THE RECONSTITUTION OF THE HISTONE OCTAMER

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CERTIFICATION OF SUPERVISOR

In terms of paragraph eight of "General regulations for the degree of Ph. D." I as supervisor of the candidate, H. J. Greyling, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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ABBREVIATIONS AND SYMBOLS

A_{230}	- Absorbance at 230nm
b.p.	- base pair
C.D.	- circular dichroism
DNase I	- Pancreatic deoxyribonuclease
DTNB	- 5,5'-dithiobis(2-nitrbenzoate)
EDC	- 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide hydrochloride
EDTA	- ethylene diamine tetra-acetic acid
EGTA	- ethyleneglycol-bis-(2-amino-ethyl ether) N,N'-tetra-acetic acid
NMR	- nuclear magnetic resonance
PMSF	- phenylmethylsulfonyl fluoride
pCMB	- p-chloromercuribenzoate
SDS	- sodium dodecyl sulfate
TAMM	- tetrakis (acetoxy-mercuri)methane
TRIS	- tris(hydroxymethyl)-aminomethane

SUMMARY

This thesis describes methodology for the reconstitution of the chicken erythrocyte octamer from acid-denatured histones or the natural H3-H4 tetramer and H2A-H2B dimers. Oligomeric properties of reconstituted octamers were elucidated during column chromatographic and chemical cross-linking studies. The conformational identity of the natural and reconstituted octamers was demonstrated by the ability of all preparations to crystallise as helical octamer tubes.

The application of the reconstitution methodology in addressing fundamental problems of chromatin research, was demonstrated during subsequent studies, namely

(i) The reconstitution of hybrid histone octamers containing a structural variant of a specific histone. These studies were undertaken to study the effect on histone-histone interactions in hybrid octamers of which erythrocyte H2B was substituted for by sea urchin sperm H2B(1) or erythrocyte H3 and H4 were substituted for by dethiolated H3 and sea urchin sperm H4 respectively.

(ii) The reconstitution of an octamer suitable for the site-specific derivatisation of a specific histone, or covalently labelled with aurothiomalate in a specific histone complex. These studies were concluded to represent general labelling strategies which may be of use in crystallographic or physico-chemical studies of nucleosome structure.

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MATERIALS AND METHODS

CHAPTER 1

1.1 THE STRUCTURE OF THE NUCLEOSOME CORE PARTICLE:

1.1.1 Introduction

The packaging of eukaryotic DNA into chromosomes requires a compaction factor in the order of 10^4. Histones are largely responsible for the compaction of the genomic DNA into chromosomal structures. Studies aimed at elucidating the mechanism by which this compaction is achieved have focused on the fundamental repeating structural unit of chromatin termed the nucleosome (Kornberg, 1977). These studies were either concerned with the internal structure of the nucleosome or the mechanisms by which nucleosomes are accommodated in higher orders of chromosomal structure (Felsenfeld, 1978; McGhee and Felsenfeld, 1980).

Several lines of experimental evidence lead to the discovery of the nucleosome as the repeating structural unit of chromatin: the demonstration that DNA is released as a set of discrete sizes upon digestion of chromatin with endogenous nucleases (Hewish and Burgoyne, 1973), the "bead on a string" electron microscopic appearance of chromatin (Olins and Olins, 1974) and the isolation of discrete nucleoprotein particles after micrococcal nuclease digestion of chromatin (Rill and Van Holde, 1973). The chemical cross-linking studies of Kornberg and Thomas (1974) demonstrated that the histones H2A, H2B, H3 and H4 are present in chromatin as protein complexes. These experiments performed on native histone complexes in solution and in chromatin, identified the histone octamer (consisting of two copies each of histones H2A, H2B, H3 and H4) as the protein core of the nucleosome.

The characterisation, with respect to DNA length and associated chromosomal proteins, of isolated products of micrococcal nuclease digestions of chromatin, yielded a more definitive description of nucleosomal composition. Initial attack of micrococcal nuclease releases the full nucleosome containing 160-240 base pairs of DNA associated with one H1 molecule and the histone octamer (Kornberg, 1977; Mirzabekov, 1980). Upon further digestion particles with 160-170 base pairs of DNA can be isolated containing the octamer and bound H1 (Simpson, 1978; Varshavsky et al. 1976). This product of digestion has been termed the chromatosome (Simpson, 1978). A model for the association of H1 with the core particle and the additional 15-25 base pair linker DNA has been proposed (Allan et al. 1979). Further digestion yields the more stable core particle consisting of approximately 146 base pairs of DNA associated with the histone octamer (Prunell et al. 1979). This structure which is universal throughout higher eukaryotic cells (Bavykin et al. 1985; Richmond et al. 1982), easily prepared and well defined in composition, has been the object of many physical and chemical studies in chromatin research.

1.1.2 Shape of the Nucleosome Core Particle and the path of the core DNA

Neutron diffraction studies established that the nucleosomal DNA is wrapped around the outside of the histone core (Pardon et al. 1975); Hjelm et al. 1977). Electron microscopic (Langmore and Wooley, 1975; Varshavsky and Bakayev, 1975; Olins et al. 1976) and in particular, neutron scattering studies (Pardon et al. 1977; Suau et al. 1977), of core particles or nucleosomes yielded descriptions of the shape of nucleosomes and core particles. These studies were not all in agreement and differed in particular with respect to the path of the DNA in the core particle (Mirzabekov, 1980). The major

deficiency of neutron scattering data (and of the electromicroscopic determinations cited above), is the lack of three dimensional information (Pardon and Richards, 1979). Analysis by appropriate techniques of ordered aggregates of single crystals of the core particle or histone complexes overcomes these problems and therefore represents the most conclusive structural information available.

Early low resolution studies (20Å resolution) by X-ray diffraction and electron microscopy on crystals of core particles yielded electron density maps in the principle projections, suggesting that the shape of the particle was that of a wedge shaped disk 57Å thick and 110Å in diameter (Finch et al. 1977). The particle was found to be strongly divided into two layers with a dyad axis of symmetry relating the two halves of the particle. A model was proposed in which the DNA was wound around the histone octamer core in about 1.75 turns of a shallow left-handed superhelix with a pitch of approximately 27Å. DNase I digestion studies of core particles (Noll, 1974; Lutter, 1978) supported the view that the core DNA was supercoiled and conclusively established the handedness of the superhelix (Lutter, 1978) used for the development of the model. Using neutron diffraction in conjunction with contrast variation, the DNA and protein components were seen as separate densities in projections at 25Å resolution (Finch et al. 1980; Bentley et al. 1981). These results were consistent with the proposed model. In conformity with spectroscopic studies (Thomas et al. 1977; Goodwin and Brahms, 1978; Cotter and Lilley, 1977) the DNA was concluded to be of the B-form (Richmond et al.1982).

The three-dimensional shape of the histone octamer was subsequently determined to a resolution of about 20Å by

image reconstruction from electron micrographs of helical tubes (Klug et al. 1980). Like the nucleosome core the histone octamer was a wedge-shaped particle of bipartite character. The surface of the octamer appeared as a system of protruding ridges that formed a nearly continuous helical ramp. This structure could provide a template onto which 1.75 turns of DNA could be wound in a left-handed superhelix. The assignment of histone locations on the map relied on DNA-histone and histone-histone crosslinking proximities obtained from chemical cross-linking studies (discussed in section 1.1.3) and suggested a division of the octamer into H2A-H2B dimers residing on opposite faces of the H3-H4 tetramer.

There had been considerable discussion in the literature regarding the way in which DNA may be deformed to allow superhelix formation. It was suggested that abrupt bends or kinks in the DNA occured at periodic intervals (Crick and Klug, 1975) and that the DNA may not be completely uniform (Bryan et al. 1979; Axel et al. 1974). No special class of DNA backbone conformation could however be detected by phosphorous or proton magnetic resonance (Cotter and Lilley, 1977; Kallenbach et al. 1978; Klevan et al. 1979). The electron density map of the core particle at higher resolution (7Å) showed that the DNA was not bent uniformly into the superhelix but exhibited several regions of tight bending or possible kinking (Richmond et al. 1984). Positions of tightest bending correlated well with the sites of greatest protection from DNase I digestion (Lutter, 1978) and enhanced reactivity with dimethyl sulphate (McGhee and Felsenfeld, 1979). As no protein density extended around the outside of the DNA at these sites it must be concluded that the reduced rates of nuclease attack arose from distortions in the DNA conformation and not through steric hindrance (Prunell et al. 1979; Klug et al. 1982). The pitch of the DNA superhelix was

not constant throughout the structure but varied between 25Å and 30Å.

From the crystallographic data (Richmond et al. 1984) and a study by Drew and Travers (1985) it is possible to make definitive statements concerning the "linking number controversy". The controversy resulted from physicochemical measurements performed on the SV40 mini-chromosome (Germond et al. 1975) which suggested only one (rather than two) superhelical turns per nucleosome. Finch et al. (1977) and Klug and Lutter (1981) argued that the change in linking number is given by the sum of the number of superhelical turns and any change in local twist induced by the protein. A change in local twist from 10.6 base pairs per turn determined for DNA when freed from the nucleosome (Rhodes and Klug, 1980) to approximately 10 base pairs per turn in the core would resolve the paradox (Klug et al. 1982). DNase 1 digestion studies of core DNA could not determine the helical twist unequivocally but an average of 10 base pairs per turn periodicity was suggested from steric considerations (Klug et al. 1982). By the method of statistical sequencing, Drew and Travers (1985) obtained a mean value of 10.17 base pairs per turn for core DNA. From this determinations and the observed 7.6 double helical turns of DNA per superhelical turn in the core (Richmond et al. 1984) the experimentally observed change in linking number of -1.25 (Germond et al. 1975) could be calculated (Drew and Travers, 1985).

The electron density map of the histone octamer at 3.3Å yielded a proposed structure (Burlingame et al. 1985) which is difficult to reconcile with that of Klug and co-workers. This structure, accounting for 90% of the expected protein mass, was found to be a prolate ellipsoid with a length of 110Å and a diameter of 65Å by 70Å. A striking feature was its tripartite organisation interpreted as representing a central

H3.H4 tetramer flanked by two H2A.H2B dimers. The volume of this structure is considerably larger than that reported by Klug et al. (1980). Knowledge of the subunit organisation of the octamer and known amino acid sequence of the histones allowed direct identification of the individual histones. Dominant features of the octamer surface are the well defined grooves and ridges traversing the octamer in a discontinuous left-handed spiral 20Å to 25Å wide. From model building it was found that two full turns of DNA (165 base pairs) saturated the probable DNA-binding surface of the protein. The pitch of the superhelix thus obtained was approximately 34A.

At present it is impossible to distinguish between the two models as the representative structure for the core particle existing in chromatin and this has been the subject of active debate (Klug et al. 1985; Moudrianakis et al. 1985). In an attempt to resolve the discrepancies in the two models Uberbacher et al. (1986), on the basis of small angle neutron scattering measurements, postulated that the elongated conformation of the octamer observed by Burlingame et al. (1985) was due to the particular salt concentrations used during crystallisation. An important consideration which has been referred to by Burlingame et al. (1985) is that the nucleosome core is a dynamic structure. Many solution studies support the view that the nucleosome core particle can undergo conformational changes (McGhee and Felsenfeld, 1980; Mirzabekov, 1980; Bavykin et al. 1985). This is also in agreement with electron microscopic studies (Dubochet and Noll, 1978) and neutron diffraction studies of core crystals (Bentley et al. 1984). It is therefore possible that the two models represent two of the biologically significant conformations of the core particle in chromatin.

1.1.3 The Histones and Histone-Histone Interactions

The amino acid sequence of a large number of the core histone variants are known (Isenberg, 1979; von Holt et al. 1979). From this data it is apparent that the variants of the respective core histones exhibit similar structural characteristics (von Holt et al. 1979; von Holt, 1985). Histones H3 (135 residues) and H4 (103 residues), present in the chromatin of all eukaryotic organisms, have shown (with the exception of yeast) virtually no changes in their primary structure throughout evolution. Both proteins are characterised by a hydrophobic region and a very basic N-terminal domain. Variations in the primary structure of these histones are all accounted for by conservative point mutations.

Histones H2A and H2B exhibit different structural characteristics to those observed for H3 and H4. All variants have a hydrophobic center but are evolutionary less well conserved. Point mutations (largely conservative) occur in H2A and H2B more frequently than in H3 and H4 (von Holt et al. 1979). In addition to these variations in sequence, variations in size are also observed for H2A and H2B (von Holt, 1985). H2A and H2B molecules from various species or cell types within an organism are characterised by either N-terminal and C-terminal extensions in the case of wheat germ H2A (Rodrigues et al. 1979; Rodrigues et al. 1985), or N-terminal variations only as observed for H2B derived from sea urchin (Strickland et al. 1977(a); Strickland et al. 1977(b); Strickland et al. 1978; Strickland et al. 1980). The number of residues of these molecules vary from 121 to 148 for H2B and 129 to 155 for H2A (von Holt, 1985).

With the limited information available on histone-histone and histone-DNA interactions (discussed in section 1.1.4) it is impossible at this stage to interpret these variations in primary structure in terms of core particle structure. Two possible explanations have been offered (McGhee and Felsenfeld, 1980):

i) That the positions of primary structure variation occur in regions of least structural importance.

ii) That the programmed synthesis of histone variants during sea urchin development (von Holt et al. 1984) and the appearance and disappearance of variants during the life span of the mouse (Zweidler, 1984) for example, may suggest different chromatin structural architectures during differentiation.

Present knowledge of histone-histone interactions is derived primarily from chemical cross-linking studies and the characterisation of histone complexes. Reconstitution experiments of histone complexes have yielded results in agreement with the studies discussed below. On the basis of the reconstitution methodology employed and the unsatisfactory characterisation of the products of reconstitution, the validity of numerous of these studies is disputable (see Chapter 2). This present discussion is solely concerned with studies on the native histone complexes.

Method of cross-linking	Length of cross-link	Observed cross-linked dimer	References
Tetranitro-methane	0Å	H4-H2B H2A-H2B	Martinson and McCarthy 1975
Ultraviolet light	0Å	H4-H2B H2A-H2B	Martinson et al.1976
Formaldehyde	2Å	H2B-H4 H2A-H2B H3-H3 H3-H2A H3-H2A H2A-H2A	Van Lente et al.1975 Jackson, 1978
Copper phenanthroline	0Å	H3-H3	Gould et al. 1980 Ausio et al. 1984

Table 1.1: Major histone dimers resulting from short-range cross-linking experiments.

The characterisation of histones isolated by the procedure of van der Westhuyzen and von Holt (1971) established the existence of discrete histone complexes. Kornberg and Thomas (1974), on the basis of chemical cross-linking and sedimentation studies identified these as the H3-H4 tetramer and the H2A-H2B dimer. It is now well established that the histone octamer core of the nucleosome comprising one H3-H4 tetramer associated with two H2A-H2B dimers can be isolated as a discrete complex in 2 M NaCl at pH 7 or 9 (Thomas and Kornberg 1975; Thomas and Bütler, 1977). Numerous studies have demonstrated that the isolated octamer can dissociate under a number of conditions. This reversible dissociation

proceeds via the loss of one H2A-H2B dimer (Godfrey et al.1980; Ruiz-Carillo and Jorcano, 1979) to yield a "meta-stable" hexamer. Subsequent loss of the remaining dimer yields the more stable H3-H4 tetramer and two H2A-H2B dimers as the natural products of octamer dissociation (Eickbush and Mondrianakis, 1978; Ruiz-Carillo and Jorcano, 1979). Dissociation of the octamer occurs at low ionic strength at pH7 and at acid pH in 2 M NaCl (Eickbush and Mondrianakis, 1978; Ruiz-Carillo and Jorcano, 1979). In addition a protein concentration dependency of histone octamer stability has been reported (Thomas and Butler, 1977; Ruiz-Carillo and Jorcano, 1979). Hydrodynamic (Godfrey et al. 1980) and calorimetric studies (Benedict et al. 1984) of octamer assembly from the tetramer and dimers strongly suggested that the two sites of tetramer-dimer interaction were allosterically linked. A possible role for histidine residues and the importance of hydrogen bonding for tetramer-dimer association were identified (Benedict et al. 1984).

Cross-linking studies of histones performed on nuclei or chromatin preparations are in agreement (but do not prove) with the subunit organisation of the octamer discussed above. This is particularly apparent from studies of histone cross-links (resulting in histone dimers) produced between residues in close proximity (table 1.1). The H2A-H2B cross-links produced by all procedures, for example, are probably all present in the H2A-H2B dimer. This is strongly suggested by the presence of a covalent link produced by ultraviolet light between Tyr-40 of H2B and Pro-26 of H2A in chromatin and the H2A-H2B dimer (Callaway et al. 1985). The H2B-H4 cross-link between residues 68-125 of H2B and 85-102 of H4 has been interpreted to be present in the site of contact between the dimer and tetramer (Klug et al. 1980). This interpretation is largely based on the appearance of an H2A-H2B-H4 ultraviolet light induced cross-linked product

(Martinson et al. 1979). The close proximity of the H3 molecules in the tetramer (and octamer) is apparent from the observed H3-H3 dimer produced by dimerisation of the Cys-110 residues (with copper phenanthroline) and the cross-linking of amino groups with formaldehyde (table 1.1). An H3-H4 dimer is detected only as a minor cross-linked product using formaldehyde (Jackson, 1978) but is more readily detected with the use of a longer cross-linker (Hardison et al. 1975; Hardison et al. 1977). The unexpected H2A-H2A cross-link formed with formaldehyde (Jackson, 1978) has been suggested to be the result of inter-nucleosomal rather than intra-nucleosomal cross-linking (Burlingame et al. 1985).

An important observation made from trypsin digestion studies (see below) by Jackson (1978) was that formaldehyde cross-links (like the other cross-links in table 1.1) were present in the C-terminal regions of the histones only. This implicated importance of the C-terminal domains of histones in histone-histone contacts is in agreement with several lines of evidence:

^{13}C and ^{1}H nuclear magnetic resonance (NMR) studies on the isolated histone octamer (Lilley et al. 1977) strongly suggested that the N-terminal domains of the histones possess considerable mobility and are thus not likely to participate in complex formation. ^{1}H nmr studies on the salt extracted H3-H4 tetramer (Moss et al. 1976(a)) and H2A-H2B dimer (Moss et al. 1976(b)) yielded similar results. The presence of intense and narrow resonances of glycine CH_2 groups (concentrated in the N-terminal domains of H3 and H4) demonstrated that these residues and hence the peptide backbones in their proximity were unrestricted (Moss et al. 1976(a)). Structured regions of all three complexes studied were concluded to be present in the C-terminal domains as ring current shifted resonances of aromatic residues assigned to

these domains could be observed (Lilley et al. 1977; Moss et al. 1976a,b). These domains were concluded to have a precise tertiary structure (Lilley et al. 1977) containing no ß - structure (Moss et al. 1976a,b) and representing the sites of histone-histone interaction.

Proteolytic digestion studies of histones (for review see Böhm and Crane-Robinson, 1984) have been suggested to be in agreement with the apparent structural differences between N- and C-terminal domains. The products of trypsin digestion were first identified by Weintraub and Van Lente (1974) as C-terminal histone peptides. These may be obtained as trypsin generated "limit peptides" using nuclei (Böhm et al. 1980), solubilised chromatin (Harborne and Allan, 1983), core particles (Whitlock and Simpson, 1977) and the histone octamer (Weintraub and Van Lente, 1974; Lilley and Tatchell, 1977) as substrates. The peptides from these different substrates exhibit similar electrophoretic mobilities. The total number of trypsin-accessible core residues amounts to approximately 20% of the total number of residues in the octamer (Böhm and Crane-Robinson, 1984). As similar trypsin "limit digests" are produced in the absence and presence of DNA, it has been concluded that the DNA plays a minor role in the protection of trypsin digestable sites (Lilley and Tatchell, 1977). Since the partially digested core histones can be extracted as an octameric complex after trypsin digestion of cores (Whitlock and Simpson, 1977), it appears that histone-histone contacts are not destroyed during digestion. This observation is in conformity with the presence of histone-histone cross-links produced in the trypsin resistant C-termini (Jackson, 1978). Although proteolytic digestion studies may reveal histone domains important in histone-histone associations in chromatin and the octamer, a correlation between protease sensitive and structurally different domains is more difficult to deduce from these experiments. Such a correlation has been proposed

on the basis of two arguments (Böhm and Crane-Robinson, 1984):

i) That since similar sets of electrophoretic bands are produced by enzymes of different specificities (Brandt et al. 1975; Rill and Oosterhof, 1981; Sollner-Webb et al. 1976), digestion is determined by the availability of the peptide chains rather than by the specificity of the enzyme.

ii) That the reduced resistance of histones to trypsin digestion in the presence of denaturing agents such as urea (Weintraub and Van Lente, 1974) attributes preferential proteolytic attack to conformational differences.

Crystallographic studies of the core particle (Richmond et al. 1984) and of the histone octamer (Burlingame et al. 1985) have not, to date, yielded a detailed description of histone-histone interactions. The assignment of the core histones in both structures are consistent with the observed pattern of octamer dissociation. This is particularly apparent in the crystal structure of the octamer where, except for a few points of contact, solvent channels separate the dimers from the H3-H4 tetramer. With the exception of the H2A-H2A cross-link all the cross-links discussed (table 1.1) are accounted for in both structures. The unaccounted 10% of the protein mass of the octamer was concluded to belong to flexible termini of the histone chains which would be invisible to X-rays (Burlingame et al. 1985).

1.1.4 Histone-DNA Interactions

The linear arrangement of histones along the core DNA was determined by the histone-DNA cross-linking procedure of Mirzabekov and co-workers (Mirzabekov et al. 1978; Shick et al. 1980). This procedure yielded the positions of histones along each strand of the DNA relative to the 5′ termini. No histones were found to be cross-linked to the first 20 nucleotides from the 5′ termini of the DNA. These represent the binding sites of high mobility group proteins (HMG) 14 and 17 (Shick et al. 1985). The primary organisation of histones determined by this methodology is consistent in core particles from repressed nuclei of sea urchin sperm and chicken erythrocytes and from nuclei active in replication and transcription isolated from Drosophila embryos and yeast (Bavykin et al. 1985). Changes in chromatin function therefore appear not to be regulated at this level of core particle structure. This methodology does not distinguish between the two copies of the core histones when assigning the positions with respect to the core DNA. Such assignments can only be made in three-dimensional space from symmetry considerations with additional information on histone-histone proximities (Klug et al. 1980; Bavykin et al. 1985). The linear arrangement of the histones along the DNA superhelix from entry to exit point then is

$$H2A_1\text{-}H2B_1\text{-}H4_1\text{-}H3_1\text{-}H3_2\text{-}H4_2\text{-}H2B_2\text{-}H2A_2$$

where subscripts 1 and 2 distinguish between the histone copies. The core dyad axis is located between $H3_1$ and $H3_2$. This model has formed the basis of histone assignments during low resolution studies (Klug et al. 1980; Richmond et al. 1982) and is in conformity with assignments deduced from

higher resolution studies (Richmond et al. 1984) or electron microscopic studies of heavy metal derivatised histones (Stoeckert et al. 1984).

A detailed description of the histone residues involved in histone-DNA contacts is unavailable. From the crystal structure of the core particle (Richmond et al. 1984) it is apparent that these occur on the inside of the superhelix with no protein density protruding between the turns of the superhelix or embracing the outside of the DNA. DNA-protein contacts are made on nearly every turn of the DNA helix between the H3-H4 tetramer and the central turn of the superhelix. This provides a structural explanation for the observed 80 base pair protection offered by the tetramer alone against micrococcal cleavage (Camerini-Otero et al. 1976; Stockley and Thomas, 1979). The H2A-H2B dimers each complexed with one half of the superhelix, make fewer contacts but increase the overall stability of the core.

Contrary to earlier proposals (De Lange and Smith, 1971; Boublik et al. 1971) several studies suggested that the basic N-termini of histones do not represent the primary binding sites to DNA. Proton nmr studies on core particles (Cary et al. 1978) demonstrated that these domains are mobile and hence not rigidly bound within the core particle. Studies on trypsinised core particles suggested that such particles remain in a "folded" state in the absence of their N-termini. The sedimentation coefficient of digested cores is reduced (Lilley and Tatchell, 1977; Whitloch and Stein, 1978) but consistent with the expected rate of sedimentation if the DNA remained folded. Trypsinised chromatin retains the "bead-on-a-string" electron microscopic appearance (Allan et al. 1982) and trypsinised cores retain buried sulhydryl groups (Wong and Candido, 1978). Furthermore, Whitlock and Stein (1978) demonstrated that the salt displaced trypsinised

histone octamer reconstituted onto core DNA to yield a complex which closely resembles the natural core. Although it may be concluded that trypsinised cores remain folded, they exhibit structural differences as compared with the native core particle. These include a change of the circular dichroism (C.D.) spectrum towards that of free DNA (Lilley and Tatchell, 1977; Grigoryev and Krasheninkov, 1982), a reduced but still biphasic thermal transition (Lilley and Tatchell, 1977) and enhanced sensitivity towards microccal nuclease (Allan et al. 1982) and DNase 1 (Whitlock and Simpson, 1977; Whitlock and Stein, 1978). These observations were suggested to be attributable to the presence of trypsin digestion not only of the N-termini but also the C-termini of H2A and H3 (Dumuis-Kervabon et al. 1986). This was concluded from studies on core particles digested with clostripain (Dumuis-Kervabon et al. 1986). This enzyme yields cleavages only in the N-termini and core particles thus obtained retains the structural features of the native particle on the basis of C.D. and thermal denaturation studies.

On the basis of the above arguments, it must be concluded that basic residues in the C-terminal domains may participate in DNA-histone contacts. Tyrosine iodination patterns (Weintraub et al. 1975) and Raman spectra (Thomas et al. 1977) discount any possible role of tyrosines in protein-DNA interactions. The role of the N-terminal histone domains in chromatin is not known. A possible role for these in stabilising chromatin higher order structures has been proposed (Strickland et al. 1977 a,b; Allan et al. 1982; von Holt, 1985).

An important issue to be considered in the discussion of histone-DNA interactions is the problem of nucleosome positioning. The essence of this problem is whether nucleosomes are located randomly or at preferred positions

along the genomic DNA. A random deposition of nucleosomes on DNA subject to limited spatial constraints only, would simplify the mechanisms of DNA packaging into higher order structures (Kornberg, 1981). Such a configuration, however, presupposes that the DNA is isotropic (Drew and Travers, 1985). This is contradictory to X-ray and solution studies which demonstrated the base sequence dependance of the structural and mechanical properties of DNA (Dickerson and Drew, 1981; Calladine, 1982; Calladine and Drew, 1984; McCall et al. 1985). Experiments designed to investigate a possible sequence dependent positioning of nucleosomes generally employ the use of nucleases (Igo-Kemenes et al. 1982; Simpson, 1986). Several experiments were undertaken before the sequence specificity of nucleases such as micrococcal nuclease (Nedospasov and Georgiev, 1980; Dingwall et al. 1981) and DNase 1 (Fedor and Daniell, 1983; Drew and Travers, 1984) were known. Experiments employing mild nuclease digestions (Wittig and Wittig, 1979; Gottesfeld, 1980) without proper controls for nuclease specificity may therefore require reinterpretation. Subsequent studies on defined DNA sequences with controls for sequence selective nuclease cutting have demonstrated sequence specific nucleosome core positioning "in vivo" and "in vitro" (Simpson, 1986). The mechanism by which such assembly is controlled is not yet understood. Recent studies (Thoma and Simpson, 1985; Linxweiler and Hörz, 1985; Thoma, 1986) provided evidence that sequence dependant interactions between histones and DNA play a decisive role. Drew and Travers (1985) provided experimental evidence strongly suggesting that in a sequence dependent positioned core particle short runs of (A,T) are preferentially positioned with minor grooves on the inside and runs of (G,C) with their minor grooves on the outside of the DNA superhelix. The crystal structure of the core particle (Richmond et al. 1984) is in agreement with this proposal as differences in widths of the minor (and major) grooves on the

inside and outside of the superhelix are discernable. The importance of positioning to cellular function has been noted by Simpson (1986). Of particular interest is that positioned cores expose only half of their DNA information to solvent componenets. This may offer an explanation of the way in which single stranded sequences are rendered readable to regulatory proteins which may read DNA sequences from one face of the double helix.

1.2 SCOPE OF THIS INVESTIGATION

The sequence specific deposition of nucleosomes during assembly, points to a mutual set of highly specific interactions at the level of core particle structure, viz. the histone-histone and histone-DNA interactions. It is also conceivable that alterations in these interactions would be central to the dynamic structural changes in the eukaryotic chromosome during mitosis, replication and transcription. A detailed description and understanding of protein-protein and protein-DNA interactions may be acquired from crystallographic studies of the core particle and the physico-chemical characterisation of well defined reconstituted histone-DNA complexes. From the above discussion, it is apparent that at present a detailed description of protein-protein and protein-DNA interaction is outstanding. Crystallographic studies on the core particle have provided insufficient data due to the limited resolution attained. An additional drawback to the crystallographic studies is the vastly different ultrastructural descriptions of the core particle yielded from the studies of Richmond et al. (1984) and Burlingame et al. (1985).

The reconstitution of histone-DNA complexes, when approached systematically, would firstly require the faithful reconstitution of the histone octamer. The histone octamer as such represents a suitable model system for the study of histone-histone interactions since thermodynamically important interactions occuring in the octamer will also occur in the core particle. Histone-DNA interactions in turn may be studied by assembling the octamer onto DNA by the method of Retief et al. (1984) for example. The present study was undertaken to systematically develop methodology for the reconstitution of the octamer from salt extracted histone complexes and acid purified histones. The potential use of this methodology in the study of histone-histone interactions is demonstrated by the construction of hybrid octamers containing a specific natural histone variant or a site-specific histone "mutant" prepared by chemical modification of a single amino acid side chain. The preparation of histone octamers containing specifically labelled histone component(s) by similar methodology was also undertaken.

CHAPTER 2

2.1 THE RECONSTITUTION OF THE HISTONE OCTAMER

2.1.1 Introduction

The reconstitution of the histone octamer may be attempted by two general methodological approaches. Firstly, the ability of the octamer to reassociate from its natural products of dissociation (discussed in 1.1.3) under suitable conditions provides a general strategy for the reconstitution of octamers from salt extracted histone complexes. As will be demonstrated in subsequent chapters, it is often desirable to attempt reconstitution from individually purified histones. Procedures employed to purify individual histones require denaturing conditions such as high urea concentrations and dilute hydrochloric acid (von Holt and Brandt, 1977). Experimental procedures for the reconstitution of octamers from histones denatured during isolation may therefore be identified as the second approach.

Controversy exists as to the ability of total acid-extracted or individually purified core histones to be renatured and result in native-like histone complexes. Roark et al. (1976) and Thomas and Kornberg (1975) have provided evidence for irreversible denaturation. Several claims in favour of reversibility and complex formation are disputable. D'Anna and Isenberg (1974) on the basis of CD and fluorescence anisotropy measurements, concluded that H3 and H4 associate in equimolar quantities upon complex formation. A reinvestigation of the experimental conditions employed during the above study (Lindsey et al. 1982) identified the product of complex formation unambiguously as an aggregate.

Investigations by Beaudette et al. (1981) and Lindsey et al. (1982, 1983) strongly suggested that the octamer and H3-H4 tetramer can be reconstituted from acid-denatured histones. The stoichiometry of all reconstituted complexes was determined by chemical cross-linking and/or gel filtration chromatography. On the basis of CD and fluorescence emission spectra all reconstituted complexes were concluded to be identical with the corresponding salt extracted native complexes.

The most valid criterion for the native state of histone complexes, would be the demonstration of biological functionality. Since this is exceedingly difficult to demonstrate with a non-catalytic molecule, the best approximation to assess functionality consists in the demonstration of structural identity between the reconstituted and native complex. The absolute test for conformational identity is the production of identical crystals from reconstituted and natural products. None of the studies cited above satisfy this criterion, since spectroscopic studies yield averaged measurements and therefore cannot demonstrate conformational identity unequivocally. The present study was undertaken to study the reconstitution of octamers from

i) native tetramers and dimers selectively extracted from chromatin or prepared by dissociation of the native octamer

ii) acid-denatured core histones, either an unfractionated mixture or individually purified proteins. Conformational states of all purified octamer preparations were probed by their crystallisation properties.

2.2 RESULTS

2.2.1 Octamer reconstitution from salt extracted complexes and Sephadex-G100 chromatography

The procedure used for the isolation of native histone octamers relies on the dissociation of the core protein from chromatin in 2 M NaCl pH 7.4 after pre-extraction of H1 and H5 (section 6.2.3.1). Residual nucleohistone was pelleted by ultracentrifugation thus yielding a crude preparation of octamers, residual H1,H5 and H2A-H2B dimers (at a total protein concentration of 0.75 mg/ml) as the supernatant. Octamers prepared by this procedure were chromatographed on Sephadex G-100 (figure 2.1). Approximately 40% of the protein eluted was recovered in fraction A. This fraction contained core histones in stoichiometric amounts free of contaminating histone H1 (fraction B) which eluted at the trailing edge of the same peak. The second peak contained H2A-H2B dimer and histone H5 (fraction C). Cross-linking proved the presence of octamer in fraction A (figure 2.4).

Octamers were reconstituted from salt extracted histone complexes prepared by the hydroxyapatite (section 6.2.3.2) and protamine displacement (section 6.2.3.3) procedures. By the hydroxyapatite procedure an increasing ionic strength successively dissociates core histone complexes from intact nucleosomes adsorbed to the matrix (figure 2.2). Histones H1 and H5 elute first (fraction A) followed by core histones (fractions B and C). The second peak fraction contains, in the leading edge, fractions enriched in H2A and H2B and in the second half those enriched in H3 and H4 (fractions B and C respectively). This suggests that core histones are displaced

from the DNA and eluted as products of octamer dissociation. This fractionation results from a salt gradient through the column generated during the elution of the column with 3 M NaCl, pH 7.4 which had been previously eluted with a low-ionic-strength (10 mM sodium phosphate) buffer (section 6.2.3.2). Fractions B and C were pooled and concentrated by ultrafiltration. Subsequent gel filtration on Sephadex G-100 in 2 M NaCl (figure 2.1b) yielded reconstituted octamer in fraction A (approximately 75% of total protein eluted) free of excess H2A-H2B dimer (fraction B).

The protamine displacement method (section 6.2.3.3) was adopted for the isolation of H3-H4 tetramers and H2A-H2B dimers. Octamers were dissociated from DNA in 2 M NaCl at neutral pH in the presence of excess protamine. Dissociation of octamers into tetramers and dimers occured on decreasing the ionic strength (Ruiz-Carillo and Jorcano, 1979) to 150 mM NaCl with a concomitant protamine precipitation of DNA (Van der Westhuizen and von Holt, 1971). Octamers reformed after dialysis of tetramers and dimers, (purified from excess protamine), against 2 M NaCl. These were separated from H1 (fraction B) and H5 and H2A-H2B dimers (fraction C) by Sephadex G-100 chromatography (figure 2.1c). Octamer present in fraction A represents 40% of total protein eluted.

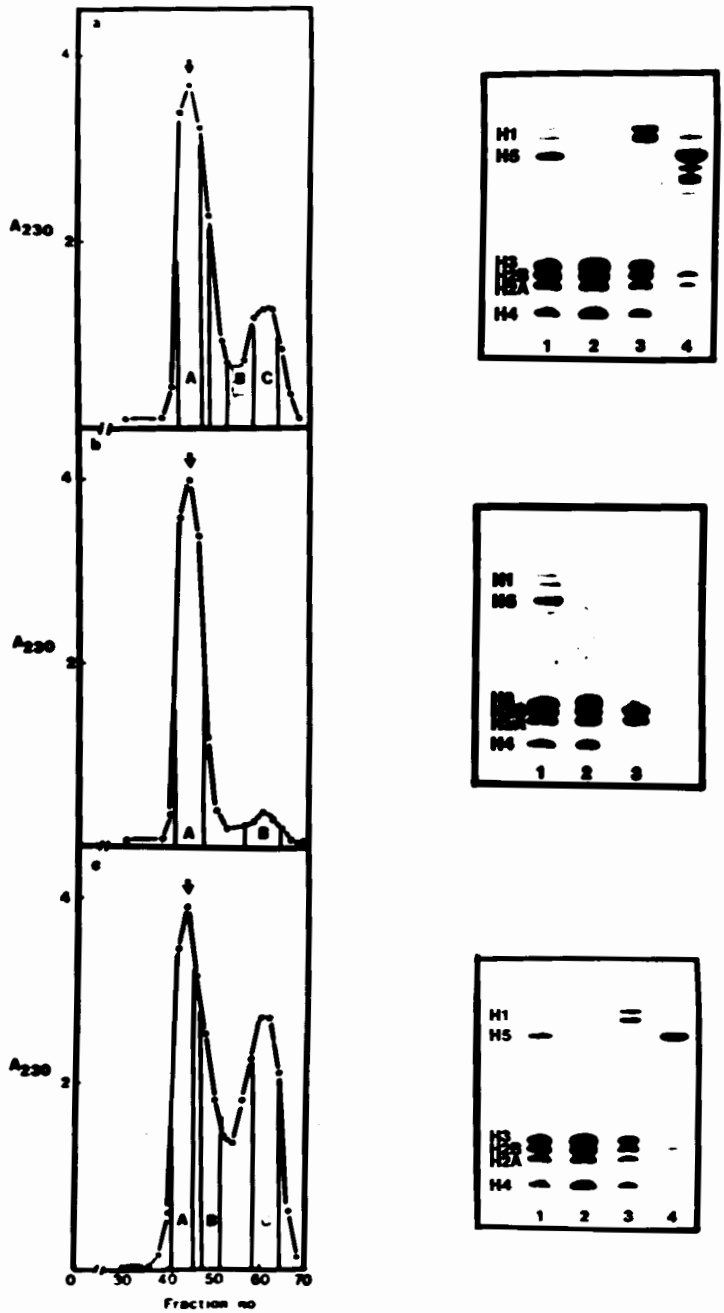


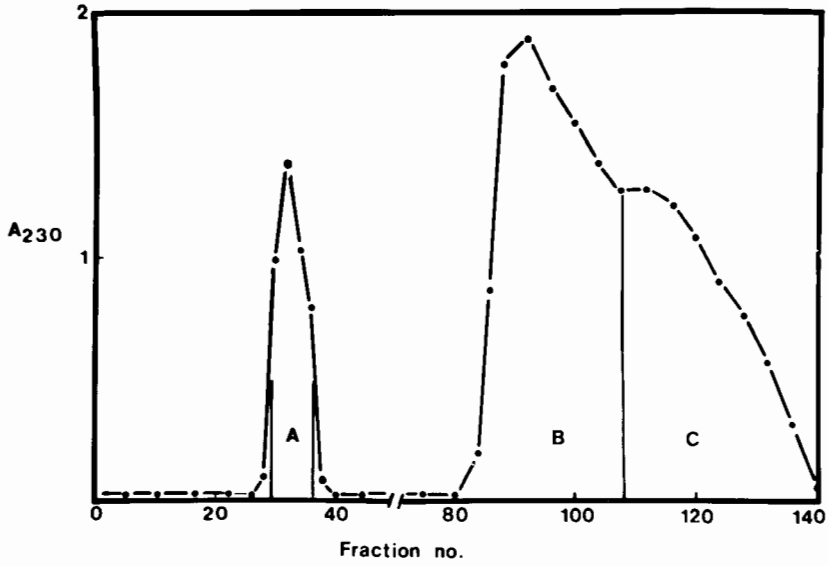
Figure 2.1

Figure 2.1 Sephadex G-100 fractionation of salt-extracted histone complexes in 2 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM PMSF. 30-40 mg protein was chromatographed. Fraction volume: 3 ml. Pressure head: 60cm. Arrows denote octamer elution volume as determined by chemical cross-linking of eluted products. (a) Native histone octamer prepared by ultracentrifugation (b) Reconstituted histone octamer from histones eluted from hydroxyapatite (c) Reconstituted octamer prepared from protamine-displaced histones. Fractions were analysed by SDS/polyacrylamide gel electrophoresis. Lane 1: histone standards; lanes 2,3 and 4: A, B and C of the corresponding chromatographs.

2.2.2 Octamer reconstitution from acid-denatured histones and Sepharose 6B chromatography

Reconstitution of octamers from acid-denatured histones was performed on total perchloric-acid-precipitated core histones (section 6.2.4.1) and individually purified histones (section 6.2.4.2). Purification of reconstituted complexes was by Sepharose 6B gel exclusion chromatography to monitor non-specific high-molecular weight aggregates (Lindsey et al. 1982; 1983). Octamer reconstitution from the unfractionated core histone mixture was achieved by dialysis against 2 M NaCl, pH 7.4 of the freeze-dried histones solubilised in acidified 8M urea (section 6.3.2). No non-specific aggregation was observed and 60% of eluted protein was recovered as pure octamer (figure 2.3a). Excess H2A-H2B dimer recovered in fraction B presumably formed because of the easier extractability of H2A and H2B over H3 and H4 in acid (Ruiz-Carillo and Jorcano, 1979).

Octamer reconstitution from individually purified histones, with the exception of H3, was performed with protein samples stored as freeze-dried fractions after isolation. Lindsey et al. (1982) demonstrated that freeze-drying of H3 causes its structure to become permanently altered. For this reason H3 was used immediately after purification or stored in the presence of 50% (v/v) glycerol at -20°C before later use. Octamer formation from stoichiometric amounts of individually purified histones subjected to the reconstitution procedure (section 6.3.2) proceeded with high efficiency. No non-specific aggregation was detectable and pure octamer recovered in fraction C (figure 2.3b) accounted for 80% of total eluted protein.



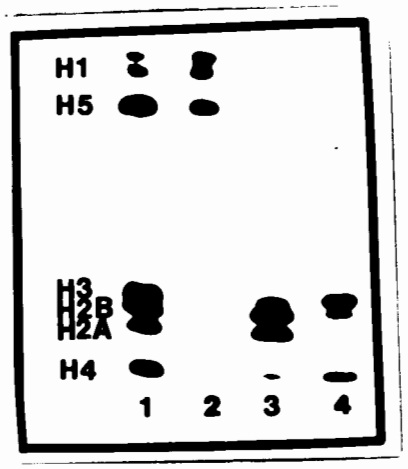


Figure 2.2 Elution of histones from hydroxyapatite. Micrococcal nuclease digest of chromatin (100 mg DNA) was loaded onto the column and the column washed with 1 vol. 10 mM sodium phosphate, 0.1 mM PMSF pH 7.4. Histones were eluted with 3 M NaCl in the same buffer; fraction volume: 2.5 ml. Fractions were analysed by SDS gel electrophoresis. Lane 1: histone standards; lanes 2,3 and 4 correspond to fractions A-C.

2.2.3 Oligomeric and conformational properties of reconstituted octamers

The octameric nature of the core histone complex prepared by the various methods was confirmed by chemical cross-linking (section 6.3.4). Kinetic analysis of the cross-linked products formed after reaction of all octamer preparations with dimethyl suberimidate identified unambiguously the major product of cross-linking as a cross-linked octamer. Shown in lanes 8-12 of figure 2.4 are the results obtained for the natural octamer. After 1 minute the major product of cross-linking is the octamer with apparent molecular weight of 100 000. Also present are products of lower apparent molecular weight. These represent expected intermediate products of octamer cross-linking corresponding to the histone hexamer (Mr = 80 000) and histone dimers (Mr = 26 000). Furthermore, the presence of cross-linked products with apparent molecular weight of 200 000 (corresponding to a 16-mer) confirms the octameric nature of the complex. After longer reaction times the octamer forms larger complexes through the 16-mer which do not enter the gradient gel (see for example lane 12). The speed of octamer formation for each preparation was found to be virtually identical within the time resolution of the method. Lanes 1-5 in figure 2.4 show the cross-linked products formed after 3 minutes for the respective octamer preparations. Clearly noticeable are the cross-linked octamer and additional 16-mer, hexamer and dimer bands, present in approximately equal relative amounts for all preparations. The equal speed by which the complexes are cross-linked to octamers suggests identical arrangements of the individual histones in the octameric complex in solution.

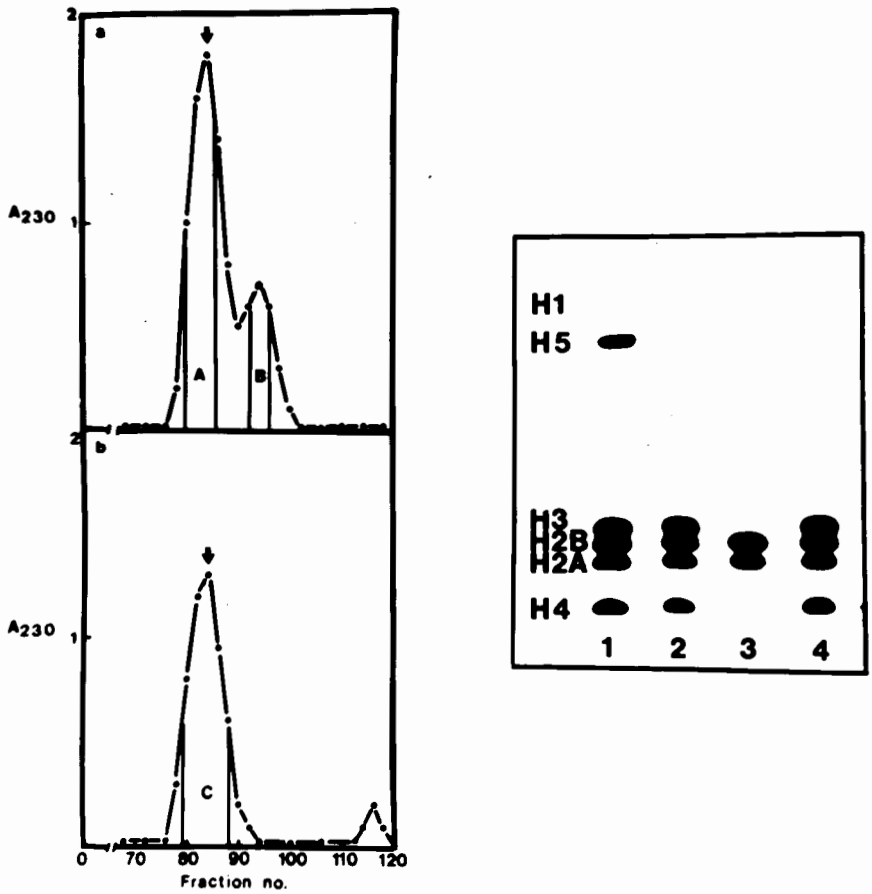

a
b
A230
A
B
C
0 70 80 90 100 110 120
Fraction no.
H1
H5
H3
H2B
H2A
H4
1 2 3 4

Figure 2.3

Figure 2.3 Sepharose 6B chromatography of reconstituted complexes prepared from acid-denatured histones. Elution buffer was 2 M NaCl, 10 mM Tris/HCl (pH 7.4), 0.1 mM PMSF. Fraction volume 3 ml. Pressure head: 60 cm. Arrows denote elution volume of octamer determined by chemical cross-linking of eluted products. (a) Octamers reconstituted from perchloric-acid-precipitated core histones. (b) Octamers resonstituted from individually purified histones. Eluted fractions were analysed by SDS gel electrophoresis, Lane 1: control histones; lanes 2 - 4 correspond to fractions A, B and C respectively.

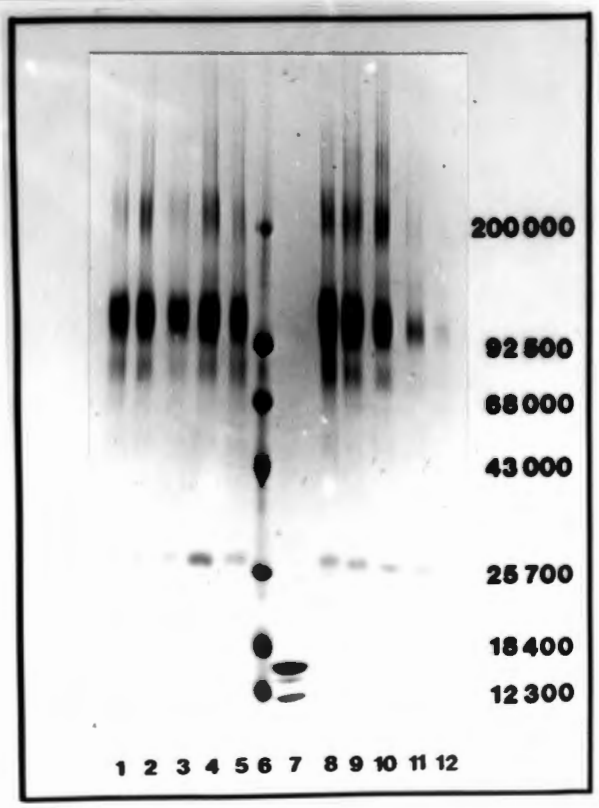

200000
92500
68000
43000
25700
18400
12300
1 2 3 4 5 6 7 8 9 10 11 12

Figure 2.4

Figure 2.4 Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of natural and reconstituted histone octamers after cross-linking with dimethyl suberimidate. Lane (1) octamer reconstituted after hydroxyapatite chromatography; lane (2) natural octamer; lane (3) octamer reconstituted from protamine displaced histones; lane (4) octamer reconstituted from perchloric-acid-precipitated core histones; lane (5) octamer reconstituted from individually purified histones; lane (6) molecular weight standards; lane (7) core histones; lanes (8-12) natural octamer cross-linked for (8) 1 min, (9) 3 min, (10) 5 min, (11) 10 min and (12) 15 min. Molecular weight standards used are, in order of decreasing electrophoretic mobility; cytochrome c , β-lactoglobulin, α-chymotrypsinogen, ovalbumin, bovine serum albumin, phosphorylase b, H chain of myosin. The molecular weights are given next to the slab.

The most discerning probe for conformational identity of the reconstituted complexes is their ability to crystallise in the same way as the natural octamer. All preparations when subjected to the crystallisation procedure (section 7.3.5) crystallised as helical octamer tubes (figure 2.5). The external diameter of the tubes obtained is approximately 30nm, identical to the dimensions reported by Klug et al. (1980). Further characterisaion of helical tubes prepared during the study was by optical diffractometry (section 6.7.2) and Fourier transformation (section 6.7.2) of the best tube obtained. From the optical transform shown in figure 2.6 axial reflections, corresponding to zero order Bessel maxima, can be observed on layer line spacings of 65Å. This measurement was confirmed during indexing of the numerical transform of the same tube (figure 2.7) and corresponds to the thickness of a ring of octamers in the helical tube (Klug et al. 1980). The numerical transform was indexed assuming the number of parallel helices (or the number of octamers per ring) to be 10. During this process the selection rule (Klug et al. 1958) was confirmed as $l = 4n' + 17m$ (where $n' = 10n$), in conformity with observation of Klug et al. (1980). From the indexed numerical transform the unit twist and helical repeat (Klug et al. 1958) of the helix was deduced. The unit twist, which describes the relative rotation of successive rings of octamers in the tube, corresponds to a rotation of $2\pi/10 \times (4/17)$. The helical repeat of the helix reflected in the layer line spacings at which the tenth and twentieth order Bessel maxima occur, was found to be approximately 280Å as also observed by Klug et al. (1980).

2.3 DISCUSSION

This study established the fidelity of reconstituted octamers prepared by different protocols including:

(i) reconstitution from dimers and tetramers extracted selectively with high sodium chloride concentrations from chromatin or produced via dissociation of the dissolved octamer;

(ii) reconstitution from a stoichiometric mixture of acid-denatured histones;

(iii) reconstitution from individually purified core histones which in the course of isolation became completely denatured. The efficiency of reconstitution for all procedures was assessed by column chromatography on appropriate matrices. In addition to the characteristic elution position of the octamer from matrices, pre-determined by chromatography of the natural octamer, chemical cross-linking with dimethyl suberimidate established the oligomeric properties of reconstituted complexes. To assess the conformational state of histones the most stringent test to probe for conformational identity, namely crystallisation, was applied.

Sephadex G-100 chromatography of histone complexes in 2 M NaCl confirmed the observations of Ruiz-Carillo and Jorcano (1979) and Godfrey et al. (1980) that octamers can be reconstituted from its natural products of dissociation. Reassociation of the octamer was accomplished in the presence of 2 M NaCl. This was achieved by dialysis against high salt concentrations of complexes prepared by the protamine procedure or pooling of selectively extracted core histone complexes (prepared by the

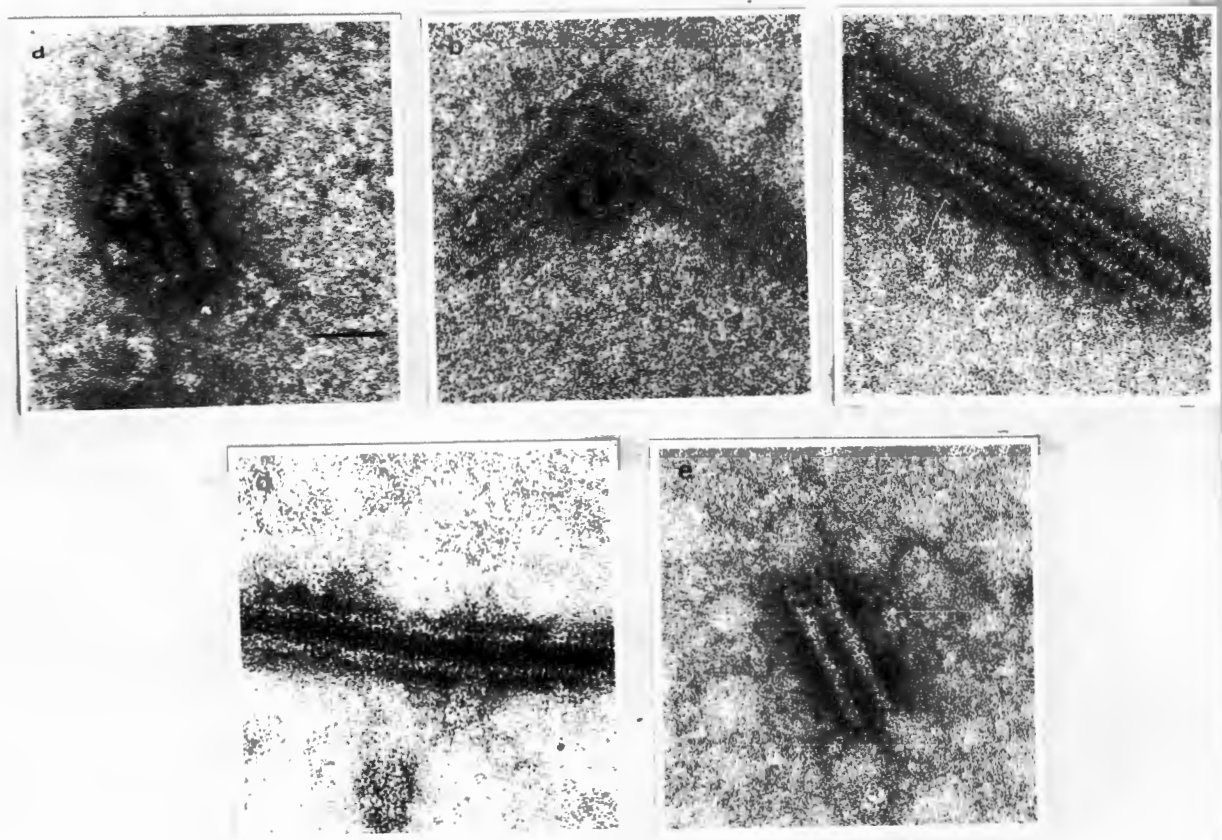


Figure 2.5 Electron micrographs of helical tubes of native and reconstituted histone octamers. Tubes prepared from native octamers (a); (b) octamers reconstituted after hydroxyapatite chromatography; (c) octamers reconstituted from protamine displaced histones; (d) octamers reconstituted from perchloric-acid-precipitated core histones (e) octamers reconstituted from individually purified histones. The bar in (a) represents 50 nm.

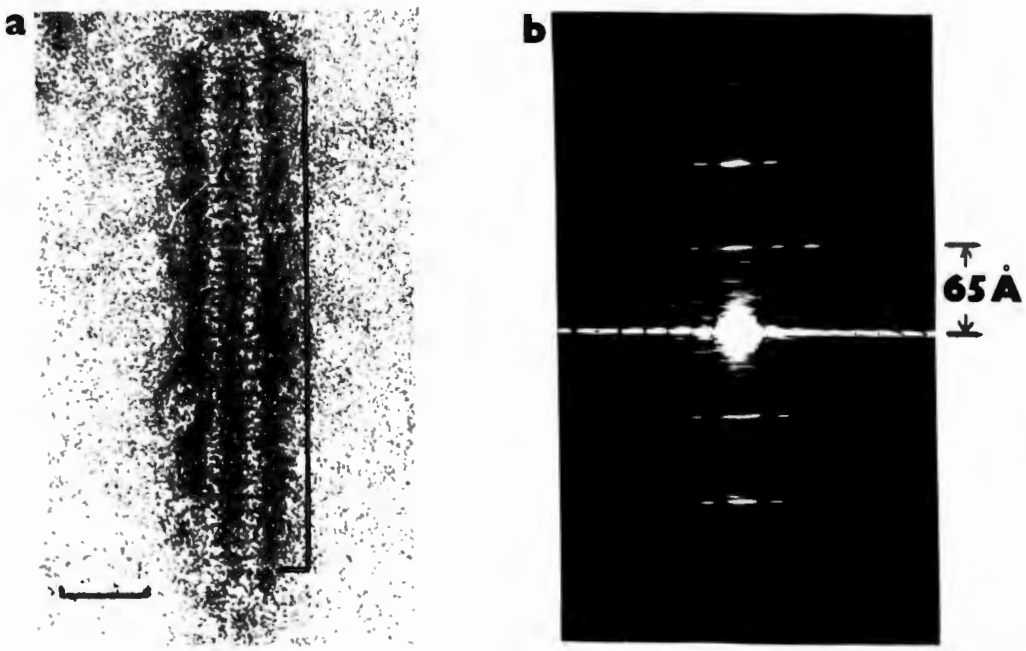


Figure 2.6 Optical diffraction pattern of helical tube shown in figure 2.5(c). The diffraction pattern (b) was recorded of a section (2 unit cells) of the helical tube as indicated in (a). The bar in (a) represents 50 nm.

hydroxyapatite procedure). Pure octamer fractions recovered after Sephadex G-100 chromatography all crystallise to the same form as the natural octamer, the helical tube described by Klug et al. (1980).

The reconstitution of octamers from a stoichiometric mixture of acid-denatured or individually purified histones proceeded with high efficiency. This was suggested by the absence of high-molecular weight aggregates during Sepharose 6B chromatography. To preclude aggregation it was considered important to dissolve histones in high concentrations of acidified urea at protein concentrations not exceeding 0.5 mg/ml. This procedure presumably results in the total unfolding and disaggregation of the polypeptide chains before subsequent renaturation and complex formation during dialysis against 2 M NaCl. It is noteworthy that reconstitution studies carried out in the absence of urea (Lindsey et al. 1982) or at high protein concentrations (Nicoli et al. 1978) yielded the reconstituted complexes with low yield as a result of aggregation. The ability of acid-denatured histones (except for H3, even after freeze-drying) not only to renature to a histone octamer but also crystallise as helical tubes, settles the long-standing debate in the literature on whether or not histones, denatured through preceeding acid or urea treatment, can be renatured or not.

The crystallisation procedure yielding octamer tubes was adopted during this study as a valid criterion to demonstrate the biological functionality of reconstituted complexes. This is based on the following considerations: A stringent evolutionary selection for the positioning of amino acid residues within the four core histones (von Holt et al. 1979; von Holt, 1985) has resulted in a specific octamer which associates with DNA to result in the nucleosome core. The highly conserved primary structure of the histones not only

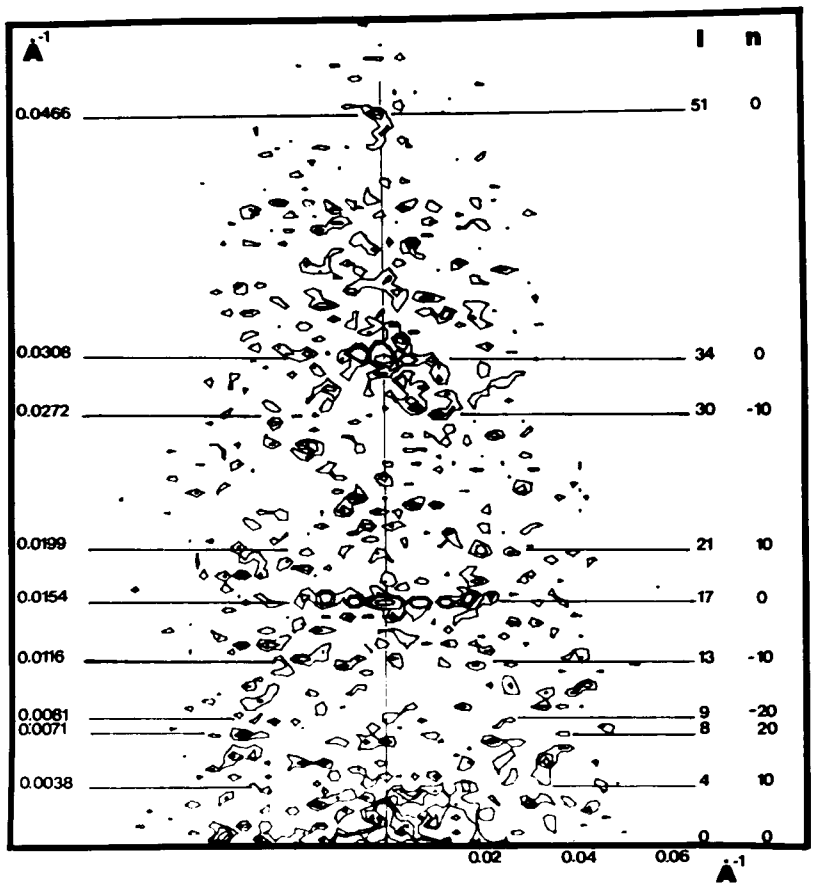


Figure 2.7 Contour map of the amplitudes of the numerical transform of the tube shown in figure 2.5(c). The numerical transform was computed of the section of the tube as shown in figure 2.6. Layer lines are marked by an index l and the orders n of the Bessel functions on each layer line are as indicated.

dictates the specific intra-core histone-histone and histone-DNA contacts but, in addition, is also responsible for the establishment of inter-octamer contacts leading to tube formation of native octamers. The ability of reconstituted octamers to crystallise into the same octamer tubes is thus indicative of the presence of a biologically competent conformation, implying an identical specific arrangement of charged and uncharged groups on the surface of the octamer. An additional criterion for the native state of reconstituted octamers is the ability of such complexes to support the assembly of nucleosome core particles indistinguishable from natural core particles. A study by Retief et al. (1984) demonstrated that octamers prepared by all the protocols reported here, satisfy this condition.

CHAPTER 3

3.1 THE RECONSTITUTION OF HYBRID HISTONE OCTAMERS

3.1.1 Introduction

Two classes of structural variation are discernable from the primary structures available for variants of the core histones (von Holt et al. 1979), namely

i) Variations in primary structure arising from point mutations or deletions of one or several amino acid residues (observed for variants of all the core histones)

ii) Variations consisting of extensive modifications in major areas of the molecules through reiteration, insertion, deletion and point mutations (predominant in variants of H2A and H2B).

The structure-function implications of these structural variations at the level of core particle structure and/or higher orders of chromatin organisation are unknown (see section 1.1.3).

The H2B variants of sea urchin sperm, characterised by variable N-terminal extensions, are representative of the second class of structural variation. Three histones H2B are present in the sperm cells of Parechinus angulosus (Strickland et al. 1977a,b; 1978a) each containing a repeating pentapeptide structure in the N-terminal domains. Similar histones are also present in the sperm of Psammechinus miliaris (Strickland et al. 1978b) and Echinolampus crassa (von Holt et al. 1979). Physico-chemical studies on the core particles from Strongylocentrotus purpuratus established the DNA content of

the particles as 145 base pairs (Simpson and Bergman, 1980) with sedimentation coefficient and C.D. properties similar to those of chicken erythrocyte core particles. The increased thermal stability and reduced rates of DNAse I digestion at specific sites observed for sperm core particles were attributed to the more basic H2B molecules. Micrococcal nuclease digestion studies by Zalenskaya et al. (1981a) lead to the proposal that the N-terminal extensions of sperm H2B are complexed with variable lengths of linker DNA. This suggests a possible role for the H2B variants in stabilising chromatin higher order structures as earlier proposed (von Holt et al. 1979). By two-dimensional gel electrophoresis Zalenskaya et al. (1981b) demonstrated the presence of mononucleosomes containing a single H2B isotype. The existence of a heterogeneous population of sperm nucleosomes of different structural properties resulting in different states of chromatin structure and stability, may therefore not be excluded.

The primary structures of histones H3 and H4 show little variation over a wide range of evolution (von Holt et al. 1979). The mutation involving cysteine in position 73 of H4 isolated from sea urchin (Strickland et al. 1974) has been suggested to possibly assign new properties to these molecules (von Holt et al. 1979). With the exception of yeast H3, generally four amino acid positions are variable for this protein. Despite fifteen amino acid substitutions present in yeast H3 (Brandt and von Holt, 1982) this histone is complexed with yeast H2A, H2B and H4 and 145 base pairs DNA to form core particles structurally similar to cores of higher eukaryotes (Thomas and Furber, 1976). Lee et al. (1982), on the basis of CD and thermal denaturation studies, concluded that yeast nucleosomes are however less constrained than chicken and calf nucleosomes. The reduced nucleosome stability observed was suggested to result from sequence changes in H3 and its

altered interaction with histone H4. It is noteworthy that nine of the fifteen substitutions are present in the C-terminal domain of H3 (Brandt and von Holt, 1982), concluded in section 1.1.3 to be the site of histone-histone interaction. Structure-function relationships as a result of the substitution of alanine for cysteine at position 110 is of particular interest to the present study.

The influence of primary structure modifications on histone-histone and histone-DNA interactions may conceivably be studied by reconstitution and characterisation of well-defined histone-histone and histone-DNA complexes. One approach to achieving this aim requires the reconstitution of hybrid histone octamers from the histone variant(s) of interest and the remaining complement of chicken erythrocytes, before subsequent assembly of hybrid octamers onto DNA. The study reported here was undertaken to attempt the reconstitution of the following hybrid octamers by the methodology for octamer reconstitution from individually purified histones described in Chapter 2:

i) a hybrid octamer containing sea urchin sperm H2B(1) and chicken erythrocyte H3, H2A and H4

(ii) a hybrid octamer containing sea urchin sperm H4, a site-specific H3 "mutant" prepared by chemical modification of cysteine to alanine (at position 110) with Raney nickel and chicken erythrocyte H2A and H2B.

3.2 RESULTS

3.2.1. Reconstitution of a hybrid octamer from sea urchin sperm H2B(1) and chicken erythrocyte H2A, H3 and H4

The reconstitution of hybrid octamers was attempted by the procedure discussed in Chapter 2 for the reconstitution of octamers from individually purified erythrocyte histones. The homogeneiety of the H2B(1) variant fraction isolated from Parechinus angulosus was confirmed by electrophoresis in the presence of urea and Triton (figure 3.1). Octamer reconstitution from the individually purified histones was achieved by dialysis against 2M NaCl, pH 7.4 of stoichiometric amounts of H2B(1) and chicken erythrocyte H3, H2A and H4 solubilised in acidified 8M urea (section 6.3.2). β-mercaptoethanol was included at high concentrations during all procedures to preclude the oxidation of H2B(1) methionine residues. The oxidation of one or both of the centrally positioned methionine residues to the sulfone imparts considerable changes on the conformation of these histones as suggested from their drastic changes in electrophoretic mobility in Triton gels (Zweidler, 1978). Purification of reconstituted complexes was by Sepharose 6B chromatography. Octamer formation proceeded with high efficiency as suggested by the absence of large molecular weight aggregates during chromatography (figure 3.2a). All protein was recovered as a single peak at the elution position of the natural salt-extracted octamer. The octameric nature of the pooled fraction (figure 3.2a) was confirmed by chemical cross-linking with dimethyl suberimidate (section 6.3.4). Electrophoretic bands corresponding to the expected products of octamer cross-linking (see Chapter 2) namely, the histone octamer, hexamer and dimer and a low intensity band corresponding to 16-mer are present after 0.5 minutes reaction time (figure 3.5a). Several hybrid octamer crystallisation attempts under conditions which lead to helical tube formation in the case of the natural octamer, were unsuccessful.

3.2.2 Reconstitution of a hybrid octamer from sea urchin sperm H4, Raney nickel-reacted erythrocyte H3 and erythrocyte H2A and H2B

3.2.2.1 Preparation of des-110-thio-histone H3

The Raney nickel-catalysed desulfurisation of cysteine, resulting in the conversion of cysteine to alanine, was performed on solutions of cysteine and erythrocyte H3 under reaction conditions as described by Perlstein et al. (1971), for other proteins. During pilot studies a 2.5 μmoles/ml solution of cysteine (4 ml) was reacted with freshly prepared Raney nickel (section 6.4.2.2.2) at pH 7.0 under a pressure of H_2 gas. The reaction was terminated after 1.5 hours by centrifugation of the reaction mixture. Amino acid analysis performed on the resulting supernatant established alanine as the only product of reaction. Conversion of cysteine to alanine, quantitated by amino acid analysis of the reaction mixture at zero time and after 1.5 hours, proceeded with 100% efficiency. This confirmed the observations of Perlstein et al. (1971).

Freshly prepared or glycerol-stored samples of H3 were used for reaction with Raney nickel. The reaction performed with H3 solubilised in H_2O only, resulted in a substantial loss of protein due to adsorption of H3 to the catalyst. Adsorption, however, was minimised when protein was solubilised in 6 M guanidinium chloride. Dethiolation of reduced samples of H3 (25 - 30 mg) was allowed to proceed for 72 hours at neutral pH before terminating the reaction by removal of the catalyst by centrifugation. The efficiency of dethiolation was assayed firstly, by selective oxidation of cysteine residues to cystinyl residues with o-iodosobenzoate (section 6.4.2.3.3). As is evident from figure 3.3b virtually no protein was recovered at the expected elution position of

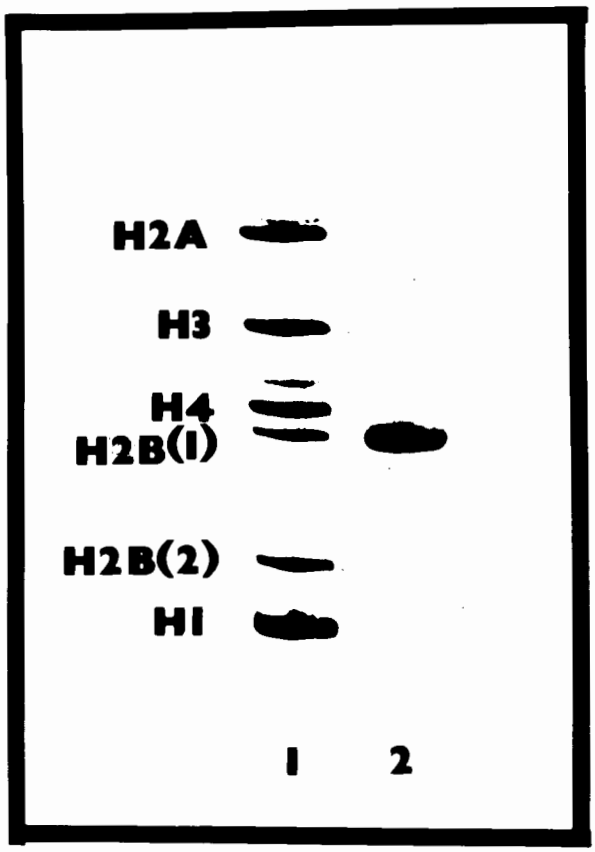


Figure 3.1 Acid-urea/Triton-polyacrylamide gel electrophoresis of purified sea urchin sperm H2B(1). Conditions for electrophoresis as detailed in section 6.5. Lane 1: sea urchin sperm total acid-extracted histones; Lane 2: purified H2B(1). No bands with increased electrophoretic mobility as a result of methionine oxidation (Zweidler, 1978) are present for the analysed sample.

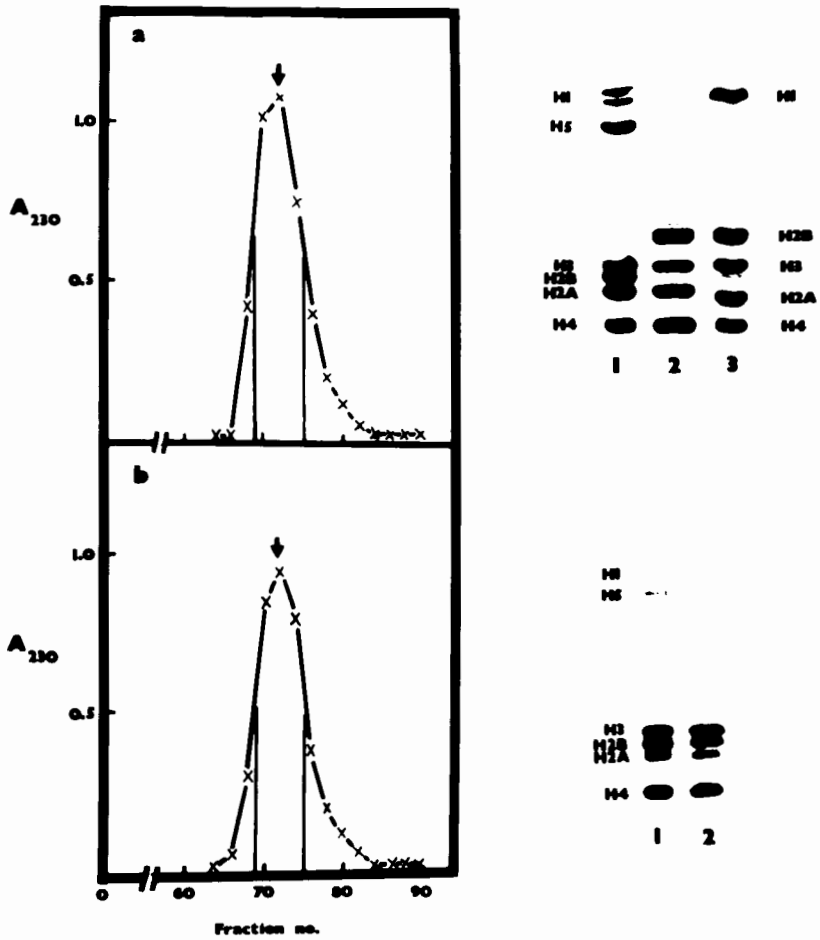


Figure 3.2

Figure 3.2 Sepharose 6B chromatography of reconstituted hybrid octamers 9 - 12 mg protein was chromatographed. Elution buffer was 2 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM $PhCH_2SO_2F$. Fraction volume: 1.5 ml. Pressure head: 60 cm. Arrows denote elution volume of natural octamer (a) Reconstituted octamer prepared from sea urchin sperm H2B(1) and chicken erythrocyte H3, H2A and H4. Pooled fraction was analysed by SDS/polyacrylamide gel electrophoresis. Lane 1: chicken erythrocyte histone standards; lane 2: pooled fraction; lane 3: sea urchin sperm histone standards. (b) Reconstituted octamer prepared from sea urchin sperm H4, dethiolated H3 and chicken erythrocyte H2A, H2B. Pooled fraction was analysed by SDS/polyacrylamide gel electrophoresis. Lane 1: chicken erythrocyte histone standards; lane 2: pooled fraction.

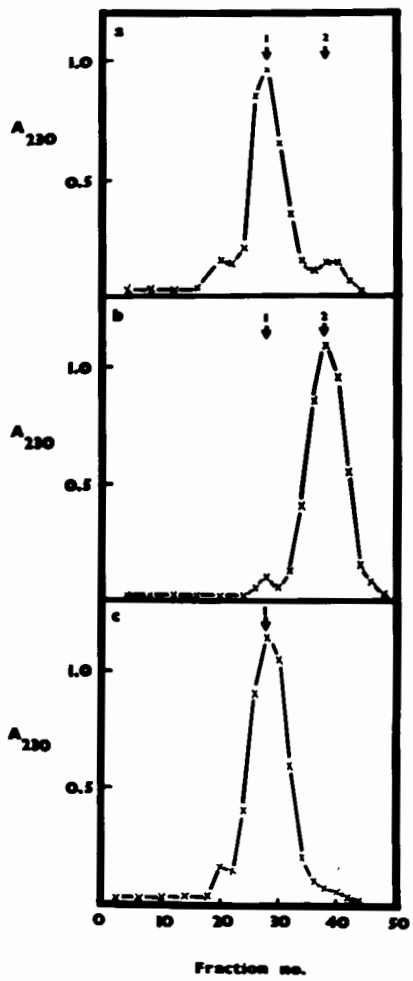


Figure 3.3 Sephadex G-100 fractionation of H3 after dimerisation with o-iodosobenzoate. Chromatography was in 10 mM HCl. Pressure head: 60cm. Fraction volume: 1.5 ml. a) Dimerisation of freshly prepared H3 (10 mg) b) Raney nickel-reacted H3 (10 mg) after reaction with o-iodosobenzoate c) Dimerisation of H3 (12 mg) after exposure to high concentrations of nickel acetate (see section 3.2.2.1). Arrows 1 and 2 denote elution positions of the dimer and monomer fractions respectively.

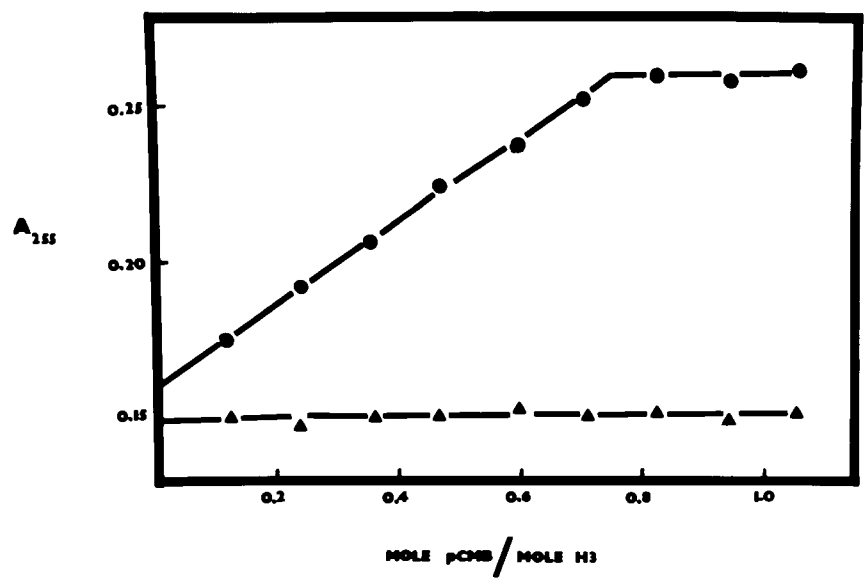


Figure 3.4 Spectrophotometric titration of histone H3 with p-chloromercuribenzoate. Titrations were performed on H3 before (-●-●-) and after (-▲-▲-) reaction with Raney nickel. H3 samples were treated with DTT before spectrophometric titration.

	Control H3	Reacted H3
mole Met / mole Val	0.28	0.27
mole Met / mole Gly	0.19	0.19

Table 3.1 Methionine content of Raney nickel-reacted and control H3. Amino acid analysis of samples was performed as detailed in section 6.4.2.3.1. Methionine content was determined as molar ratios of methionine to amino acids that are stable during hydrolysis (section 6.4.2.3.1).

the H3 dimer after Sephadex G-100 chromatography of the oxidised sample. This demonstrates that dethiolation was virtually complete since approximately 95% of the H3 which was not reacted with Raney nickel, is recovered as H3 dimer after oxidation with o-iodosobenzoate under identical experimental conditions (figure 3.3a). The possible reaction of free nickel acetate with protein sulfhydryl groups was considered as an alternative explanation for the absence of a dimer fraction in figure 3b. Nickel acetate may have been present during the desulfurisation reaction as a result of improper washing of the catalyst during its preparation (section 6.4.2.2.1). The affinity of nickel acetate for cysteine was examined by incubating 10 mg. reduced H3 in 6 M guanidinium chloride with a 10% molar excess of nickel acetate at neutral pH for 3 hours before subsequent dialysis against 0.25 N HCl. Oxidation of this sample with o-iodosobenzoate yielded all the protein as dimer during chromatography on Sephadex G-100 (figure 3c). The reaction of nickel acetate with cysteine can therefore be discounted as an alternative explanation for the inability of H3 to dimerise after reaction with Raney nickel. The cysteine content of H3 samples after dethiolation was determined by a second assay namely, the spectrophotometric titration of sulfhydryl groups with p-chloromercuribenzoate (section 6.4.2.3.2). By this method, the cysteine content of reacted protein recovered in the monomer fraction after dimerisation was found to be zero (figure 3.4).

Quantitation of the cysteine content of reacted H3 by amino acid analysis was not attempted because of the low efficiency of carboxymethylation of cysteine (section 6.4.2.3.1). Detection of increases in alanine content (as a result of dethiolation) by amino acid analysis is also limited by the high alanine content (18 residues) of H3. Although the dethiolation of cysteine could not be determined by amino acid analysis during the present study, the reaction specificity of

Raney nickel towards sulfur amino acids may be determined by this method. As is evident from table 3.1, no difference in the methionine content of reacted and control H3 is discernable.

3.2.2.2 Oligomeric and conformational properties of the reconstituted octamer

The hybrid octamer was reconstituted from the individually purified histones (section 6.3.2). Samples of purified Parechinus angelosus sperm H4 which had not been subjected to freeze-drying after isolation, were used for reconstitution (section 6.2.4.2). Stoichiometric amounts of Raney nickel-reacted H3 (recovered as monomer after dimerisation of unconverted H3), sperm H4 and erythrocyte H2A and H2B were solubilised in 8 M urea before dialysis against 2 M NaCl, pH 7.4. Reconstituted complexes were characterised by Sepharose 6B chromatography. All histones were recovered in equimolar amounts at the expected elution position of the histone octamer (figure 3.2b). The octameric nature of the eluted product was confirmed by chemical cross-linking studies with dimethyl suberimidate. Cross-linking proceeded via the formation of expected intermediate products of octamer cross-linking, namely the hexamer and dimer (figure 3.5b). The formation of larger aggregates of cross-linked octamer via the 16-mer confirmed the major product of cross-linking as an octamer. Reconstituted octamers recovered after Sepharose 6B chromatography when subjected to the crystallisation procedure detailed in section 6.3.5 crystallised to the same form as the natural octamer, namely the helical tube (figure 3.6). The

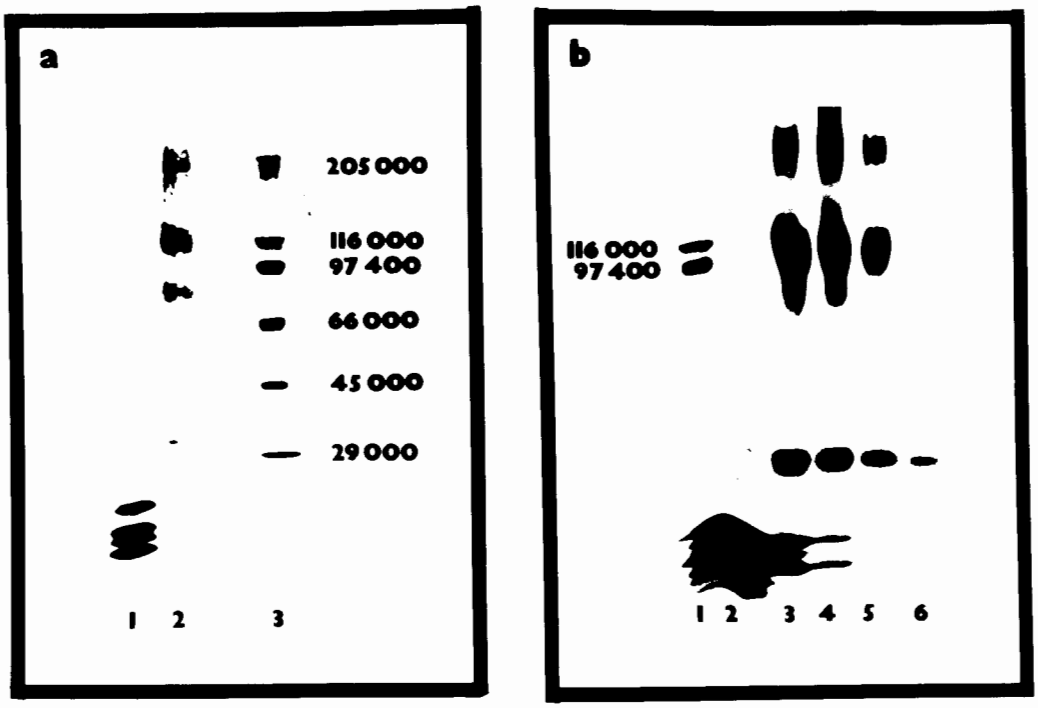

a
205 000
116 000
97 400
66 000
45 000
29 000
1 2 3
b
116 000
97 400
1 2 3 4 5 6

Figure 3.5

Figure 3.5 Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of reconstituted histone octamers after cross-linking with dimethyl suberimidate. a) Cross-linking of sea urchin sperm H2B(1) - chicken erythrocyte H3, H2A, H4 hybrid octamer for zero min. (lane 1) and 0.5 min (lane 2). Molecular weight standards used (lane 3) are, in order of decreasing mobility, myosin, α-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase. b) Cross-linking of hybrid octamer reconstituted from desulfurised H3, sea urchin sperm H4 and chicken erythrocyte H2A, H2B. Lanes 2 - 6, hybrid octamer cross-linked for 0, 0.5, 1.0, 2.0 and 4.0 min. respectively. Molecular weight standards used, in order of decreasing electrophoretic mobility, are α-galactosidase and phosphorylase b (lane 1). Identification of dimer, hexamer, octamer and 16-mer bands are as shown.

diameter of the helical tubes, obtained in high yields during crystallisation (figure 3.6a) was 30 nm. By this criterion, the hybrid octamer is therefore structurally indistinguishable from the natural chicken erythrocyte octamer.

3.3 DISCUSSION

The structure-function implications as a result of sequence variations observed for the core histone variants are unknown. The characterisation of well-defined histone-histone and histone-DNA complexes may contribute to the understanding of the structural implications of the sequence variations at the level of core particle structure. The present study was undertaken to characterise reconstituted chicken erythrocyte hybrid octamers, that is, octamers of which one erythrocyte histone molecule was substituted for by i) a variant of the substituted histone and/or ii) a chemically modified "mutant" of the substituted histone. Hybrid octamers were reconstituted from individual polypeptides by the methodology described (chapter 2) for the reconstitution of erythrocyte octamers, capable of tube formation, from individually purified histones. The oligomeric properties of the reconstituted complexes were probed by Sepharose 6B chromatography and chemical cross-linking with dimethyl suberimidate. Purified hybrid octamers were subjected to the crystallisation procedure leading to helical tube formation in the case of the natural octamer, to establish possible conformational and structural differences between the hybrid complexes and the natural octamer.

Since Zalenskaya et al. (1981b) had demonstrated the existence of a heterogeneous population of sea urchin sperm core particles (with respect to H2B content) it was considered

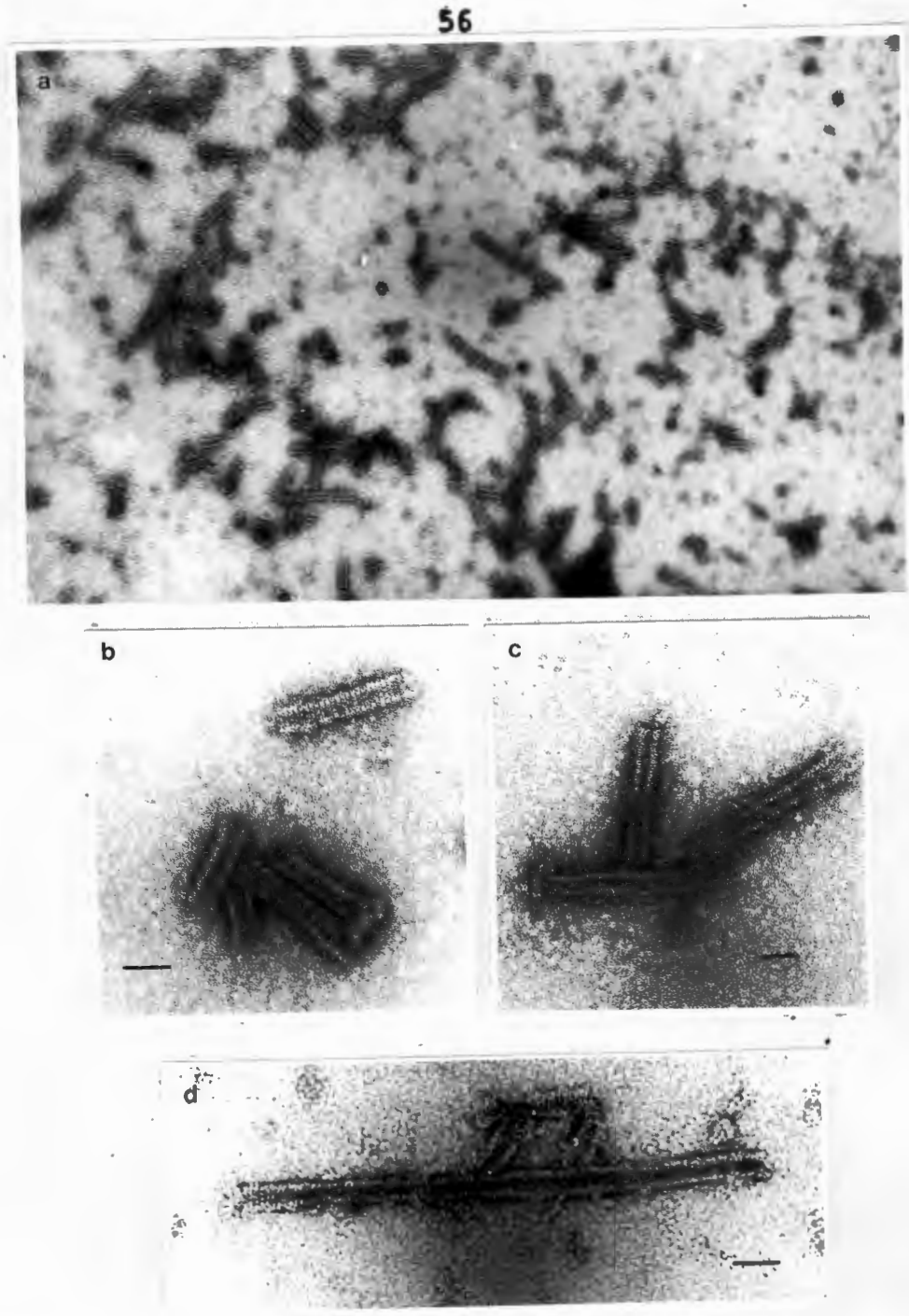


Figure 3.6 Electron micrographs of helical tubes of hybrid octamers reconstituted from Raney nickel-reacted H3, sea urchin sperm H4 and chicken erythrocyte H2A, H2B. Helical tubes were negatively stained with uranyl acetate. Grids were examined at magnification of 10 000 x (a) and 50 000 x (b,c,d). The bar in (b-d) represents 50 nm.

important to study the effect on octamer structure of a single H2B isotype, namely H2B(1). This was achieved by characterising the hybrid octamer reconstituted from sperm H2B(1) and chicken erythrocyte H2A, H3 and H4. The absence of high-molecular weight aggregates during Sepharose 6B chromatography of the reconstituted complex strongly suggested that the hybrid octamer reconstitution proceeded with similar efficiency as was observed for the reconstitution of erythrocyte octamers from individually purified histones. Complex formation between H2B(1) and the specified erythrocyte histones to result in an octameric complex was also confirmed by chemical cross-linking with dimethyl suberimidate. Purified hybrid octamer preparations were however not capable of tube formation. With the available information on histone-histone interactions (discussed in section 1.1.3) it is possible to formulate a plausible explanation for the observed characteristics of this hybrid octamer. When the amino acid sequences of H2B(1) and calf H2B are alligned for homologies (figure 3.7) it is apparent that the sequence from residue 54 to the C-terminal end of H2B(1) is virtually identical to the corresponding region of calf H2B. This sequence in calf H2B has been identified, on the basis of cross-linking studies, to represent the sites of the H2B-H4 and H2A-H2B contacts in the histone octamer. As these contacts constitute the major contacts between H2B and the core histones in the octamer, H2B(1), on the basis of the identified sequence homology, may be expected to associate with erythrocyte H3, H2A and H4 to form an octamer. The remainder of the H2B(1) molecule is considerably different to calf H2B. In H2B(1), the amino terminal region shows an extension of 19 amino acids beyond the amino terminal proline of calf H2B (figure 3.7). It is also only from position 39 to 53 in H2B(1) that the two sequences begin to show some homology. The sequence differences observed for these domains are unlikely to influence complex formation since the histone

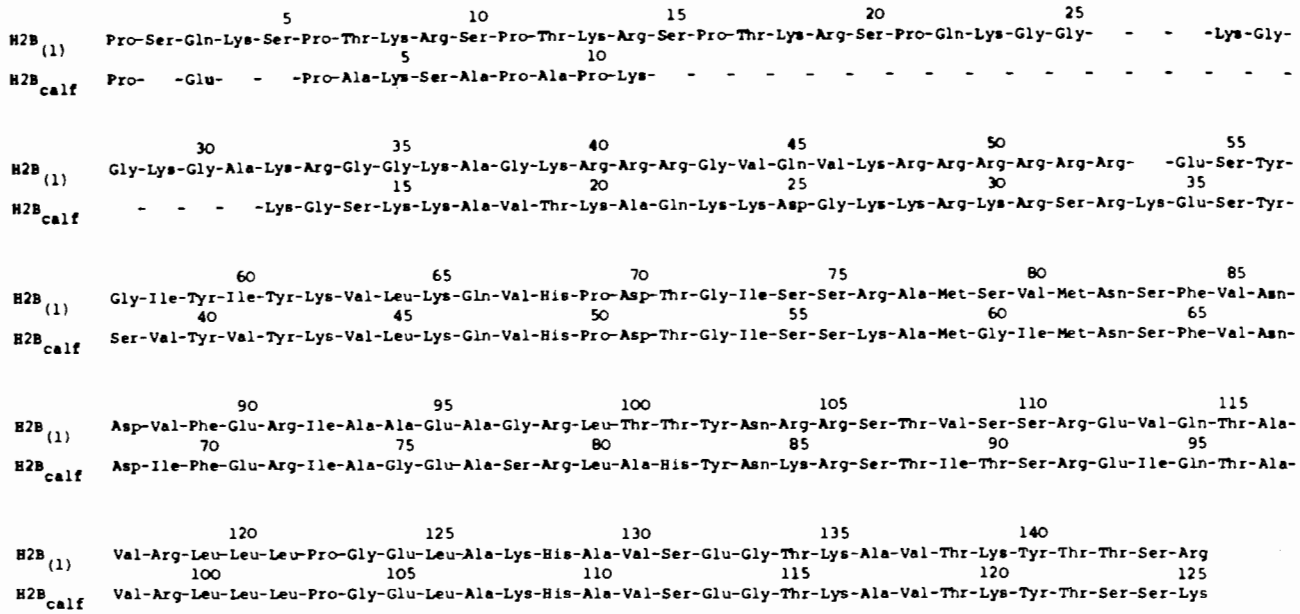


Figure 3.7 Amino acid sequences of sperm H2B(1) and calf H2B alligned for homologies according to Strickland et al. (1977a).

N-termini apparently do not participate in histone-histone interactions (see section 1.1.3). Although the above arguments may account for the ability of H2B(1) to participate in complex formation with erythrocyte histones, the conformational properties of the hybrid octamer, probed by the crystallisation procedure remain unclear in this study. A possible explanation for the inability of the reconstituted octamer to crystallise as helical tubes is that the extended N-terminal region of H2B(1), although not required for, or altering complex formation, sterically inhibited tube formation. Conformational and structural properties of the hybrid octamer may however become apparent from a characterisation of core particles assembled from the hybrid octamer by the procedure of Retief et al. (1984).

In order to label octamers specifically at H4 (see chapter 5), the reconstitution of a hybrid octamer of which erythrocyte H4 is substituted for by sea urchin sperm H4 was attempted. The cysteine residue at position 73 of sperm H4, if accessible in the reconstituted octamer, would provide a suitable site for the introduction of a single cysteine-specific label. To preclude the dual labelling of cysteine residues present in sperm H4 and erythrocyte H3 of the octamer, only samples of H3 which had been reacted with Raney nickel were used for the reconstitution of the hybrid octamer. The Raney nickel reaction had previously been demonstrated to result in the desulfurisation of cysteine residues in proteins (Perlstein et al. 1971). In the present study the Raney nickel reaction resulted in the complete desulfurisation of a cysteine solution yielding alanine as the only reaction product. The cysteine content of H3 samples after reaction with Raney nickel at pH 7.0 was determined by spectrophotometric titration with pCMB and oxidation of residual cysteine residues to disulfide bonds with o-iodosobenzoate. Virtually

no protein was recovered as dimerised H3 after oxidation with o-iodosobenzoate. Spectrophotometric titration of the H3 fraction resistant to dimerisation confirmed the absence of cysteine in this sample. The methionine contents of reacted and control H3, determined by amino acid analysis, were identical. This observation confirmed the reaction specificity at neutral pH of this procedure towards cysteine (Perlstein et al. 1971). The possible reaction of nickel acetate (contaminating the Raney nickel preparation) with protein sulfhydryl groups was considered as an alternative explanation for the results obtained during determinations of the cysteine content of reacted H3. This possibility may be discounted since

i) H3 samples were treated with large excesses of DTT to displace sulfhydryl blocking agents before spectrophotometric titration, and

ii) H3 samples exposed to high concentrations of nickel acetate retained the ability to dimerise with high efficiency when oxidised with o-iodosobenzoate.

Reconstitution of the hybrid octamer from individually purified sperm H4, dethiolated H3 and erythrocyte H2A and H2B, proceeded with high efficiency. This was evident from the absence of high molecular weight aggregates during Sepharose 6B chromatography of the products of reconstitution. The octameric nature of the reconstituted complex was confirmed by chemical cross-linking with dimethyl suberimidate. The ability of the hybrid octamer to crystallise as helical octamer tubes demonstrated unequivocally that the reconstituted octamer is structurally indistinguishable from the natural erythrocyte octamer. It is therefore concluded that the conversion of cysteine to alanine at position 110 in H3 and the substitution at position 73 in sperm H4 do not alter inter-histone contacts in the octamer. This in turn implies that the substitution of alanine for cysteine in

yeast H3 does not account for the altered stability of yeast core particles (Lee et al. 1982). It is noteworthy that, in the case of H3 this observation is in agreement with observations made by Burlingame et al. (1985) during crystallographic studies of the octamer. The ability of the cysteine and at least some of the backbone of each H3 molecule to move over a distance of 2.5Å in the octamer crystal provides additional evidence that the cysteine residues do not participate in histone-histone contacts.

CHAPTER 4

4.1 THE HEAVY METAL DERIVATISATION OF THE HISTONE OCTAMER

4.1.1 Introduction

The labelling of specific nucleosomal components is of importance to physico-chemical and crystallographic studies of nucleosome structure. Several physico-chemical studies for example, have relied on the fluorescent labelling of histones and/or DNA in elucidating the dynamics of structural changes within nucleosomes accompanying changes in chromatin function (Prior et al. 1980; Prior et al. 1983) or induced by alterations in ionic strength (Eshaghpour et al. 1980; Dieterich and Cantor, 1981). Crystallographic studies of the octamer (Burlingame et al. 1985) and core particle (Richmond et al. 1984) required heavy metal derivatisation of histones to allow structural determination by the method of isomorphous replacement. In addition to satisfying the criteria for isomorphous replacement, the specific derivatisation of H3 aided the identification of this polypeptide during both crystallographic studies.

For physico-chemical studies by fluorescence techniques and the method of isomorphous replacement used in X-ray crystallography, it is desirable that the labels suitable to the study occupy unique positions. Procedures for the derivatisation of DNA fragments at the 5′ (Richmond et al. 1982) and 3′ termini (Eshaghpour et al. 1979) have been described. These procedures are however limited in their application to nucleosomal studies because of the spread of core DNA lengths generated during the preparation of nucleosomes or cores. The labelling of histones at unique sites has therefore been the preferred strategy during core particle labelling studies.. These studies have however been

limited to two polypeptides only, namely H3 and H4. Two sites in H4 have proved suitable for derivatisation with fluorescent dyes namely the methione at position 84 (Lewis, 1979) and the cysteine at position 73 in sea urchin sperm H4 (Eshaghpour et al. 1980). The cysteine residue at position 110 in all variants of H3 (except yeast H3) characterised to date (von Holt et al. 1979) has been a favourite target for chemical probing. The crystallographic studies of Richmond et al. (1984) and Burlingame et al. (1985) relied on the labelling of these residues with the mercury cluster TAMM. Several fluorescent studies have also been performed on nucleosome cores after derivatisation of the H3 cysteine residues with fluorescent reporter groups. Controversy exists over the structural integrity of nucleosome cores labelled with fluorescent groups at these residues. During the studies of Dieterich et al. (1979), Daban and Cantor (1982), Eshaghpour et al. (1980) and Dieterich and Cantor (1981) fluorescent labelling could only be achieved after disruption of native core particles in the presence of urea and/or high salt concentration. These authors claimed that core particles labelled in this way could be renatured to the natural state after removal by dialysis of the denaturing agents. Lewis and Chiu (1980) and Wingender et al. (1981) however, concluded that fluorescent labelling of H3 resulted in the destabilisation of core particles.

In this chapter two labelling strategies for the covalent derivatisation of a specific histone and histone complex of the octamer are demonstrated. The first strategy discussed describes a procedure for the covalent labelling of protein amino groups with aurothiomalate. This label contains a single gold atom which is covalently linked to the thiomalate moiety. Covalent coupling of aurothiomalate to protein amino groups after the prior carbodiimide activation of one carboxyl group of the thiomalate moiety was performed by the two-step

procedure, generally applicable to protein substitution with carboxylic acid in aqueous environment, described by Davis and Preston (1981). In order to reconstitute an octamer labelled in its H2A-H2B dimer only, native octamers were labelled with aurothiomalate before subsequent octamer dissociation and purification of the labelled dimer. The ability of octamers reconstituted from the labelled dimers and native H3-H4 tetramers to crystallise as helical tubes strongly suggests that the labelling procedure had resulted in minimum conformational changes in the H2A-H2B dimer. The second labelling strategy discussed involves the derivatisation of cysteine 73 of sperm H4 before or after reconstitution of a hybrid octamer of which erythrocyte H4 is substituted for by this histone.

4.2 RESULTS

4.2.1 The reconstitution of an octamer from histones labelled with aurothiomalate

4.2.1.1 Carbodiimide activation of sodium aurothiomalate

The carbodiimide coupling of aurothiomalate to protein amino groups was initiated by the carbodiimide activation of one carboxyl group of aurothiomalate at low pH to yield an O-acylisourea (Carraway and Koshland, 1972). This was achieved by reacting equimolar amounts of EDC and aurothiomalate in 10 mM sodium phosphate pH 5.0. By this procedure the activation of both carboxyl groups was precluded which may have resulted in the cross-linking of protein amino groups when added to the protein solutions. The formation of the O-acylisourea during the activation reaction was confirmed by the addition of hydroxylàmine as a trapping reagent (Hestrin, 1949) to the reaction mixture (section 6.4.1.1). Optical density determinations at 540 nm after the addition of

$FeCl_3$ to the solution measured the amount of O-acylisourea formed and allowed the optimal time for the activation reaction to be determined (section 6.4.1.1). The optimal reaction time was found to be from 0 to 2 minutes (figure 4.1) whereafter a rapid decrease in the amount of activated complex formed was observed. Decreases in the amount of activated complex formed after longer time periods were presumably due to hydrolysis of the O-acylisourea in the aqueous buffer used.

4.2.1.1 Coupling of activated aurothiomalate to octamer

The coupling of activated aurothiomalate to histone octamers was performed at high pH (in order to provide the nucleophile) and in the presence of high concentrations of sodium phosphate (Davis and Preston, 1981). In addition, in order to avoid the coupling to ε-NH_2 groups involved in inter-histone contacts, a high concentration of NaCl was maintained. Coupling of activated aurothiomalate to histone octamers in 2 M NaCl, 200 mM sodium phosphate pH 8.0 resulted in protein precipitation. No precipitation however, was evident when the reaction was performed at pH 7.4. Increasing yields of aurothiomalate coupling were observed at increasing concentrations of activated aurothiomalate (figure 4.2a). A maximum molar ratio of approximately 14 moles aurothiomalate incorporated/mole octamer was achieved at a final concentration of 4 mM of aurothiomalate and EDC (figure 4.2a). This represents a 12% occupancy of amino groups present in the histone octamer. No further increase in occupancy (figure 4.2a) was achieved at higher input of EDC and aurothiomalate. This suggests that all sterically accessible and reactive amino groups were derivatised under the experimental conditions.

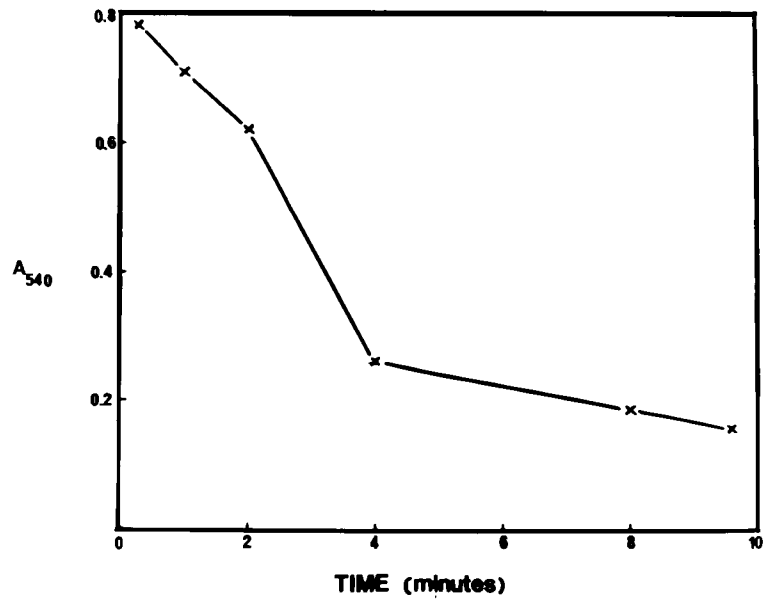


Figure 4.1 Carbodiimide activation of aurothiomalate. The concentrations of EDC and aurothiomalate in 10 mM sodium phosphate were 50 mM. The reaction was allowed to proceed for the time intervals as shown whereafter the degree of activation was determined (section 6.4.1.1).

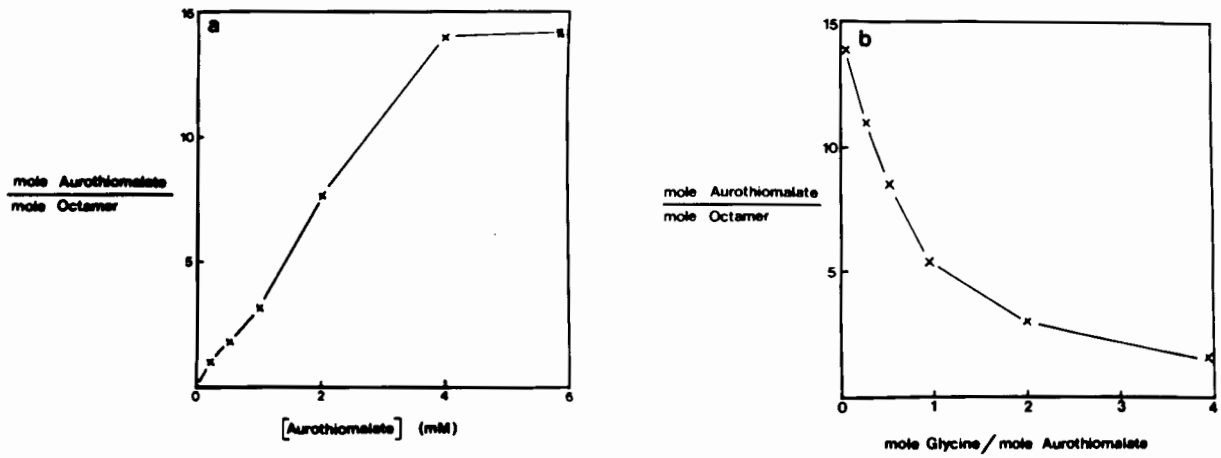


Figure 4.2 Covalent coupling of aurothiomalate to histone octamers.

a) Carbodiimide coupling of aurothiomalate at different final concentrations of aurothiomalate and EDC. 2 mg of octamers was labelled in 2 M NaCl, 200 mM sodium phosphate pH 7.4, 0.2 mM PMSF as described in section 6.4.1.2.

b) Inhibition of aurothiomalate coupling to histone octamers. Labelling of 2 mg. octamers with aurothiomalate at a concentration of 6 mM was carried out as in (a). In all determinations glycine was added as a competing nucleophile for activated aurothiomalate at different concentrations.

In control experiments in which protein was exposed to identical conditions in the absence of EDC, aurothiomalate was not incorporated into the octamer. Therefore the molar ratios of aurothiomalate to protein observed for figure 4.2a accounted for aurothiomalate covalently coupled to protein. This observation was confirmed when glycine was included in the labelling reaction as a competing nucleophile for activated aurothiomalate. Glycine inhibits the coupling of aurothiomalate to histone octamers (figure 4.2b).

All labelled protein samples were analysed by SDS gel electrophoresis to detect possible products of protein-protein cross-linking. As is evident from figure 4.3 no cross-linking of octamers could be observed for all conditions studied. This confirms that only one carboxyl group of aurothiomalate had been activated during the activation reaction. This observation also confirms that the labelling procedures precluded the activation of protein carboxyl groups by unreacted carbodiimide which may facilitate protein-protein cross-linking (Davis and Preston, 1981).

4.2.1.3 Purification of labelled histones and histone complexes

The purification of labelled individual histones or labelled histone complexes from gold labelled histone octamers was attempted. The purification of labelled individual histones (section 6.4.1.4) was attempted by the methods of von Holt and Brandt (1977). Desalted labelled octamers containing 14 to 16 aurothiomalate molecules per octamer were made 20 mg/ml in 8 M urea, 50 mM NaCl, 10 mM HCl before application to the Biogel P60 column. Approximately 70% to 80% of eluted protein was recovered in the outer column volume (figure 4.4b) presumably as an aggregate. The remaining protein exhibited a similar elution pattern to that obtained when unlabelled histones were

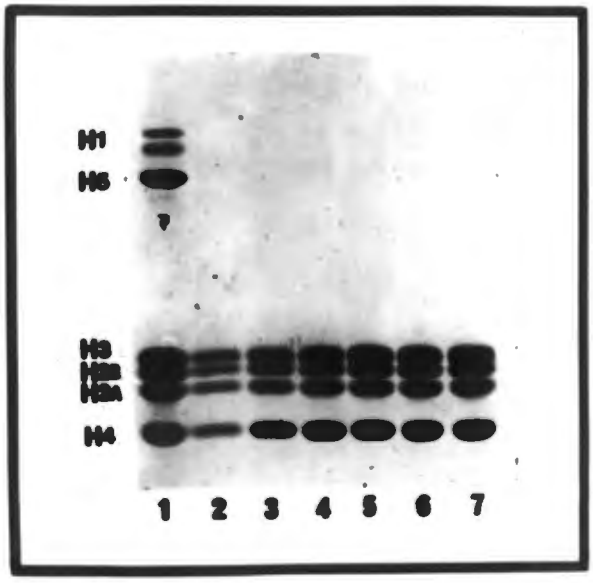


Figure 4.3 SDS/polyacrylamide gel electrophoresis of histone octamers after labelling with aurothiomalate. Lane 1: control core histones. Lanes 2-7 correspond to histone octamers labelled with aurothiomalate at final concentrations of 0.25 mM, 0.5 mM, 1.0 mM, 2,0 mM, 4.0 mM and 6.0 mM respectively.

chromatographed on the same column (figure 4.4a). Gold determinations (section 6.4.1.6) performed on the four fractions obtained in figure 4.4b indicated a high gold to protein ratio for the first fraction and substantially lower ratios for the remaining fractions. H4 which can be isolated in pure form by this procedure (fraction 4 of figure 4.4b) for example, exhibited a low occupancy of 0.5 gold atoms per H4 molecule. These findings suggest that the aggregation of the bulk of the protein eluted in peak 1 of figure 4.4b was the result of the high gold incorporation observed for this fraction and that only the histones exhibiting low occupancies can be recovered at the expected elution positions.

The purification of labelled histone complexes was achieved by gel filtration on Sephadex G-100 in 2 M NaCl pH 5.5 (section 6.4.1.4). Labelled octamers containing 14 to 16 aurothiomalate molecules per octamer were dissociated by chromatography on Sephadex G-50 in 2 M NaCl pH 5.5. The natural products of dissociation of the native octamer under these conditions have been identified as the H3-H4 tetramer and the H2A-H2B dimers (Eickbusch and Moundrianakis, 1978). The elution profile yielded when labelled histone complexes were chromatographed on Sephadex G-100 in 2 M NaCl pH 5.5 is shown in figure 4.5. The bulk of the histone pairs H3-H4 eluted as an aggregate (peak 1 of figure 4.5b) and the remaining H3-H4 was recovered as tetramer (peak 2 of figure 4.5b). Peak 3 of figure 4.5b contained H2A and H2B in apparent equimolar amounts at the elution position of the H2A-H2B dimer. A ratio varying from 2 to 4 aurothiomalate molecules per dimer was calculated from gold and protein determinations for this fraction during different isolations. This implies that approximately 50% of the aurothiomalate coupled to the histone octamer during labelling was associated with the two H2A-H2B dimers. Gold determinations on the aggregate fraction containing only H3 and H4 confirmed this observation.

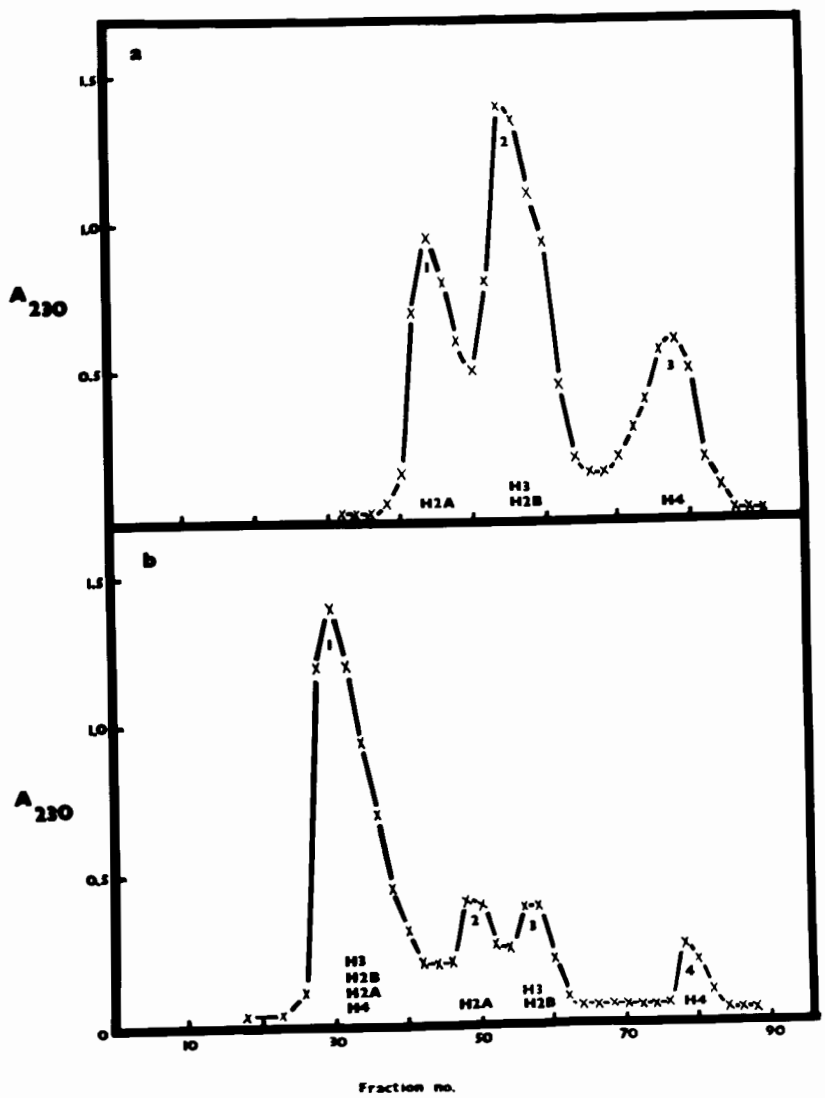


Figure 4.4 Biogel P60 chromatography of acid extracted erythrocyte histones and gold labelled core histones. Histones were eluted with 50 mM NaCl, 10 mM HCl. Fraction volume: 3 ml. Pressure head: 60 cm. The histones eluted in each fraction are as shown.

a) Elution of acid extracted core histones.

b) Elution of labelled core histones.

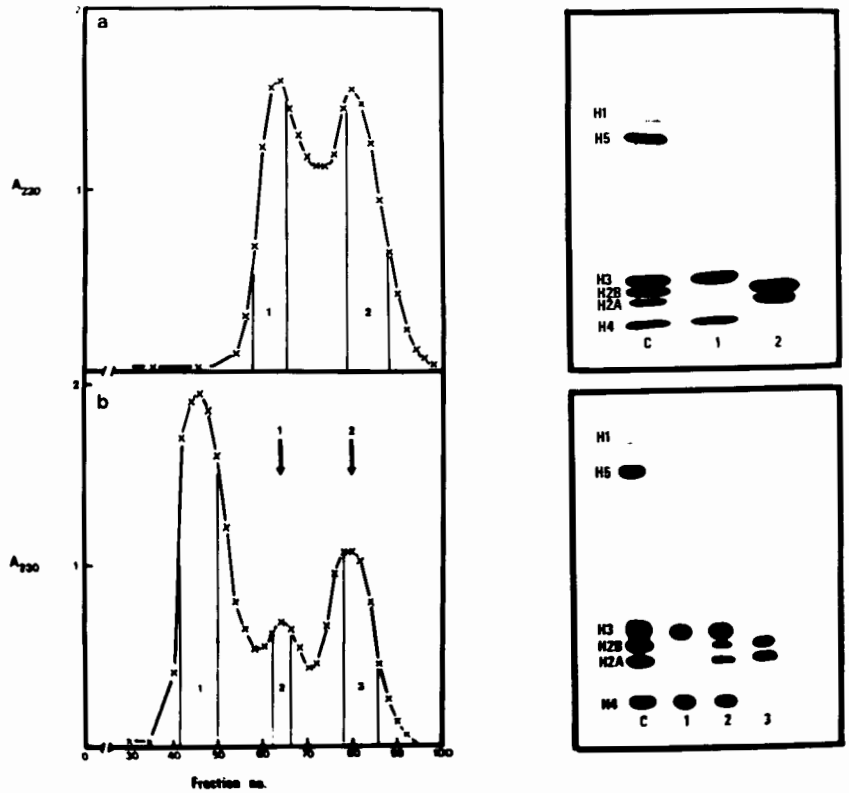


Figure 4.5 Sephadex G-100 chromatography of native and labelled histone complexes. Chromatograms were developed in 2 M NaCl, 10 mM sodium phosphate pH 5.5, 0.1 mM PMSF. Fraction volume: 3 ml. Pressure head: 60 cm.

a) Native histone complexes.

b) Labelled histone complexes. Arrows 1 and 2 denote the elution positions of the H3-H4 tetramer and H2A-H2B dimers respectively as determined by chromatography of native tetramers and dimers under the identical experimental conditions. Fractions were analysed by SDS/polyacrylamide gel electrophoresis. Lane C: histone standards; Lanes 1,2 and 3: fractions 1,2 and 3 of the corresponding chromatograms.

4.2.1.4 Reconstitution of a labelled octamer

In order to produce a histone octamer labelled with gold in a specific histone or histone complex, reconstitution experiments were undertaken. Since individually purified labelled histones could only be recovered in low yields and exhibited low occupancies, the labelled octamer was reconstituted from the native tetramer and labelled dimers to yield an octamer labelled in its dimer only. Native H3-H4 tetramer was isolated by Sephadex G-100 chromatography (figure 4.5a) as detailed previously (Eickbusch and Mondrianakis, 1978).

Reconstitution of the labelled octamer was achieved by mixing stoichiometric amounts of labelled H2A-H2B dimer (3 aurothiomalate residues/dimer) and native H3-H4 tetramer before raising the pH to 7.4 by dialysis (section 6.4.1.5). Chromatography of the products of reconstitution on Sepharose 6B yielded the elution profile shown in figure 4.6a. All the protein eluted as a single peak at an elution volume identical to that observed for the native histone octamer. The gold to protein ratio of the peak fraction was approximately 6 aurothiomalate residues per octamer.

The octameric nature of the core protein complex eluted from the Sepharose 6B column was confirmed by chemical cross-linking (section 6.3.4) with dimethyl suberimidate (figure 4.6b). Cross-linking to octamer proceeds rapidly with the appearance of protein bands arising from the expected intermediate products of octamer cross-linking. Furthermore, an increase in the relative amounts of cross-linked octamer to unreacted histone monomer could be observed when the reaction was allowed to proceed for longer time intervals. The major product of reconstitution is therefore concluded to be a histone octamer.

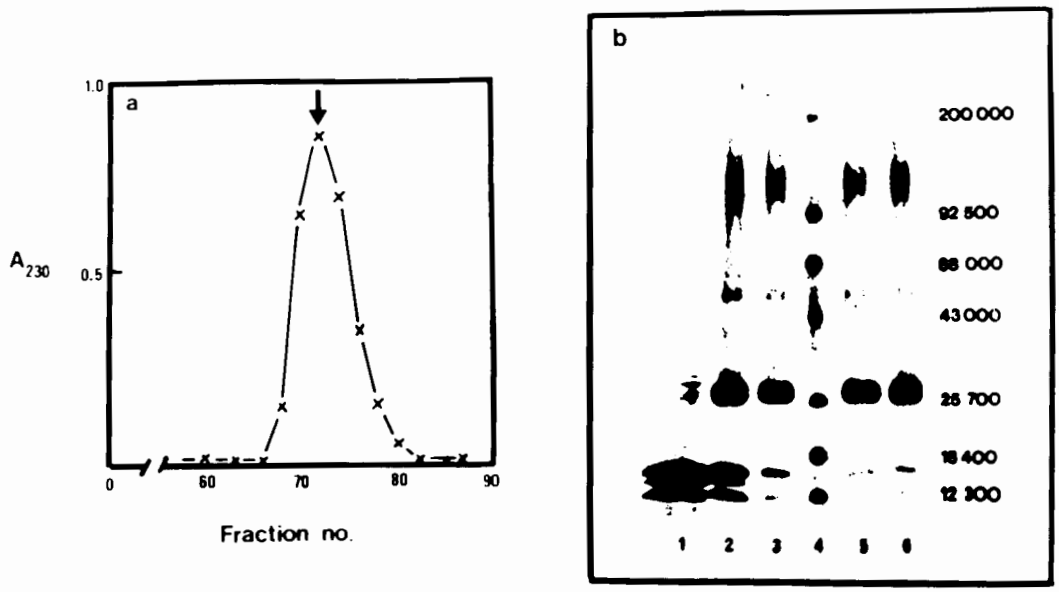


Figure 4.6 Gel exclusion chromatography and chemical cross-linking of reconstituted labelled histone octamers.

a) Sepharose 6B chromatography of labelled reconstituted octamer (7 mg) in 2 M NaCl, 10 mM Tris-HCl pH 7.4, 0.1 mM PMSF. Fraction volume: 1.5 ml. Pressure head: 60 cm. Arrow denotes elution volume of native histone octamer.

b) SDS/polyacrylamide gel electrophoresis of labelled reconstituted octamer after cross-linking with dimethylsuberimidate. Lanes 1-6 (excluding lane 4); octamer cross-linked for 0 min, 30 seconds, 1 minute, 2 minutes and 4 minutes. Molecular weight standards (lane 4) are, in order of decreasing electrophoretic mobility: cytochrome C, β-lactoglobulin, α-chymotrypsinogen, ovalbumin, bovine serum albumin, phosphorylase b, H chain of myosin. The molecular weights are given next to the slab.

Reconstituted octamers were subjected to the crystallisation procedure detailed in section 6.3.5. A bright yellow precipitate formed over a period of one week. The precipitate contained helical octamer tubes in high concentration as was revealed by electron microscopic investigation of the precipitate (figure 4.7). The external diameter of the tubes obtained were approximately 30 nm, identical to dimensions reported for helical tubes of the native octamer (Klug et al. 1980). Gold determinations on the precipitate yielded the expected molar ratio of 6 aurothiomalate residues per octamer. No gold or protein could be detected in the supernatant fraction obtained after crystallisation.

4.2.2 Strategies for the labelling of H4 in the octamer

The first strategy investigated required the derivatisation of the cysteine of sperm H4 before reconstitution of an octamer from the labelled histone and erythrocyte H3, H2B and H2A. Labelling of sperm H4 with TAMM was achieved by reaction of equimolar amounts of label and reduced protein in 8 M urea pH 7.4 (section 6.4.2.1). TAMM was solubilised in 8 M urea, 10 mM Tris-HCl pH 7.4 containing pennicillamine at 3 times the molar concentration of TAMM, before addition to the H4 solution. The inclusion of pennicillamine at this molar ratio has previously been demonstrated to result in the blocking of three Hg atoms of TAMM (Lipka et al. 1979), thereby rendering only one Hg atom reactive towards protein sulfhydryl groups. The specificity of TAMM towards cysteine was confirmed by amino acid analysis of H4 preparations before and after reaction with TAMM. As is evident from table 4.1 cysteine could only be detected (as the carboxymethyl derivative) before reaction with TAMM. Octamer reconstitution from the labelled histone and individually purified histones was attempted by dialysis of the histones (solubilised in acidified urea) against 2 M NaCl pH 7.4. Reconstituted

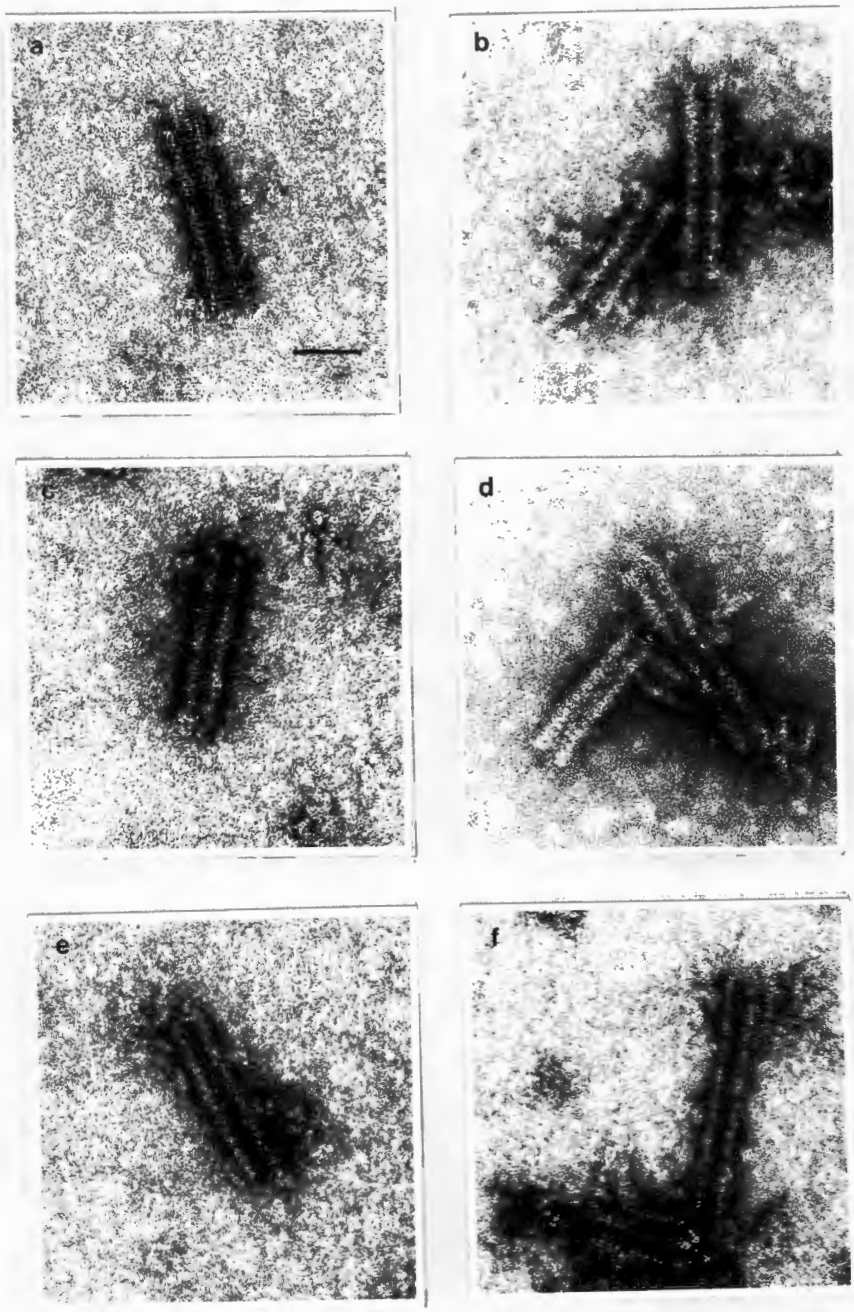


Figure 4.7 Electron micrographs of helical tubes of labelled reconstituted octamers. The bar in (a) represents 50 nm.

complexes were analysed by chromatography on Sepharose 6B in 2 M NaCl. Octamer reconstitution by this criterion was unsuccessful as the histones were eluted as high-molecular weight aggregates and complexes of molecular weight lower than that expected of an octamer (figure 4.8).

For this reason the reconstitution of a hybrid octamer from sperm H4, dethiolated H3 and erythrocyte H2A and H2B (discussed in chapter 3) was undertaken to provide an alternative labelling strategy. This octamer was shown to be structurally identical to the natural octamer. The accessibility of the cysteine residues of the sperm H4 molecules in the hybrid octamer towards sulfhydryl-specific reagents was determined quantitavely by reaction of the octamer with dithio-bis-nitrobenzoic acid (section 6.4.2.3.4). The sulfhydryl groups of both H4 molecules in the octamer were fully reactive towards this reagent.

Control H4		Labelled H4	
Residue	mole %	Residue	mole %
Lys	10.9	Lys	11.3
His	1.9	His	1.9
Arg	14.0	Arg	14.3
Pro	1.6	Pro	1.6
Asp	5.0	Asp	4.9
Thr	6.2	Thr	5.6
Ser	2.6	Ser	2.9
Glu	6.7	Glu	6.8
Gly	16.5	Gly	16.3
Ala	6.7	Ala	6.7
Val	7.5	Val	7.6
Met	0.9	Met	1.0
Ile	5.2	Ile	5.1
Leu	7.8	Leu	7.9
Tyr	3.8	Tyr	3.8
Phe	1.9	Phe	2.0
CMC	0.9	CMC	-

Table 4.1 Amino acid analysis of sperm H4 before and after labelling with ŤAMM. Cysteine was detected (section 6.4.2.3.1) as carboxymethyl cysteine (CMC).

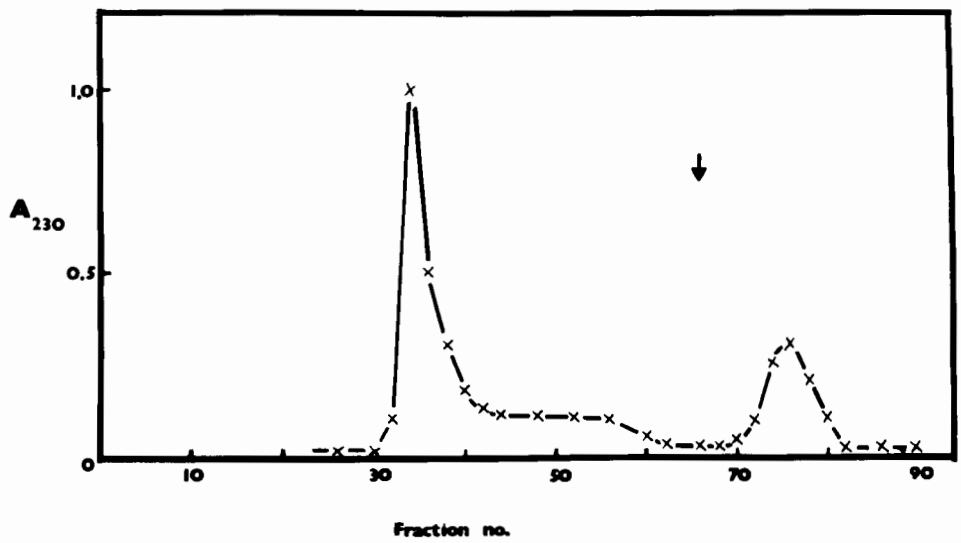


Figure 4.8 Sepharose 6B chromatography of reconstituted TAMM-labelled complexes in 2 M NaCl, 10 mM Tris-HCl pH 7.4. Fraction volume: 1.5 ml. Pressure head: 60 cm. Expected elution position of the octamer is denoted by the arrow.

4.3 DISCUSSION

The labelling of a specific histone or histone complex is of importance to physico-chemical and crystallographic studies of nucleosome structure. This may be achieved by reconstitution of an octamer from the labelled histone or histone complex (derivatised with labels appropriate to the study) and the remaining complement of native complexes or histones. Subsequent assembly of the reconstituted octamer onto DNA in turn may yield the labelled core particle. In the present study two experimental strategies for the covalent heavy metal derivatisation of the histone octamer in a specific histone complex and individual histone were developed.

By the first strategy an octamer labelled in its H2A-H2B dimer was reconstituted from histones gold labelled with aurothiomalate. Covalent coupling of aurothiomalate to protein amino groups was achieved by a two-step procedure similar to that described by Davis and Preston (1981) for the coupling of carboxylic acids to protein amino groups. The reaction was initiated by the carbodiimide activation of one carboxyl group of the aurothiomalate moiety in 10 mM sodium phosphate pH 5.0. A low concentration of phosphate buffer was used to minimise the competition between phosphate and aurothiomalate for EDC (Davis and Preston, 1981). The optimal reaction time for the activation reaction was found to be approximately between 0 and 2 minutes whereafter a decrease in the concentration of activated complex formed was observed, probably due to hydrolysis. The covalent coupling of aurothiomalate to amino groups was achieved by addition of the activated complex to the octamer solution after raising the pH to 7.4. The high phosphate concentration on the one side, mopping up any unreacted carbodiimide (Davis and Preston, 1981) and the initial activation at equimolar ratios of

EDC:aurothiomalate prevented protein-protein crosslinking. The absence of aurothiomalate coupling when EDC was excluded from the reaction and the ability of glycine to inhibit the reaction confirmed that this labelling procedure resulted in the specific covalent coupling of aurothiomalate to protein amino groups.

Attempts to purify labelled histones by Biogel P-60 chromatography, which requires the histones to undergo dramatic conformational changes (von Holt and Brandt, 1977), were unsuccessful. Purification of labelled histone complexes after dissociation of the labelled octamer into tetramers and dimers however, were successful. Sephadex G-100 chromatography in 2 M NaCl pH 5.5 of the dissociated complexes yielded labelled H2A-H2B dimers with maximum gold occupancy as a pure fraction. Octamer reconstitution from stoichiometric mixtures of these labelled dimers and native tetramers proceeded with high efficiency as was confirmed by Sepharose 6B chromatography and chemical cross-linking. This strongly suggests that labelling had resulted in minimum conformational changes in the dimer. Subsequent crystallisation of this labelled octamer yielded helical tubes thus establishing the identity in conformational states of this reconstituted and native histone octamers. It may be argued that the calculated ratio of 3 aurothiomalate molecules/dimer used in these experiments represents the average molar ratio for a population of dimers containing different amounts of label. Although all the H2A-H2B dimers were recovered in the octamer fraction after reconstitution, a selective crystallisation of octamers containing dimers with a low occupancy may have resulted. This argument may be discounted firstly, because the labelling was carried out at pH 7.4 which is well below the optimal pH for the chemical modification of protein amino groups (Carraway and Koshland, 1972). Under these conditions the labelling at unique sites would become likely as only the

most reactive amino groups would be labelled. Furthermore, the final precipitate obtained after crystallisation exhibited the expected ratio of 6 aurothiomalate residues/octamer thus precluding the possibility of a selective crystallisation. It may therefore be concluded that the experimental strategy described here yielded an octamer, covalently labelled with aurothiomalate in its H2A-H2B dimers (containing 3 aurothiomalate molecules/dimer), which is structurally indistinguishable from the natural octamer.

The labelling procedure reported here for the covalent derivatisation of histones with aurothiomalate has been suggested to be of general use in electron microscopic and crystallographic studies of proteins (Greyling et al. 1986). This procedure was demonstrated to result in the specific covalent coupling through a single bond of a heavy metal atom label to protein amino groups. Although this procedure caused the H3-H4 tetramer to aggregate, the ability of the reconstituted octamer to crystallise to the same form as the natural octamer establishes this method as a gentle procedure resulting in minimum changes of protein conformation. Furthermore, the general reaction scheme, carried out here under similar conditions as described previously (Davis and Preston, 1981), would be feasible under various solvent conditions (Carraway and Koshland, 1972). These properties make this procedure a suitable heavy metal labelling procedure for localisation studies by scanning transmission electron microscopy, or the preparation of heavy metal derivatives of protein crystals as required for the method of isomorphous replacement used in X-ray crystallography (Greyling et al. 1986).

The second labelling strategy was developed to specifically label histone H4 of the octamer. This was attempted firstly by derivatising the cysteine residue of sperm H4 with TAMM

before reconstitution of an octamer from the labelled histone and erythrocyte H3, H2B and H2A. Reconstitution from stoichiometric amounts of these histones by the methodology described for octamer reconstitution from individually purified histones (chapter 2) yielded no octamer. The labelling of sperm H4 in the hybrid octamer reconstituted from dethiolated H3, sperm H4 and erythrocyte H2B and H2A was considered as an alternative approach. This hybrid octamer was demonstrated to be capable of tube formation (chapter 4) and therefore concluded to be structurally identical to the natural octamer. The accessibility of the sperm H4 cysteine residues in the octamer was quantitatively determined by reaction of the octamer with DTNB. By this method both sulfhydryl groups were concluded to be reactive, therefore strongly suggesting that these residues may be accessible not only towards TAMM but also sulfhydryl-specific fluorescent reporter groups.

CHAPTER 5

CONCLUSIONS

A detailed description of protein-protein and protein-DNA interactions at the level of core particle structure is central to the understanding of structural changes in the core particle accompanying mitosis, replication and transcription. X-ray crystallographic studies of nucleosome structure which ultimately will provide a sound structural basis for the interpretation of these interactions at the highest level of detail have, to date, proved unsuccessful. This has primarily been due to the limited resolution attained during studies of the core particle (Richmond et al. 1984) and the vastly different ultrastructural descriptions of core particle structure yielded from the studies of Richmond et al. 1984 and Burlingame et al. 1985. The reconstitution and physico-chemical characterisation of well defined histone-DNA complexes represents an alternative approach to the study of histone-histone and histone-DNA interactions. Such studies undertaken in parallel with crystallographic studies at the present and improved resolution, will increase the level of insight into the underlying mechanisms of biologically significant structural changes in the core particle.

A systematic approach to the reconstitution of histone-DNA complexes would firstly require the reconstitution of the histone octamer. It is well established that the core histones H3, H2B, H2A and H4 associate in pairs to result in the octameric structure with subunit organisation $(H2A\text{-}H2B)\text{-}(H3\text{-}H4)_2\text{-}(H2A\text{-}H2B)$ (Kornberg and Thomas, 1974). The biological significance of the intra- and inter-subunit interactions, defined by the above proposed subunit organisation, in the core particle and octamer is apparent

from several lines of evidence: The altered interactions between tetramer and dimers during transcription (Baer and Rhodes,1983) and the "in vivo" nucleosome assembly (Worcel et al. 1978) strongly suggest that biologically significant changes in core particle structure are accompanied by altered tetramer-dimer subunit interactions. Similarly, for the octamer, several authors have provided evidence for the reversible dissociation of the octamer to yield the H3-H4 tetramer and H2A-H2B dimers as natural products (Godfrey et al. 1980; Ruiz-Carillo and Jorcano, 1979). These observations would therefore strongly suggest that thermodynamically favoured interactions occuring in the octamer will also occur in the core particle. This establishes the histone octamer as a suitable model system for the study of histone-histone interactions in the core particle. The study of histone-DNA interactions, in turn, may be facilitated by the "in vitro" assembly of the octamer onto unique DNA through the agency of polyglutamic acid to yield the core particle (Retief et al. 1984; 1987).

This thesis described firstly, methodology for the reconstitution of the chicken erythrocyte histone octamer by different protocols. During these investigations and subsequent studies it was considered important to:

i) assess the efficiency of complex formation during reconstitution, by column chromatographic procedures. This was achieved by gel exclusion chromatography of reconstituted complexes on Sephadex G-100 or Sepharose 6B. During these procedures reconstituted octamers were purified from contaminating histone or histone complexes (Sephadex G-100 chromatography) or products that may have resulted from non-specific aggregation (Sepharose 6B chromatography).

ii) verify the stoichiometry of the reconstituted complexes by chemical cross-linking with dimethyl suberimidate.

iii) demonstrate unequivocally the conformational and structural identity of reconstituted and the natural histone octamers. This problem, a major drawback to many studies reported in the literature, was overcome by demonstrating the ability of reconstituted octamers to crystallise to the same form as the natural octamer, namely the helical tube.

The reconstitution of the erythrocyte octamer was attempted from

i) the natural products of octamer dissociation namely the H3-H4 tetramer and H2A-H2B dimers prepared either by selective extraction from chromatin or by dissociation of the extracted octamer.

ii) acid-denatured histones, available either as a stoichiometric mixture of core histones or individually purified histones.

Octamer reconstitution from tetramers and dimers required an increase in ionic strength and/or an increase in pH to yield the conditions under which inter-subunit interactions in the octamer are stabilised. Reconstitution from acid-denatured histones was achieved similarly by dialysis of the disaggregated histones against 2 M NaCl, pH 7.4. Although not proved during this study, the gradual increase in ionic strength and pH during dialysis is likely to have resulted in the association of the histone pairs H3, H4 and H2A, H2B before subsequent association of the subunits to form an octamer. The ability of reconstituted octamers prepared by the different protocols to crystallise as helical tubes

demonstrated unequivocally the identity of conformational states of reconstituted and native histone octamers. This observation settled the long-standing debate in the literature on whether or not histones, denatured through preceding acid or urea treatment, can be renatured or not (Greyling et al. 1983).

The application of the reconstitution methodology developed in addressing fundamental problems of chromatin research, was demonstrated during subsequent studies. Firstly, in order to establish the influence of variations in the primary structures of the core histones on histone-histone interactions in the octamer, the reconstitution of hybrid octamers was undertaken. To this end octamers, of which one erythrocyte histone molecule was substituted for by a structural variant or chemically modified "mutant" of the substituted histone, were reconstituted. Octamer reconstitution during these studies was undertaken from individual polypeptides by methodology developed for the reconstitution of erythrocyte octamers from individually purified histones. To establish the influence of the extended N-terminus of sperm $H2B_1$ on histone-histone interactions, the reconstitution of a hybrid octamer from this histone and erythrocyte H3, H2A and H4 was attempted. The high efficiency of octamer complex formation during reconstitution was interpreted to strongly suggest that the H2A-H2B and H2B-H4 interactions in the octamer were maintained despite the extended N-terminus of the substituted histone. The high sequence homology of the C-terminal domain of $H2B_1$ when compared with the same domain of calf H2B, supports this conclusion. This domain in calf H2B had previously been identified as the site of closest contact between H2B and neighbouring histones in the octamer (Callaway et al. 1985). The conformational properties of this hybrid octamer probed by the crystallisation procedure, however, remained unclear during this study.

A second hybrid octamer was reconstituted from des-110-thio-H3, sea urchin sperm H4 and erythrocyte H2A and H2B. The Raney nickel catalysed dethiolation of cysteine 110 of H3 (yielding alanine as product) was confirmed by spectrophotometric titration with pCMB and oxidation with o-iodosobenzoate of sulfhydryl groups in the reacted protein. The ability of the reconstituted octamer to crystallise as helical tubes, proved unequivocally that the reconstituted octamer was structurally and conformationally indistinguishable from the natural octamer. It may therefore be concluded that the substitution involving the cysteine residue at position 73 of sperm H4 and the conversion of cysteine to alanine in H3 do not alter histone-histone interactions in the octamer.

Finally, experimental strategies relying on the application of appropriate octamer reconstitution procedures to yield octamers labelled in a specific histone complex or individual histone, are reported in this thesis. Although developed to yield heavy metal derivatised octamers suitable for crystallographic studies, it is conceivable that these strategies may prove useful for the derivatisation of specific components of the octamer with labels suitable to physico-chemical studies. This certainly applies to the labelling strategy developed for the specific derivatisation of H4. The accessibility towards DTNB of the sperm H4 cysteine residues in the hybrid octamer discussed above established these residues as suitable targets for chemical probing with sulfhydryl-specific labels. A second labelling strategy developed, yielded octamers gold labelled in a specific histone complex. The gold labelling procedure developed during this study relied on the covalent coupling of aurothiomalate to histone amino groups after the prior carbodiimide activation of aurothiomalate. Octamers labelled with aurothiomalate were dissociated into tetramers and dimers

before subsequent purification of labelled H2A-H2B dimers. Octamer reconstitution from labelled dimers and the native tetramer yielded the octamer labelled in its H2A-H2B dimer only. The ability of the labelled reconstituted octamer to crystallise as helical tubes demonstrated the labelling method as a gentle procedure resulting in minimal changes of protein conformation. The labelling procedure was therefore concluded to be of general use in electron microscopic and crystallographic studies. It is also noteworthy that the gold derivative prepared during this study satisfies the criteria of Richmond et al. (1982) for heavy-atom derivatives for use in crystallographic studies of the nucleosome core particle.

CHAPTER 6

MATERIALS AND METHODS

6.1 MATERIALS

All operations were carried out at 0-4°C unless otherwise stated.

Centrifugation was performed with a Sorvall RC-2B centrifuge and appropriate rotors, or with a Beckman L2-75B ultracentrifuge.

Ultrafiltration was accomplished using an Amicon ultrafiltration cell (10ml or 200ml), fitted with a PM-10 membrane. Ultrafiltration was performed under a nitrogen pressure of 40 psi.

Different grades of Sephadex were obtained from Pharmacia. Sepharose 6B and Biogel P-60 were obtained from Pharmacia and Biorad respectively. When not in use, columns were stored in the presence of 0.02% (w/v) sodium azide to prevent microbial growth.

Glass double-distilled water was used for all solutions except for dialysis water which was once-distilled.

Hydroxyapatite was prepared and stored as detailed by Bernardi (1971).

Micrococcal nuclease and 1-Ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Sigma. Tetrakis (acetoxy-mercuri)methane (TAMM) and (1,2-dicarboxyethylthio)gold, disodium salt (sodium aurothiomalate) were obtained from Aldrich.

All chemicals not further described were of Analar grade or equivalent supplied by various companies.

6.2 HISTONE ISOLATION

6.2.1 Preparation of cells and nuclei

Nuclei were prepared from chicken erythrocytes by the method of Murray et al. (1968) with some modifications. Blood from mature hens was collected at the local abattoir using 140 ml anticoagulant (0.016 M citric acid, 0.089 M trisodium citrate, 0.016 M sodium dihydrogen phosphate, 0.13 M glucose) per litre blood. The suspension was cooled on ice and filtered through four layers of cheesecloth. Erythrocytes were sedimented by centrifugation at 5000 x g for 10 minutes. The sediment was suspended in 5 volumes 0.014 M NaCl, 0.01 M trisodium citrate and centrifuged as before. This washing procedure was repeated twice. Lysis was achieved by suspending the washed cells in a buffer (Burgoyne et al. 1974) containing 15 mM Tris, 65 mM KCl, 65 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM EDTA, 0.5 mM EGTA, 5 mM 2-mercaptoethanol and 0.1 mM PMSF to which had been added 1% (w/v) Nonidet-LE. The total volume of this suspension was equivalent to the original volume of blood. Nuclei were washed by repeated suspension in 5 volumes of the above buffer (from which the Nonidet had been omitted), followed by centrifugation as before until the supernatant was clear. Batches of nuclei were also stored at -20°C after dilution of the nuclear suspension with an equal volume of glycerol. Such stored nuclei were processed further after extensive washing with the buffer required for the particular subsequent methodology.

6.2.2 Purification of sea urchin sperm

Mature, Parechinus angulosus, ready to spawn, were collected from rock pools along the local coastline. Sperm was collected from male sea urchins after intracoelomic injection of 0.5 M KCl by inverting individuals on 100 ml beakers filled with sea water. The sea water was decanted and the sperm slurry filtered through a plastic mesh (pore size = 250 µm before centrifugation at 2000 x g for 5 minutes. The pelleted sperm was suspended in 5 volumes 0.14 M NaCl, 0.01 M trisodium citrate and centrifuged as before. This washing procedure was repeated until the supernatant was clear.

6.2.3 Isolation of histone complexes

Natural histone complexes were isolated by the following procedures:

6.2.3.1 Histone - DNA separation via ultracentrifugation

The natural histone octamer complex was isolated essentially according to the procedure of Ruiz-Carillo and Jorcano (1979). Washed nuclei were extracted (4 mg DNA/ml) with 0.75 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.2 mM PMSF for 1 hour to remove H1 and H5. The residual nucleohistone was sedimented by centrifugation at 46 000 rpm in a 60 Ti rotor for 16 hours. The resulting pellet was homogenised (Ultra-Turrex homogenisation) in 2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.2 mM PMSF and the DNA concentration adjusted to 4 mg/ml with the same buffer. Extraction was allowed to proceed for 1 hour and the DNA pelleted by centrifugation as before. The supernatant, containing core histones, was concentrated to 12 mg/ml using an Amicon PM-10 ultrafiltration membrane. The natural octamer was isolated from this solution via exclusion

chromatography on Sephadex G-100 (2.5 x 95 cm) in 2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM PMSF.

6.2.3.2 Histone elution from hydroxyapatite

Histones were isolated from digested chromatin by the method of Simon and Felsenfeld (1979) with some modifications. Nuclei were suspended (5 mg DNA/ml) in 25 mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.4, 0.2 mM PMSF after repeated washings in the same buffer. Digestion with micrococcal nuclease (40 units/mg DNA) was performed at 37° C for 30 minutes. The reaction was terminated by the addition of EDTA from a 250 mM stock solution to a final concentration of 5 mM, followed by centrifugation at 6000 x g for 5 minutes. To extract soluble chromatin, the pellet was homogenised in 0.25 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.2 mM PMSF and dialysed against the same buffer for 16 hours. Digested chromatin was recovered in the supernatant after centrifugation at 10 000 x g for 20 minutes. The soluble, partially digested chromatin (100 mg DNA in 20 ml of extraction buffer) was loaded on an hydroxyapatite column (4.5 cm x 10 cm) which had been pre-equilibrated with 10 mM sodium phosphate, pH 7.4, 0.1 mM PMSF. Unbound material was eluted with one column volume of the same buffer. The column was then eluted with 3M NaCl, 10 mM sodium phosphate (pH 7.4), 0.2 mM PMSF. Fractions containing the core histones were pooled and concentrated to 12 mg/ml by ultrafiltration.

6.2.3.3 Protamine displacement of histones

The histone pairs H2A-H2B and H3-H4 were isolated by a modification of the method of van der Westhuizen and von Holt (1971). All purification procedures were carried out at neutral pH and not at pH 5 as in the original method. This

precluded endogenous protease activity as described before (Lindsey et al. 1981). Nuclei were dissolved (4 mg DNA/ml) in 2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.2 mM PMSF and allowed to equilibrate for 1 hour. Protamine was dissolved at a concentration of 20 mg/ml in a separate volume of the same buffer. Equal volumes of the two solutions were mixed together and dialysed against 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.2 mM PMSF until all the DNA was precipitated as a deoxyribonucleoprotamine complex. The suspension was centrifuged at 10 000 x g for 20 minutes and the supernatant, containing histone and excess protamine, was concentrated by ultrafiltration through a PM-10 membrane. The concentrate (150 mg histone in 15 ml) was then fractionated into histone and protamine by gel filtration on a column of Sephadex G-50 (5 x 90 cm) equilibrated and eluted with 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM PMSF. The histone fraction was concentrated to 12 mg/ml by ultrafiltration.

6.2.3.4 Purification of natural tetramers

Natural H3-H4 tetramers were purified after dialysis of histone complexes isolated by hydroxyapatite chromatography against 2 M NaCl, 10 mM sodium phosphate pH 5.5, 0.2 mM PMSF for 24 hours at 4 C. The solution containing tetramers and dimers (50 mg protein) was concentrated by ultrafiltration to yield a sample volume of 1.5 ml. H3-H4 tetramers were purified by subsequent chromatography on a Sephadex G-100 column (2.5 cm x 90 cm) in the presence of 2 M NaCl, 10 mM sodium phosphate pH 5.5, 0.1 mM PMSF.

6.2.4 Purification of acid-extracted histones

6.2.4.1 Preparation of acid-extracted core histones

Total acid-extracted histones were prepared from washed nuclei or sperm (10 mg DNA/ml) by extraction in 0.25 M HCl. The supernatant obtained after centrifugation at 10 000 x g for 15 minutes was dialysed against water before freeze-drying. Acid-denatured erythrocyte core histones were isolated by extraction of histones H1 and H5 from total acid-extracted histones with 5% (w/v) perchloric acid (Oliver et al. 1972). To this end, total acid-extracted histones were suspended (10 mg/ml) in 5% perchloric acid and centrifuged at 10 000 x g for 15 minutes. The pellet was re-extracted and the final sediment dissolved in 0.25 M HCl and dialysed against the same solvent for 24 hours to convert the histone perchlorates into the hydrochlorides. Subsequently the core histones were dialysed against distilled water, freeze-dried and stored at -20°C.

6.2.4.2 Purification of individual histones

Histones H2A, H2B and H4 were purified by two-step column chromatography procedures as detailed previously (von Holt and Brandt, 1977). These histones were freeze-dried and stored at -20°C. The purification of H3 involved the dimerisation of H3 coeluted with the H2B fraction after chromatography on a Biogel P-60 column (5 cm x 90 cm) in the presence of 50 mM NaCl, 10 mM HCl (Brandt and von Holt, 1974). The dimer was eluted from Sephadex G-100 (2.5 cm x 90 cm) with 0.01 M HCl. This procedure yielded pure H3 dimer that was subsequently reduced by addition of mercaptoethanol to a final concentration of 1% (v/v). Freeze-drying of samples during the purification of H3 was omitted (Lindsey et al. 1983). Samples were concentrated instead by ultrafiltration using an

Amicon ultrafiltration cell of appropriate volume fitted with a PM-10 membrane.

Sea urchin sperm H2B(1) was a generous gift of Dr. M. Strickland. Sea urchin sperm H4 was purified by a two-step column chromatography procedure. Sperm H4 was purified from total acid extracted sperm histones by chromatography on a Biogel P-60 column (5 x 90 cm) in 50 mM NaCl, 10 mM HCl as detailed before (Brandt and von Holt, 1977). The final purification procedure involved the dimerisation of sperm H4 with o-iodosobenzoate as previously detailed (Brandt and von Holt, 1977) before gel filtration on a Sephadex G-100 column (2.5 x 90 cm) in the presence of 10 mM HCl. This procedure was undertaken to purify the sperm H4 variant containing cysteine from the variant for which this amino acid is absent (Dr. G.G. Lindsey, personal communication). Purified H4 was reduced with 2-mercaptoethanol and concentrated by ultrafiltration.

6.3 RECONSTITUTION OF HISTONE OCTAMERS

6.3.1 Octamer reconstitution from salt extracted complexes

Histone octamers were reconstituted from the stoichiometric core histone mixture isolated after elution from hydroxyapatite (section 6.2.1.3) or protamine displacement (section 6.2.1.4) by either directly applying them to a Sephadex G-100 column (2.5 x 95 cm) (complexes isolated in section 6.2.1.3) or after prior dialysis of isolated complexes (section 6.2.1.4) against 2 M NaCl, 10 mM Tris-HCl pH 7.4, 1% (v/v) 2-mercaptoethanol, 0.2 mM PMSF for 24 hours. In either case the column was eluted with 2 M NaCl, 10 mM Tris-HCl pH 7.4, 0.1 mM PMSF.

6.3.2 Octamer reconstitution from acid-denatured histones

Octamer reconstitution from acid-denatured histones was essentially as described by Lindsey et al. (1983). An approximately stoichiometric mixture of erythrocyte core histones prepared by perchloric acid precipitation (section 6.2.2.1) was dissolved in freshly prepared 8 M urea, 0.25 N HCl, 1% (v/v) 2-mercaptoethanol at a concentration of 0.5 mg/ml. This solution was allowed to stand for several hours to facilitate disaggregation and dialysed against 2 M NaCl, 10 mM Tris-HCl pH 7.4, 1% (v/v) 2-mercaptoethanol for 14 hours. The sample was concentrated by ultrafiltration to 12 mg/ml and loaded on a Sepharose 6B column (2.5 x 90 cm) equilibrated with 2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM PMSF and eluted with the same solution.

For octamer reconstitution from individually purified erythrocyte histones, freeze-dried histones H2A, H2B and H4 were dissolved separately (0.5 mg/ml) in freshly prepared 8 M urea, 0.25 N HCl containing 1% (v/v) 2-mercaptoethanol. Histone H3 was brought to a concentration of 0.5 mg/ml in 8 M urea, 1% (v/v) 2-mercaptoethanol by ultrafiltration. The solutions were allowed to stand for 16 to 24 hours and then added together before dialysis against 2 M NaCl, 10 mM Tris-HCl,
pH 7.4, 1% (v/v) 2-mercaptoethanol, 0.2 mM PMSF for 24 hours. Gel filtration of the sample (26 mg in 2 ml) on Sepharose 6B was as described above.

The reconstitution of hybrid octamers from stoichiometric amounts of

(i) Sperm H2B(1) and erythrocyte H3, H2A and H4 and
(ii) Sperm H4, dethiolated H3 (section 6.4.2.2) and erythrocyte H2A and H2B

was undertaken by the same methodology as described for the reconstitution of octamers from individually purified erythrocyte histones. Sperm H4, erythrocyte H3 and desulfurised H3, not freeze-dried during isolation, were made 0.5 mg/ml in 8 M urea, 0.25 N HCl, 1% (v/v) 2-mercaptoethanol before dialysis (during reconstitution experiments) by ultrafiltration.

6.3.3 Storage of histone octamers

Some of the natural and reconstituted octamers in 2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.2 mM PMSF were diluted with an equal volume of glycerol and stored at -20°C. Such octamers were dialysed against the buffer required for the subsequent methodology.

6.3.4 Chemical cross-linking of octamers

Purified histone octamers were dialysed exhaustively against 2 M NaCl, 50 mM sodium borate, pH 9.0. The final protein concentration after dialysis was 2 mg/ml. Dimethyl suberimidate was dissolved in a separate volume of 2 M NaCl, 250 mM sodium borate, pH 9.8. The final pH of this solution was 9. Equal volumes of both solutions were mixed and the cross-linking reaction allowed to proceed for the time intervals as described in the text. The reaction was terminated by addition of 250 mM glycine to a final concentration of 25 mM and the reaction mixture chilled on ice.

6.3.5 Crystallisation of the octamer

Crystalline histone octamer tubes were prepared essentially as described by Klug et al. (1980). Pure histone octamer (2-3 mg/ml), natural or reconstituted, was brought to 20% saturation with ammonium sulfate in the presence of 2 M NaCl, 100 mM Tris-HCl, pH 7.4, by dialysis for 16 hours at room temperature. The dialysed sample was clarified by centrifugation at 2000 x g for 2 minutes before dialysis of the supernatant against 2 M NaCl, 100 mM Tris-HCl, pH 7.4, 45% saturated ammonium sulfate at 4°C. Crystallisation occured within 4 days at 4°C.

6.4 LABELLING OF HISTONES

6.4.1 Gold labelling of histones

6.4.1.1 Carbodiimide activation of aurothiomalate

The carbodiimide activation of sodium aurothiomalate was performed by a similar procedure to that described by Davis and Preston (1981). This was achieved by mixing equimolar amounts of EDC dissolved in H_2O and sodium aurothiomalate solubilised in 20 mM sodium phosphate pH 5.0. The final concentrations of EDC and aurothiomalate in the reaction mixture were 50 mM. The reaction was allowed to proceed for 1.5 minutes at room temperature before addition of the activated complex to the octamer solution.

The optimal reaction time for carbodiimide activation was determined by the procedure of Hestrin (1949) for carbodiimide and aurothiomalate at a final concentration of 50 mM in 10 mM sodium phosphate. The activation reaction was carried out in samples with a total volume of 1 ml and the reaction allowed

to proceed for different time periods. Addition of the hydroxylamine and $FeCl_3$ solutions to the samples, to determine the degree of activation was performed as detailed by Hestrin (1949). The absorbance of the solutions was promptly determined at 540 nm. Blank solutions were prepared by excluding either EDC or sodium aurothiomalate or both reagents from the reaction mixture. These solutions exhibited similar absorbances at 540 nm.

6.4.1.2 Coupling of aurothiomalate to octamers

The coupling of activated aurothiomalate to protein amino groups was essentially as the procedure detailed by Davis and Preston (1981) for the coupling of carboxylic acids to protein amino groups. During pilot studies activated aurothiomalate (activation time = 1.5 minutes) was added to 2 ml of a 1 mg/ml solution of histone octamers in 2 M NaCl, 200 mM sodium phosphate buffer pH 7.4, 0.2 mM PMSF. The final concentrations of EDC and aurothiomalate were varied from 0.25 mM to 6 mM. The coupling reaction was allowed to proceed for 16 hours at room temperature. Unreacted activated aurothiomalate was separated from the labelled protein fraction by gel exclusion chromatography on Sephadex G-50 in the presence of 2 M NaCl, 10 mM sodium phosphate pH 5.5, 0.1 mM PMSF. The elution of the two fractions was monitored by the absorbance of all fractions at 230 nm.

6.4.1.3 Inhibition of aurothiomalate coupling

Inhibition of gold labelling of histone octamers was achieved by including glycine as a competing nucleophile in the reaction mixture. Labelling of 2 ml aliquots of histone octamer solutions with EDC and aurothiomalate at a final concentration of 6 mM was performed as detailed in Section

6.4.1.2. Glycine was added to the protein solutions prior to the coupling reaction from a 10 mg/ml stock solution to yield a molar ratio of glycine to activated aurothiomalate ranging from 0.25 : 1 to 4 : 1. The coupling reaction was allowed to proceed and the labelled octamers desalted as detailed above.

6.4.1.4 Purification of labelled histones and histone complexes

50 mg octamers (1 mg/ml) were labelled in 2 M NaCl, 200 mM sodium phosphate buffer pH 5.5, 0.2 mM PMSF at a final concentration of 6 mM EDC. For the purification of individual labelled histones, labelled octamers were desalted on a Sephadex G-50 column (4.5 x 80 cm) in the presence of 2 M NaCl, 10 mM Tris-HCl pH 7.4, 0.1 mM PMSF. Desalted octamers were dissociated in 8 M urea, 50 mM NaCl, 10 mM HCl and concentrated to 10 mg/ml by ultrafiltration, before chromatography on a Biogel P60 column (2.5 x 90 cm) in the presence of 50 mM NaCl, 10 mM HCl.

Purification of labelled histone complexes was undertaken after exclusion chromatography of labelled octamers on a Sephadex G-50 column (4.5 x 80 cm) in 2 M NaCl, 10 mM sodium phosphate pH 5.5, 0.1 mM PMSF. Labelled octamers, thus dissociated into H3-H4 tetramers and H2A-H2B dimers, were concentrated to 3.5 ml by ultrafiltration before gel exclusion chromatography on a Sephadex G-100 column (2.5 x 90 cm) in 2 M NaCl, 10 mM sodium phosphate pH 5.5, 0.1 mM PMSF.

6.4.1.5 Reconstitution of gold-labelled octamers

Labelled histone octamers were reconstituted from labelled H2A-H2B dimers (section 6.4.1.4) and native H3-H4 tetramers (section 6.2.3.4). This was achieved by mixing stoichiometric amounts of tetramers and dimers (final protein concentration was 0.5 mg/ml) before dialysis against 2 M NaCl, 10 mM sodium phosphate pH 7.4, 0.2 mM PMSF at 4°C for 24 hours.

The products of reconstitution were characterised by gel exclusion chromatography on a Sepharose 6B column (1.5 x 80 cm) equilibrated and eluted with 2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM PMSF. Chemical cross-linking and crystallisation of the reconstituted labelled octamers were as detailed in Sections 6.3.4 and 6.3.5.

6.4.1.6 Gold determinations

Molar ratios of aurothiomalate : protein were calculated from gold and protein (section 6.6) determinations. The aurothiomalate concentration in protein samples was determined by atomic absorption using a Varian Techtron AA6 model 70 spectrometer. A standard curve was constructed for the concentration range 1-4 μg/ml (sample volumes = 1.5 ml) using a standard solution of $AuCl_3$. A buffered solution of histone octamers was used as the blank solution in all determinations. Standard solutions were prepared by addition of an appropriate volume of the gold standard solution to the protein blank solution.

6.4.2 Labelling of sea urchin sperm H4

Labelling strategies to result in the derivatisation of sperm H4 before (section 6.4.2.1) or after (section 6.4.2.2) reconstitution of an octamer containing this histone were developed.

6.4.2.1 Labelling of H4 with TAMM

4 mg sperm H4 was made 1 mg/ml in 8 M area, 10 mM Tris-HCl, pH 7.4, by ultrafiltration before addition of an equimolar amount of TAMM solubilised in the same buffer containing penicillamine at molar ratio of 3 : 1 for penicillamine : TAMM. The solution was magnetically stirred for 1 hour at room temperature before gel exclusion chromatography on Sephadex G-25 in 10 mM HCl. Derivatisation of the cysteine residue of H4 after labelling was confirmed by amino acid analysis (section 6.4.2.3.1). Reconstitution of an octamer from the derivatised histone and erythrocyte H3, H2B and H2A was attempted by the methodology detailed in section 7.3.2 for octamer reconstitution from individually purified histones.

6.4.2.2 Dethiolation of erythrocyte H3

In order to reconstitute an octamer containing sperm H4 suitable for the derivatisation of only the H4-cysteine, the dethiolation of erythrocyte H3 was undertaken.

6.4.2.2.1 Preparation of Raney nickel

Raney nickel was prepared as described by Perlstein et al. (1971) Sodium borohydride (1 g) was added to a 1% (w/v)

aqueous solution (600 ml) of nickel acetate. The black amorphous precipitate was filtered and washed on the filter (Watman No. 1) with 4 litres distilled water. The precipitate was not allowed to dry and used directly after preparation.

6.4.2.2.2 Dethiolation of cysteine and erythrocyte H3

Dethiolation of solutions of cysteine or H3 was performed essentially as detailed by Perlstein et al. (1971). The reaction was carried out on an aqueous solution (2 ml) of cysteine (2.5 μmol/ml) after adjustment of the pH of the solution to 7.0 with 0.2 M NaOH. Raney nickel prepared as detailed in section 6.4.2.2.1 was added and the reaction allowed to proceed under a pressure of H_2 at room temperature for 1.5 hours. The pH was maintained at 7.0 by the addition of 0.2 M HCl using a pH-stat. The reaction was terminated by centrifugation at 7000 x g for 20 minutes and the supernatant frozen.

Dethiolation of H3 was carried out on 25 to 30 mg of reduced H3 made 7-10 mg/ml in 6 M guanidinium chloride by ultrafiltration. The pH of the solution was adjusted to 7.0 with 0.2 M NaOH before addition of Raney nickel catalyst. The reaction was allowed to proceed for 72 hours at pH 7.0 as detailed above. Fresh preparations of Raney nickel were added to the reaction mixture at 12 to 16 hour intervals. The reaction was terminated by centrifugation at 7000 x g for 30 minutes and the supernatant dialysed against 0.25 M HCl (2 changes) for 24 hours and finally against H_2O.

6.4.2.3 Determination of cysteine content

6.4.2.3.1 Amino acid analysis

The cysteine content of sperm H4 reacted with TAMM (section 7.4.2.1) was determined after carboxymethylation of free sulfhydryl groups with iodoacetic acid before subsequent quantitation of the derivatised amino acid by amino acid analysis. Carboxymethylation of unreacted sperm H4 was undertaken during control experiments. To this end protein samples were made 1 mg/ml in 4 M urea, 100 mM Tris-HCl, pH 8.3 by ultrafiltration before addition of an equimolar amount of iodoacetic acid solubilised in 200 mM Tris-HCl, pH 8.3. The reaction mixture was magnetically stirred in the dark for 2 hours, dialysed against H_2O and freeze-dried. Protein samples (0.2 mg - 0.4 mg) were hydrolysed in twice distilled constant boiling 5.7 M HCl at 110°C for 24 hours. Oxidative modifications of various amino acids were reduced by the inclusion of phenol as an anti-oxidant during hydrolysis. The HCl was evaporated over NaOH and the hydrolysates dissolved in pH 2.2 buffer containing norleucine as an internal standard. Analyses were performed on either a Beckman Model 116 or 119 two-column system amino acid analyser connected to a Waters Associates HPLC ion-exchange amino acid analyser, using orthopthalaldehyde (OPA) post column fluorescence detection. Since OPA does not react with secondary amino acids, proline was not determined during analyses. Destruction of amino acids during hydrolysis were not corrected for during determinations.

The conversion of cysteine to alanine by reaction of Raney nickel with a solution of cysteine (section 6.4.2.2.2) was quantitated by amino acid analysis of samples before and after reaction. This was achieved by dilution of the sample with an appropriate volume of pH 2.2 buffer before analysis as detailed above. Quantitation of the cysteine content of

erythrocyte H3 after reaction with Raney nickel by amino acid analysis was not attempted due to the low efficiency (50%) of carboxymethylation of unreacted H3. By comparison, carboxymethylation of sea urchin sperm was virtually 100% (see Table 4.1). Analyses of H3 before and after reaction with Raney nickel were undertaken to determine the methionine content of the analysed samples.

6.4.2.3.2 Spectrophotometric titration of protein sulfhydryl groups with p-chloromercuribenzoate

The cysteine content of H3 before and after reaction with Raney nickel was determined by spectrophotometric titration with p-chloromercuribenzoate. Approximately 100 nmoles of protein were dissolved in 0.3 ml 6 M urea to which 2.7 ml of 0.05 M sodium phosphate buffer pH 7 was added. The blank consisted of the same solutions except protein. A 1.75 mM PCMB solution was prepared by dissolving p-chloromercuribenzoic acid in a sufficient amount of 0.1 M NaOH to bring the pH of the solution just above 7. Ten µl aliquots of pCMB reagent were added to both solutions and, after mixing, the difference in absorbance of the blank and protein solution measured at 255 nm. This procedure was repeated until no further change in absorbance could be detected.

6.4.2.3.3 Oxidation of H3 sulfhydryl groups to disulfide bonds

The cysteine content of Raney nickel reacted samples of H3 was estimated after dimerisation of unreacted sulfhydryl groups to disulfide bonds with equimolar amounts of o-iodosobenzoate. The reaction was carried out on 10 to 15 mg protein at pH 7.0 as detailed by von Holt and Brandt (1977), before exclusion chromatography on a Sephadex G-100 column (1.5 x 90 cm) in 10 mM HCl to separate dimerised H3 from monomer.

6.4.2.3.4 Reaction of protein sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoate)

The accessibility of the sperm H4 cysteine residue in reconstituted octamers was determined quantitatively by reaction of octamers with DTNB as detailed by Glazer et al. (1975). The reaction was carried out on octamers (0.02 to 0.05 μmoles) in 2.5 ml 2 M NaCl, 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA. The blank consisted of the same solution except protein. DTNB was solubilised at a final concentration of 0.01 M in 50 mM sodium phosphate buffer pH 7.0. A 100 μl aliquot of DTNB was added to both solutions and the absorbance of each solution determined at 412 nm. The sulfhydryl content was calculated assuming an extinction coefficient for 3-carboxylato-4-nitrothiophenolate of 13600 M^{-1} cm^{-1} (Ellman, 1959).

6.5 GEL ELECTROPHORESIS OF HISTONES

The purity of individual histones and histone complexes was assessed by SDS gel electrophoresis according to the method of Laemmli (1970). SDS/polyacrylamide gel electrophoresis was carried out on 15% slab gels (25 x 12.5 x 0.15 cm) with a 3% stacking gel. Gels were run vertically at constant voltage (100V) for approximately 16 hours and stained in 0.25% Coomassie brilliant blue, 45% methanol, 10% acetic acid. Destaining was by diffusion in 25% ethanol, 7% acetic acid.

Cross-linked histones were examined on polyacrylamide gradient slabs as SDS complexes as described by Laemmli (1970). Slab gels (25 x 12.5 x 0.15 cm) consisted of a 5-20% linear gradient of polyacrylamide running gel with an acrylamide to bisacrylamide ratio of 30 : 0.8. The stacking gel was identical to that described above. Gels were electrophoresed,

stained and destained as detailed above.

The purity of sea urchin sperm H2B(1) was determined by Triton X-100/urea polyacrylamide (15%) gel electrophoresis (Zweidler, 1978). Slab gels (25 x 12.5 x 0.15 cm) contained urea and Triton X-100 at final concentrations of 3.8 M and 0.3% (w/v) respectively. Gels were run vertically at constant current (15 mA) with acetic acid (0.9 M) as the tray buffer. Protein samples were dissolved in 8 M urea, 0.9 M acetic acid for electrophoresis. Staining and destaining of gels were as described above.

6.6 DNA AND PROTEIN DETERMINATIONS

The DNA concentrations of nuclei and digested chromatin was determined spectrophotometrically in 4 M NaCl assuming a 1 mg/ml DNA solution to exhibit an absorbance of 20 at 260 nm.

Protein determinations were performed in triplicate by the procedure of Lowry et al. (1951) as detailed by Layne (1956). Acid-extracted histones were used as protein standards in all determinations.

6.7 ELECTRON MICROSCOPY

6.7.1 Specimen preparation and electron microscopy

Negatively stained specimens for electron microscopy were prepared by mixing 200 µl of the sample with 200 µl of a 0.4% (w/v) freshly prepared uranyl acetate solution. The mixing time period was not longer than 20 seconds to prevent dissociation of the octamer tubes. A carbon coated grid was floated on a drop of the mixed sample on parafilm. After 1

minute excess solution was removed by blotting with filter paper and the grid floated on a drop of freshly prepared 0.2% (w/v) uranyl acetate. Excess solution was removed after 15 seconds by blotting and this procedure repeated again. Grids were examined in a Zeiss EM 109 electron microscope at magnifications of 10 000 x and 50 000 x.

6.7.2 Helical analysis

The helical parameters of octamer tubes were deduced from the optical and numerical transforms of electron micrographs (section 2.2.3). Optical diffractometry was carried out as detailed previously by De Rosier and Klug (1968).

The computations of numerical transforms were performed on an IBM 4381 using computer programs made available by Dr. L. Amos (MRC Laboratory of Molecular Biology, Cambridge, U.K.). For the computation of numerical transforms, electron micrographs were digitised on an Optronics P1200 densitometer using a step size of 25 μm which corresponded to a spacing in the images of 5.16Å. Digitised images of the helical tubes were masked from their surroundings and Fourier transformed as detailed by De Rosier and Moore (1970). The Fourier transforms were displayed as contour maps of the amplitudes and indexed as detailed by Klug et al. (1958).

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