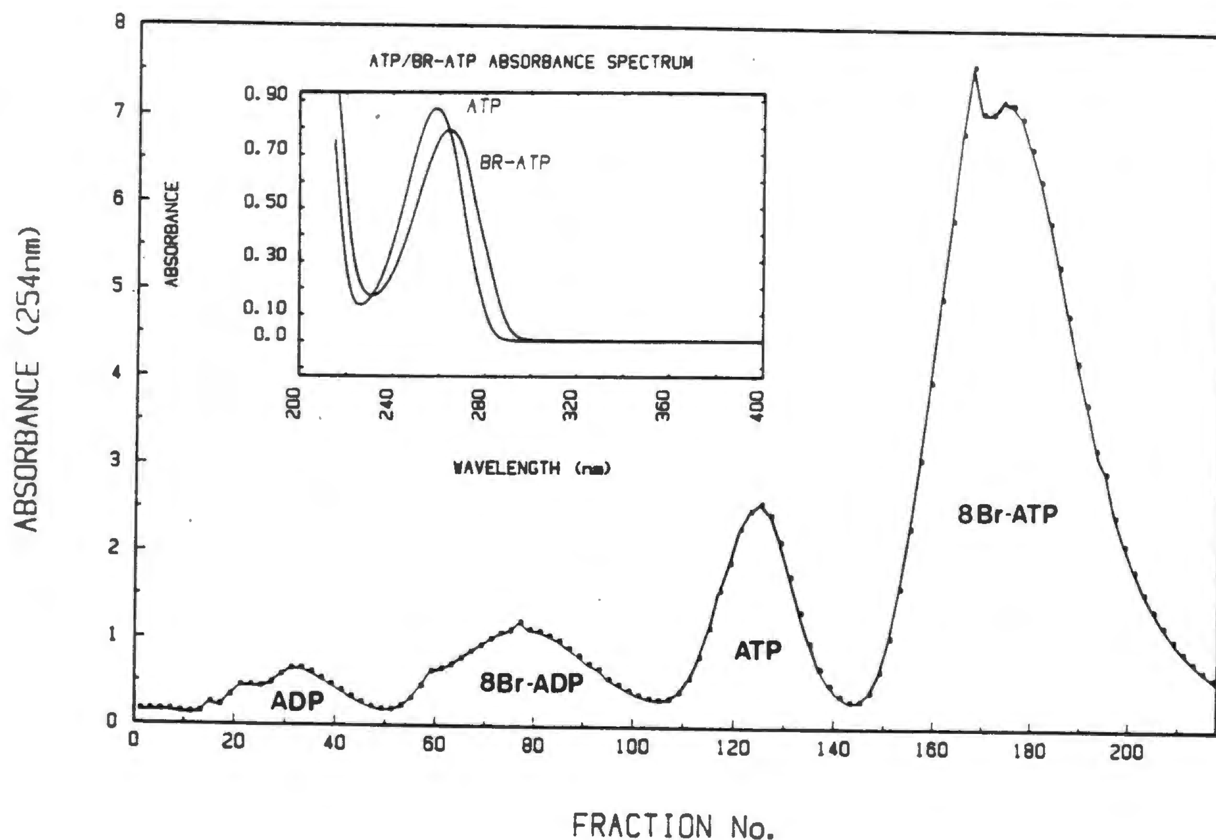


Figure 3.1 <u>**Anion Exchange Separation and Spectral Properties of Brominated Nucleotides**</u>.

Nucleotides (5 mmol) were applied to a column (3 x 25 cm) of Dowex 1 X8 and eluted with a linear gradient from 0.13 M LiCl + 0.001 N HCl to 0.25 M LiCl + 0.001 N HCl (4 l + 4 l) followed by 2.5 l of 0.25 M LiCl + 0.001 N HCl at a flow rate of 300 ml/h. Fractions were collected and the absorbance measured at 254 nm.

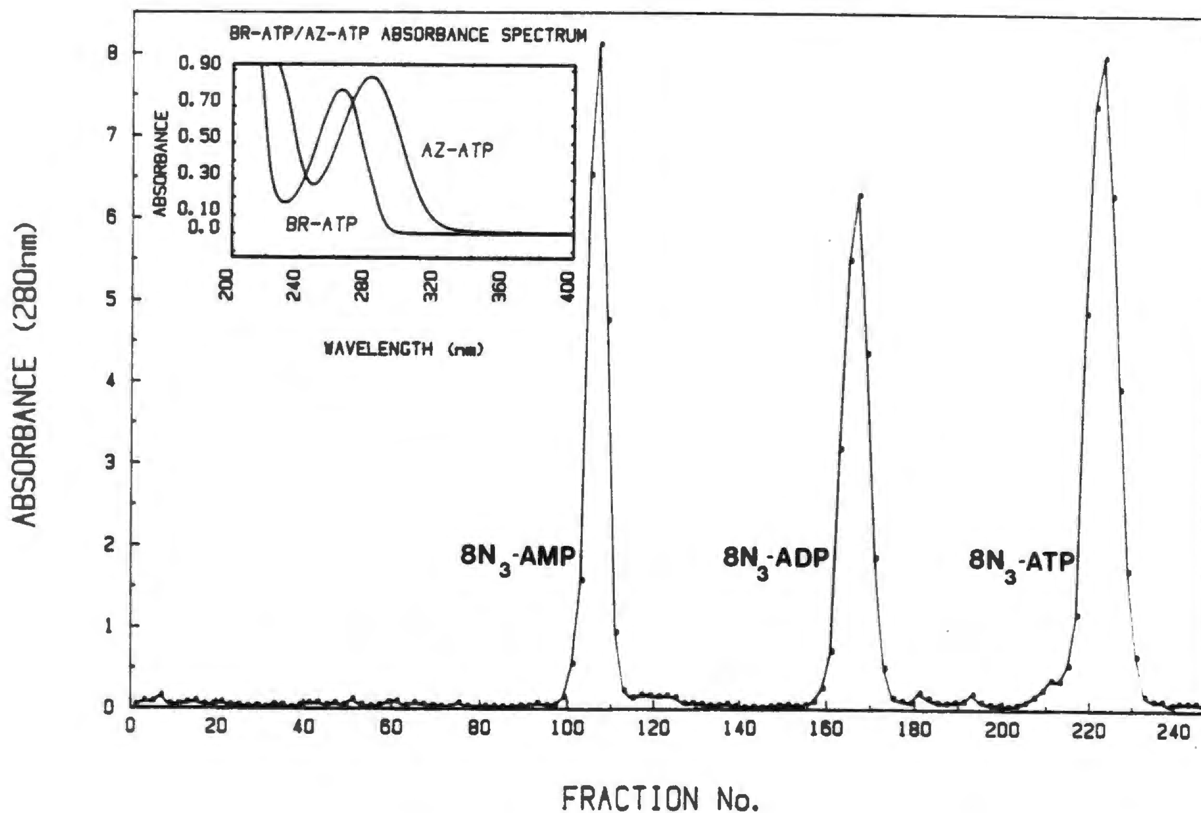


Figure 3.2 <u>Anion Exchange Separation and Spectral Properties of Azido Nucleotides</u>.

Nucleotides (1.0 mmol) were appliied to a column (3 x 60 cm) of DEAE-Sephadex A-25 and eluted with a linear gradient from 0.06 - 0.6 M TEA.HCO_3^- (3 l + 3 l) at a flow rate of 150 ml/h. Fractions were collected and the absorbance measured at 280 nm.

3.1.3 Synthesis and purification of TNP-$8N_3$-nucleotides

Initially, we performed the synthesis exactly as described by Hiratsuka (1982) but substituting $8N_3ADP$ for ADP. The reaction products were purified on Sephadex LH20 as described by Hiratsuka (1982) (Fig. 3.3). The priciple reaction product had a reduced UV absorbance maximum at 273 nm in addition to the visible spectrum characteristic of the TNP moeity (Fig. 3.4). However, the substituted adenine ring absorbance was unaffected by irradiation with UV light and the compound was postulated to be TNP-$8NH_2$-ATP. A compound with UV absorbance maximum at 273 nm following reduction of 8-azido-adenine has previously been postulated to be $8NH_2$-ATP (Schafer et al., 1978; Fitzpatrick et al., 1985). The reduction of the azido group to the amino group is probably caused by the sulfite anion released by TNBS before attachment of the trinitrophenyl group to the ribose moeity (Fitzpatrick et al., 1985) (Fig. 3.5). None of the other peaks could be identified as TNP-$8N_3ADP$.

Using a more rapid HPLC system with better resolving power to separate the reaction products, a small amount of TNP-$8N_3$-nucleotide could be obtained if the reaction mixture was fractionated shortly after mixing. A typical profile following separation of the mixture by HPLC and using $8N_3ATP$ as starting material is shown in Fig. 3.6. The peak identified as TNP-$8N_3ATP$ had absorbance maxima at 281 nm, 408 nm, and 468 nm. The yield of TNP-$8N_3ATP$ was only 3%, however, and the principle reaction products were considered to be $8NH_2$-ATP and TNP-$8NH_2$-ATP.

To increase the yield of TNP-$8N_3$-nucleotides, we experimented with the introduction of an oxidising agent into the reaction mixture to react with the released SO_3^{2-} and so prevent it's reaction with the azido group. Out of a number of agents tested, DTNB was found to be the best. Inclusion of DTNB in the reaction mixture not only prevented the unwanted reduction from occurring but also improved the yield of the TNP reaction from the published value of 50% (Hiratsuka, 1982) to about 80% (Fig. 3.7). This latter improvement could be due to a mass action

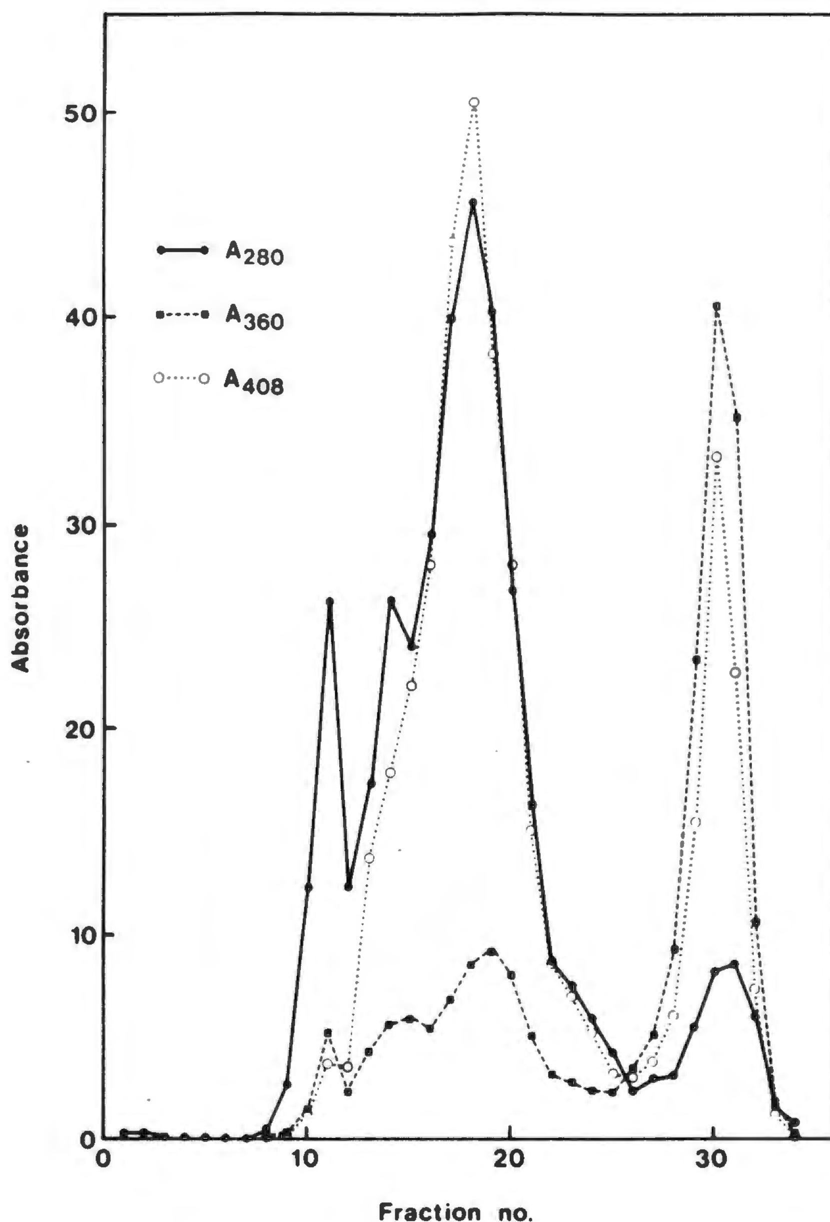


Figure 3.3 Separation of Trinitrophenylation Reaction Mixture on Sephadex LH 20.

Nucleotides (25 µmol) were applied to a column (2 x 16 cm) of Sephadex LH 20 and eluted with H_2O at a flow rate of 15 ml/h. Fractions (4 ml) were collected and the absorbance measured at 280, 360 and 408 nm.

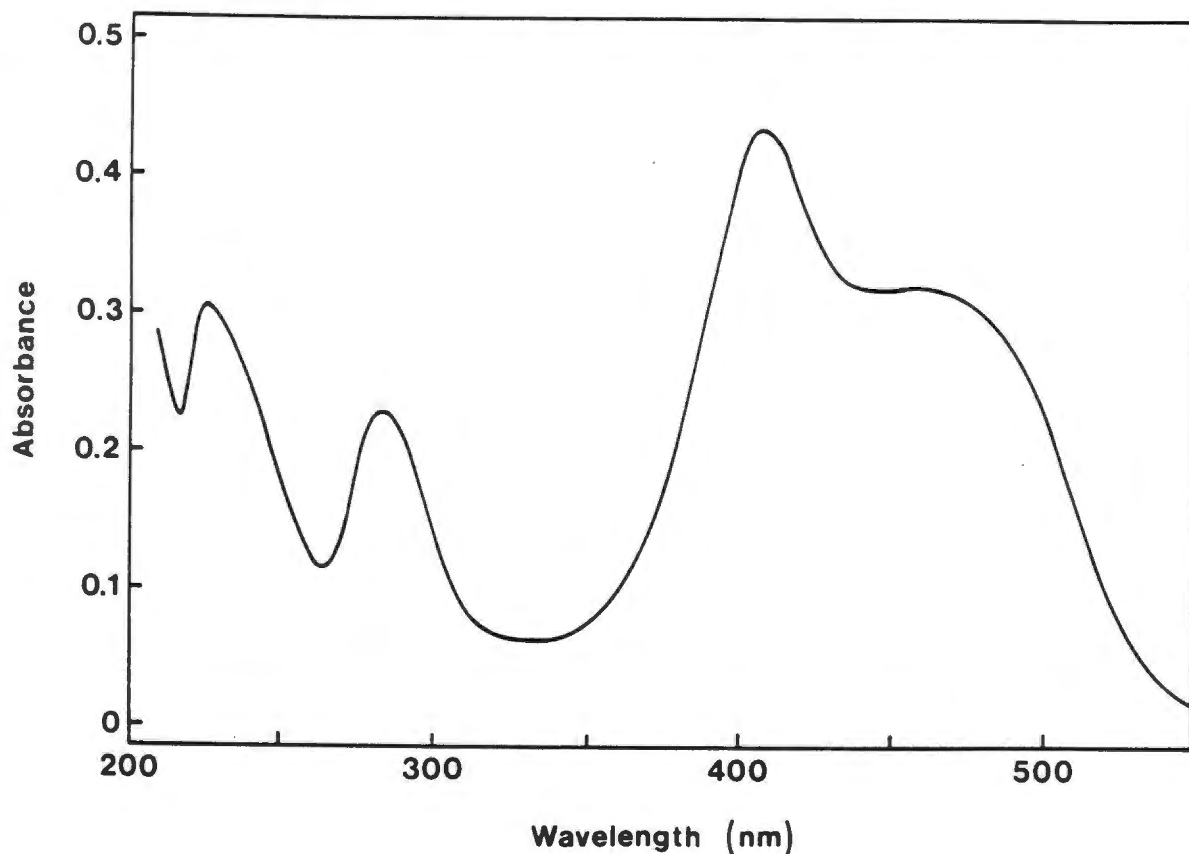


Figure 3.4 <u>Absorbance Spectrum of the Principle Reaction Product of the Trinitrophenylation Reaction.</u>

Fractions 16 - 22 from the separation shown in Fig. 3.3 were pooled and lyophilized. The lyophilizate was redissolved in 10 mM TRIS/MOPS pH 8.0 and scanned.

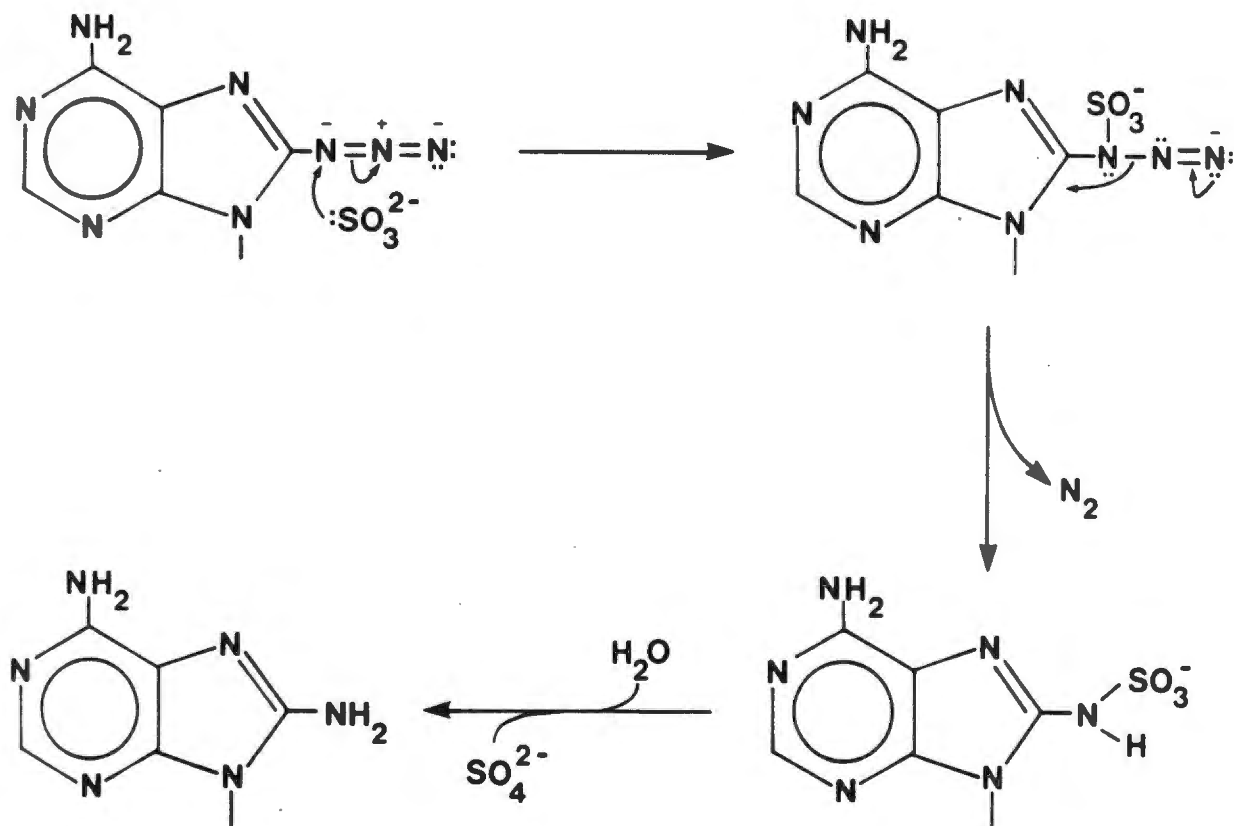


Figure 3.5 <u>Mechanism of Azido Group Reduction</u>.

The mechanism of reduction of 8-azido-adenosine by sulfite as postulated by Fitzpatrick <u>et al</u>. (1985).

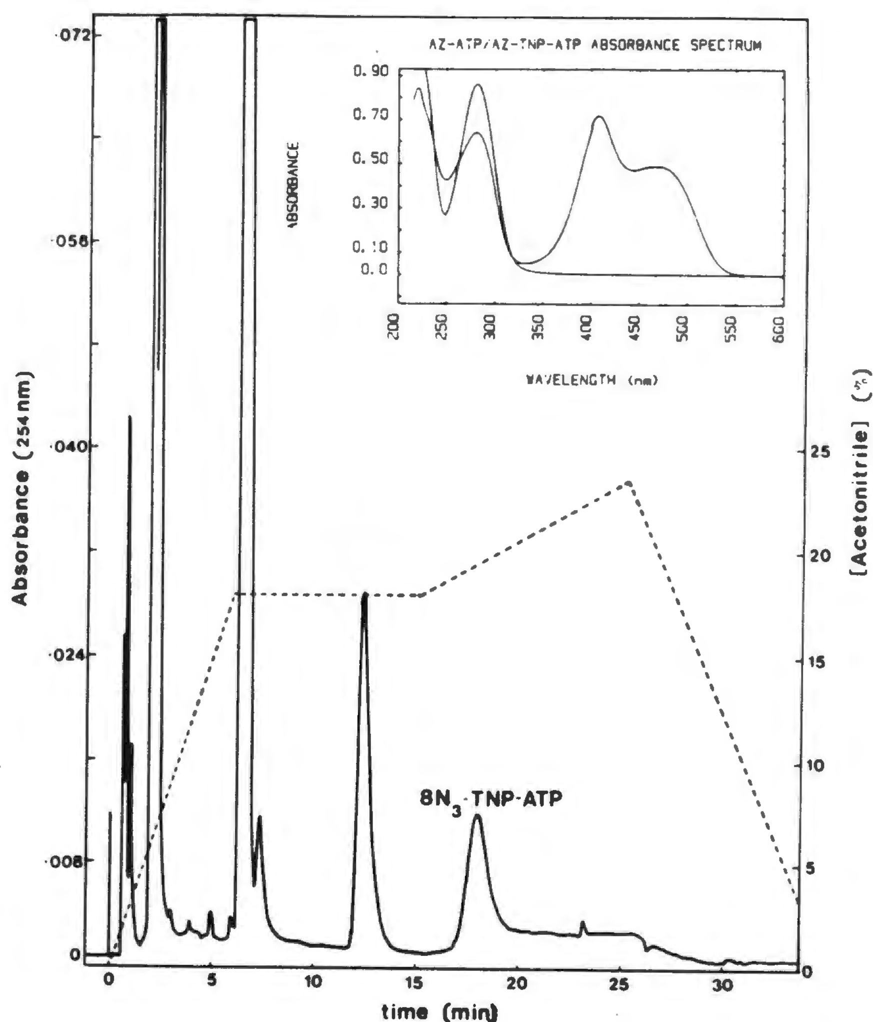


Figure 3.6 HPLC Separation of Trinitrophenylation Reaction Mixture.

The trinitrophenylation reaction mixture, using $8N_3ATP$ as the starting material was carried out in the absence of DTNB as described in EXPERIMENTAL PROCEDURES. The products of the reaction mixture were separated on a Radial Pak C18 column with solvent A as 75 mM KH_2O_4, pH 5.0 and solvent B as as for solvent A with 60% acetonitrile. The peak identified as TNP-$8N_3ATP$ was lyophilized, desalted and then scanned in 10 mM MOPS/TRIS, pH 8.0 resulting in the spectrum shown in the inset.

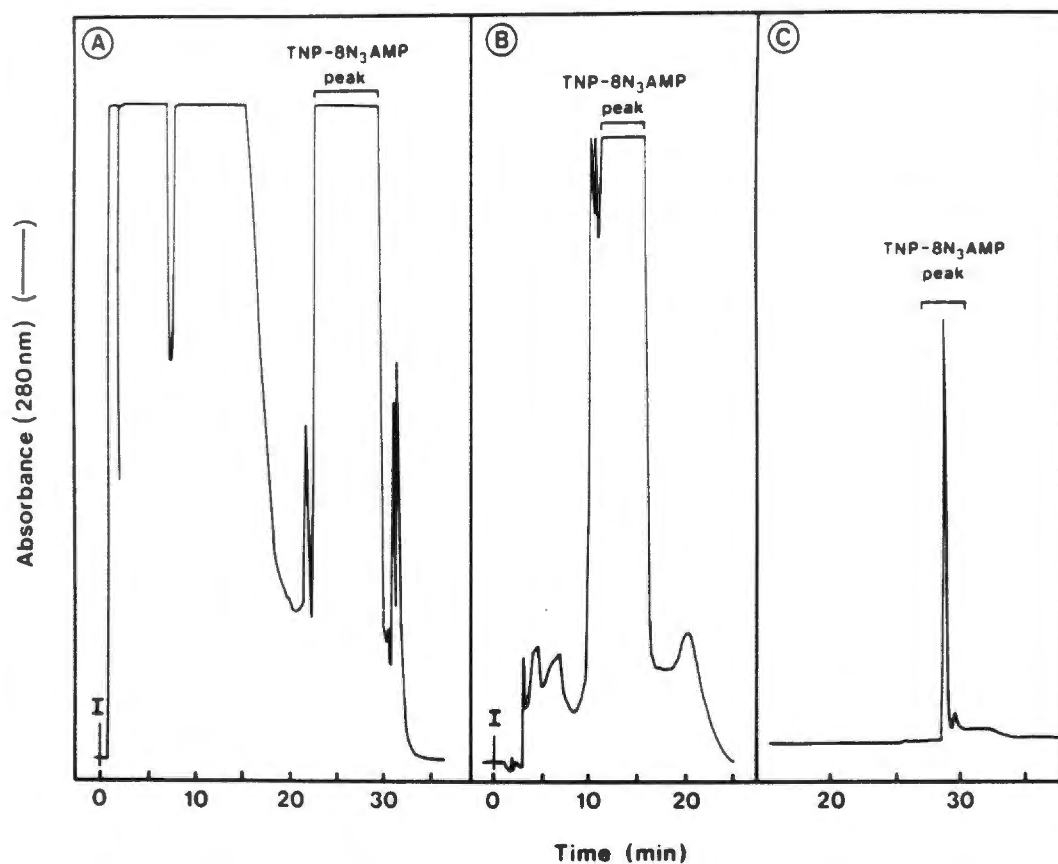


Figure 3.7 <u>HPLC Separation of trintrophenylation reaction mixture in the presence of DTNB.</u>

Conditions were exactly as described in Fig. 3.6 except that DTNB was included in the reaction mixture. Aliquots, 1.2 ml, of the reaction mixture were then fractionated by HPLC as described above and the appropriate peak collected. A and B represent two successive purifications of the TNP-8N_3-AMP reaction mixture, and C is an analytical run.

effect on the reaction following the removal of product, SO_3^{2-}.

TNP-8N_3ADP and TNP-8N_3AMP were prepared in a similar way with similar yields. We optimized the nucleotide separation by changing the salt concentration within the 20-100 mM range.

The absorbance spectrum and proposed structure of TNP-8N_3ATP are shown in Fig. 3.8. The spectra of TNP-8N_3ADP and TNP-8N_3AMP are similar (data not shown) although we found small differences in extinction coefficient (see below). The measured absorbance maxima are 281 nm for the 8-azido-adenine group and 408 and 468 nm for the TNP group. The absorbance at 281 nm is light dependent (Fig.3.8) and diminishes after irradiation with UV light. As reported for TNP-ATP (Hiratsuka and Uchida, 1973), the absorbance of the TNP group is pH dependent and disappears at acid pH. Irradiation with the same light source (Xe, 150 W) that produces complete disappearance of the 281 nm peak but after passing through a 406 nm cuton filter giving about 45% transmittance has only a slight effect after 60 min demonstrating that light of the wavelength of absorbance of the 8-azido-adenine group is required to activate the azido group.

TNP-8N_3-nucleotides were stored as a 15 - 20 mM solution in H_2O at -20 ^{o}C, and were stable for at least two years, on the basis of their spectral characteristics.

3.2. SYNTHESIS AND PURIFICATION OF RADIOLABELED DERIVITIVES

The method for synthesis of the [8-^{14}C] and [2-^{3}H] derivatives of the TNP-8N_3-nucleotides was developed because of the small amounts of nucleotide involved as well as to minimize the number of manipulations. The main differences between this method and that used for the large scale preparation of unlabeled nucleotides were, firstly, the brominated derivatives were eluted from the HPLC column with a gradient of acetonitrile in TEA.HCO_3^- and evaporated to dryness. This obviated the necessity for a separate desalting step and also left the 8-Br-ATP as the TEA salt. Secondly, the separation after the azido reaction was omitted and the nucleotides trinitrophenylated directly. A new gradient system was developed which resolved mono-, di- and

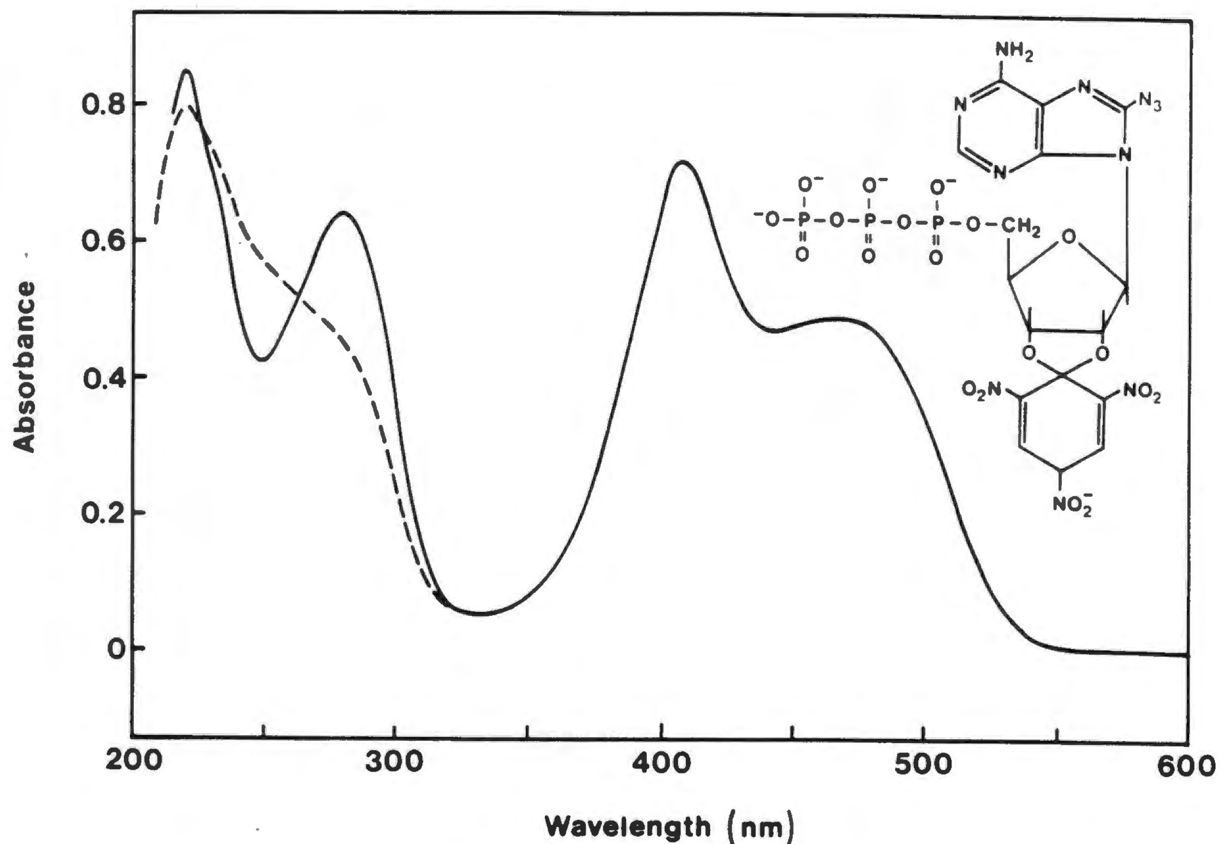


Figure 3.8 <u>Absorption spectra of unphotolysed and photolysed $TNP-8N_3ATP$</u>.

$TNP-8N_3ATP$ was scanned in 10 mM MOPS/TRIS, pH 7.0 (continuous line), then the cuvette was removed and irradiated for 3 min (dashed line). The proposed structure of $TNP-8N_3ATP$ is shown alongside the spectra.

triphosphate derivatives. Although a good separation was obtained, a second HPLC purification was always carried out even if it served only to demonstrate purity. In this way we were able to obtain good yields from a three step organic synthesis. Yields from starting [8-^{14}C]-ATP were: 11% (TNP-8N_3-[8-^{14}C]-AMP), 4% (TNP-8N_3-[8-^{14}C]-ADP) and 10% (TNP-8N_3-[8-^{14}C]-ATP) and the specific radioactivity of each was 6300 cpm/nmol. Purity was checked by the following criteria: a spectrophotometric peak with UV absorbance maximum between 281 and 283 nm, a single peak on HPLC and single radioactive and UV absorbing spots on TLC (Fig. 3.9).

3.3. CHEMICAL PROPERTIES OF TNP-8N_3-NUCLEOTIDES

Elemental analysis of TNP-8N_3ATP was carried out: calculating for $C_{16}H_{12}N_{11}K_5O_{19}P_3.7.8H_2O$ (1091.29) (for TNP-ATP see Hiratsuka and Uchida, 1973): C, 17.61; H,1.11; N,14.12. Found: C, 18.75; H, 2.3; N, 14.1. The extinction coefficients for the three compounds were calculated by comparing the specific activity of the synthesized TNP-8N_3-[8-^{14}C]-AXP with that of the starting [8-^{14}C]-ATP using an extinction coefficient for ATP of 15.4 $mM^{-1}.cm^{-1}$ at 260 nm. The calculated results are ($mM^{-1}.cm^{-1}$):

$\varepsilon(mM^{-1}cm^{-1})$

	280 nm	408 nm	468 nm
TNP-8N_3ATP	24.2	28.0	19.3
TNP-8N_3ADP	25.0	27.6	19.0
TNP-8N_3AMP	22.3	25.9	18.1

The R_f values on PEI-cellulose TLC plates developed in 2 M formic acid and 0.5 M LiCl were: TNP-8N_3ATP (0.02), TNP-8N_3ADP (0.33), TNP-8N_3AMP (0.59).

An attempt to analyze the samples by mass spectroscopy was unsuccessful as we were unable to volatilize the nucleotides.

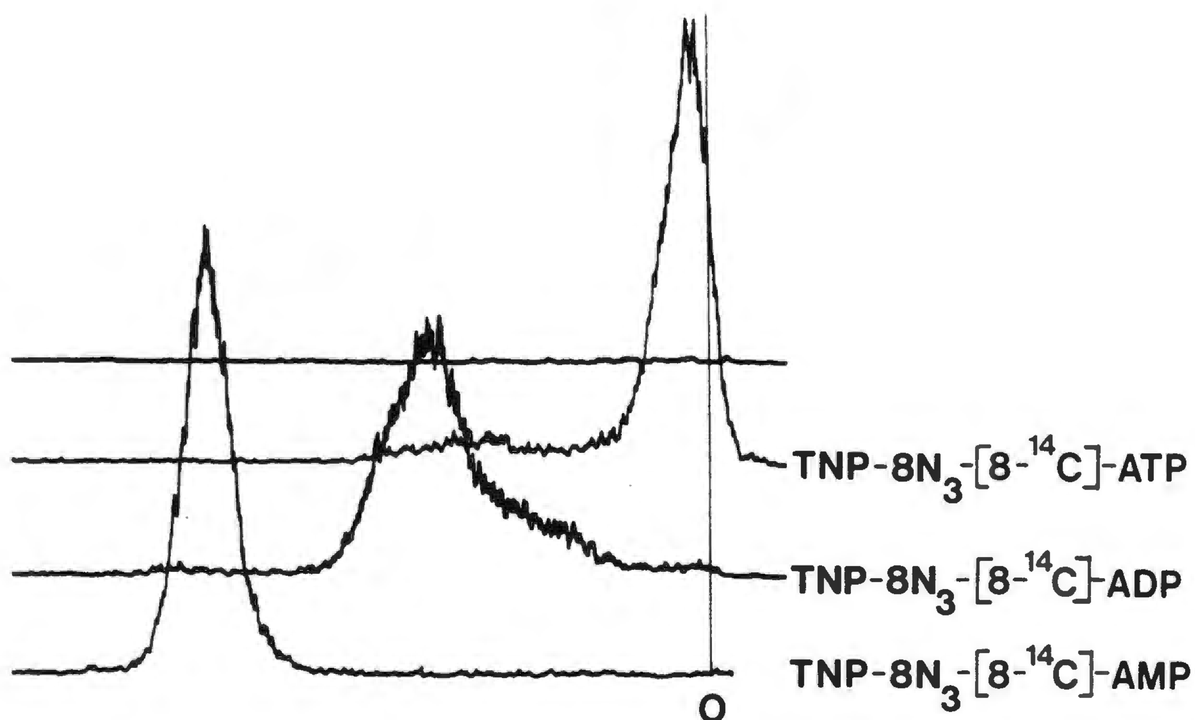


Figure 3.9 Separation of TNP-8N_3-[8-^{14}C]-nucleotides by TLC.

TNP-8N_3-[8-^{14}C]-ATP, -ADP, and -AMP were spotted on a PEI-cellulose TLC plate (Merck) and developed in 2 M formic acid + 0.5 M LiCl. The plate was then dried and exposed to NH_4OH fumes. Spots could be seen in visible light (orange) and UV light (blue). The plate was then scanned on a proportional gas flow scanner to identify areas of radioactivity.

3.4. SR PROTEIN COMPOSITION

The protein content of an SR vesicle preparation stained with Coomassie Blue is shown in Fig. 3.10. The predominant protein species present is the M_r 110 kDa Ca^{2+}-ATPase and M_r 50 kDa calsequestrin.

3.5 EFFECT OF TNP-8N_3-NUCLEOTIDES ON ATPase ACTIVITY

The effect of TNP-8N_3ATP on the hydrolysis of [γ-^{32}P]-ATP by the Ca^{2+}-ATPase, under conditions of vesicle loading in the presence of oxalate, as assayed by the charcoal method, is shown in Fig. 3.11. At all three concentrations of ATP used, TNP-8N_3ATP in the 25-100 µM range, had little effect on the hydrolysis of ATP but at higher concentrations, the activity was inhibited. This pattern suggests that the interaction of TNP-8N_3ATP with the Ca^{2+}-ATPase is complex and cannot simply be described by competitive inhibition.

The effect of TNP-8N_3-nucleotides on ATP hydrolysis under conditions of maximal enzyme turnover in the presence of Ca^{2+} ionophore, was examined using a pH-stat procedure by which Dupont et al. (1985) showed that TNP-ATP and TNP-AMPPNP accelerated ATPase activity approximately two-fold. Using this method, TNP-8N_3AMP, TNP-8N_3ADP, and TNP-8N_3ATP (5 µM each) accelerated the hydrolysis of ATP (75 µM) 1.0-, 1.4-, and 1.5-fold respectively. Higher concentrations of nucleotides did not result in greater acceleration. Dupont et al.. (1985) found that TNP-ADP had no accelerating effect on ATP hydrolysis, and it appears that the azido derivative may differ in this respect. However, it has been found that TNP-ADP accelerates E_2-P hydrolysis approximately 2-fold (Champeil et al., 1988).

TNP-8N_3ATP itself is hydrolysed at a low rate by SR vesicles (< 10 nmol/min/mg of protein at 25 ^{o}C) in a medium including 50 mM MOPS.TRIS, pH 7.0, 0.1 M KCl, 5 mM $MgCl_2$, 200 µM TNP-8N_3-[γ-^{32}P]-ATP, 4% (w/w) A23187, 0.1 mg of SR protein/ml and either with or without 100 mM $CaCl_2$.

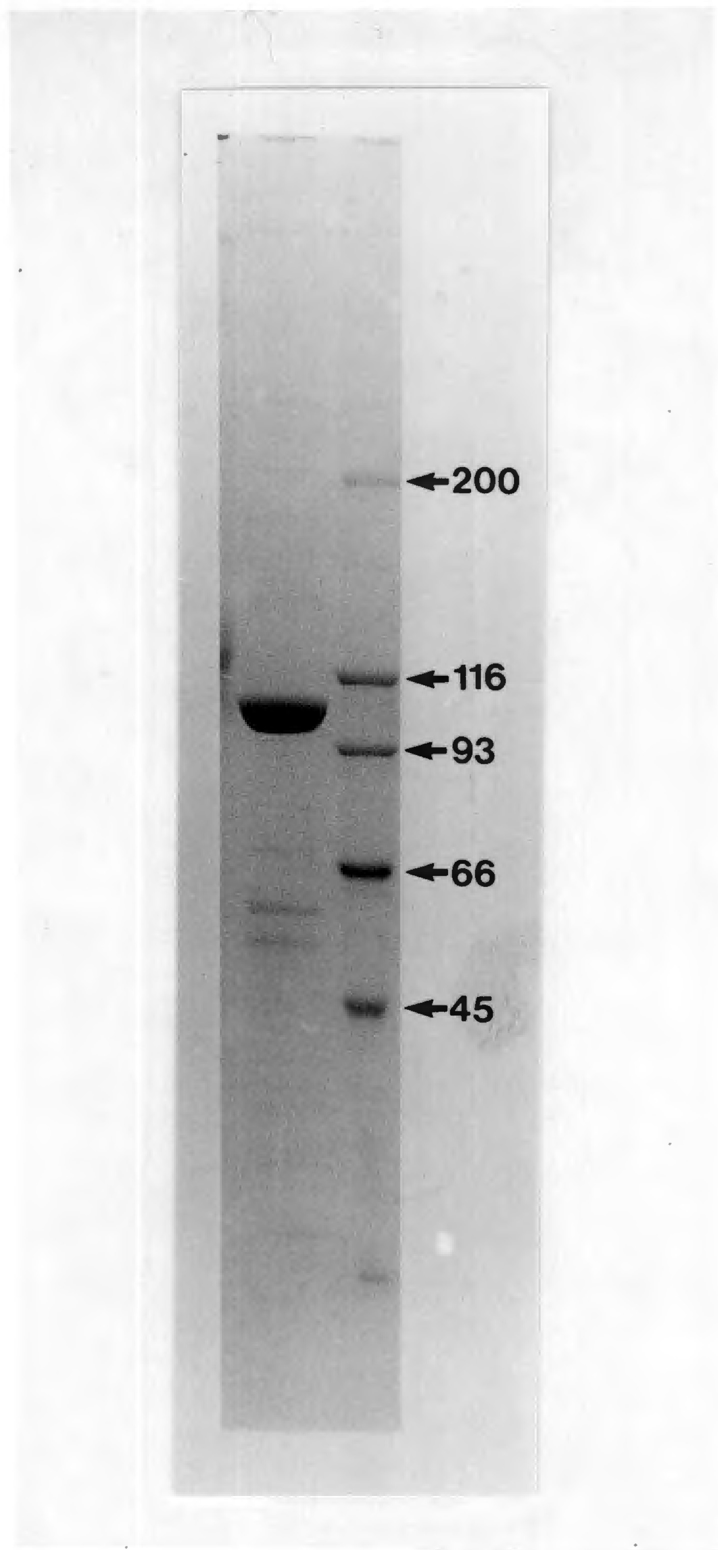


Figure 3.10 Electrophoretic Analysis of SR Vesicle Preparation.

SR vesicles (10 µg of SR protein) were loaded onto a 7% polyacrylamide gel (Laemmli, 1970) and electrophoresed at 5 ^{o}C. The gel was then stained with Coomassie Blue, destained and dried.

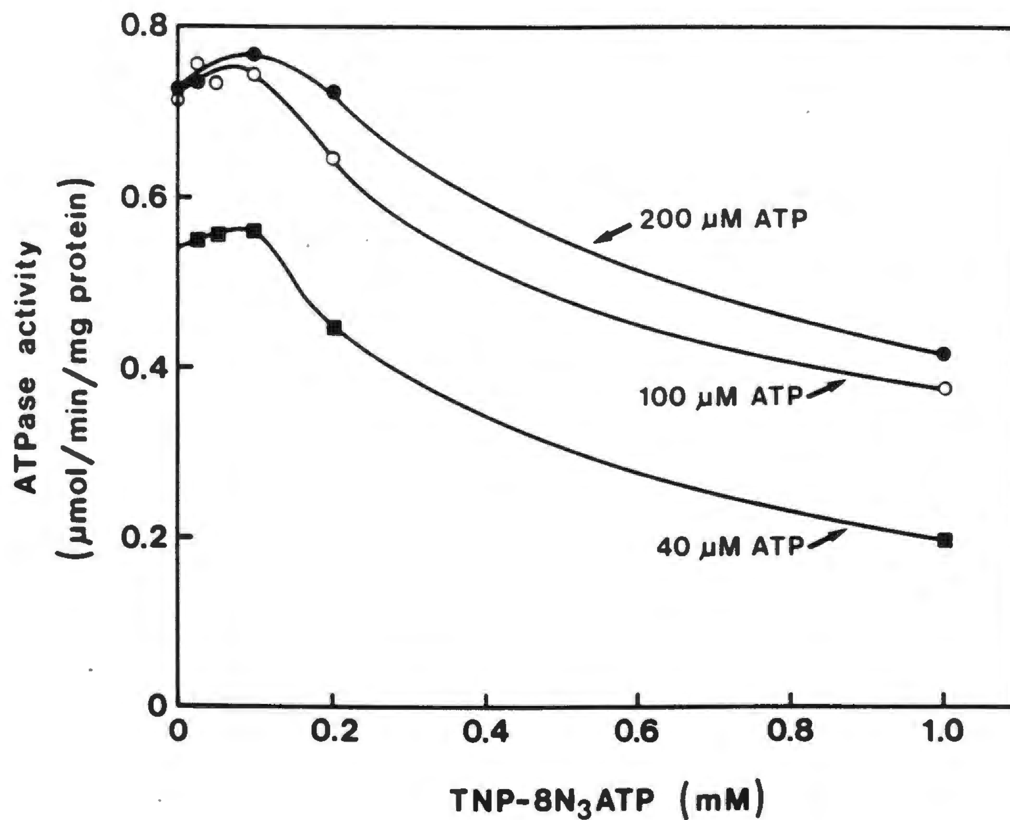


Figure 3.11. Effect of TNP-$8N_3$-ATP concentration on ATPase activity.

Ca^{2+}-stimulated ATPase activity was measured using [γ-^{32}P]ATP and the charcoal extraction procedure in a medium of 20 mM MOPS pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 5 mM oxalate, 0.1 mM $CaCl_2$ and 0.1 mg of SR protein/ml and the indicated concentrations of TNP-$8N_3$-nucleotides.

3.6. BINDING TO SR VESICLES

3.6.1 Binding under nonturnover conditions

Binding of TNP-8N_3-nucleotides to SR vesicles in the absence of ATP using a filtration method is shown in Fig. 3.12. In the low concentration range, namely 10^{-8}-10^{-6} M (see inset for linear scale), the nucleotides bound in a saturable manner to the extent of 4 - 6 nmoles/ mg protein, an amount equivalent to the active ATPases as judged by the levels of phosphoenzyme obtained from P_i in the presence of 30% (v/v) Me_2SO (McIntosh and Ross, 1988). The monophosphate species exhibited the highest affinity ($k_{0.5}$ = 0.1 µM) and the di- and triphosphate having approximately equal affinity ($k_{0.5}$ = 0.2 µM). At concentrations in excess of 10^{-5} M additional binding was observed, which is greatest in the case of the triphosphate species and appears to be nonsaturable.

We were unable to measure binding in the presence of EDTA using this technique as the absence of divalent cation appeared to destabilize the vesicles under the conditions used resulting in low levels of binding being measured.

On account of this, the effect of $MgCl_2$ on nucleotide binding was examined by means of a fluorescence assay (Fig. 3.13). In the presence of EDTA (Fig. 3.13A), binding of all three nucleotides to a saturable site could be discerned in the 0 - 2 µM range. In these experiments the concentration of ATPases was approximately 1 µM and the binding curves are consistent with the titration of the high affinity catalytic site in this concentration range. The binding of TNP-ATP was indistinguishable from that of the azido nucleotides (results not shown). In the presence of 20 mM $MgCl_2$, additional binding, especially in the case of the triphosphate species, is evident (Fig. 3.13B). The effect of the cation was less marked with TNP-ATP. The binding curves in the presence of 1 mM $MgCl_2$ were similar to those with EDTA (results not shown). The results indicate that high concentrations of $MgCl_2$ enhance the binding of the azido nucleotides to SR vesicles.

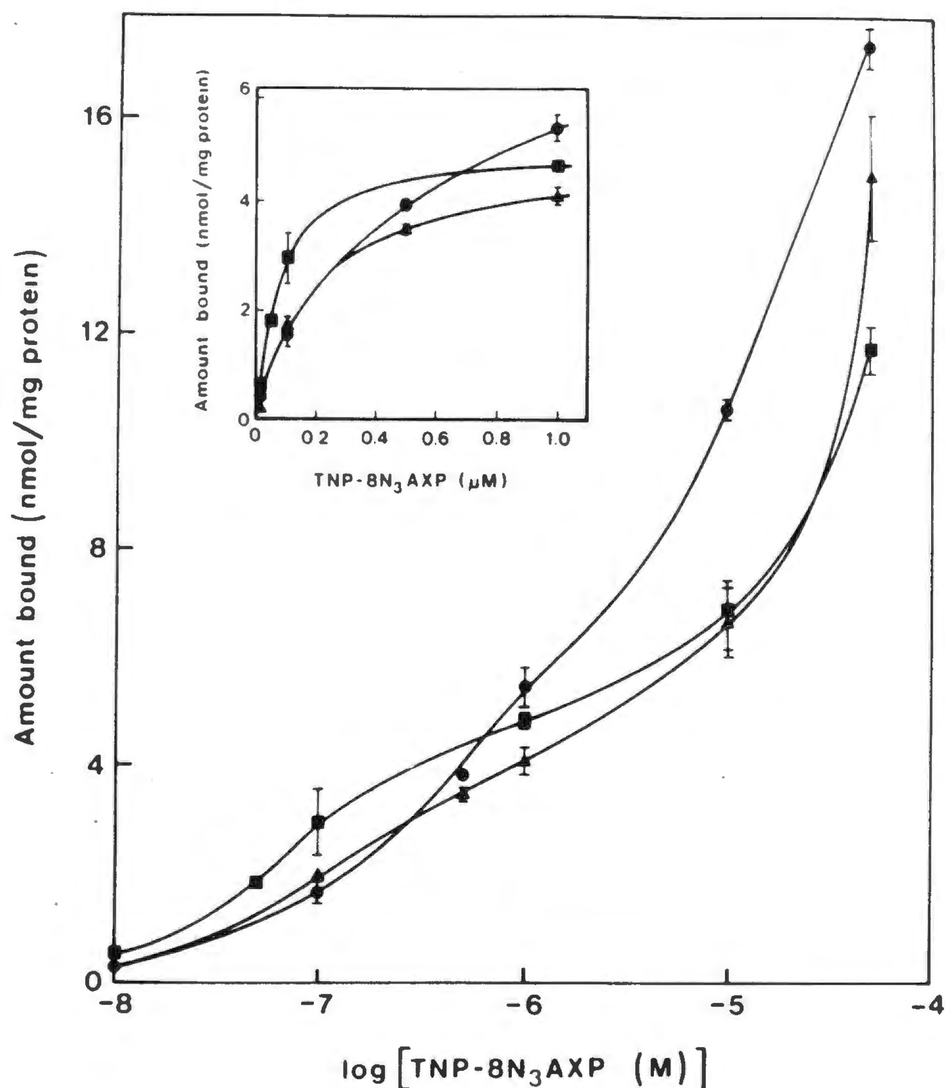


Figure 3.12. <u>Binding of TNP-8N$_3$-nucleotides measured by a filtration procedure.</u>

The binding of TNP-8N$_3$-[8-^{14}C]ATP (circles), TNP-8N$_3$-[^{14}C]ADP (triangles) and TNP-8N$_3$-[8-^{14}C]AMP (squares) was measured in 50 mM MOPS/TRIS, pH 7.0, 5 mM MgCl$_2$, trace of [^{3}H] sucrose, the concentrations of TNP-8N$_3$AXP indicated and 0.3 mg of SR protein. The binding curve for TNP-ATP was similar to that for TNP-8N$_3$AMP and is not shown. A linear plot of the data points up to 1 μM nucleotide is shown in the inset.

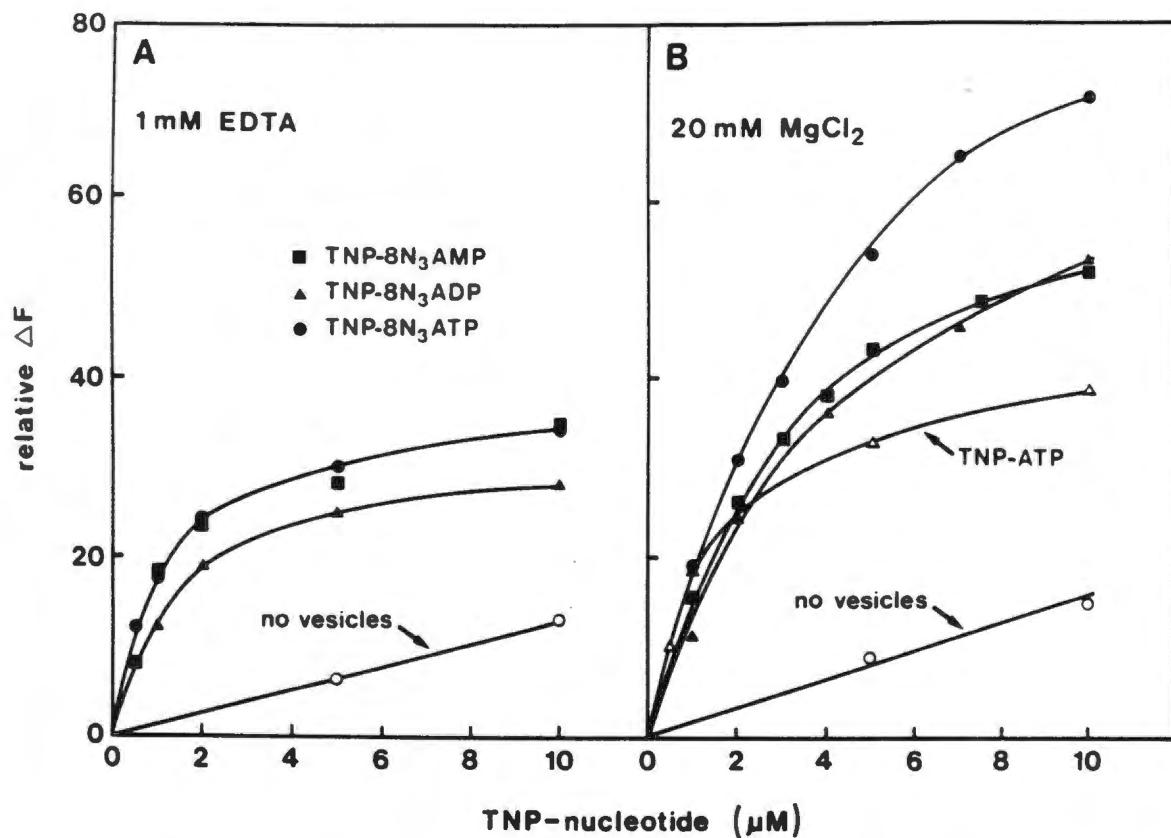


Figure 3.13. <u>Binding of TNP-$8N_3$-nucleotides measured by fluorescence.</u>

Binding was measured in 50 mM MOPS/TRIS, pH 7.0, 0.2 mg of SR protein/ml and either 1 mM EDTA (A) or 20 mM $MgCl_2$ (B) and the indicated concentration of TNP-$8N_3$-nucleotides. The difference in fluorescence intensity before and after addition of nucleotide is plotted against nucleotide concentration.

Specific binding of nucleotides to the active site of the Ca^{2+}-ATPase can be measured by inhibition of active site cross-linking with glutaraldehyde (Ross and McIntosh, 1987a). The cross-link alters the hydrodynamic properties of the enzyme in sodium dodecyl sulfate such that it migrates on electrophoresis in polyacrylamide with an apparent molecular weight of 125 kDa instead of the usual 110 kDa (Ross and McIntosh, 1987b). The binding of TNP-$8N_3$AMP and inhibition of cross-linking is shown in Fig. 3.14. The glutaraldehyde concentration and the time of cross-linking were adjusted to give approximately 40% E(125) formation in the absence of nucleotide (lanes 2 and 10), so that initial rates of cross-link formation were measured. The concentration of protein was kept low in these experiments (20 µg of SR protein/ml) in order to ensure that the bound/unbound nucleotide ratio was low. TNP-$8N_3$AMP caused increasing inhibition of E(125) formation in the presence of EDTA (lanes 3-9) and in the presence of 5 mM $MgCl_2$ (lanes 11-17). The results obtained in this way, for the mono-, di-, and triphosphate species in the presence of 5 mM $MgCl_2$, and expressed as a percentage of control rate of E(125) formation are shown in Fig. 3.15. The data fit well to a mechanism in which there is a single noninteractive nucleotide binding site. The K_d values obtained for the best fit of the data are in reasonable agreement with those obtained by the filtration method (Fig. 3.12). In the presence of 1 mM EDTA K_d values of 0.8, 1.0, 0.3 and 0.12 µM were obtained for the mono-, di-, and triphosphate azido nucleotides and TNP-ATP respectively. $MgCl_2$, therefore, only affected the binding of TNP-$8N_3$AMP significantly.

3.6.2 BINDING UNDER TURNOVER CONDITIONS

The fluorescence increase observed with TNP-ATP was compared with that of the azido derivatives measured under turnover conditions as a function of TNP nucleotide concentration (Fig. 3.16). All of the azido derivatives displayed approximately equal superfluorescence, but the amplitude of the fluorescence increase was less than that of TNP-ATP. The TNP-$8N_3$-nucleotides

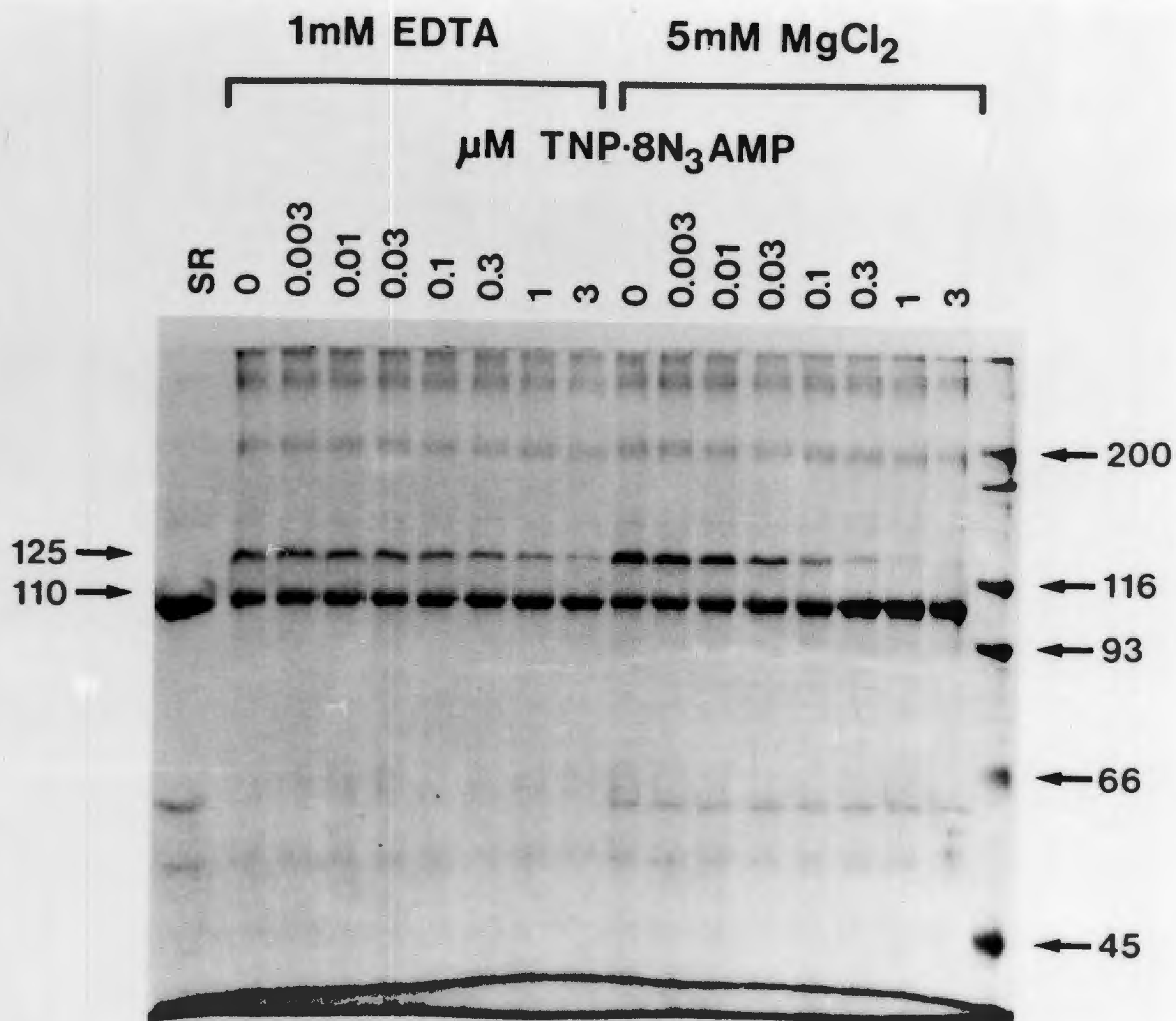


Figure 3.14. Binding of TNP-$8N_3$AMP measured by inhibition of active site cross-linking : gel.

SR vesicles (5 μg of protein/ml) were equilibrated for 5 min at 25 ^oC in 50 mM MOPS/TEA, pH 8.0, either 1 mM EDTA or 1 mM EGTA + 5 mM $MgCl_2$ and the concentrations of TNP-$8N_3$AMP indicated. Samples were then reacted with 0.5 mM glutaraldehyde and quenched after 2.5 min with 25 μl hydrazine. Aliquots were electrophoresed on 7% gels.

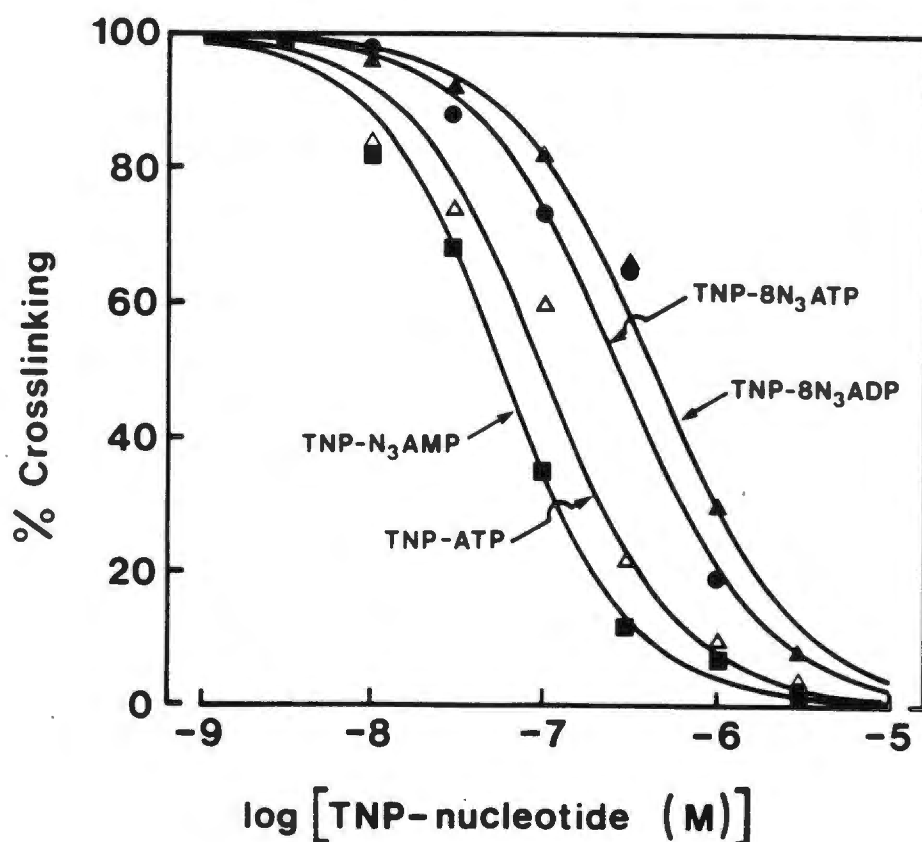


Figure 3.15. <u>Binding of TNP-8-N_3-nucleotides measured by inhibition of active site cross-linking : inhibition curves.</u>.

Conditions are as described in Fig. 3.14 in the presence of 5 mM $MgCl_2$ and 1 mM EGTA. Inhibition curves were fitted to the percentage decrease in E(125) species according to the equation $k_{0.5}$ = (E-EL)(L-EL)/EL where E = total site concentration (5.5 nmol/mg of protein), L = total nucleotide concentration and EL = concentration of enzyme.nucleotide complex and using the following $k_{0.5}$ values: TNP-8N_3ATP, 0.3 µM; TNP-8N_3ADP, 0.4 µM; TNP-8N_3AMP, 0.04 µM; TNP-ATP, 0.1 µM.

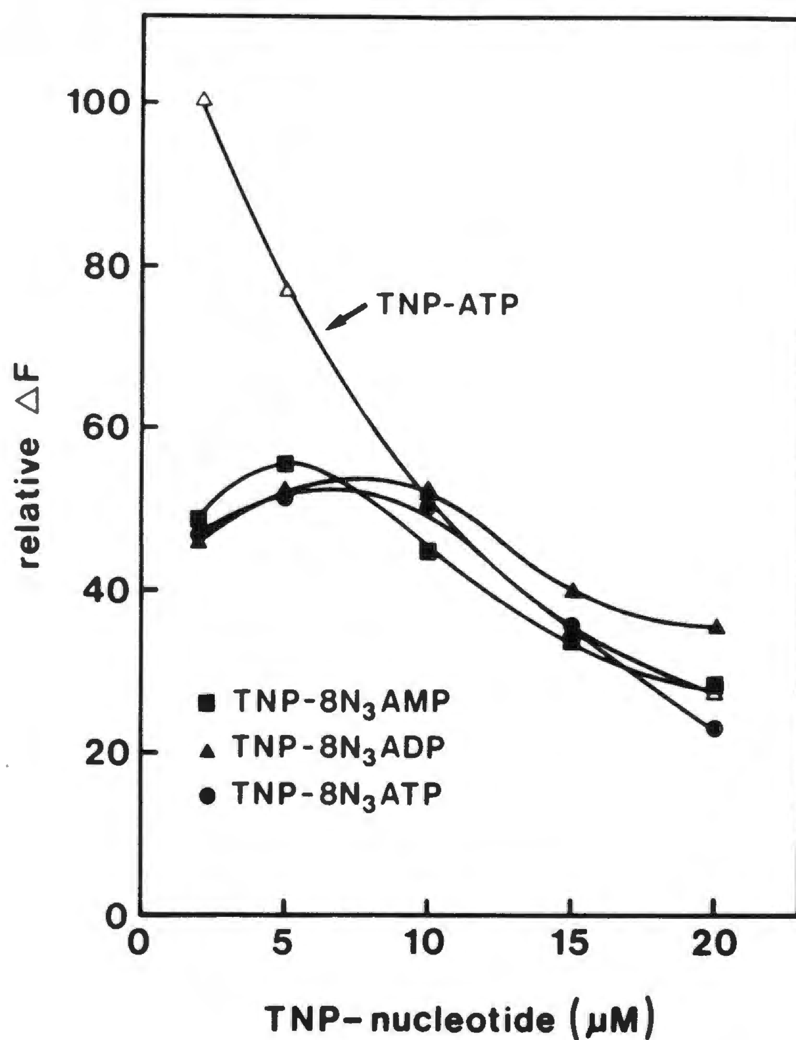


Figure 3.16. Turnover-dependent fluorescence increase of TNP-8N_3-nucleotides.

Final concentrations were: 20 mM TRIS/MOPS, pH 8.0, 20 % (v/v) glycerol, 5 mM $MgCl_2$, 0.2 mg of SR protein/ml, 50 μM $CaCl_2$, 100 μM ATP and 2-20 μM TNP-8N_3ATP (circles), TNP-8N_3ADP (closed triangles), TNP-8N_3AMP (squares) or TNP-ATP (open triangles). Enzyme turnover was stopped by addition of 1 mM EGTA and the decrease in fluorescence as a result of this addition is plotted as a function of nucleotide concentration.

appear to have a lower affinity for the superfluorescent site compared with TNP-ATP and the competition with ATP may account for the lower fluorescence yield. Alternative explanations are that the compound has a lower level of intrinsic fluorescence, or a slightly different orientation in the nucleotide site.

3.7. LABELING CONDITIONS

Irradiation of SR vesicles in quartz cuvettes with an unfiltered Xenon light source resulted in inactivation of enzyme activity to the extent of about 50% within 3 min. It was thus apparent that it would be necessary to filter out the shorter wavelength light in the region of protein absorbance. By carrying out the illumination in glass cuvettes, inactivation was completely prevented but activation of the azido group within the times used was also very low. Out of a number of organic solvents tested, toluene, which has a cutoff in the UV at approximately 285 nm, gave the best result. The filter did not appear to substantially affect activation of the azido group and a curve of photolabeling of SR vesicles with time showed photoincorporation to the extent of 90% within 3 min (Fig. 3.17). Inactivation of SR vesicles in the absence of azido nucleotide under these conditions was less than 3%.

Glycerol was included in the irradiation medium to stabilize the ATPase, and obviates the need to add glycerol in the fluorescence experiments described later in Section 3.10. The effect of glycerol on incorporation was found to be slight (Fig. 3.18) although there appeared to be a slight decrease in nonspecific labeling.

3.8. PHOTOAFFINITY LABELING OF SR VESICLES

The results of irradiation of SR vesicles in the presence of TNP-$8N_3$AMP under four sets of conditions, namely, (A) in the presence of Ca^{2+}, (B) in the presence of Ca^{2+}, Mg^{2+}, and ATP (only binding to the regulatory and superfluorescent sites), (C) in the presence of excess EGTA, and (D) as in (C) but including

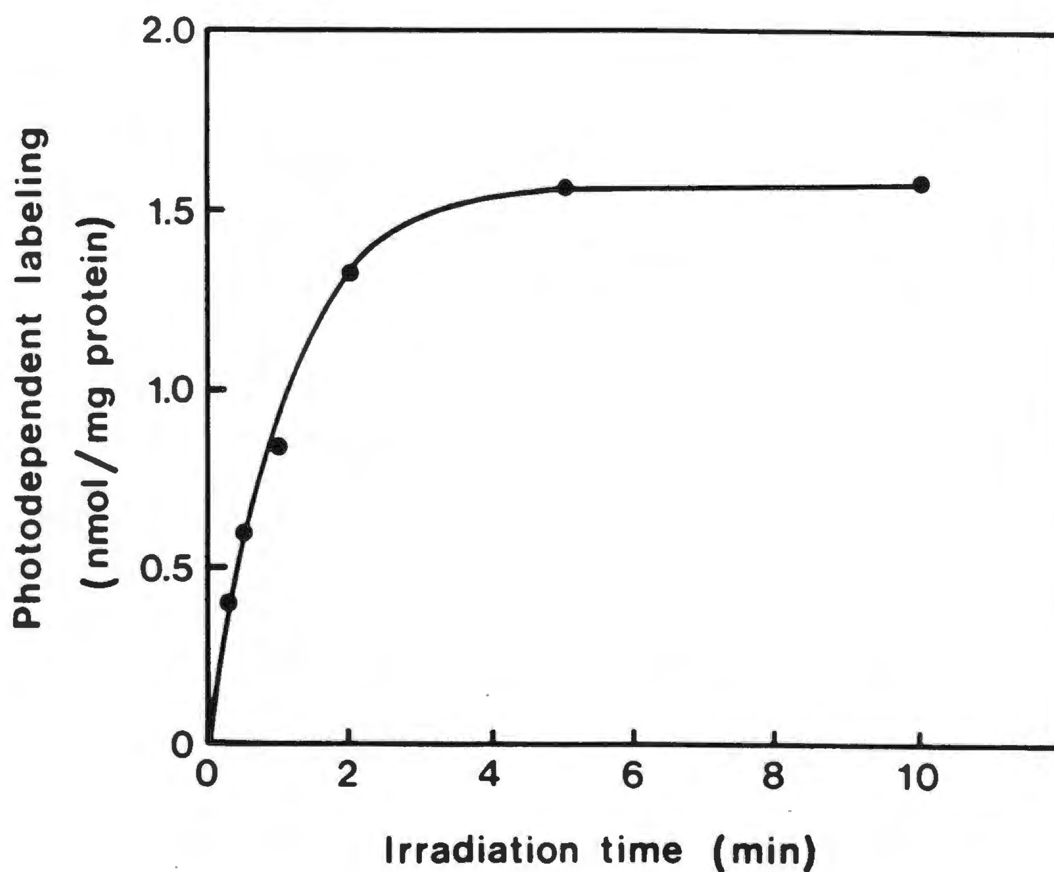


Figure 3.17. Time dependence of TNP-8N_3-AMP labeling with toluene filters.

SR vesicles were irradiated with a Xenon light source through toluene filters in a medium containing 20 mM MOPS, pH 8.0, 20% glycerol and 2 μM TNP-8N_3-[2-^{3}H]-AMP. The vesicles were collected by filtration and then counted for radioactivity.

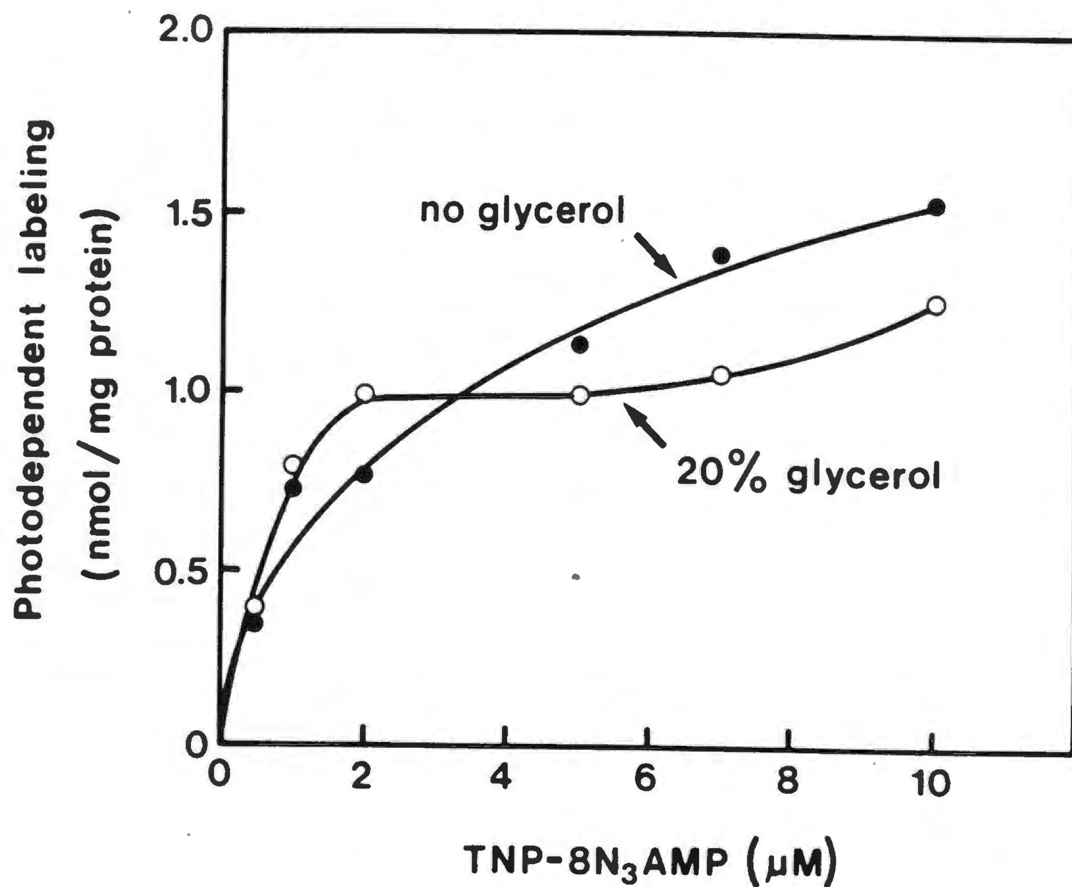


Figure 3.18. <u>Photoaffinity labeling of SR vesicles in the presence and absence of glycerol.</u>

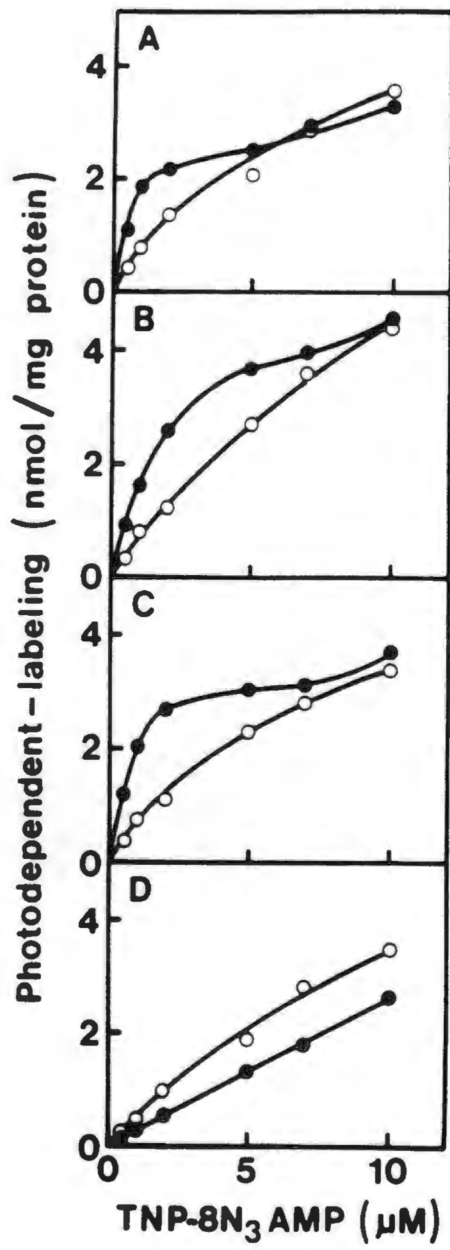
SR vesicles were irradiated in a medium containing 20 mM MOPS, pH 8.5, 2 μM TNP-8N_3AMP and either with or without 20% glycerol. The vesicles were collected by filtration and then assayed for radioactivity.

100 μM ATP, is shown in Fig. 3.19. In the series of panels on the left, irradiation was done at pH 6.0 (open circles) and 9.0 (closed circles) as a function of TNP-8N_3-nucleotide concentration. Under each of the conditions, (A), (B), (C), and (D), the labeling at pH 6.0 was similar and nonsaturable in the 0-10 μM concentration range. At pH 9.0, under nonturnover conditions, namely (A) and (C), there was a sharp rise in the amount of labeling at low concentrations of nucleotide (0-2 μM) to reach a plateau level of 2-2.5 nmol/mg of protein. The concentration of active ATPase polypeptides in these experiments was approximately 1 μM (0.2 mg of protein/ml and approximately 5 nmol of ATPases/mg of protein). Hence occupancy of the catalytic site, which can be expected to be saturated at approximately 2 μM, results in the covalent incorporation of nucleotide to the extent of about half the sites. This labeling was substantially reduced in the presence of 100 μM ATP (D). Under turnover conditions (B), a plateau level of labeling is only obtained at 5 μM TNP-8N_3AMP, which correlates with the concentration of TNP-nucleotide required to saturate the superfluorescent (Fig. 3.16) and regulatory (see above) sites. The level of labeling reached was 4.0 nmol/mg of protein, or approximately 80% of active ATPases.

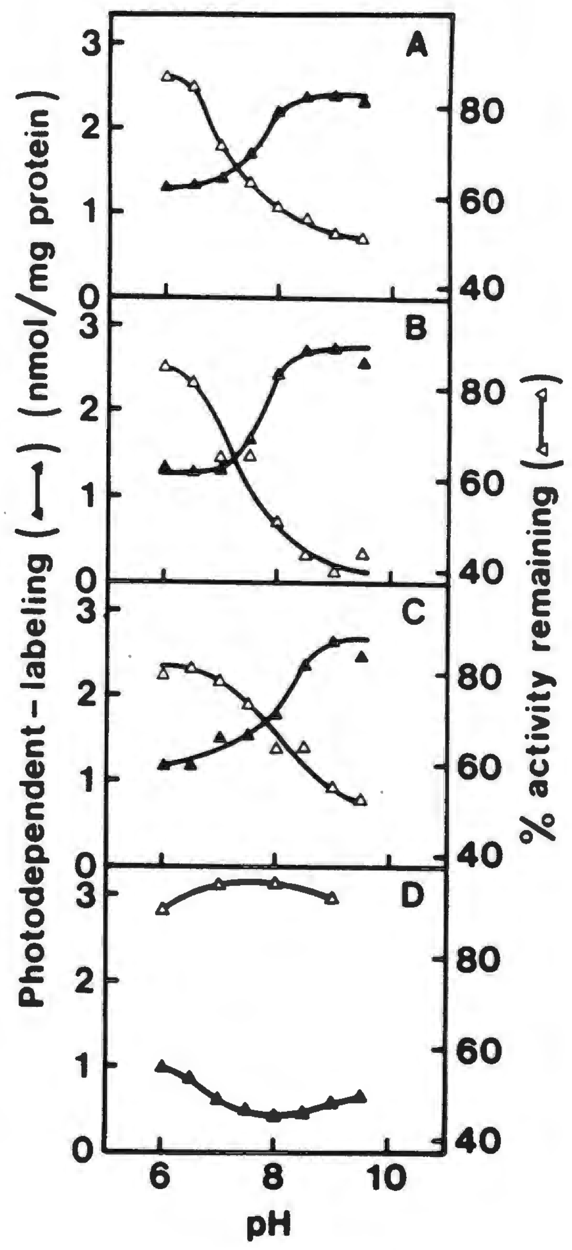
The pH dependence of photolabeling in the presence of 2 μM TNP-8N_3AMP and the effect on ATPase activity is shown in the series of panels on the right-hand side of Fig. 3.19. As already seen, there is increased labeling as the pH is increased at this concentration of nucleotide irrespective of whether the enzyme is turning over (B) or not (A and C). The pH dependence under the different conditions was similar. The sites labeled in the presence of ATP and EGTA (D) do not show this pH dependence. At pH 6.0, there was approximately 10% inactivation of ATPase activity despite the fact that there was labeling to the extent of about 1.3 nmol/ mg of protein (25% of active ATPases). Clearly, most of the labeling was at a site (s) which does not affect activity. As the pH was increased, inactivation of ATPase activity increased. At pH values above 8.0, there was a close correlation between the percentage ATPase labeling (taking

Figure 3.19. Covalent incorporation of $TNP-8N_3AMP$ into SR vesicles.

In the left hand series of panels, SR vesicles (0.2 mg of protein/ml) were irradiated in 20 mM Buffer pH 6.0 (open circles) or pH 9.0 (closed circles), 5 mM $MgCl_2$, 20 % (v/v) glycerol, the indicated concentrations of $[^3H]TNP-8N_3AMP$ and either (A) 50 µM $CaCl_2$, (B) 50 µM $CaCl_2$ + 100 µM ATP, (C) 0.5 mM EGTA, or (D) 0.5 mM EGTA + 100 µM ATP. Aliquots containing 0.2 mg of protein were then filtered, washed, and counted. On the right hand side, the vesicles were irradiated in a similar manner except that the pH was varied as indicated and the concentration of $TNP-8N_3AMP$ was 2 µM. Aliquots were then filtered as above or assayed for ATPase activity using the coupled enzyme method. The various buffers used are described in Experimental Procedures.



A
B
C
D
Photodependent - labeling (nmol/mg protein)
TNP-8N3 AMP (µM)



A
B
C
D
Photodependent - labeling (nmol/mg protein)
% activity remaining
pH

5 nmol/ mg of protein as the amount of active ATPases) and percentage inactivation of ATPase activity, again, irrespective of whether the enzyme was turning over (B) or not (A and C). Note that the pH dependence of inactivation of ATPase activity and of labeling were not identical, the former being shifted to a slightly lower range. This is due to the fact that in the 6-7 pH range the total amount of labeling does not increase appreciably but the locus of labeling is shifted increasingly to the catalytic site. This means that in the lower pH range the catalytic site was not the only site labeled and the curve cannot be used to obtain the pK of the ionizing group. This value is obtained from the inactivation of ATPase activity which is due only to labeling of the catalytic site and is approximately 7.4

The above experiments were carried out with 2 µM TNP-$8N_3$AMP. According to Fig. 3.19B (left side), a higher level of labeling could be obtained under turnover conditions at higher concentrations of nucleotide (up to 4.0 nmol/ mg of protein or 80% of active ATPases). In keeping with this, we found that ATPase activity was inhibited to the extent of 80% using 10 µM TNP-$8N_3$AMP (Fig. 3.20). A second irradiation, following removal of the photolysed nucleotide with Dowex anion exchange resin and addition of fresh azido nucleotide, resulted in further inactivation of the enzyme (results not shown). This indicated that the reason why all ATPases are not labeled in a single irradiation period is because the efficiency of labeling is less than 100% and not because a population of ATPases were resistant to labeling. A similar finding has been reported for the photoaffinity labeling of tightly bound $2N_3$-nucleotides to the chloroplast F_1 ATPase (Xue _et al_., 1987). The close correlation between extent of labeling at alkaline pH and inactivation of ATPase activity suggests that under these conditions there was almost exclusive labeling of the catalytic site, whereas at acid pH another site(s) is apparently preferentially labeled.

The photolabeling experiments described above were carried out using TNP-$8N_3$AMP. In a less extensive series of experiments, we found that the di- and triphosphate species gave similar results.

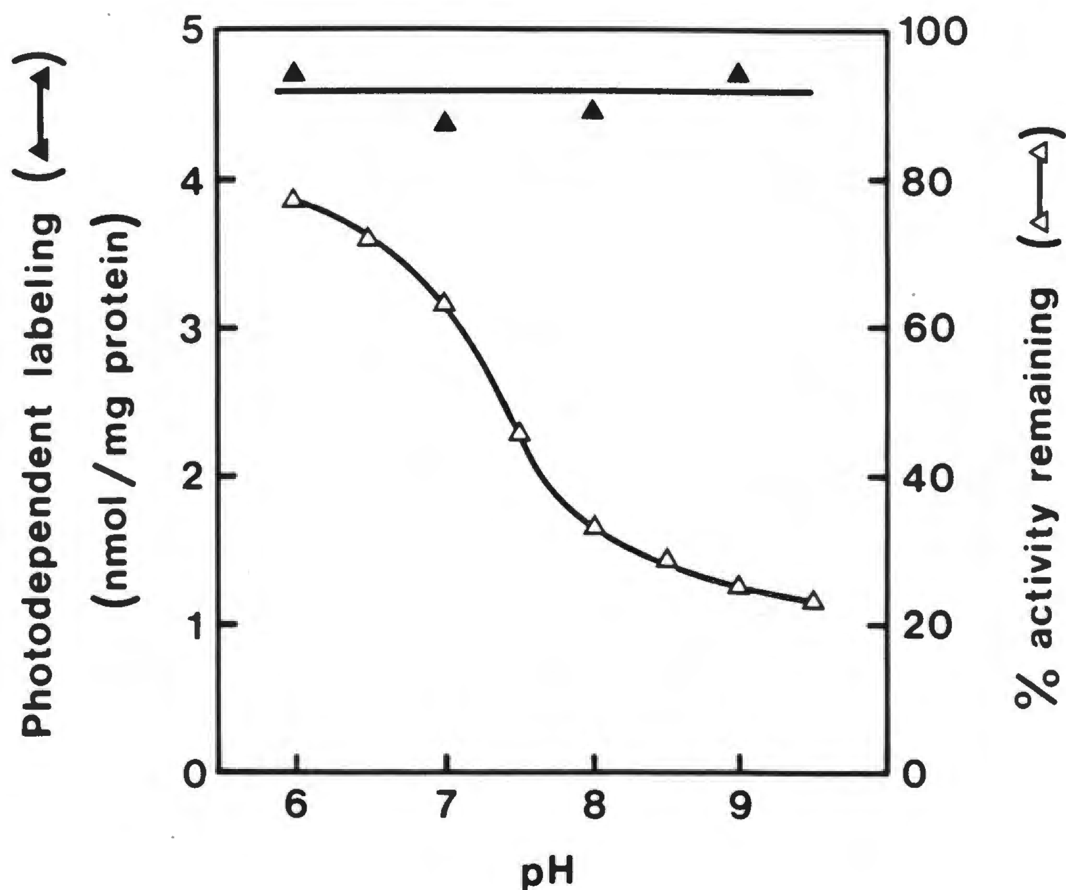


Figure 3.20. <u>SR photoaffinity labeling with 10 µM TNP-8N_3AMP</u>

SR vesicles were irradiated in the same medium described in Fig. 3.19(B) (turnover conditions) except that the concentration of TNP-8N_3-[2-^{3}H]-AMP used was 10 µM. Samples were taken and assayed for covalent incorporation and ATPase activity as described above (Fig. 3.19).

The effect of photolabeling on active site cross-linking was also investigated (Fig. 3.21). Under the conditions used, which were designed to maximize cross-linkage, 80-90% of ATPases were cross-linked to the E(125) species in the absence of irradiation (lanes 2-5). The remaining ATPases were mainly cross-linked intermolecularly. In the control samples (lanes 2 - 5), there was a very small amount of E(110) in the pH range 6 - 9, under all conditions used. Note that the medium was diluted 100 fold prior to initiating the glutaraldehyde reaction so that the concentration of noncovalent nucleotide was well below the K_d for binding to the catalytic site, ensuring that only covalent nucleotide located at the catalytic site could inhibit cross-linkage. Irradiation, under nonturnover conditions in the absence of ATP (gels labeled "Ca^{2+}" and "EGTA") and under turnover conditions (gel labeled "Ca^{2+}+ATP") resulted in inhibition of cross-linkage, the level of which increased as the pH increased from 6 to 9. The presence of EGTA and 100 µM ATP (gel labeled "EGTA+ATP") prevented inhibition. At pH 9.0, the percentage inhibition of cross-linkage (40-60%, depending on labeling conditions, last lane) correlates well with the percentage of ATPases modified and percentage inhibition of ATPase activity (Fig. 3.19).

3.9. FLUORESCENT PROPERTIES OF COVALENTLY ATTACHED NUCLEOTIDES

The ability of a TNP-$8N_3$-nucleotide, covalently attached at the catalytic site, to exhibit superfluorescence, was investigated using ATP (Fig. 3.22, panel A), acetyl phosphate (panel B), and P_i (panel C) as substrates. It was anticipated that, since the nucleotide was attached at the catalytic site, ATP may not be accommodated, but possibly a smaller substrate such as acetyl phosphate, or P_i, may gain entry, analogous to the situation following FITC modification of the ATPase (Pick, 1981; Champeil et al., in press). The vesicles were irradiated in a medium containing 5 µM TNP-$8N_3$AMP at pH 8.6 under nonturnover conditions, with EGTA (traces A iii, B iii, and C iii), and under

Figure 3.21. Inhibition of active site cross-linking by photoincorporated TNP-8N_3AMP.

SR vesicles (0.2 mg of protein/ml) were irradiated in the same media as in Fig. 8, right hand series of panels, A (labeled "Ca^{2+}"), B (labeled "Ca^{2+} + ATP"), C (labeled "EGTA"), and C (labeled "EGTA + ATP") at the pH values indicated. The samples were then diluted 100-fold, reacted with glutaraldehyde, collected on glass fiber filters, washed, dissolved in solubilizing solution and finally electrophoresed in 7% gels, as described in Experimental Procedures. In the control samples, TNP-nucleotide was added immediately after the irradiation period (as indicated). The lane labeled "SR" represents a sample which went through a similar procedure but to which hydrazine was added prior to the addition of glutaraldehyde.

Irradiated without TNP·$8N_3$AMP

Irradiated with TNP·$8N_3$AMP

pH

pH

SR 6 7 8 9 6 7 8 9

125 →

110 →

Ca^{2+}

125 →

110 →

Ca^{2+} + ATP

125 →

110 →

EGTA

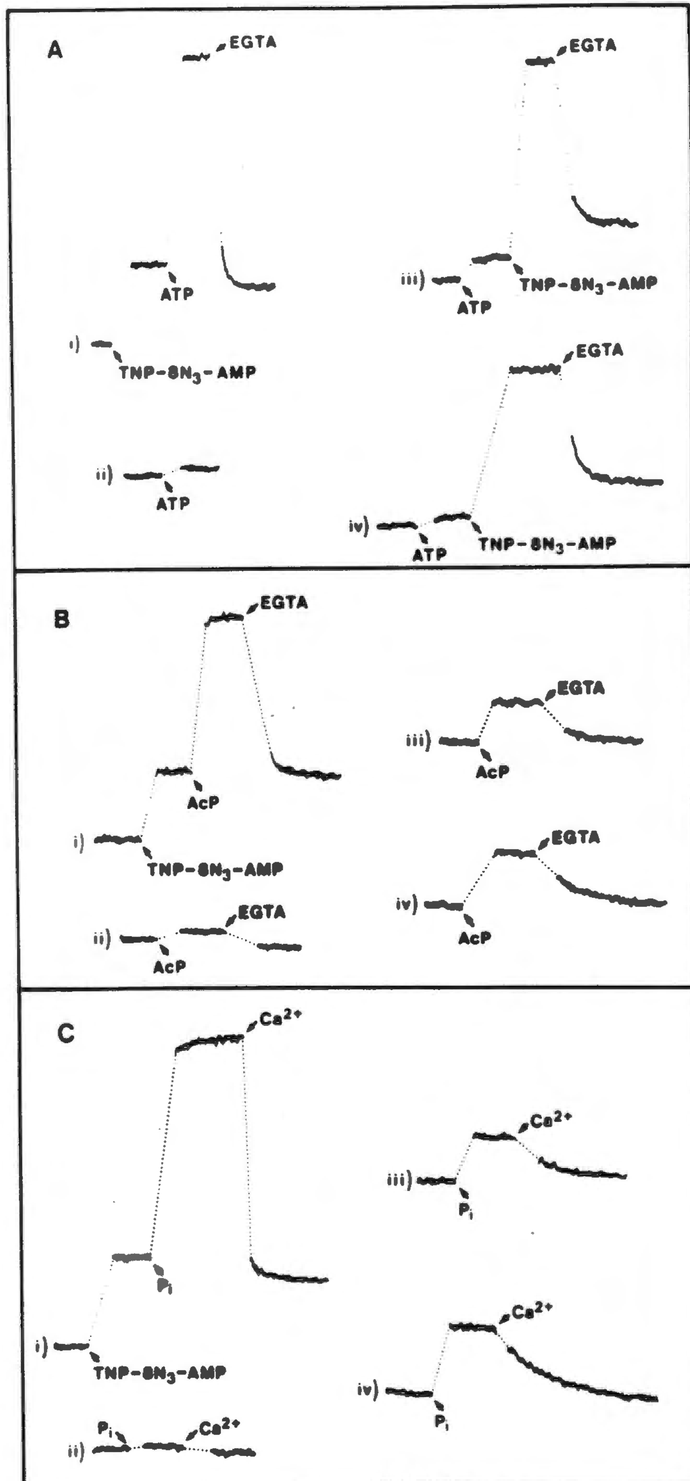
125 →

110 →

EGTA + ATP

Figure 3.22. Fluorescence properties of photoincorporated nucleotide.

SR vesicles (0.2 mg of protein/ml) were irradiated in 20 mM CHES/TMAH, pH 8.6, 5 mM $MgCl_2$, 20 % (v/v) glycerol, without (traces i and ii) or with (traces iii and iv) 15 µM TNP-$8N_3$AMP under nonturnover (0.5 mM EGTA, trace iii) or turnover (50 µM $CaCl_2$ and 100 µM ATP, trace iv) conditions. In the control samples (traces i and ii) TNP-$8N_3$AMP (5 µM) was added immediately following the irradiation period. Nucleotide not covalently attached was removed and the fluorescence measured on undiluted samples. In (A) ATP (100 µM), (B) acetyl phosphate (AcP, 5 mM) and (C) P_i (5 mM) were used as substrates. In the latter case, the pH was adjusted to 6.5 with maleic acid just prior to the fluorescence measurements. Where TNP-$8N_3$AMP, EGTA and Ca^{2+} are indicated the concentrations were 5 µM, 0.5 mM and 50 µM respectively.


A
EGTA
ATP
i)
TNP-8N3-AMP
ii)
ATP
iii)
ATP
TNP-8N3-AMP
EGTA
iv)
ATP
TNP-8N3-AMP
B
EGTA
AcP
i)
TNP-8N3-AMP
ii)
AcP
EGTA
iii)
AcP
EGTA
iv)
AcP
EGTA
C
Ca2+
Pi
i)
TNP-8N3-AMP
Pi
ii)
Ca2+
iii)
Pi
Ca2+
iv)
Pi
Ca2+

turnover conditions, with Ca^{2+}, Mg^{2+}, and 100 µM ATP (traces A iv, B iv, and C iv). The amount of labeling expected was 2 nmol/mg of protein (or 30% of active ATPase polypeptides) and 2.5 nmol/mg of protein (or 50% of active ATPase polypeptides) respectively (values derived from Fig. 3.19). Following irradiation, noncovalently attached nucleotides were removed by passage through an anion exchange resin. The effectiveness of the latter procedure is shown by the lack of superfluorescence of control preparations which had not been irradiated but to which TNP-8N_3AMP had been added prior to the column (traces A ii, B ii, and C ii). The removal of noncovalently bound TNP-nucleotides is difficult on account of their high affinity and slow rate of dissociation from the ATPase. Effective removal required the addition of ATP and very slow passage of the mixture through the column. Nevertheless, even with this procedure , in the case of the nonturning over enzyme a small amount of free nucleotide eluted with the vesicles (see below). In panel A, it is shown that the covalently attached nucleotide does not exhibit superfluorescence with ATP as substrate irrespective of whether the labeling was carried out under nonturnover (iii) or turnover (iv) conditions. In the case of (iii), the small amount of fluorescence increase seen upon addition of ATP and Ca^{2+}, but before addition of extra TNP-8N_3AMP, is attributed to the presence of a small amount of free TNP-nucleotide. Addition of TNP-8N_3AMP to preparations irradiated in EGTA (iii) and Ca^{2+} + ATP (iv), resulted in superfluorescence to the extent of 70% and 50% of the control (i) respectively, which is equivalent to the amount of ATPases expected not to contain attached nucleotide.

Activation of enzyme turnover with acetyl phosphate, resulted in superfluorescence of the covalently attached nucleotide if the irradiation was done in EGTA and in Ca^{2+} and ATP (panel B, traces iii and iv respectively). The amplitude of superfluorescence was 25% and 40% of the control respectively, which is slightly less than the percentage of ATPases labeled.

A similar result was obtained if the phosphoenzyme was formed from P_i in excess EGTA at pH 6.5 (Fig. 3.22, panel C). Superfluorescence, in amounts proportional to labeling levels,

was observed when the irradiation is performed under nonturnover (iii) and turnover (iv) conditions of irradiation. The amplitude, expressed as a percentage of the control value, namely 25 % and 40% respectively, was slightly less than the percentage of active sites modified (Fig. 3.19). ATP, 2 mM, completely inhibited the superfluorescence of control preparations but had no effect on that of preparations with the covalently attached nucleotide (results not shown), showing that ATP is unable to bind to the catalytic site under these conditions.

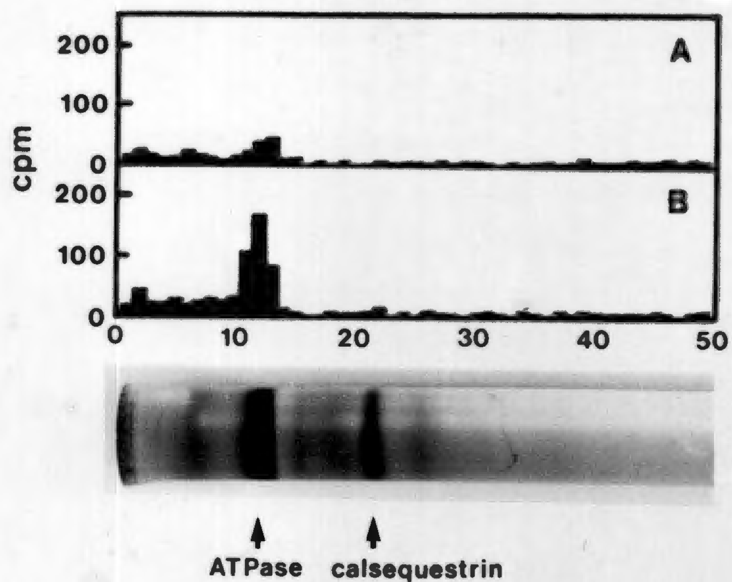
The results obtained with a preparation irradiated in the presence of Ca^{2+} were essentially the same as those for the preparation irradiated in the presence of excess EGTA and are not shown. This is in accord with the similar extents of labeling under these conditions (Fig. 3.19).

3.10 LOCATION OF LABELED SITES

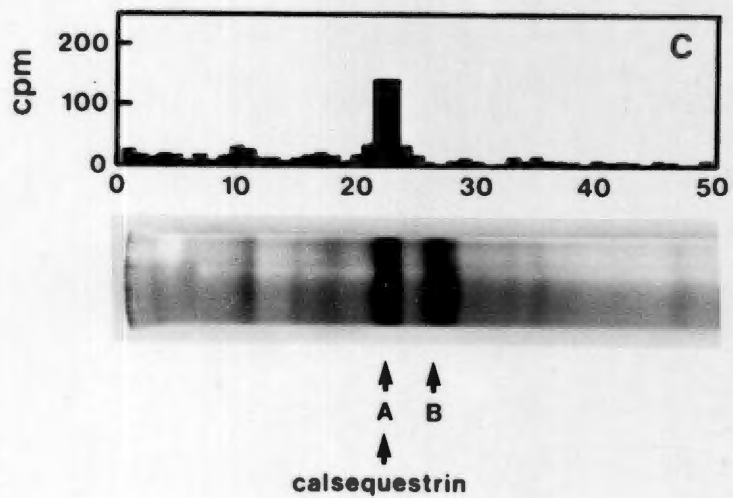
The extent of covalent labeling by TNP-8N_3ADP of the protein components of SR vesicles and of the primary tryptic fragments following SDS-PAGE are shown in Fig. 3.23. A relatively high concentration (10 μM) of azido nucleotide was used so that the vesicles were labeled to approximately the same extents at pH 6.0 and at pH 9.0 (see Fig. 3.20). At pH 6.0, the ATPase was not labeled to a significant extent (Panel A) and most of the labeling was presumably on phospholipid. This conclusion is in agreement with the small inhibition of ATPase activity (Fig. 3.19) and of active site cross-linking (Fig. 3.21) under these conditions. In contrast, at pH 9.0 (Panel B), the ATPase was labeled to a much higher degree. Tryptic cleavage of the ATPase at the T1 site to produce fragments A and B (Fig. 3.23, second gel and Panel C) shows that the label is entirely associated with the A fragment. At a higher ratio of trypsin to SR protein (1:100), which cleaved the A fragment into the A1 and A2 subfragments, only A1 is labeled (Panel D). The lack of labeling of the B and A2 fragments confirms that the nonspecific labeling was negligable. Similar results were obtained when the labeling was carried out under nonturnover conditions in the

Figure 3.23. SDS-PAGE of photolabeled SR proteins and tryptic fragments.

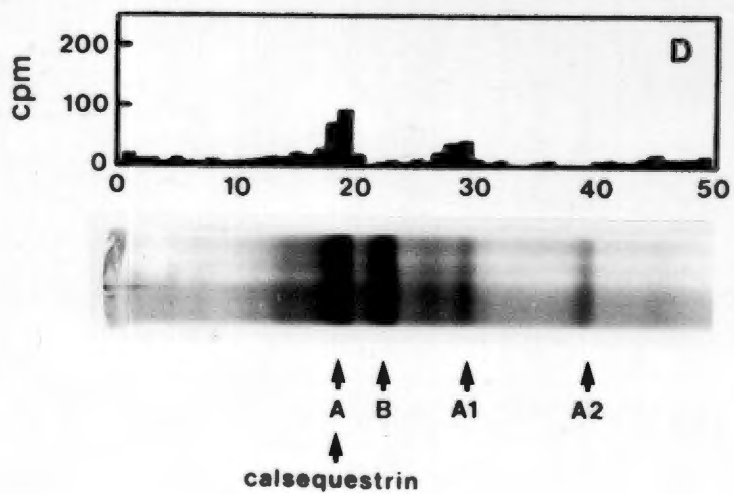
SR vesicles (0.2 mg of protein/ml) were irradiated in 20 mM CHES/TMAH, pH 6.0 (A) or pH 9.0 (B, C and D), 5 mM $MgCl_2$, 20 % (v/v) glycerol, 50 µM $CaCl_2$, 100 µM ATP and 10 µM TNP-8N_3-[2-^{3}H]-ADP for 3 min. The suspension was then incubated without (A and B) or with (C, 1:1000 and D, 1:200 mg / mg of SR protein, respectively) trypsin for 15 min at 25 ^{o}C. Aliquots (50 mg of SR protein) were then taken for electrophoresis.



cpm
200
100
0
A
B
0
10
20
30
40
50
ATPase
calsequestrin



cpm
200
100
0
C
0
10
20
30
40
50
A
B
calsequestrin



cpm
200
100
0
D
0
10
20
30
40
50
A
B
A1
A2
calsequestrin

presence of 1 mM EGTA and without added Ca^{2+} and ATP (results not shown).

3.11. PEPTIDE MAPPING

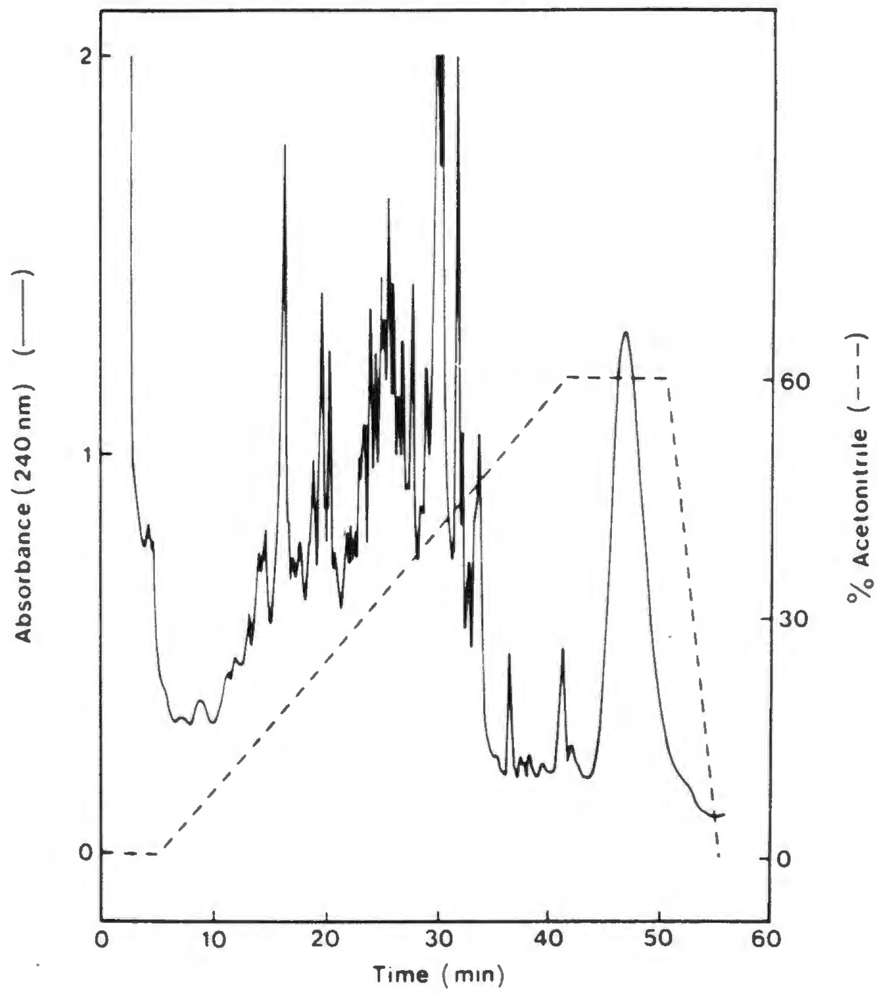
In this section we describe a preliminary attempt to locate the locus of attachment of the TNP-$8N_3$-nucleotide to the ATPase. The analysis was not completed but is included to indicate the direction that the research is going and points to some of the difficulties which need to be overcome.

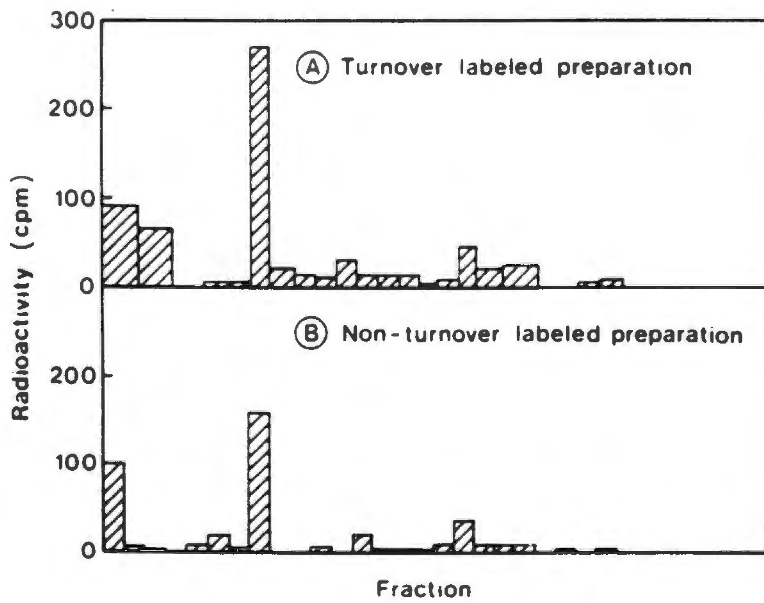
3.11.1 Purification of labeled peptides

SR vesicles were labeled under conditions resulting in specific incorporation of TNP-$8N_3$-[2-^3H]-AMP into the turning over and nonturning over enzyme (see Fig. 3.19). The extent of covalent incorporation of the probe was found to be 1.5 nmol/mg of SR protein, whether labeling was carried out under turnover or nonturnover conditions. This value is slightly lower than expected from previous results (see Fig. 3.19). Following trypsinization of the resuspended vesicles, only 2-5% of the counts were pelleted with the membrane fraction, consistent with most of the label being associated with the hydrophilic extra membranous portion of the ATPase. An initial fractionation by reversed phase HPLC of the tryptic peptides released from the turnover-labeled preparation, is shown in Fig. 3.24. The profile obtained for the nonturnover-labeled preparation was similar. The radioactivity profiles for the turnover and nonturnover labeled preparations are also shown in Fig. 3.24a and b. Two major peaks of radioactivity were found with both labeling conditions, the first corresponded to the column void volume and the second to species eluting at about 15% acetonitrile. A number of smaller peaks of radioactivity were seen, but these were not investigated further, at this stage of the investigation. The large peak of radioactivity eluting in the void volume was also not investigated further. It is possible that the TNP-$8N_3$AMP or tritium label is unstable under

Figure 3.24. HPLC Purification of TNP-8N_3AMP labeled tryptic peptide.

TNP-8N_3AMP labeled tryptic peptides were injected onto a µBondapak C18 column in 5 mM PIC A (Buffer A) and eluted with a gradient of acetonitrile + 5 mM PIC A at a flow rate of 3 ml/min. Absorbance of the effluent was monitored at 240 nm. Fractions of 2 ml were collected and 50 µl aliquots were assayed for radioactivity in 10 ml Insta-gel. The absorbance trace shown here is for the turnover labeled preparation with the radioactivity profile drawn below. The radioactivity profile for the nonturnover labeled preparation is drawn above.


Absorbance (240 nm) (——)
% Acetonitrile (- - -)
Time (min)
0
1
2
0
30
60
0
10
20
30
40
50
60


Radioactivity (cpm)
Fraction
Ⓐ Turnover labeled preparation
Ⓑ Non-turnover labeled preparation
300
200
100
0
200
100
0

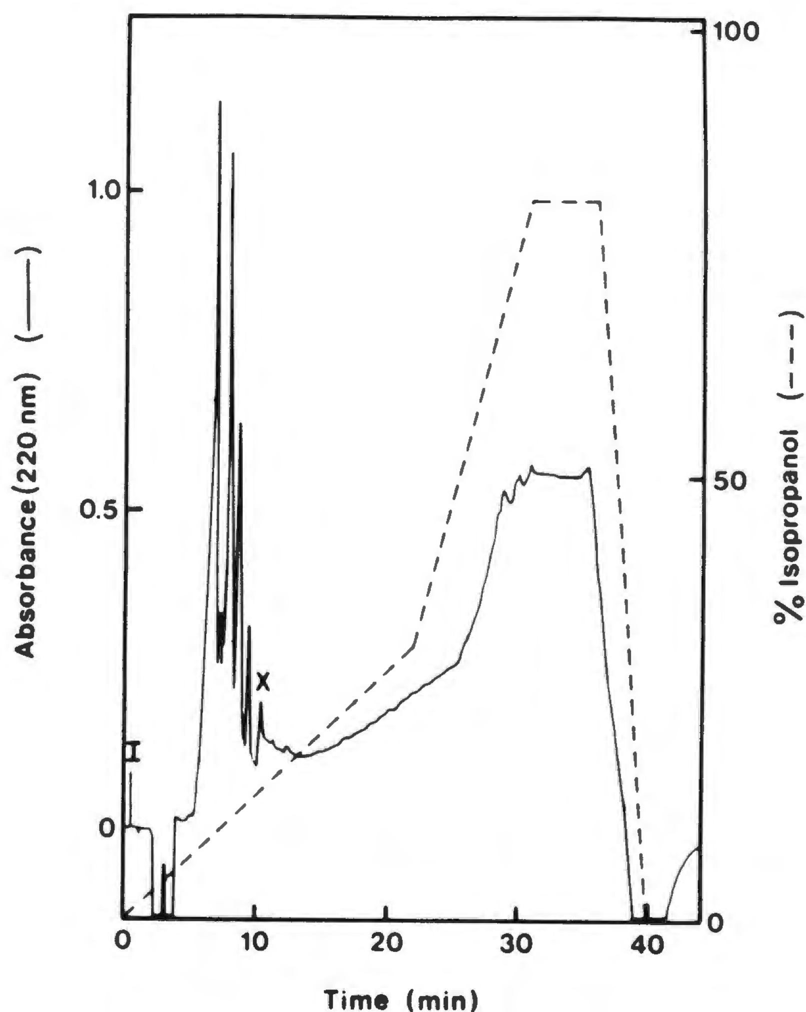
these conditions. Approximately 35-40% of the radioactivity applied to the column eluted in the peak at 15% acetonitrile.

This peak, in both cases, was collected, lyophilized to dryness, then reinjected onto the same column using a different mobile phase. The absorbance trace at 220 nm for the second purification of the turnover labeled preparation is shown in Fig. 3.25. The elution profile for the nonturnover labeled preparation was similar. In both cases, the peak of radioactivity eluted after several larger peptide peaks at about 13% isopropanol. This peak was collected and lyophilized to dryness. The similarities between the two preparations as regards the HPLC elution of the labeled peptides suggests a single site of labeling under both turnover and nonturnover conditions.

SR vesicles were labeled with FITC and treated in the same way as the TNP-8N_3AMP labeled preparation to act as a control and to check on the methodology. The FITC labeled tryptic peptide was isolated in a single HPLC elution (Fig. 3.26). A single peak with strong absorbance at 254 nm was collected, eluting at about 31% isopropanol. The characteristic absorbance spectrum of FITC was seen on scanning the fraction at alkaline pH. A second HPLC run of the lyophilized peak showed a single peak.

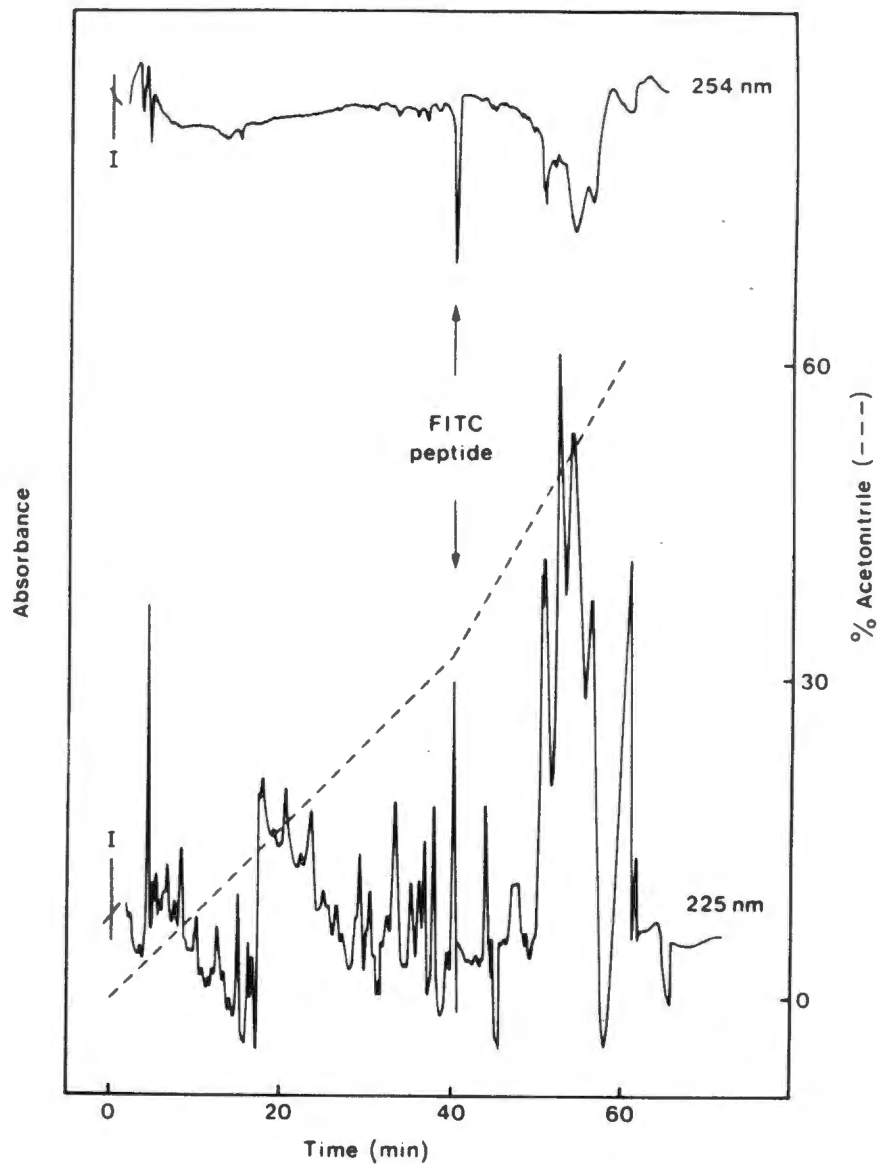
3.11.2. Amino acid analysis and sequencing of labeled peptides

HPLC separation of seventeen standard PITC-amino acids is shown in Fig. 3.27. Asn and Gln are hydrolysed to their respective acids during the acid hydrolysis. Trp is labile and does not appear in the elution profile. A clear separation between Asp and Glu as well as betwen Arg and Thr was not obtained, but approximate quantitation was achieved using a peak separation method in the integrator. The pattern obtained was reproducible and retention times of standards did not vary by more than a few seconds between successive injections. A preparation of amino acids which had been subjected to the acid treatment was routinely analyzed to obtain the percentage



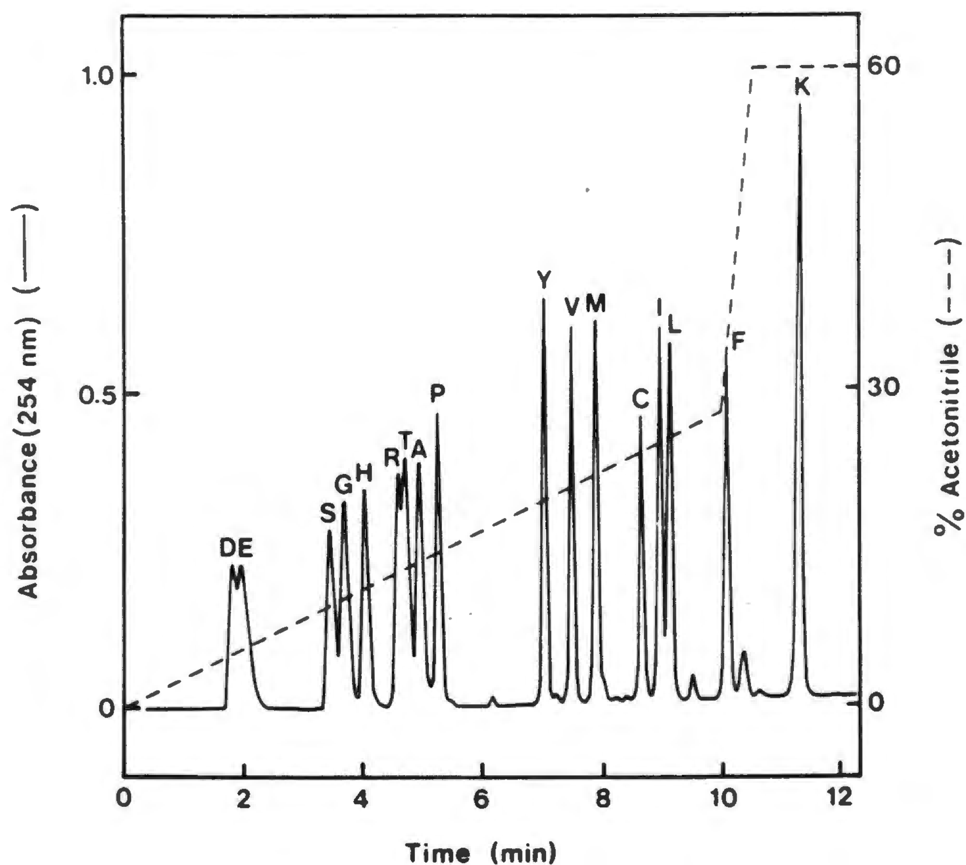
3.25. <u>Second Purification of TNP-8N_3AMP labeled tryptic peptide by HPLC.</u>

The major peak of radioactivity from the initial fractionation was reinjected onto the same column in 0.01 M ammonium acetate, pH 4.0 and eluted with a gradient of isopropanol in buffer A. The absorbance of the effluent was monitored at 220 nm. The peak of radioactivity is shown with a cross. The results shown here represent the preparation labeled under turnover conditions, that labeled under nonturnover conditions was similar.



3.26. HPLC purification of FITC-labeled tryptic peptide

FITC-labeled tryptic peptides were injected onto a uBondapak C18 column in 0.01 M ammonium acetate, pH 4.0 (Buffer A) and eluted with a gradient of isopropanol in Buffer A at aflow rate of 2 ml/min. Absorbance of the effluent was monitored at 254 nm. The main peak of absorbance was collected, scanned at alkaline pH and identified as containing fluorescence.



3.27. <u>HPLC separation of major PITC-amino acids.</u>

10 nmoles of PITC derivitized amino acids (Pierce amino acid standard H) were injected onto a Picotag column in Picotag Eluent A and eluted with a gradient of Picotag Eluent B. Detection was at 254 nm.

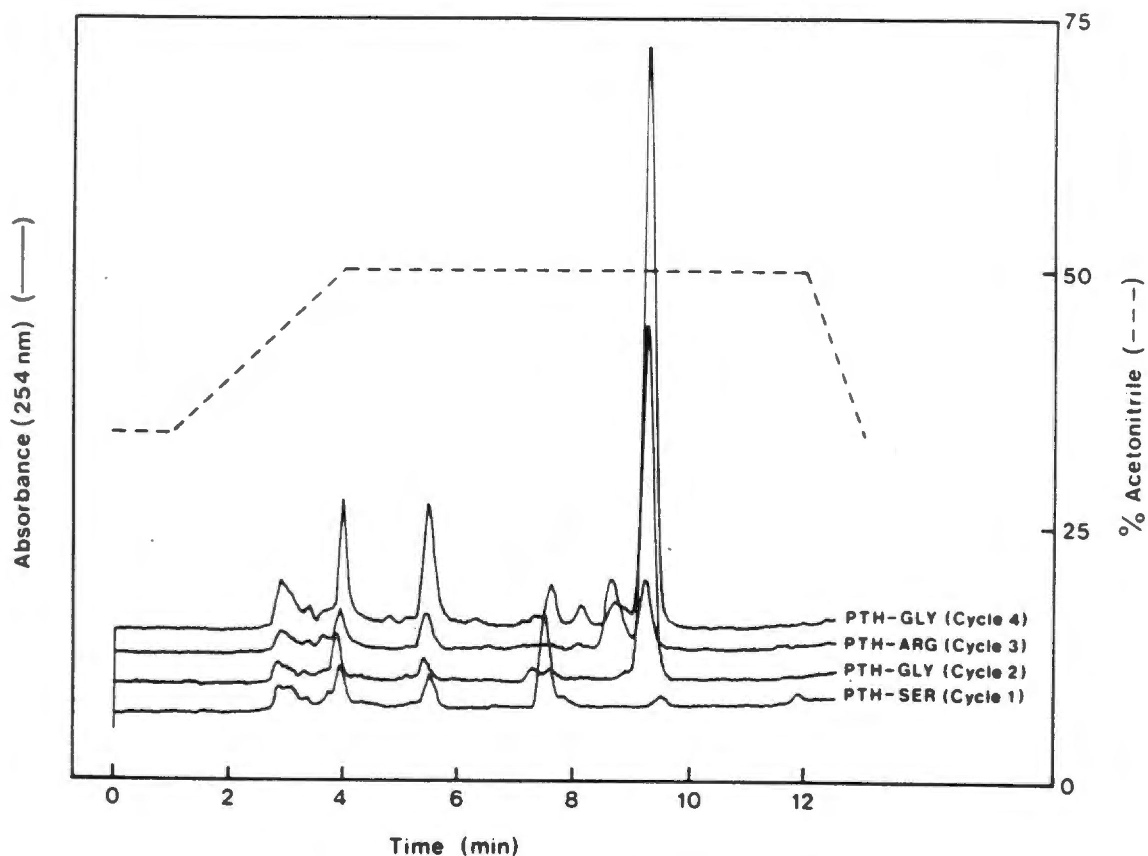
decomposition of individual amino acids during the acid hydrolysis. The main species giving lower yields were Tyr (80% yield) and Cys (55% yield).

The manual method we used for the sequencing of standard peptided has the important advantage that a vapour phase Edmann degradation is carried out resulting in lower levels of background contamination. This results in simplification of the identification process. Fig. 3.28 shows four traces superimposed on one another representing the first four cycles of degradation of the standard peptide AC9. Material from the cleavage reaction was washed into pyrrolysed tubes, treated with acid and then the PTH-amino acids analysed by HPLC. Data was collected on a benchtop computer and then superimposed in a single trace staggered with respect to the preceding cycle. Unfortunately, analysis of the radioactive material obtained from the labeling experiment in Fig. 3.25, using the same procedure, did not result in any detectable PTC-amino acids. There are several possible reasons for this, but the principle one is possibly the lack of sufficient material. A larger amount of material was used to sequence the standard peptides than was used in the amino acid analysis. As noted above, the covalent adduct may be unstable, and more rapid and less severe conditions may have to be developed to obtain greater yields of radioactive peptides. We plan to invesigate these possibilities in the future. Another possibility is that the peptide may contain a cyclized N-terminal residue which would prevent it's being sequenced by this method. This phenomenon has been noticed before, in addition to other problems with the sequencing of azido labeled peptides (Knight and McEntee, 1985a, c).

3.11.3 Amino acid analysis of labeled peptides

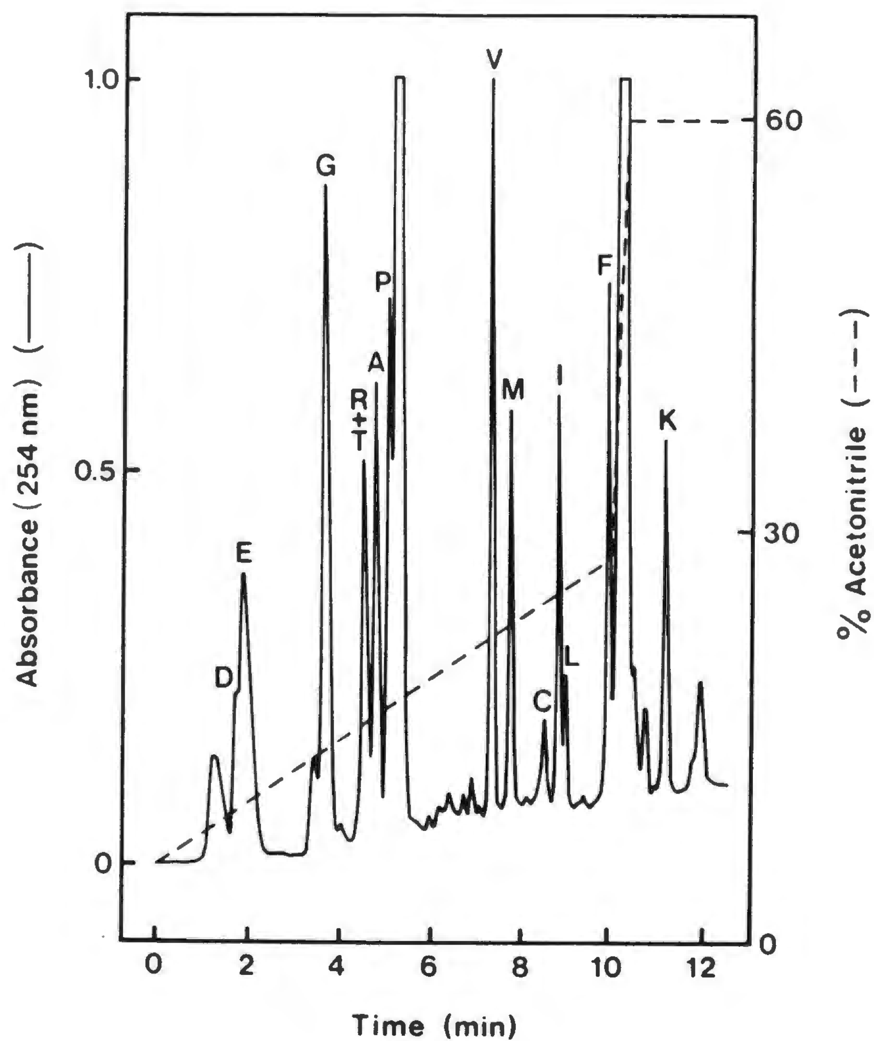
The amino acid analysis of the FITC-labeled peptide is shown in Fig. 3.29. The relative levels obtained are consistent with those in the published sequence (Brandl et al., 1986).

The profile obtained for the TNP-8N$_3$AMP labeled peptide (nonturnover conditions) is shown in Fig. 3.29. The traces



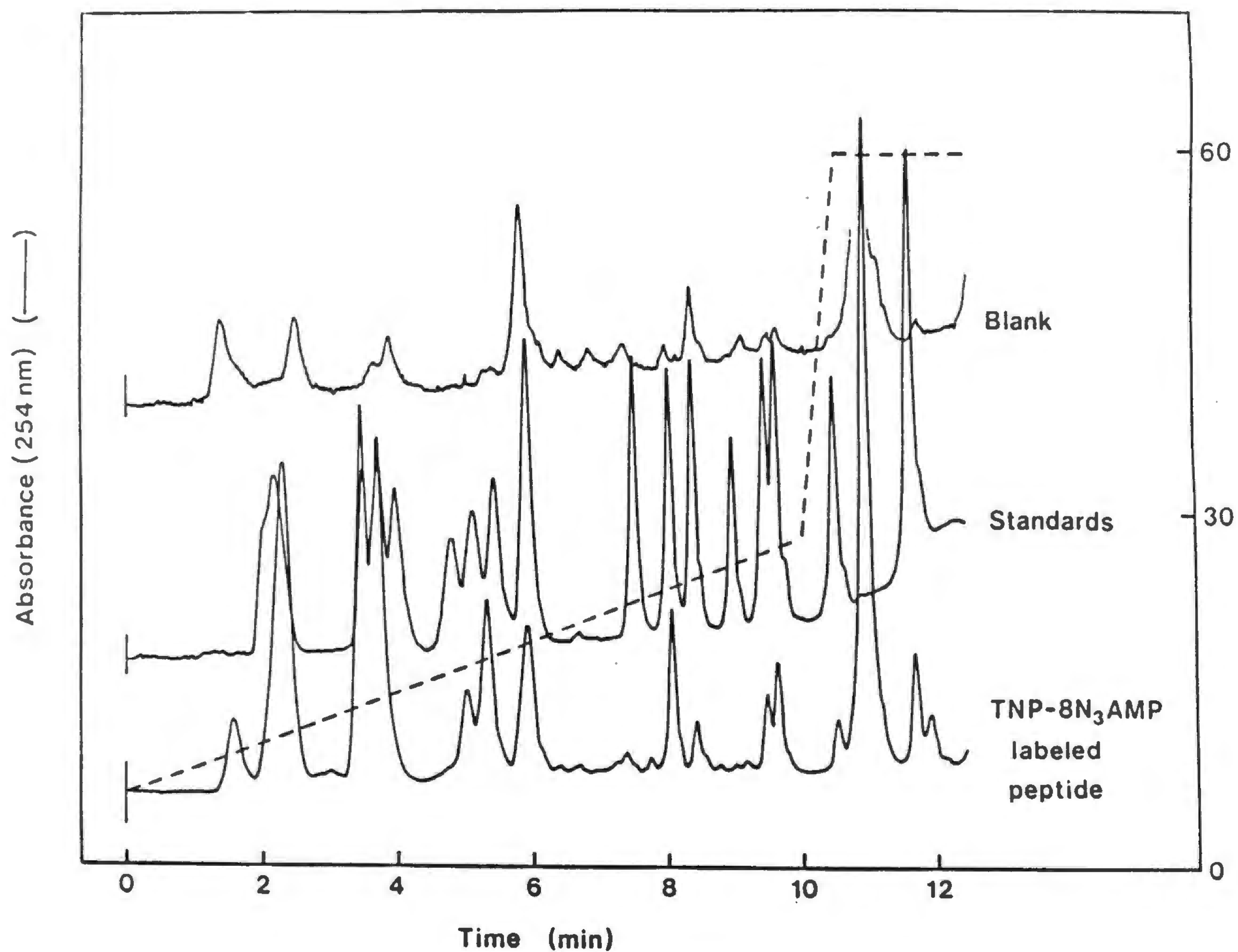
3.28. <u>4-cycle sequence determination of a model peptide.</u>

The synthetic peptide AC9 was subjected to manual sequencing by Edmann degradation for 4 cycles then the amino acids derivitized and analyzed by HPLC on a Vydac C18 protein and peptide column.



3.29. <u>Amino acid analysis of FITC labeled tryptic peptide.</u>

The isolated FITC labeled tryptic peptide was hydrolysed to completion, derivitized and PITC-amino acids analysed by HPLC as described.



3.30. <u>Amino acid analysis of TNP-8N_3AMP labeled tryptic peptide.</u>

The isolated TNP-8N_3-AMP labeled tryptic peptide was hydrolysed to completion, derivatized and analyzed by HPLC as described. Also shown are the derivatized commercial amino acid standards and a blank which consisted of the same amount of sample diluent without amino acids.

shown represnt sample diluent (A), standards (C), and the radiolabeled peak (D), from the purification shown in Fig. 3.25. The major amino acids identified are Glu/Gln, Ser, Gly, Thr, and Val, but no region on the A1 fragment, or in any other part of the protein, could be identified consistent with this composition.

4 DISCUSSION

In this study we have developed a new class of photoaffinity analogs of ATP, ADP and AMP. The compounds combine a light sensitive azido group covalently attached to the 8-position of the adenine ring with a fluorescent trinitrophenyl group attached to the 2' and 3' positions of the ribose moeity. We have used these compounds to probe the nucleotide binding sites of the sarcoplasmic reticulum Ca^{2+}-ATPase.

Both 2- and 8-azido-ATP derivatives are in common use as photoaffinity probes, but our choice of the 8-position for the azido group was dictated by reports that the 2-azido derivative spontaneously forms an unreactive tetrazole isomer in solution. Furthermore, alkaline pH has been shown to alter the 2-azido/tetrazole equilibrium in favor of the latter isomer, while the absorption of visible light by the TNP group is decreased as the pH of the medium is lowered (pK = 5.1) (Hiratsuka and Uchida, 1973). The disadvantage of the 8-azido derivative is that the 8-azido adenine group adopts a _syn_ conformation about the N-glycosidic bond instead of the _anti_ conformation favored by ATP. However, in preliminary experiments with 8-BrATP and $8N_3ATP$, it was found that these derivatives are as good substrates as ATP for the Ca^{2+}-ATPase (see Champeil _et al_., 1988). It is possible that binding induces the sterically unfavorable _anti_ conformation in these nucleotides bringing the position of the adenine in line with that of ATP in the binding cleft. Alternatively, it is also possible that the binding site can accommodate this orientation or that derivatization with the TNP group results in further conformational changes. This should be borne in mind when the site of covalent attachment is assessed in relationship to amino acid side chains comprising the binding site of ATP. This is a general problem when using probes for investigating biological function.

The trinitrophenyl group was introduced for several reasons. It has been found that the TNP derivatives of ATP, ADP, and AMP

all bind with high affinity to the catalytic site of the Ca^{2+}-ATPase with k_D values of about 0.1 - 0.3 μM (Watanabe and Inesi, 1982; Dupont et al., 1982, 1985; Nakamoto and Inesi, 1984; Davidson, 1986). Thus the affinity is significantly higher than that for ATP (k_D about 2 μM). This has two advantages for photoaffinity labeling. Firstly, lower levels of nonspecific labeling are obtained as the photolabeling reaction can be carried out at a lower concentration of probe, and secondly, tighter binding increases the probability of obtaining covalent attachment to the protein.

The affinity of TNP-nucleotides for the regulatory site is also much greater than that of ATP. The k_D values for the phosphorylated Ca^{2+}-ATPase have been reported as 0.95 μM (Davidson, 1986) for TNP-ATP and 3.3 μM for TNP-AMP (Bishop et al., 1986), while the regulatory site responsible for acceleration of activity has an affinity for ATP of about 1 mM (de Meis and Vianna, 1979). Thus TNP-nucleotides have an affinity for this site which is about three orders of magnitude greater than that of ATP.

Furthermore, the fluorescence of TNP-ATP is increased approximately seven-fold on binding to the phosphorylated Ca^{2+}-ATPase. The nature of this site is still unknown. It may represent a modified catalytic site, the regulatory site, or another site. One of the major aims of this study is to attempt to differentiate between these possibilities.

The absorbance and fluorescence properties of the TNP group are advantagous in that they provide a very useful means for characterizing the interaction of the probe with the enzyme. Thus, difference absorbance spectra have been used to monitor the binding of TNP-ATP to the Ca^{2+}-ATPase while the TNP group fluorescence has been used to monitor changes in the nucleotide site(s) during catalysis. Other advantages include the fact that it is possible to get selective binding of TNP-nucleotides to the catalytic, regulatory and superfluorescent sites under

well established conditions and also the fact that the interaction of TNP-nucleotides with SR vesicles has been well studied. Finally, TNP-nucleotides have been used in many other enzymes which means that their photoaffinity analogs may be of more general use.

In this study, we have found that the TNP-8N_3-nucleotides exhibit many of the characteristics of the parent non-azido TNP-nucleotides. When incubated with SR vesicles they bind with high affinity to the catalytic site of the Ca^{2+}-ATPase. Under conditions leading to phosphorylation of the enzyme they undergo a large increase in fluorescence and under the same conditions, the di-, and triphosphate species accelerate ATP hydrolysis. Efficient, specific labeling of the enzyme under both turnover and nonturnover conditions was obtained at alkaline pH to the A1 tryptic fragment, resulting in modification of the catalytic site in both cases. The covalently incorporated TNP-8N_3-nucleotide, whether turnover or nonturnover labeled, increases it's fluorescence in the presence of P_i and Me_2SO or AcP and Ca^{2+} but not in the presence of ATP and Ca^{2+}. We conclude that the catalytic and regulatory nucleotide sites share the same locus, and the results are consistent with a model in which the A1 tryptic fragment forms part of the nucleotide binding domain.

4.1 Synthesis of TNP-8N_3-nucleotides

The TNP-8N_3-nucleotides were synthesized from ATP in essentially three steps involving bromination of the adenine ring, displacement with the azido group and addition of the TNP group. 8N_3-nucleotides may also be synthesized by phosphorylation of commercial 8N_3-adenosine. The method we chose, however, (Schafer et al., 1978b) has two advantages in that it uses a less expensive starting material and also generates a good yield of 8N_3ATP, 8N_3ADP and 8N_3AMP in a single step. Trinitrophenylation of the 8-substituted nucleotides proceeds readily under the same conditions described for ATP (Hiratsuka, 1982), although the inclusion of an oxidising agent

is essential to prevent reduction of the azido group by the released sulfite anion. DTNB is particularly useful in this regard as it not only almost completely prevents reduction of the azido group, but also increases the yield of trinitrophenylation to about 80% compared to published yields of about 50% for ATP (Hiratsuka, 1982).

TNP-8N_3-[8-^{14}C]-, and TNP-8N_3-[2-^{3}H]-nucleotides were synthesized from [8-^{14}C]-, and [2-^{3}H]-ATP using a scaled down version of the method used for synthesis of the nonradioactive TNP-8N_3-nucleotides, with a few modifications. While the yield obtained for the [8-^{14}C] derivatives was good, that for the [2-^{3}H] derivatives was poor. This was on account of the exchangeability of the label under conditions leading to bromination in the 8-position. An alternative method for the synthesis of 8N_3-[2-^{3}H]-ATP from [2-^{3}H]-ATP has been published (Boos _et al_., 1978) with overall radiochemical yields of between 30 and 40%.

The criteria used for judging purity of the synthesized compounds include spectrophotometry, anion exchange TLC and reversed phase HPLC. Disruption of the extended azido adenine ring structure by photolysis or reduction of the azido group results in a shift in the absorption maximum from 281 nm to 273 nm. Thus any decrease in UV absorbance maximum of a stock solution is an indication that a degree of decomposition of the azido group has taken place. The anion exchange TLC system readily separates TNP-8N_3ATP from TNP-8N_3ADP and from TNP-8N_3AMP as well as from the non-trinitrophenylated adenine nucleotides. The most powerful technique, however, is reversed phase HPLC which separates all the compounds as in the TLC system and, in addition, separates out the derivatives with photolyzed or reduced azido group.

Stock solutions of TNP-8N_3-nucleotides are stable for at least two years when stored frozen in aqueous solution at neutral pH, based on the above criteria. With time, some reduction of

the azido group does occur as well as loss of the γ-phosphate group in the case of TNP-8N_3ATP.

4.2 Interaction of TNP-8N_3-nucleotides with SR vesicles

Evidence for binding of the TNP-8N_3-nucleotides to the catalytic site of the Ca^{2+}-ATPase was obtained by several methods. The filtration procedure was used to measure directly the binding of TNP-8N_3-nucleotides to SR vesicles (Fig. 3.12). This method revealed a single class of high affinity binding sites for all three nucleotides. The $k_{0.5}$ values for binding to this site were found to be 0.1 µM for TNP-8N_3AMP, and 0.2 µM for TNP-8N_3ADP and TNP-8N_3ATP. This high affinity binding plateaued at a value of about 4 nmol/mg protein after which a secondary increase was seen which was nonsaturable within the limits used here. For this reason, we postulate that this represents nonspecific partitioning of the compounds into the SR membrane as a result of the increased hydrophobicity conferred on the molecule by the azido group. In the presence of EDTA very low levels of binding were measured. This could be due to the instability of the vesicles under vacuum in the absence of divalent cations or, alternatively, the binding sites may not exist in EDTA. Binding to the catalytic site was also conveniently measured by means of the inhibition of intramolecular cross-linking method recently developed in our laboratory (Ross and McIntosh, 1987b) (Fig. 3.14). These authors have shown that equilibrium nucleotide binding to the catalytic site can be measured from the initial rate of formation of the species intramolecularly cross-linked with glutaraldehyde as determined by SDS PAGE (Ross and McIntosh, 1987a). In this study, the usefulness of this technique was extended into the nanomolar ligand concentration range by using low protein concentrations and large volumes, and collecting the protein on glass fiber filters prior to electrophoresis. The K_i values obtained with this method are 0.04 µM (TNP-8N_3AMP), 0.3 µM (TNP-8N_3ADP) and 0.4 µM (TNP-8N_3ATP), and agree closely with the K_d values obtained by equilibrium binding measurements. Slightly

lower K_d values have been reported for the parent TNP-nucleotides (Watanabe and Inesi, 1982; Dupont, 1982, 1985; Nakamoto and Inesi, 1984; Davidson, 1986), although, again, the monophosphate derivative has the highest affinity (Nakamoto and Inesi, 1984). The slightly higher k_D values obtained for the TNP-8N_3-nucleotides with respect to their TNP-nucleotide counterparts is probably a reflection of the more bulky azido group hindering access to the binding site. Lastly, we used the fluorescence properties of the TNP group to titrate the catalytic site, both in the presence and absence of Mg^{2+} (Fig. 3.13). In the presence of a high concentration of Mg^{2+}, additional binding of the TNP-8N_3-nucleotides, and particularly TNP-8N_3ATP, was detected to a fluorescent site and which was not found with TNP-ATP. Again, this probably represents partitioning of the more hydrophobic, chelated TNP-8N_3-nucleotides into the SR membrane.

Evidence for the binding of TNP-8N_3ATP to the catalytic site of the Ca^{2+}-ATPase was also obtained by measuring ATPase activity in the presence of increasing concentrations of TNP-8N_3ATP (Fig. 3.11). The results obtained are complicated by the fact that at low concentrations, TNP-8N_3ATP accelerates activity by binding to a regulatory site. Increasing concentrations progressively inhibit activity, following competition at the catalytic site. The parent TNP-nucleotides have alternately been reported to accelerate ATPase activity (Dupont et al., 1985; Champeil et al., 1988), or to block acceleration by ATP (Bishop et al., 1987). We used a pH-stat procedure to measure acceleration of ATPase activity and found that TNP-8N_3AMP, TNP-8N_3ADP, and TNP-8N_3ATP accelerated activity 1.0-, 1.4-, and 1.5-fold, respectively. TNP-nucleotide acceleration of ATPase activity is only evident using the pH-stat procedure and not with the coupled enzyme method. The reasons for this are not yet understood. Binding of TNP-8N_3-nucleotides to this regulatory site was determined by measuring the increase in fluorescence of the bound TNP-8N_3-nucleotide upon addition of ATP and Ca^{2+} (Fig. 3.16). Similarly to binding to the catalytic site, the TNP-8N_3-nucleotides have a lower apparent affinity ($k_{0.5}$ = 5 - 8 μM) for

this site with respect to TNP-ATP and also have a lower yield of fluorescence. This may either be an intrinsic property of the fluorophore, or it may be a result of the decreased affinity for the site. The reason for the increased affinity of the monophosphate species may be related to a decrease in the steric properties of the probe. It is also interesting that the monophosphate does not accelerate ATPase activity. Again, this could be a result of the increased affinity for the catalytic site which would inhibit ATP binding. The conditions under which acceleration is obtained with TNP-8N_3ATP and TNP-8N_3ADP are the same as those for obtaining the high fluorescence state, identifying the high fluorescence site with a regulatory site responsible for acceleration of turnover. A number of studies with TNP-ATP have correlated the high fluorescence site with binding to E_2-P (Dupont and Pougeois, 1983; Andersen et al., 1985; Wakabayashi et al., 1986). It has also been postulated that the binding of ATP to the phosphorylated enzyme intermediate results in acceleration of turnover (McIntosh and Boyer, 1983). From these studies we concluded that the TNP-8N_3-nucleotides bind with high affinity to a moderately fluorescent catalytic site and a highly fluorescent regulatory site. Identification of the highly fluorescent site with the regulatory site responsible for ATP modulation of turnover, is suggested from the similar conditions required for binding to both sites.

4.3 Photoaffinity labeling

Nitrenes are capable of a number of reactions including addition reactions, insertion into C-H bonds either directly or via proton abstraction, rearrangements and nucleophilic attack (Knowles, 1972). For an efficient reaction with a protein it is necessary that the azido group is in close contact with the amino acid side chains, possibly within 2 Å (Guillory, 1979). Nitrenes react readily with H_2O, and this reaction could compete with that for the protein (Knowles, 1972). In the experiments described in this work, we noted a marked pH dependence of photoincorporation. This finding may be of more widespread

significance in photoaffinity labeling, and has not previously been described as a necessary requirement for specific photoincorporation; it may, generally, be unnecessary, depending on the nucleophile involved. A low degree of labeling is obtained at neutral pH which had little effect on ATPase activity. At higher pH, however, highly specific and efficient labeling (40 - 80 %) was obtained. The affinity of the catalytic site for the TNP-$8N_3$-nucleotides is approximately the same at pH 7 and 8, demonstrating that this effect is due to enhanced efficiency of the photoincorporation reaction, rather than increased occupancy of the site. Although nitrenes are theoretically capable of insertion into C-H bonds they are more reactive with nucleophilic centres. A number of studies have reported the specific modification of Tyr residues (Garin et al., 1986; Cross et al., 1987; Wise et al., 1987). A possible candidate for labeling from our observed pH dependence would be a sulfhydryl residue (usual pK = 7.5), but a Tyr residue with a lowered pK for the functional group cannot be ruled out (usual pK = 10). The pK values of functional groups are known to depend on the protein environment (Barker, 1971). This pH dependence of labeling may account for the low level of specific labeling of the Ca^{2+}-ATPase with $8N_3$ATP (Campbell and MacLennan, 1983; Cable and Briggs, 1984). Both 8-BrATP and $8N_3$ATP are good substrates for the Ca^{2+}-ATPase (Champeil et al., 1988; Seebregts and McIntosh, unpublished observations), indicating that the enzyme has no difficulty in accomodating the syn structured nucleotides. NMR measurements have established that ATP bound to both the high and low affinity nucleotide sites of the Ca^{2+}-ATPase is in the anti conformation (Clore et al., 1982). Our results suggest that at neutral pH, water or buffer in the active site represents a preferred target for the reactive nitrene radical, while at more alkaline pH a reactive nucleophile on the protein becomes the target. This would also explain the increased efficiency of incorporation obtained under turnover conditions, where it has been postulated that water activity in the active site is lower (Dupont, 1983). The affinity of this site for TNP-$8N_3$-nucleotides is slightly lower than the catalytic site as we were

able to obtain increased labeling, up to 80% incorporation, at higher concentrations of probe (10 μM). Under all three conditions, specific modification of the catalytic site was confirmed by inhibition of glutaraldehyde cross-linking (Fig. 3.21). Low glutaraldehyde concentrations and extended reaction times were used to obtain almost complete conversion to the cross-linked species. Under all conditions, the extent of inhibition of the cross-link correlated with the percentage inhibition of activity.

The photoaffinity labeling levels obtained in this study may be compared favorably with the recent results obtained for labeling the tight nucleotide sites of the F_1-ATPase with $2N_3$ATP/ADP, where free nucleotide may be removed prior to labeling because of the extremely high affinity of the site, and long irradiation times are used (Cross et al., 1987). These authors typically report values of between 50 and 70% photoincorporation, as assayed by inhibition of ATPase activity, with no apparent nonspecific labeling, as judged by extrapolation of the inhibition curve to integral values of sites (one in the case of the catalytic sites and two in the case of the noncatalytic sites) (Cross et al., 1987). It was estimated that nearly all the bound nucleotide became incorporated with little or no nonspecific labeling (Xue et al., 1987; Melese et al., 1988). We obtained levels of 50 - 80% photoincorporation under steady state conditions at alkaline pH as assayed by inhibition of ATPase activity, with little nonspecific labeling.

We have localized the site of labeling of the Ca^{2+}-ATPase with TNP-$8N_3$AMP to the A1 tryptic fragment (residues 199-505). Furthermore, the region from residues 328-505 appears a more likely site as it occurs in the postulated phosphorylation domain. Residues 238-328 are located in a postulated transmembrane region and residues 199-238 are in the so-called transduction domain. This section of the A1 fragment contains two regions of conserved primary structure, 296-316 and 326-359. Neither of these two regions contain Tyr residues and the latter

contains two Cys residues, Cys-344 and Cys-349. Cys-344 has been identified as the site labeled with NEM and, with Cys-364 is one of the 2 sulfhydryl residues making up SH_D, in the nomenclature of Kawakita et al., (1980). It may represent a uniquely reactive residue and thus be more susceptible to labeling.

TNP-8N_3-nucleotides, like the parent TNP-nucleotides, undergo a large increase in fluorescence upon binding to the phosphorylated Ca^{2+}-ATPase, in the dark. Similarly, the covalently incorporated TNP-8N_3-nucleotides, following removal of the free nucleotide, undergo an increase in fluorescence. The extent of this increase correlates well with the amount of photoincorporation and does not depend on whether photoincorporation is carried out under nonturnover or turnover conditions. In an analogous way to the situation with FITC (Pick and Bassilian, 1981), phosphorylation, and the consequent fluorescent increase, of the labeled enzyme can only be achieved either with AcP as substrate in the presence of Ca^{2+}, or with P_i as substrate in the presence of Me_2SO. These results are consistent with a model in which the covalently incorporated TNP-8N_3-nucleotide blocks ATP binding but allows entry of the smaller substrates, AcP and P_i. A similar conclusion was reached for FITC. The importance of our results stems from the finding that inhibition of ATP binding occurs whether the enzyme is labeled at the catalytic site or at the regulatory site. The most simple conclusion is that the two sites share the same locus.

4.4 Location of the Regulatory Nucleotide Site of the Ca^{2+}-ATPase

One of the more important goals of this study was to determine the relationship of the regulatory nucleotide site, responsible for the acceleration of enzyme activity, to the catalytic site. The models which have been postulated to explain the regulation can be divided into those which invoke a second site spatially distinct from the catalytic site to which

the accelerating ATP binds and those which require only a single site to explain the kinetics. In this latter model, the same site is seen to bind both catalytic and regulatory nucleotide but at different stages of the catalytic cycle or on a subunit of a dimer, i.e. the catalytic and regulatory sites reside on different intermediates. This model is the one supported by the data presented in this work as the site of labeling under conditions of turnover and of nonturnover have a number of similar characteristics. (1) The pH dependence of labeling both sites is similar, (2) the same tryptic fragment is labeled in both cases, (3) TNP-$8N_3$AMP covalently incorporated into both sites has indistinguishable fluorescent properties and blocks ATP binding and (4) a single labeled tryptic peptide eluting in the same place is obtained by HPLC. Furthermore, in both cases we were able to titrate only one high affinity TNP-$8N_3$-nucleotide binding site. Even though the same site houses both catalytic and regulatory nucleotides, the known conformational changes during the cycle could lead to a different site of labeling, which would not necessarily constitute evidence for another site. This possibility appears unlikely, however, as the pH dependence of incorporation argues in favor of a single reactive residue being labeled. In fact, a more likely criticism is that the probe seeks out the reactive residue and thus may not reflect the nearest amino acid to the 8 position of the adenine ring, but rather the nearest reactive site. Identification of the highly fluorescent site with the regulatory site responsible for acceleration of activity by TNP-$8N_3$ATP and TNP-$8N_3$ADP has been demonstrated in a number of ways.

It has been postulated that the increase in fluorescence of the TNP group is due to an increase in the hydrophobicity of the environment surrounding the probe in the active site (Watanabe and Inesi, 1982), or due to an interruption of base stacking between the TNP and adenine moeities by adenine stacking with the aromatic functional group of another amino acid (Hiratsuka, 1975). In this study, we consistently obtained an increase in the extent of incorporation under turnover conditions, i.e. under

conditions of binding to the regulatory site. This appears to be consistent with the postulate that the increase in fluorescence is due to expulsion of water as nitrenes are known to be highly reactive towards water and a decrease in water activity would lead to an increase in the efficiency of labeling.

There have been kinetic descriptions of more than two types of site as well as more than two types of modulation. With this in mind, it must be pointed out that we have not excluded the possibility of other sites in addition to the two we have labeled. In fact, in the presence of Mg^{2+} we have detected binding to another site, although we have ascribed this to nonspecific partitioning into the membrane.

Our preliminary attempts to identify the amino acid labeled by TNP-8N_3AMP were hindered by an apparent instability of the probe during the isolation procedures. We found that following covalent incorporation, the TNP group was lost from the protein during the tryptic digestion, as evidenced by a loss of colour and absorbance in the visible region. This was not entirely paralleled by a loss of radioactivity, although an amount eluting in the void volume of the HPLC column was seen. It thus appears that the adenine ring remains partially attached to the protein. The yields were lower than expected, so some loss does occur. We were, however, still able to isolate a single labeled peak from both nonturnover and turnover labeled preparations. This finding is consistent with a conclusion that the same peptide is labeled although it is also possible that the elution of the peptide is determined by the presence of the TNP group. Our atttempts to sequence the labeled peptide yielded no result even though we were able to detect the presence of amino acids following total amino acid analysis. Problems with the sequencing technique do not appear to be the cause of this as we were able to perform a four-cycle analysis of a standard synthetic peptide. One possibility is that the peptide has a blocked N-terminus, preventing the PITC coupling reaction, or that the azido group is situated near the N-terminus and prevents

reaction occuring.

4.5 Nucleotide site structure

A common nucleotide binding site has been found to exist in a number of nucleotide binding proteins including dehydrogenases, kinases, and, more recently, F_1-ATPases, elongation factors, and oncogene products. This common folding pattern is not immediately evident from the primary structure as there is little similarity apart from a few regions of homology. This gives rise to the question as to whether these supersecondary structures have arisen as a result of divergent evolution from a common ancestor, or whether they represent a convergence of functional evolution from disparate sources. There seems no doubt that within families eg. the aspartophosphoryl ATPase family, differences represent divergence from a common proto-enzyme (Fig. 1.2), but is there a common ancestor between families? In addition, in view of the structure of the encoding genes, in which functional domains appear to be separated by introns, or, in the protein, by trypsin sensitive sites, there is the possibility that these enzymes may have arisen by duplication of common functional domains and attachment to eg. domains of differing ion specificity. Within families, there also appear to be differences in the mechanism of nucleotide binding. Apart from the low degree of accuracy inherent in the predictive method, it has also been claimed that the consensus sequences are rather loose and incorporate a large range of possibilities (Serrano, 1988). The presence of any homology at all is indicative of a common ancestor, however, on account of the unlikely event of it appearing fortuitously, especially in a number of different enzymes.

Walker et al., (1982) have presented a model for the nucleotide binding site of F_1 based on that of adenylate kinase (Fig. 1.9). A region of homology has been located between the $8N_3ATP$ binding site of the F_1 ß-subunit and the phosphorylation site of the P-type ATPases (Modyanov et al., 1985; Ernster et

al., 1986). The site of labeling the Ca^{2+}-ATPase with AP_3-PL occurs in a region homologous to a portion of the myosin heavy chain (Yamamoto et al., 1988). Two models has been postulated for the nucleotide site of the Ca^{2+}-ATPase based primarily on that of adenylate kinase (Green et al., 1988) and that of phosphofructokinase (Serrano, 1988) (Fig. 4.1). In view of the fact that both adenylate kinase and phosphofructokinase share two consensus sequences, it follows that, in fact, all these enzymes may have a nucleotide site which has arisen from a common ancestor. According to this idea, then, the differences have arisen as a result of a divergent evolutionary process. It also appears that a similar site may also evolve a different mode of binding. The mechanism of the different enzymes does not necessarily prevent this sort of argument as it has eg. been claimed that the phosphorylated aspartic acid of the P-type ATPases is analogous to the tightly bound nucleotides of the F-type ATPases. We have localized the binding of TNP-8N_3ATP to the A1 tryptic fragment (Fig. 3.23). This fragment has previously been identified as the site of labeling with BzATP (Cable and Briggs, 1984), although the authors also reported substantial labeling of the B fragment and phospholipid. A model has been postulated in which the A and B fragments span the length of the molecule abutted to one another (Squier et al., 1987). Our results agree with this model and we conclude that a portion of the A1 fragment must constitute a constant part of the nucleotide site of the Ca^{2+}-ATPase. More recently, the A2 fragment has been specifically labeled following irradiation of enzyme bound UTP (Ferreira and Verjovski-Almeida, 1988). Thus, it appears that the A2 fragment is also involved in making up the nucleotide binding site.

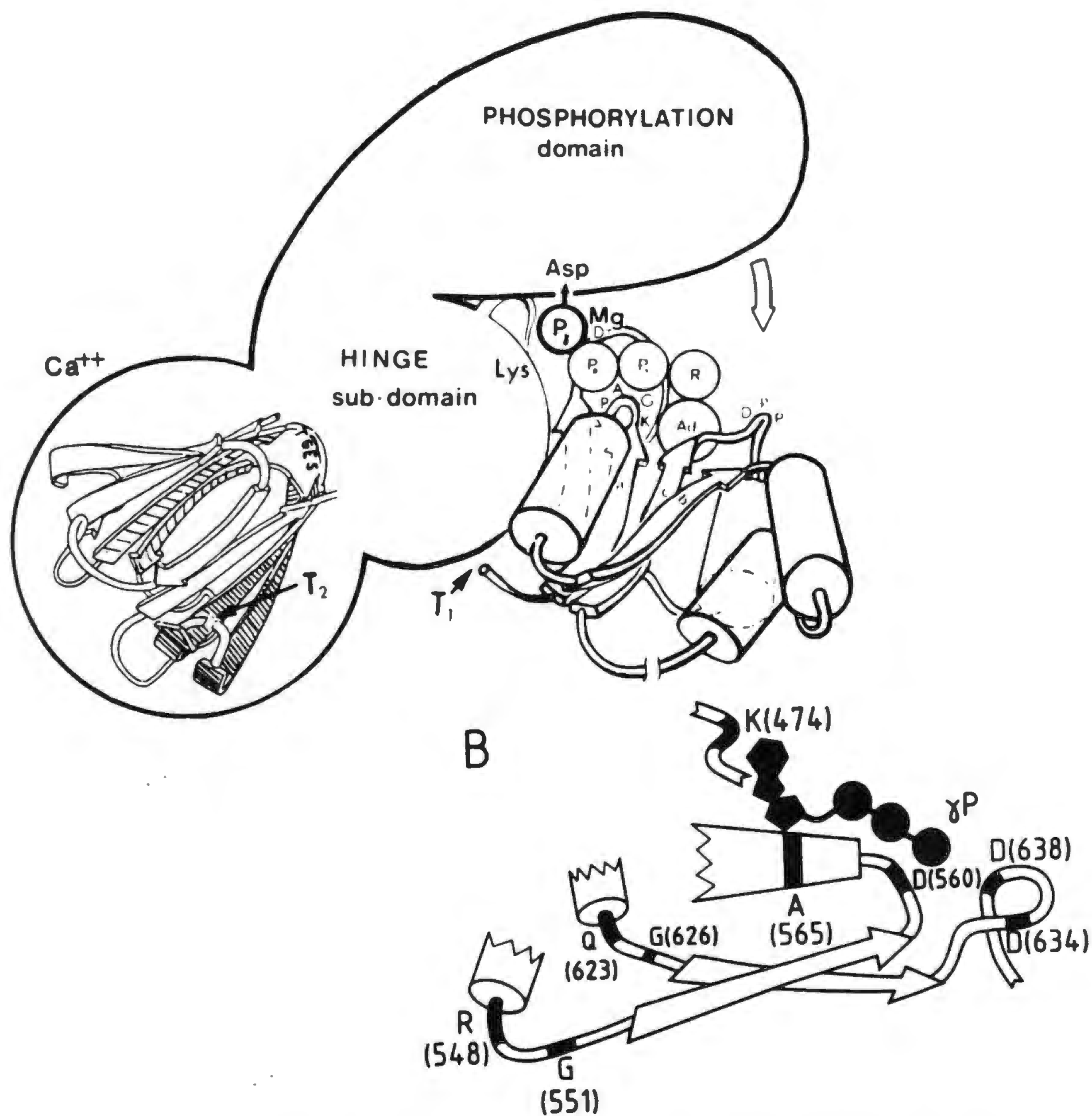


Figure 4.1 <u>Postulated models of the tertiary structure of two P-type ATPases in the region of the nucleotide binding site</u>

Two models of the postulated folding patterns in P-type ATPases in the region of the nucleotide binding site are shown. The one above which refers to the Ca^{2+}-ATPase (Green <u>et al</u>., 1988) is based on the structure postulated for adenylate kinase (Fry <u>et al</u>., 1986), while the one below referring to the fungal plasma membrane H^+-ATPase(Serrano, 1988) is based on the structure postulated for phosphoglycerate kinase (Evans <u>et al</u>., 1981). Shaded areas in the lower figure represent conserved regions.

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