THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 17, Issue of September 10, pp. 10433-10438, 1983
Printed in U.S.A.

Interaction of Nucleotides and Cations with the (Ca^{2+},Mg^{2+})-ATPase of Sarcoplasmic Reticulum As Determined by Fluorescence Changes of Bound 1-Anilino-8-naphthalenesulfonate*

(Received for publication, February 22, 1983)

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The changes in fluorescence of 1-anilino-8-naphthalenesulfonate (ANS^-) have been used to determine binding of ligands to the (Ca^{2+},Mg^{2+})-ATPase of sarcoplasmic reticulum vesicles, isolated from rabbit skeletal muscle. ANS^- binds to sarcoplasmic reticulum membranes with an apparent K_d of 3.8×10^{-5} M. The binding of ANS^- had no effect on Ca^{2+} transport or Ca^{2+}-dependent ATPase activity. EGTA, by binding endogenous Ca^{2+}, increased the fluorescence intensity of bound ANS^- by 10–12%. Subsequent addition of ATP, ADP, or Ca^{2+}, in the presence or absence of Mg^{2+}, reversed this change of fluorescence. The binding parameters, as determined by these decreases in fluorescence intensity, were as follows: for ATP, $K_d = 1.0 \times 10^{-5}$ M, $n_H = 0.80$; for ADP, $K_d = 1.2 \times 10^{-5}$ M, $n_H = 0.89$; and for Ca^{2+}, $K_d = 3.4 \times 10^{-7}$ M, $n_H = 1.8$. The binding parameters for ITP and for the nonhydrolyzable analogue, adenyl-5′-yl-β,γ-methylene)diphosphate, were similar to those of ATP, but GDP, IDP, CDP, AMP, and cAMP had lower apparent affinities. Millimolar concentrations of pyrophosphate also decreased the fluorescence of bound ANS^-, whereas orthophosphate caused a small (2–3%) increase in fluorescence in Ca^{2+}-free media. Vanadate, in the presence of EGTA, decreased the fluorescence of bound ANS^- with half-maximal effect at 4×10^{-5} M. The changes of fluorescence intensity of bound ANS^- appear to reflect conformational changes of the (Ca^{2+},Mg^{2+})-ATPase, consequent to ligand binding, with the low and high fluorescence intensity species corresponding to the E_1 and E_2 conformations, respectively. These appear to reflect similar conformational states of the (Ca^{2+},Mg^{2+})-ATPase to those reported by changes in intrinsic tryptophan fluorescence (DuPont, Y. (1976) *Biochem. Biophys. Res. Commun.* 71, 544–550).

The (Ca^{2+},Mg^{2+})-ATPase (Ca^{2+}-ATPase) of SR[1] actively transports Ca^{2+} ions from the sarcoplasm against a high concentration gradient (>1000:1). Calcium transport is coupled to Ca^{2+}-dependent hydrolysis of ATP with a Ca^{2+}/ATP ratio of two (1). There is substantial evidence that the ATPase alternates between two major conformational states, designated E_1 and E_2, during the catalytic cycle (for review see Ref. 2). The E_1 form is phosphorylated by ATP and has a high affinity Ca^{2+}-binding site ($K_d = 3 \times 10^{-7}$ M) that is accessible to the cytoplasm. The E_2 conformation may be phosphorylated by P_i and has a low affinity site ($K_d = 1 \times 10^{-3}$ M) that is accessible to the interior of the tubular system. The transition $E_1 \sim P \cdot Ca^{2+}{}_{out} \rightarrow E_2\text{-}P \cdot Ca^{2+}{}_{in}$ provides a basis for active transport.

Binding parameters for the high affinity Ca^{2+}- and the ATP-binding sites have been derived from studies of ligand dependence of Ca^{2+} transport, steady state ATPase activity, and rapid kinetics of E-P formation (3–5). In the case of Ca^{2+} binding, these functional studies do not distinguish positive cooperativity between identical sites from obligatory binding of two Ca^{2+} ions prior to phosphorylation. Ca^{2+}-binding sites with similar characteristics appear to be responsible for stabilization against inactivation of transport under acid conditions (6) or by EGTA (7) and of inactivation of catalytic activity by dicyclohexylcarbodiimide (8). Recently, evidence has been provided, relating conformational changes to Ca^{2+} binding, from studies on changes in intrinsic tryptophan fluorescence (9) and from quenching of fluorescence of fluorescein bound at the active center (10). These and more direct equilibrium binding studies, using column chromatographic procedures (11), indicate a highly cooperative binding mechanism ($n_H = 1.6$–2.0).

The fluorescence intensity of ANS^- is sensitive to the dielectric properties of its environment and is enhanced under hydrophobic conditions. This characteristic is the basis for its use as a conformational probe of hydrophobic binding sites of soluble proteins (12–14). ANS^- has been used as a probe of the functional state of mitochondria (15), myelin (16), ascites cells (17), and erythrocytes (18). However, the relative contributions of protein and of phospholipids to fluorescence changes, mediated by alterations in ionic composition, temperature, or pH of the medium on these membranous systems, are uncertain.

Studies on the interaction of ANS^- with sarcoplasmic reticulum membranes have led to conflicting interpretations. Vanderkooi and Martonosi (19) attributed significant contributions of phospholipids to enhanced fluorescence that may be dominant under certain conditions, particularly those favoring binding of divalent cations. Chiu *et al.* (20) have used the binding of ANS^- to measure surface potential and surface charge densities of SR membranes, based upon the premise that the fluorescence signal originates primarily from lipid

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; E∼P and E-P, phosphorylated forms of enzyme; ANS^-, 1-anilino-8-naphthalenesulfonate; FITC, fluorescein 5′-isothiocyanate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

bilayer regions. These authors have concluded that conformational changes of the ATPase are not responsible for increased fluorescence under conditions of active calcium transport by isolated SR vesicles (21). Augustin and Hasselbach (14) have, however, shown that the quantum yield of ANS^- is virtually unaffected by delipidation of SR vesicles and that the observed fluorescence energy transfer efficiency of 95% from a tryptophan donor to ANS^- acceptor indicates a donor-acceptor distance of 20 Å, leading to their conclusion that ANS^- is located in a hydrophobic pocket of the ATPase.

We report here on the effects of known ligands of the Ca^{2+}-ATPase on fluorescence of ANS^-, bound to isolated SR vesicles. The findings indicate that fluorescence changes of bound ANS^- may be used as a convenient method for the study of Ca^{2+} and of nucleotide binding and that these changes appear to reflect the reversible $E_1 \leftrightarrow E_2$ transition of the ATPase. A preliminary communication of this work has been presented (22).

MATERIALS AND METHODS

Preparation of Sarcoplasmic Reticulum Vesicles—Vesicles of fragmented SR were prepared from longissimus dorsi muscle of white rabbits by the method of Eletr and Inesi (23) and were stored at 0 °C in 0.3 M sucrose, 10 mM imidazole, pH 7.4, at concentrations of 15 to 25 mg/ml. The Ca^{2+}-ATPase accounted for approximately 80 to 85% of total protein, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue (24), in suspensions of SR employed in this study, *i.e.* approximately 8 nmol/mg of protein. All measurements on SR vesicle suspensions were made within 5 days of isolation.

Labeling of SR Vesicles with ANS^-—SR vesicles (1 mg/ml) were preincubated for 1–2 h at 0 °C in 50 mM imidazole, pH 7.0, 50 mM KCl, 40 μM ANS^-, and 3 μM valinomycin. The valinomycin facilitates permeation of the ion pair, K^+ANS^-, through the vesicular phospholipid leaflet of SR membranes (21).

Fluorescence Measurements—Fluorescence was measured with an Aminco SPF-500 spectrophotofluorimeter. Measurements were carried out at 20 °C in a temperature-controlled cell holder, thermostatted with a Kryo-Thermostat WK5 circulating bath. Excitation and emission wavelengths were 367 and 481 nm, respectively, except when otherwise indicated.

Measurement of ANS^- Binding to SR—SR vesicles, 0.02 mg/ml, were preincubated in 50 mM imidazole, pH 7.0, 50 mM KCl, 1 mM EDTA, and 3 μM valinomycin for 10 min. Increasing amounts of ANS^-, 1 mM in water, were added, and fluorescence emission was measured. Relative fluorescence values were corrected for emission of free ANS^-, measured in the absence of SR vesicles.

Assay of ATP-dependent Calcium Uptake and Ca^{2+}-dependent ATPase Activity—Calcium uptake in the presence of oxalate was determined by the Millipore filtration method, based on measurement of radioactivity remaining on the filter (25). Ca^{2+}-dependent ATPase activity was determined by the NADH-coupled method (26).

Cation Binding to SR—ANS^--labeled SR vesicles, 0.02 mg/ml, were suspended in 50 mM imidazole, pH 7.0, 1 mM EDTA, 50 mM KCl, 40 μM ANS^-, and 3 μM valinomycin. Additions of 100 mM Ca^{2+}, Mg^{2+}, or Sr^{2+}, giving a total cation concentration of up to 3 mM, were added and the changes in fluorescence intensity were measured. Free cation concentration was calculated at pH 7.0 from the kP_a values of H-EDTA, M-EDTA, and MH-EDTA, where H and M indicate bound H^+ and metal ions, respectively (27). Values for K_d and n_H were obtained by least squares analysis of the data, according to the method of Atkins (28).

Treatment of the SR-ANS^- Complex with N-Ethylmaleimide—ANS^--labeled SR, 1 mg/ml, in 50 mM imidazole, pH 7.0, 50 mM KCl, 40 μM ANS^- and 3 μM valinomycin was incubated in the presence of 1 mM *N*-ethylmaleimide for 1 h at 20 °C. Under these conditions 95% of the ATPase activity is abolished (29).

RESULTS

Fluorescence Changes on Binding of ANS^- to SR in the Absence of Divalent Cations—The effects of interaction of ANS^- with isolated SR vesicles was measured in the presence of EGTA. The fluorescence intensity was increased more than 5-fold in the presence of SR vesicles (Fig. 1). In addition, the peak of fluorescence emission was shifted from 520 to 480 nm. The affinity of ANS^- for SR membranes was determined from the increase in fluorescence with increasing concentrations of ANS^- in the presence of SR vesicles (Fig. 2*a*). The apparent K_d for binding of ANS^- was found to be 3.8×10^{-5} M. This value is similar to that obtained previously (14, 19, 21) in the absence of EGTA. Binding of increasing concentrations of SR in 1 μM ANS^- showed an initial linear titration curve with saturation at a ratio of 1 mol of ANS^- per 3 mol of Ca^{2+}-ATPase (Fig. 2*b*). Such behavior indicates an additional site of high affinity for ANS^-. Similar data were obtained when fluorescence titration curves were measured in the presence of EDTA, indicating that endogenous Mg^{2+} is not involved in ANS^- binding. The stoichiometry of the lower affinity site could not be determined. Augustin and Hasselbach (14) have described nonlinear Scatchard plots of ANS^- binding data, indicating functional heterogeneity of ANS^- binding sites.

Effects of ANS^- and Valinomycin on Calcium Transport and ATPase Activity of SR—The possibility was considered that binding of ANS^- to the Ca^{2+}-ATPase might modify catalytic function. Equilibration of ANS^- with SR vesicles has previously been shown to be facilitated by valinomycin *plus* K^+, presumably by co-permeation of the K^+ANS^- ion pair (21). The effects of ANS^- and valinomycin on calcium transport and ATPase activity are shown in Fig. 3, *a* and *b*. ANS^- alone had no effect on either transport or ATPase activity, but valinomycin did decrease both these parameters at saturating levels of MgATP and Ca^{2+} by approximately 30%. At saturating levels of MgATP, valinomycin inhibition was unaffected over a range of calcium concentrations, spanning the high affinity ($K_{d(Ca^{2+})} \simeq 0.3$ μM) calcium stimulatory site (Fig. 3*a*). Valinomycin had little effect on calcium transport when

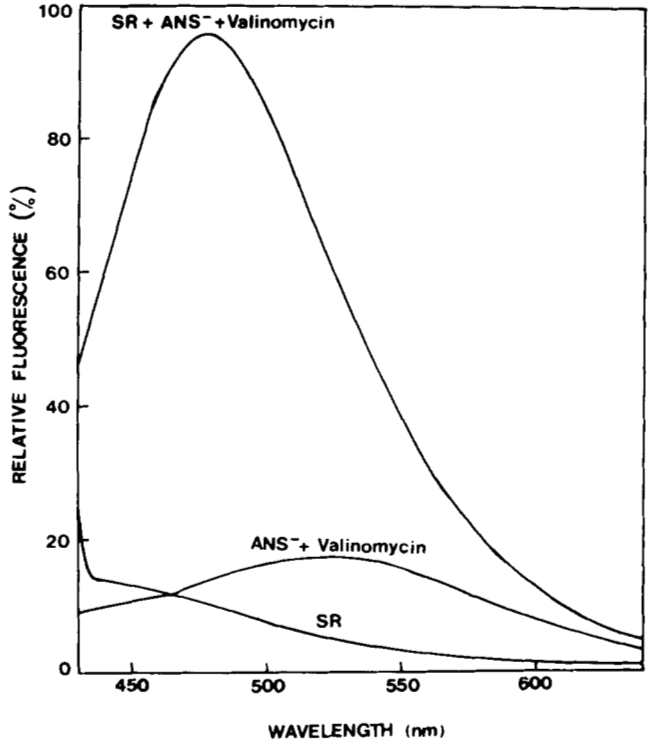


FIG. 1. **Fluorescence changes on binding of ANS^- to isolated SR vesicles.** The medium, at 20 °C, contained 50 mM imidazole, pH 7.0, 50 mM KCl, and 5 mM EDTA. The excitation wavelength was set at 367 nm. ANS^-, 40 μM, valinomycin, 3 μM, and SR vesicles, 0.02 mg/ml, were added where indicated.

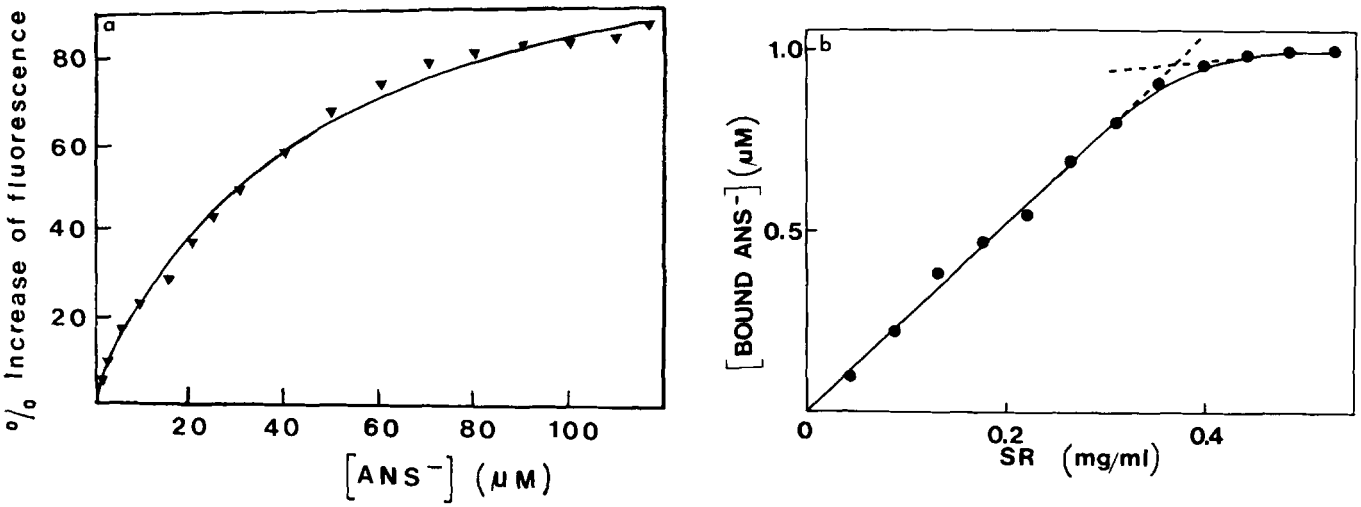


FIG. 2. **Binding of ANS⁻ to SR vesicles.** *a*, SR vesicles, 0.02 mg/ml, were preincubated in 50 mM imidazole, pH 7.0, 50 mM KCl, 1 mM EDTa, and 3 μM valinomycin for 10 min. Increasing amounts of ANS⁻, 1 mM in buffer, were added and fluorescence emission was measured. Relative fluorescence values were corrected for emission of free ANS⁻, measured in the absence of SR vesicles. *b*, fluorescence emission at 481 nm was monitored in a medium, as described in Fig. 1, containing in addition, 1 μM ANS⁻. Increasing amounts of SR vesicles were added. The amounts of bound ANS⁻ were calculated from the fluorescence yield obtained when further addition of SR did not increase the fluorescence signal.

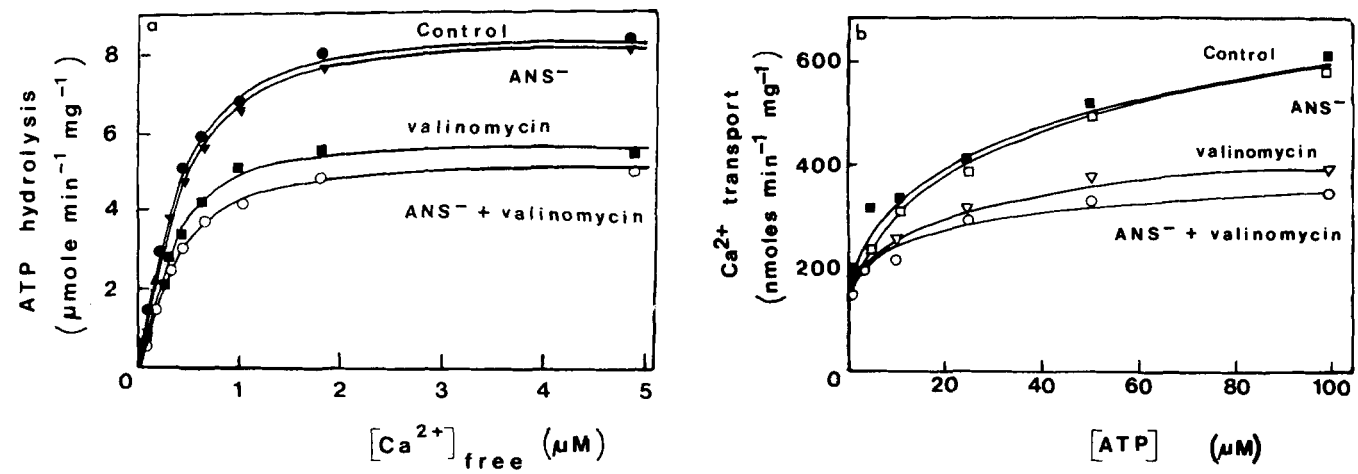


FIG. 3. **Effect of ANS⁻ and valinomycin on Ca^{2+} transport and Ca^{2+}-ATPase activity.** Ca^{2+}-stimulated ATPase activity, in the presence of 2 μM of the ionophore, A23187 (*a*), and Ca^{2+} uptake (*b*) were measured as described under "Materials and Methods." Valinomycin (3 μM) and ANS⁻ (40 μM) were included in the assay mixtures, where indicated.

measured at concentrations of MgATP below 10 μM. However, transport was inhibited by approximately 30% in the range of 20 to 100 μM ATP (Fig. 3*b*). Addition of ANS⁻ in the presence of valinomycin did not influence the inhibitory effect of the ionophore, nor alter its substrate dependence.

Calcium transport and Ca^{2+}-ATPase activity are known to exhibit complex substrate dependence. Following saturation of a high affinity ($K_d \simeq 10^{-5}$ M) catalytic site, higher concentrations of MgATP result in secondary activation via a low affinity regulatory site with a K_d in the millimolar range (30). It appears, therefore, that valinomycin blocks the regulatory effect but does not affect the high affinity catalytic site.

The effect of EGTA and of Calcium on the Fluorescence of Bound ANS⁻—The effects of EGTA and of free calcium ions, in the micromolar range, on the fluorescence intensity of bound ANS⁻ are shown in Fig. 4. EGTA enhanced fluorescence intensity by 10–12%. Further addition of $CaCl_2$ ($[Ca^{2+}]_{free} \simeq 10$ μM) decreased the fluorescence signal to the original value, measured prior to adding EGTA. Addition of 10 μM $CaCl_2$ to ANS⁻-labeled SR in the absence of EGTA did not cause a change of fluorescence. Scans of both excitation and emission showed that peak wavelengths were unaffected by either EGTA or $CaCl_2$ (data not shown). Changes in fluorescence intensity were complete within the mixing time of the procedure (<5 s).

These experiments indicate that low (micromolar) concentrations of free calcium ions result in changes in fluorescence of ANS⁻, bound to SR, such that calcium-bound and calcium-free forms of ANS⁻-labeled SR vesicles correspond to low and high fluorescence intensity species, respectively. Enhanced fluorescence, following initial addition of EGTA, is readily explained by calcium ion contamination of buffers and of SR vesicles themselves, resulting in measured free Ca^{2+} levels of approximately 5–15 μM (31).

A typical calcium binding curve, determined from decreasing fluorescence intensity with increasing $[Ca^{2+}]_{free}$, is shown in Fig. 5. A best fit of the data gave a value of 3.4×10^{-7} M for the apparent dissociation constant, K_d, with a Hill coeffi-

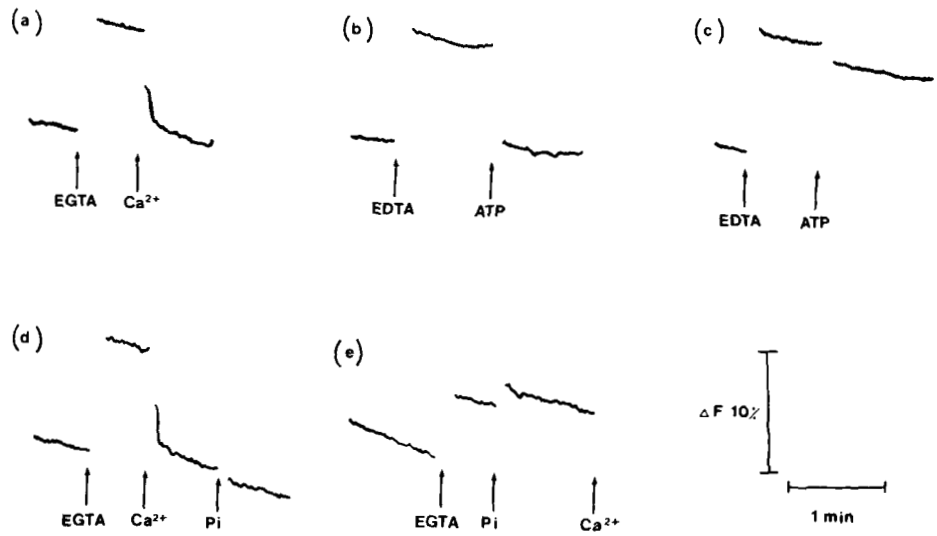


FIG. 4. **Effects of Ca^{2+}, ATP, and P_i on fluorescence of ANS^--labeled SR vesicles.** SR vesicles, 0.02 mg/ml in 50 mM imidazole, pH 7.0, 50 mM KCl, 40 μM ANS^-, and 3 μM valinomycin were present in the incubation medium at 25 °C. EDTA or EGTA, 1 mM, 30 μM ATP, 1 mM $CaCl_2$, and 10 mM P_i were added where indicated. In the presence of both EGTA and $CaCl_2$, the free Ca^{2+} concentration was calculated to be 16 μM. In *b*, ANS^--labeled SR was preincubated in 1 mM *N*-ethylmaleimide for 1 h at 20 °C, as described under "Materials and Methods."

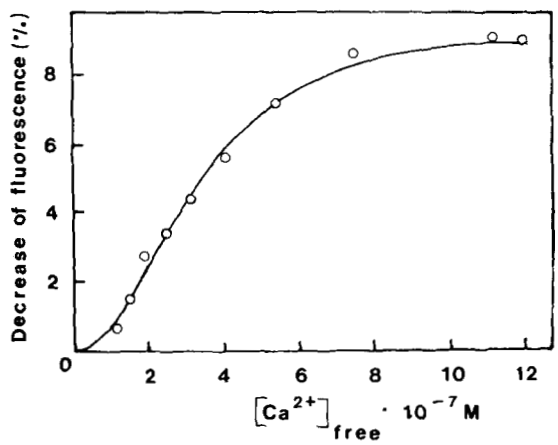


FIG. 5. **Binding of Ca^{2+} to SR vesicles as measured by changes in ANS^- fluorescence.** Increasing concentrations of $CaCl_2$ were added to a medium containing 50 mM imidazole, pH 7.0, 50 mM KCl, 3 mM EGTA, 40 μM ANS^-, 3 μM valinomycin, and SR vesicles, 0.026 mg/ml, to give the concentrations of free Ca^{2+} shown. The *solid line* is the best fit of the data, assuming $K_{0.5(Ca^{2+})} = 3.4 \times 10^{-7}$ M, $n_{H(Ca^{2+})} = 1.8$.

cient, $n_{H(Ca^{2+})}$, of 1.8. Sr^{2+} also decreased the fluorescence when added in the presence of either EGTA or EDTA. A similar analysis, in the presence of 1 mM EDTA, gave an apparent dissociation constant for Sr^{2+} binding of 6.7×10^{-6} M and Hill coefficient of 0.93 (Table I). Unlike Ca^{2+} and Sr^{2+}, Mg^{2+} and La^{3+} both increased fluorescence intensity when added in the presence or absence of EGTA or EDTA, as reported previously (19). We have also confirmed that Ca^{2+}, at free ion concentrations greater than 100 μM, increases ANS^- fluorescence.

It appears, therefore, that the binding characteristics of the high affinity divalent cation binding site that mediates decreases in fluorescence intensity of ANS^-, bound to SR vesicles, are similar to those of the site that supports transport and stimulates ATPase activity (32). This effect is readily distinguishable from that of high concentrations of divalent cations that lead to increases in fluorescence and which are compatible with the proposed mechanism of deshielding of surface negative charges (19).

The Effects of Nucleotides and Their Analogues on the Fluorescence of ANS^- Bound to SR Vesicles—The effects of a number of nucleotides and their analogues, known to bind to the Ca^{2+}-ATPase, have been studied by their effects on the fluorescence of bound ANS^- in the presence of EDTA. ATP (50 μM) decreased fluorescence to a similar extent as did Ca^{2+} (Fig. 4). This effect was decreased from 10.2 to 1.2% by prior treatment of SR vesicles with *N*-ethylmaleimide under conditions, previously shown to modify five thiol groups, includ-

TABLE I

Ligand binding parameters as determined from changes in fluorescence intensity of ANS^- bound to SR vesicles

The medium contained 50 mM imidazole (pH 7.0), 50 mM KCl, 40 μM ANS^-, 3 μM valinomycin, and SR vesicles, 0.02 mg/ml. EGTA, 1 mM, was added in all assays, except where otherwise indicated. Cation binding was determined as described under "Materials and Methods." Nucleotides, pyrophosphate, and vanadate binding were determined by adding aliquots of substrate and measuring changes of fluorescence intensity. Relative fluorescence values were corrected for dilution. Values for K_d and n_H were obtained by least square analysis of the data according to the method of Atkins (28). The figures in parentheses refer to data obtained by other methods.

Ligand	ΔF_{max}	K_d	n_H
	%	M	
Ca^{2+}	−10	3.4×10^{-7} (4.4×10^{-7}); Ref. 11	1.8 (1.82); Ref. 11
Sr^{2+} [a]	−10	6.7×10^{-6}	0.93
La^{3+} [b]	+25	1.3×10^{-5} (6.5×10^{-6}); Ref. 42	0.85
ATP	−12	1.0×10^{-5} (3.3×10^{-6}); Ref. 43 7.0×10^{-6} [c]	0.80
ITP	−10	2.2×10^{-5} (3.1×10^{-5}); Ref. 44	1.01
AMP-PCP [d]	−12	8.9×10^{-5} (1.5×10^{-5}); Ref. 45	0.78
ADP	−13	1.2×10^{-5} (3.3×10^{-6}); Ref. 45 7.4×10^{-6} [c]	0.89
GDP	−6.6	3.3×10^{-4}	0.88
IDP	−4.5	6.6×10^{-4}	0.92
CDP	−4.5	8.2×10^{-4}	0.78
AMP	−11	1.8×10^{-3}	
cAMP	−6.0	6.5×10^{-3}	
PP_i	−6.5	5.5×10^{-4} (2×10^{-3}); Ref. 35	
VO_3^-	−15	4.0×10^{-5} (1×10^{-5}); Ref. 35 2.0×10^{-5} [c]	

[a] Measured in the presence of 1 mM EDTA.
[b] Measured in the absence of chelating agents.
[c] Measured in the presence of 2.5 mM $MgCl_2$.
[d] AMP-PCP, adenyl-5′-yl-(β,γ-methylene)diphosphate.

ing three goups that are believed to be located at the substrate binding site, on the basis that their derivitization is specifically blocked by ATP and its analogues (29). *N*-Ethylmaleimide treatment did not affect Ca^{2+}-induced fluorescence quenching (data not shown).

The binding parameters of ATP, ADP, and a number of analogues, as determined by decreased fluorescence of bound ANS^-, are shown in Table I. Fluorescence changes were rapid and complete during the mixing time of the experiments (<5 s). No fluorescence changes were noted in the absence of calcium-chelating agents. The binding parameters and specificity for ATP and its hydrolyzable analogues, determined from ANS^- fluorescence changes, are in agreement with those previously determined for the high affinity catalytic site by a variety of methods, including substrate dependence of transport (30), Ca^{2+}-ATPase activity (33), and Ca^{2+}-dependent E-P formation (34). In general, highest affinities were associated with an intact adenine ring and with the presence of a pyrophosphate linkage. The nonhydrolyzable analogue, adenyl-5′-yl-(β,γ-methylene)diphosphate, also decreased fluorescence by binding to a high affinity site.

The Effects of Phosphate, Pyrophosphate, and Vanadate on Bound ANS^- Fluorescence—Since both Ca^{2+} and ATP appear to stabilize the low fluorescence form of ANS^--labeled SR that corresponds to the E_1 conformation of the Ca^{2+}-ATPase (1, 2), the effects of phosphate and vanadate that are presumed to bind to the alternate E_2 form of the enzyme (35) and of pyrophosphate were also studied. Phosphate, in the presence of EGTA, caused a further increase in fluorescence (Fig. 4*e*). Presuming that the fluorescence signal, in the presence of ANS^-, represents the equilibrium mixture of the low fluorescence, $E_1 \cdot ANS^-$, and the high fluorescence, $E_2 \cdot ANS^-$, forms and that P_i stabilizes $E_2 \cdot ANS^-$, it can be calculated that in the presence of Ca^{2+}, the equilibrium constant, K, for the transition $E_1 \cdot ANS^- \rightleftharpoons E_2 \cdot ANS^-$ is 3.1 in favor of $E_2 \cdot ANS^-$. Vanadate and pyrophosphate, in contrast, decreased fluorescence intensity. The apparent binding parameters, so determined, are shown in Table I, together with results previously obtained by different methods.

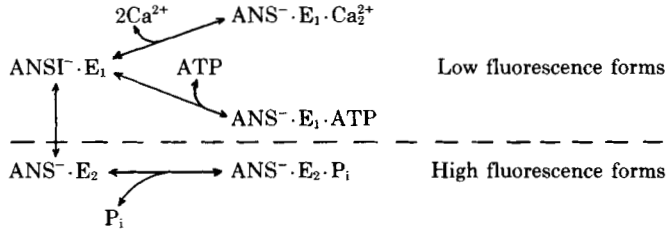
DISCUSSION

The ligand dependence of increase in fluorescence of ANS^-, bound to SR vesicles in the absence of Ca^{2+}, is in good agreement with the specificity and binding characteristics of the Ca^{2+}- and nucleotide-binding sites of the Ca^{2+}-ATPase, as determined by a wide range of functional and equilibrium methods. Since binding of ANS^- has no effect on Ca^{2+} or ATP dependence of transport or on ATPase activity, it can be concluded that the alterations in fluorescence are mediated by an indirect mechanism, such as a conformational change following binding of ligand. Augustin and Hasselbach (14) have suggested that ANS^- is located within a protein component of SR membranes. This conclusion was based on fluorescence polarization measurements that indicated limited mobility of the probe and on energy transfer studies that give a distance of 20 Å between tryptophan donor and ANS^- acceptor.

The changes in fluorescence (decreasing) with $[Ca^{2+}]_{out}$ are opposite to those noted by Vanderkooi and Martonosi (19) and Chiu and Haynes (21). The simplest explanation for this discrepancy is that the experimental conditions differed in that in this study the reference condition was in the presence of EGTA ($Ca^{2+}{}_{free} \simeq 10^{-9}$ M), whereas previous data were obtained in the presence of endogenous Ca^{2+}, which is expected to be in excess of 5 μM $[Ca^{2+}]_{free}$ and would have saturated the high affinity Ca^{2+}-binding site. We have confirmed that further addition of Ca^{2+}, in the presence of EGTA to give $[Ca^{2+}]_{free}$ in the millimolar range, leads to an increase in fluorescence as does Mg^{2+}, in the same concentration range, and La^{3+}, 20 μM.

It appears, therefore, that changes in ANS^- fluorescence in the presence of SR membranes are due to two readily distinguishable mechanisms. At low ($<1.0 \times 10^{-5}$ M) $[Ca^{2+}]_{free}$, the probe reports conformational changes of the Ca^{2+}-ATPase, and at high divalent cation concentrations there is increased binding of ANS^- to the lipid bilayer, due to counteraction of fixed negative charges, as has previously been suggested (19).

Micromolar concentrations of both ATP and Ca^{2+} are expected to stabilize the E_1 conformation of the enzyme. The present data suggest that E_1 is the low fluorescence form and that the transition to the E_2 conformation is followed by an increase of fluorescence intensity,



Loomis *et al.* (36), on the basis of Ca^{2+} inhibition of P_i-dependent phosphorylation of the ATPase in the absence of a calcium gradient, has concluded that the equilibrium constant, K, for interconversion of the Ca^{2+}-free forms of the enzyme, $E_1 \leftrightarrow E_2$, is very much in favor of E_1 ($K \simeq 10^3$). However, the experiments of Pick, using the FITC-modified Ca^{2+}-ATPase, suggest a K equilibrium value nearer to unity, based upon the assumption that vanadate ions bind to and stabilize the E_2 conformation (35). Similarly, vanadate stabilizes the analogous E_2 conformation of the FITC-modified (Na^+,K^+)-ATPase, presumably by acting as a phosphate transition state analog (37). FITC inhibits both the (Na^+,K^+)-ATPase and Ca^{2+}-ATPase by binding to the nucleotide binding site, since ATP specifically protects against FITC modification (38).

Pick (35) has concluded that the FITC-modified Ca^{2+}-ATPase of SR behaves identically with the native enzyme, with respect to the equilibrium, $E_1 \leftrightarrow E_2$. The increase in fluorescence caused by phosphate binding to ANS^--labeled SR (Fig. 4) also suggests that significant amounts of the E_1 form of the ATPase are present in the absence of Ca^{2+} ions. These findings are similar to those reported by DuPont (39) from studies of the $E_1 \leftrightarrow E_2$ equilibrium, as monitored by changes in intrinsic fluorescence.

FITC- and ANS^--labeled preparations of SR differ, however, in their response to the binding of VO_3^-. Vanadate appears to favor the E_1 (high fluorescence) form of ANS^--labeled SR and thus acts analogously to Ca^{2+} and to substrates for the ATPase. One possible explanation is that both E_1 and E_2 forms bind VO_3^-, but that E_1 has a higher affinity for the anion in the native enzyme. Since FITC blocks the substrate binding site of the E_1 conformation, binding is then only possible to the weaker site of the E_2 conformation.

In conclusion, ANS^- is a sensitive and convenient probe for monitoring Ca^{2+} and substrate binding to the ligand-free form of the Ca^{2+}-ATPase of sarcoplasmic reticulum. The low and high fluorescence forms of bound ANS^- reflect the nonphosphorylated E_1 and E_2 conformations of the enzyme. An example of its potential is the ready demonstration of positively cooperative passive binding of Ca^{2+} to the enzyme, confirming previous reports, using intrinsic tryptophan fluo-

rescence (9), an equilibrium column chromatographic procedure (11), and the protection by Ca^{2+} against thermal inactivation of Ca^{2+} transport (7). Although it is still uncertain whether ANS^- binds predominantly to protein or phospholipid components of biological membranes (for review see Ref. 40), the present studies indicate that changes in conformation of an intrinsic membrane protein, such as the Ca^{2+}-ATPase, can have significant effects on the environment of the probe. The possibility that the effects of such conformational changes may be transmitted out laterally into the plane of the lipid bilayer cannot be excluded.

REFERENCES

1. Tada, M., Yamamoto, T., and Tonomura, Y. (1978) *Physiol. Rev.* **58,** 1–79
2. Inesi, G. (1978) in *Membrane Transport in Biology* (Tosteson, D. C., ed) Vol. II, pp. 357–393, Springer-Verlag, Berlin
3. Martonosi, A. (1971) in *Biomembranes* (Manson, L. A., ed) Vol. 1, pp. 191–256, Plenum Press, New York
4. Inesi, G. (1972) *Annu. Rev. Biophys. Bioeng.* **1,** 191–210
5. Panet, R., Pick, U., and Selinger, Z. (1971) *J. Biol. Chem.* **246,** 7349–7356
6. Berman, M. C., McIntosh, D. B., and Kench, J. E. (1977) *J. Biol. Chem.* **252,** 994–1001
7. McIntosh, D. B., and Berman, M. C (1978) *J. Biol. Chem.* **253,** 5140–5146
8. Pick, U., and Racker, E. (1979) *Biochemistry* **18,** 108–113
9. DuPont, Y. (1976) *Biochem. Biophys. Res. Commun.* **71,** 544–550
10. Pick, U. (1981) *Eur. J. Biochem.* **121,** 187–195
11. Inesi, G., Kurzmack, M., Coan, C., and Lewis, D. E. (1980) *J. Biol. Chem.* **255,** 3025–3031
12. Stryer, L. (1965) *J. Mol. Biol.* **13,** 482–495
13. Weber, G., and Daniel, E. (1966) *Biochemistry* **5,** 1900–1907
14. Augustin, J., and Hasselbach, W. (1973) *Eur. J. Biochem.* **35,** 114–121
15. Vanderkooi, J. M., and Chance, B. (1972) *FEBS Lett.* **22,** 23–26
16. Feinstein, M. B., and Felsenfeld, H. (1975) *Biochemistry* **14,** 3041–3048
17. Loeser, C. N., Clark, E., Maker, M., and Tarkwell, H. (1970) *Arch. Biochem. Biophys.* **141,** 762–763
18. Radda, G. K., and Vanderkooi, J. (1972) *Biochim. Biophys. Acta* **265,** 509–549
19. Vanderkooi, J., and Martonosi, A. (1969) *Arch. Biochem. Biophys.* **133,** 153–163
20. Chiu, V. C. K., Mouring, D., Watson, B. D., and Haynes, D. H. (1980) *J. Membr. Biol.* **56,** 121–132
21. Chiu, V. C. K., and Haynes, D. H. (1980) *J. Membr. Biol.* **56,** 219–239
22. Arav, R., and Berman, M. C. (1983) *Proceedings of the 7th South African Biochemistry Congress, Stellenbosch, January, 1983,* Tafelberg Publishers, Cape Town
23. Eletr, S., and Inesi, G. (1972) *Biochim. Biophys. Acta* **282,** 174–179
24. Inesi, G., and Scales, D. (1974) *Biochemistry* **13,** 3298–3306
25. Martonosi, A., and Feretos, R. (1964) *J. Biol. Chem.* **239,** 648–658
26. Horgan, D. J., Tume, R. K., and Newlands, R. B. (1972) *Anal. Biochem.* **48,** 147–152
27. Sillen, L. G., and Martell, A. E. (1964) *Stability Constants of Metal-Ion Complexes,* The Chemical Society, London
28. Atkins, G. L. (1973) *Eur. J. Biochem.* **33,** 175–180
29. Yamada, S., and Ikemoto, N. (1978) *J. Biol. Chem.* **253,** 6801–6807
30. Verjovski-Almeida, S., and Inesi, G. (1979) *J. Biol. Chem.* **254,** 18–21
31. Berman, M. C., and Aderem, A. A. (1981) *Anal. Biochem.* **115,** 297–301
32. Verjovski-Almeida, S., and Silva, J. L. (1981) *J. Biol. Chem.* **256,** 2940–2944
33. Vianna, A. L. (1975) *Biochim. Biophys. Acta* **410,** 389–406
34. Kanazawa, T., Yamada, S., Yamamoto, T., and Tonomura, Y. (1971) *J. Biochem. (Tokyo)* **70,** 95–123
35. Pick, U. (1982) *J. Biol. Chem.* **257,** 6111–6119
36. Loomis, C. R., Martin, D. W., McCaslin, D. R., and Tanford, C. (1982) *Biochemistry* **21,** 151–156
37. Smith, R. L., Zinn, K., and Cantley, L. C. (1980) *J. Biol. Chem.* **255,** 9852–9859
38. Pick, U., and Karlish, S. J. D. (1980) *Biochim. Biophys. Acta* **26,** 255–261
39. DuPont, Y. (1978) *Biochem. Biophys. Res. Commun.* **82,** 893–900
40. Slavik, J. (1982) *Biochim. Biophys. Acta* **694,** 1–25
41. Deleted in press
42. Chevallier, J., and Butow, R. (1971) *Biochemistry* **10,** 2733–2737
43. Meissner, G. (1973) *Biochem. Biophys. Acta* **298,** 906–926
44. de Meis, L., and Fialho de Mello, M. C. (1973) *J. Biol. Chem.* **248,** 3691–3701
45. Pang, D. C., and Briggs, F. N. (1977) *J. Biol. Chem.* **252,** 3262–3266

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J. Biol. Chem. 1983, 258:10433-10438.