Conformational Transitions in the Calcium Adenosinetriphosphatase Studied by Time-Resolved Fluorescence Resonance Energy Transfer†

Woubalem Birmachu, Franz L. Nisswandt, and David D. Thomas*

Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455

Received June 14, 1988; Revised Manuscript Received December 20, 1988

ABSTRACT: We have used time-resolved fluorescence to study proposed conformational transitions in the Ca-ATPase in skeletal sarcoplasmic reticulum (SR). Resonance energy transfer was used to measure distances between the binding sites of 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (IAEDANS) and fluorescein 5-isothiocyanate (FITC) as a function of conditions proposed to affect the enzyme's conformation. When 1.0 ± 0.15 IAEDANS is bound per Ca-ATPase, most (76 ± 4%) of the probes have an excited-state lifetime (τ) of 18.6 ± 0.5 ns, and the remainder have a lifetime of 2.5 ± 0.9 ns. When FITC is bound to a specific site on each IAEDANS-labeled enzyme, most of the long-lifetime component is quenched into two short-lifetime components, indicating energy transfer that corresponds to two donor–acceptor distances. About one-third of the quenched population has a lifetime τ = 11.1 ± 2.5 ns, corresponding to a transfer efficiency E = 0.40 ± 0.07 and a donor–acceptor distance R_1 = 52 ± 3 Å. The remaining two-thirds exhibit lifetimes in the range of 1.2–4.2 ns, corresponding to a second distance 31 Å ≤ R_2 ≤ 40 Å. Addition of Ca^{2+} (in the micromolar to millimolar range), or vanadate (to produce a phosphoenzyme analogue), had no effect on the donor–acceptor distances. Addition of decavanadate results in the quenching of IAEDANS fluorescence but has no effect on the energy-transfer distance. Formation of phosphoenzyme from inorganic phosphate at pH 6.2 produced no change when performed in aqueous solution but did produce a small (6.4 ± 3.2 Å) decrease in R_1 when performed in 40% DMSO. The lack of large changes in the energy-transfer distance upon the binding of the ligands studied leads us to propose that the B domain, and perhaps the ATPase as a whole, does not undergo large changes in its tertiary structure upon ligand binding.

The central question in elucidating the mechanism of active calcium transport in sarcoplasmic reticulum (SR)[1] is how the energy derived from ATP hydrolysis is transduced by the Ca-ATPase into the translocation of calcium against its electrochemical gradient. Several lines of evidence strongly indicate the role of specific ligands in inducing protein conformational changes that are essential in calcium translocation. Kinetic studies (Inesi et al., 1980), sulfhydryl group reactivity (Yamada & Ikemoto, 1978), changes in intrinsic fluorescence intensity (Dupont, 1978; Fernandez-Belda et al., 1984), changes in fluorescence intensity of the FITC- and IAEDANS-labeled enzyme (Pick, 1981; Pick & Karlish, 1982; Andersen et al., 1982; Suzuki et al., 1987; Yamashita & Kawakita, 1987), and changes in the nanosecond mobility of the spin-labeled enzyme (Coan, 1983) have all indicated that the binding of calcium to the high-affinity site induces changes in protein structure or flexibility. Phosphorylation by inorganic phosphate or ATP (Pick, 1981; Watanabe & Inesi, 1982; Fernandez-Belda et al., 1984; Blasie et al., 1985) and the binding of nucleotide analogues such as AMPPNP (Coan & Keating, 1982; Lewis & Thomas, 1987) also induce conformational changes. Vanadate, presumably acting as a phosphate analogue, has also been shown to induce detectable changes in the enzyme (O'Neil et al., 1979; Pick, 1982; Dupont & Bennett, 1982; Highsmith et al., 1985; Lewis & Thomas, 1986).

Although several methods have been used to detect structural or dynamic changes in the Ca-ATPase, most of these do not provide direct information about the nature or scale (spatial and temporal) of the structural changes occurring. Previous studies in this laboratory have focused on the measurement of overall rotational motions of the Ca-ATPase, because these measurements could be interpreted quantitatively without the requirement for detailed structural information (Lewis & Thomas, 1986; Squier et al., 1988; Squier & Thomas, 1988). Recent advances in structural analysis of the Ca-ATPase have made it feasible to ask more focused questions about the internal structure of the enzyme (MacLennan et al., 1985). In the present study, we have used time-resolved fluorescence to measure resonance energy transfer, which provides direct information on the change in distance between reporter groups and hence on the magnitude of the conformational change in the protein. Even though energy transfer has been used to study distances between functional groups in the Ca-ATPase (Scott, 1985; Herrmann et al., 1986; Squier et al., 1987), the effects of functionally important perturbations on these distances have not been studied to date. We have used SR vesicle preparations, in which the ATPase was labeled with the fluorescent energy donor IAEDANS and the acceptor FITC, to measure intramolecular distances and to investigate the effects of physiologically important ligands and other perturbations on these distances. This is the first study of this problem to employ subnanosecond time resolution, permitting

†This work was supported by grants to D.D.T. from the National Institutes of Health (GM 27906 and RR01439) and the American Heart Association. W.B. was supported by a postdoctoral fellowship and a research grant from the American Heart Association (Minnesota Affiliate), and D.D.T. was supported by an established investigatorship from the American Heart Association.

[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; FITC, fluorescein 5-isothiocyanate; IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; BSA, bovine serum albumin.

0006-2960/89/0428-3940$01.50/0

us to resolve quantitatively the distinct states of the system.

Materials and Methods

Membrane Preparations. SR vesicles were prepared from rabbit skeletal white muscle (fast twitch) and partially purified on a discontinuous sucrose gradient essentially as described by Fernandez et al. (1980). The light and intermediate fractions in the 35–40% zone (excluding the heavy fraction at the 40–50% interface) of the gradient were pooled, diluted 5-fold, and centrifuged at 100000*g*. The pellet was resuspended in solution A (0.3 M sucrose/30 mM MOPS, pH 7.0) and stored in liquid nitrogen. All preparations were done at 4 °C. Partial purification of SR vesicles using deoxycholate was performed according to the method of Meissner et al. (1973).

Assays. Ca-dependent ATP hydrolysis was measured in a solution containing 0.05 mg of protein/mL, 60 mM KCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 μM ionophore A23187, and 30 mM MOPS (pH 7.0) essentially as described before (Lewis & Thomas, 1986). Protein concentrations were determined by the Biuret method, using bovine serum albumin (BSA) as a standard (Lewis & Thomas, 1986).

Labeling with Fluorescent Probes. 5-[[2-[(Iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonate (IAEDANS) and fluorescein 5-isothiocyanate (FITC) were obtained from Molecular Probes, Inc. (Eugene, OR). The Ca-ATPase was labeled with IAEDANS in a solution containing 5 mM $MgCl_2$, 100 mM KCl, 30 mM MOPS, pH 7.0 (solution B), and 3–5 mg of SR protein/mL for 3.0 h at 25 °C at specified concentrations of the probe. Unreacted probe was usually removed by diluting the reaction mixture with 5 times the volume of solution B containing 1 mg of BSA/mL, followed by centrifugation at 62000*g* for 30 min at 4 °C. The inclusion of BSA ensured the removal of noncovalently bound probe, as assayed by the absorbance of the supernatant after centrifugation. The pellet was washed once more in solution B and resuspended in solution A if no further chemical modification was desired. In other experiments, 10.0 mg of SR protein/mL was prelabeled with 65 μM NEM in 40 mM MOPS, 100 mM KCl, and 50 μM $CaCl_2$, pH 7.0, for 50 min at 0 °C, to block fast-reacting sulfhydryl groups (Hidalgo & Thomas, 1977) and thus improve the specificity of subsequent labeling with IAEDANS. The sample was then diluted to 3.0 mg of protein/mL and reacted with specified concentrations of IAEDANS at 25 °C for 5 h. Unreacted probe was removed as described above.

The stoichiometry of labeling with IAEDANS was determined directly from the fluorescence of the labeled membranes, using a SPEX Fluorolog II fluorometer, and indirectly from the absorbance of the supernatant (after the first centrifugation), using a Hewlett Packard diode-array spectrophotometer. In the fluorescence determination, a known concentration of IAEDANS was incubated overnight with SR vesicles at a IAEDANS/ATPase ratio less than 1, ensuring complete reaction. This solution was used as a standard from which the concentration of dye in the unknown labeled SR vesicles was determined. In the absorbance method, an extinction coefficient of 6100 M^{-1} cm^{-1} at 334 nm (determined in this laboratory) was used to determine binding stoichiometry from the supernatant. Labeling stoichiometries determined from the above two procedures were consistent with each other. In contrast, we found that the values determined directly from the absorbance of the labeled vesicles in 2% DOC gave unreliable results, especially at low concentrations of bound dye, due to interference from protein absorption and light scattering. The molar concentration of Ca-ATPase was determined from the protein concentration (Biuret assay), assuming a molecular weight of 115K (Meissner et al., 1973) and assuming that 80% of the protein was Ca-ATPase (Bigelow et al., 1986).

IAEDANS-labeled samples were labeled with FITC using a modification of the method of Pick and Bassilian (1980). The AEDANS-SR pellet was resuspended in solution C (5 mM $MgCl_2$, 100 mM KCl, and 30 mM Tris, pH 8.7), at a protein concentration of 2–3 mg/mL, and then incubated for 45 min at 25 °C with specified concentrations of FITC. Unreacted probe was removed by centrifugation as described above, once in the presence of BSA in solution C followed by resuspension in solution A and subsequent centrifugation. The final pellet was resuspended in solution A. The stoichiometry of labeling was measured directly from the labeled vesicles in the presence of 2% DOC using an extinction coefficient of 47 000 M^{-1} cm^{-1} (at 498 nm and pH 7.0) determined in this laboratory.

The extinction coefficients of the fluorescent probes when bound to SR were determined as follows. SR samples, at 8–10 mg of protein/mL, were incubated with the probes at a total ratio of less than 0.5 mol of reagent/mol of ATPase, as described above for each probe. The reaction was allowed to proceed at 25 °C, for 45 min at pH 8.7 in the case of FITC and for 3 h at pH 7.0 in the case of IAEDANS. The samples were further incubated for 16 h at 4 °C to ensure complete reaction. At the end of the reaction period, the FITC-labeled SR samples were titrated down to pH 7.0 with 0.1 M HCl. The labeled stock solutions were successively diluted for absorbance measurements at 334 nm for IAEDANS and at 498 nm for FITC.

Vanadate Solutions. Mono- and decavanadate solutions were prepared from sodium vanadate (Fisher) as described by Lewis and Thomas (1986).

Trypsin Digestion and Gel Electrophoresis. SR vesicles suspended at a concentration of 0.5–2 mg of protein/mL, in 30 mM MOPS buffer, pH 7.0, containing 0.3 M sucrose, were preincubated at 25 °C for 30 min prior to digestion with trypsin. The vesicles were incubated at 25 °C with trypsin at a ratio of 1 trypsin molecule per 400 ATPase molecules. After various incubation times, the digestion was stopped by the addition of soybean trypsin inhibitor at an inhibitor/trypsin ratio of 10. SR samples for gel electrophoresis were diluted one-to-one into a solution containing 10 mM Tris-HCl, pH 8.0, 1% β-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue prior to loading onto 6–18% gradient SDS–polyacrylamide slab gels. Bromophenol blue was omitted from samples containing fluorescently labeled samples. Fluorescent bands were photographed through a 480-nm band-pass filter and a 550-nm cutoff filter for IAEDANS and FITC, respectively. These bands were quantitated by scanning the negatives with an absorption spectrometer.

Time-Resolved Fluorescence Measurements. Fluorescence lifetimes were measured by the time-correlated single-photon-counting method (Ware, 1971). The excitation source was a pulsed laser system (Spectra-Physics, Palo Alto, CA). The excitation light was a pulse of light tunable from 310 to 340 nm, with a repetition rate of 821 kHz. The pulse was obtained by frequency doubling (using a lithium iodate crystal) the output of a DCM-dye laser (Model 375), synchronously pumped by the 514-nm line of a Model 165 argon ion laser, which was mode-locked at 41 MHz. The repetition rate of the dye laser was controlled by a Model 344S cavity dumper. Emission was selected through an interference filter, detected by a Hamamatsu R1564U-01 microchannel plate photomultiplier tube and timed with an Ortec 457 time-to-amplitude

converter. Fluorescence decays were accumulated on a Tracor Northern TN-7200 multichannel analyzer at a time resolution of 0.102 ns/channel and transferred to a Zenith Z-158 microcomputer for storage and analysis. These decays were analyzed by an iterative nonlinear least-squares method. The excitation function used for the deconvolution of the observed fluorescence decay was obtained by using a scattering solution viewed at the excitation wavelength. The data were fit to the function

$$F(t)/F(0) = \sum_{i=1}^{n} a_i \exp(-t/\tau_i) \quad (1)$$

where $F(0)$ is the fluorescence intensity at time 0, τ_i is the fluorescence lifetime of the ith component, a_i is the mole fraction of this component, and n is less than 5. The goodness of fit was determined from the reduced χ^2 value, $\sum(F_e - F_f)^2/\sqrt{F_e}$, and by inspection of the modified residual plot, defined as $(F_e - F_f)/\sqrt{F_e}$, where F_e is the experimental fluorescence and F_f is the fit to eq 1. The value of n reported generally corresponds to the lowest value for which a decrease in n increases the reduced χ^2 value significantly.

During fluorescence lifetime measurements, samples of IAEDANS-labeled SR (AEDANS–SR) vesicles normally contained 0.2–0.5 mg/mL protein. Samples were excited at the absorption maximum of IAEDANS (336 nm), and the emission was collected through a Corion S10 (480 ± 10)-nm interference filter. In order to determine the contribution of FITC and intrinsic fluorescence to SR–AEDANS samples labeled with FITC (IAEDANS–FITC–SR), fluorescence decays were measured from control samples containing the equivalent amount of FITC and protein, but no IAEDANS. At 1 mol of FITC bound per mole of ATPase, the maximum background intensity was less than 4% of the control AEDANS–SR emission. Subtracting the decays of control FITC-SR samples from the corresponding decays of AEDANS–FITC–SR samples did not significantly alter the fit parameters a_i and τ_i (eq 1).

Analysis of Energy-Transfer Data. The theory and application of dipole–dipole energy transfer between a donor–acceptor pair separated by a fixed distance R have been well documented (Stryer, 1978). The energy-transfer efficiency is given

$$E = 1 - \tau_{DA}/\tau_D = \frac{R_0^6}{R_0^6 + R^6} \quad (2)$$

$$R/R_0 = (1/E - 1)^{1/6} \quad (3)$$

where τ_{DA} and τ_D are the fluorescence lifetimes of the donor in the presence and absence of the acceptor, respectively. R_0, the distance at which $E = 0.5$, is given by

$$R_0 = (9.79 \times 10^3)(k^2 n^{-4} Q_D J)^{1/6}$$

where n is the refractive index of the membrane, Q_D is the fluorescence quantum yield of the donor in the absence of the acceptor, k^2 is the orientation factor, and J is the overlap integral. An R_0 value of 49 Å was used for the IAEDANS–FITC donor–acceptor pair, determined under conditions similar to those used in the present study (Squier et al., 1987). Whenever conditions were changed (e.g., by addition of a ligand or change of solvent), R_0 was corrected for any change in the donor quantum yield Q_D according to $R_0'/R_0 = (\tau_D'/\tau_D)^{1/6}$, since Q_D is proportional to τ_D.

If there is only one donor–acceptor distance R, then the addition of acceptor should produce only a single new lifetime τ_{DA}, and eq 2 and 3 can be applied in a straightforward way to determine E and R. If there is more than one R value, due to either heterogeneity of labeling or conformational states, each different resolved τ_{DA} value can be used to calculate a different E (and R), using eq 2. To obtain maximum precision in analyzing the overall decay, we also calculated an average transfer efficiency from the total intensity:

$$I = \sum_{i=1}^{n} a_i \tau_i \qquad E = 1 - I_{DA}/I_D \quad (4)$$

which produces a result equivalent to a steady-state fluorescence measurement.

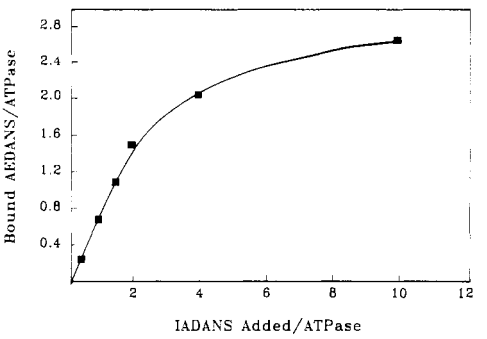

FIGURE 1: Covalent modification of the Ca-ATPase with IAEDANS. Samples containing 2.0 mg/mL SR in 30 mM MOPS (pH 7.0), 5 mM $MgCl_2$, and 100 mM KCl were covalently modified with IAEDANS at the total probe/ATPase ratio indicated on the abscissa. The ordinate indicates the amount of label incorporated, as determined from fluorescence measurements. The values plotted are averages from two independent experiments.

Results

Specificity of Labeling with IAEDANS. Labeling conditions were varied to maximize site-specific labeling of SR with IAEDANS. Labeling was carried out in 100 mM KCl, 5 mM $MgCl_2$, and 30 mM MOPS at an SR protein concentration of 3.0 mg/mL (20 μM Ca-ATPase) and a dye concentration of 40 μM unless otherwise specified. The pH was varied from 7 to 8.5, the pCa from 5.7 to 8.3, and the reaction time from 30 min to 3 h. Labeling specificity was judged by three criteria: (1) labeling stoichiometry (should be ≤1 dye/ATPase); (2) the pattern of fluorescent bands on SDS–polyacrylamide gels of samples subjected to mild tryptic digestion (should be specific for one tryptic fragment); (3) the time-resolved fluorescence decay (should be a single exponential).

The extent of reaction of SR vesicles with IAEDANS at pH 7.0 in 30 mM MOPS buffer containing 0.1 M KCl and 5 mM $MgCl_2$ is shown in Figure 1. At a 10-fold excess of IAEDANS, approximately 3 IAEDANS/ATPase (mol/mol) are incorporated, with full retention of enzymatic activity. The reaction does not have a simple 1/1 stoichiometry, as evidenced by the lack of saturation of binding sites. Subsequent labeling was done at a total IAEDANS per ATPase ratio of 2, resulting in a bound AEDANS/ATPase ratio of 1.0 ± 0.15.

Mild proteolysis with trypsin results in two polypeptide fragments, A and B, with molecular weights of 57K and 52K, respectively (Mitchinson et al., 1982). Scans of fluorescence photographs showed that at least 80% of the label was localized on the B fragment. The distribution of label between the A and B fragments was not affected significantly by varying the pH, calcium concentration, or reaction time.

Time-Resolved Fluorescence of AEDANS–SR. Figure 2 shows a typical fluorescence decay of SR labeled with IAEDANS at a stoichiometry of 1.0 ± 0.15 AEDANS bound per Ca-ATPase, along with results of fits to eq 1. Fit parameters, averaged over all data sets, are summarized in the first three rows of Table I. The fluorescence decay of AEDANS–SR is poorly fit by a single-exponential function (row 1 of Table

Table I: Fluorescence Decay Parameters of AEDANS–SR with and without FITC[a]

FITC/ ATPase	n	τ_1 (ns)	a_1	τ_2 (ns)	a_2	τ_3 (ns)	a_3	τ_4 (ns)	a_4	χ^2
0	1	15.2 (0.3)	1.0							4.5 (1.5)
0	2	18.6 (0.5)	0.76 (0.04)			2.5 (0.9)	0.24 (0.04)			1.37 (0.10)
0	3	19.1 (0.4)	0.57 (0.04)			6.7 (2.1)	0.17 (0.04)	0.7 (0.4)	0.29 (0.05)	1.26 (0.08)
0.9 (0.1)	2	14.6 (0.3)	0.39 (0.04)			2.3 (0.4)	0.61 (0.04)			3.7 (1.0)
0.9 (0.1)	3	15.0 (0.7)	0.27 (0.07)			4.9 (0.7)	0.29 (0.03)	1.0 (0.3)	0.44 (0.06)	1.43 (0.15)
0.9 (0.1)	4	19.2 (0.5)	0.13 (0.06)	11.1 (2.5)	0.21 (0.10)	4.2 (1.3)	0.32 (0.14)	1.2 (0.5)	0.34 (0.08)	1.26 (0.16)

[a] All samples had 1.0 ± 0.15 AEDANS bound per ATPase, and the ratio of FITC bound per ATPase is given in the first column. Fluorescence decays were measured and fit to eq 1 as illustrated in Figures 2 and 4, where τ_i and a_i are the lifetimes and mole fractions, respectively, and n (second column) is the number of exponential terms assumed. Each value is the mean from 10–15 experiments (SEM in parentheses).

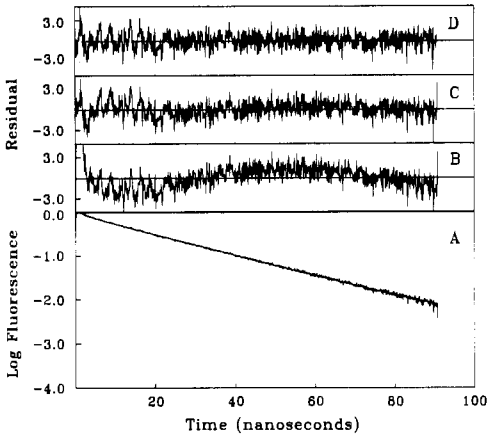

FIGURE 2: Fluorescence decays of AEDANS–SR. SR samples were labeled with IAEDANS as described in Figure 1, at an IAEDANS/ATPase ratio of 2.0, resulting in 1.0 ± 0.15 mol of AEDANS bound/mol of ATPase. (A) The fluorescence decay of AEDANS-labeled SR is superimposed on the best fit to a two-exponential function (eq 1, n = 2). (B), (C), and (D) show the residuals for the fits to one-, two-, and three-exponential functions, respectively.

I), as judged by the large magnitude of the χ^2 value (4.5 ± 1.5) and the nonrandom residual (Figure 2B). The two-exponential fit (row 2 of Table I) is much better, resulting in a substantial improvement in χ^2 and a residual that fluctuates randomly about zero (Figure 2C), and Figure 2A shows this fit superimposed on the experimental decay. Most (76 ± 4%) of the decay is characterized by a lifetime of 18.6 ± 0.5 ns. A three-exponential fit (row 3 of Table I) does not improve the χ^2 value or the residual (Figure 2D) significantly, indicating that the two-exponential fit is adequate.

By the criteria illustrated in Figure 2 and Table I (first three rows), the fluorescence decays of AEDANS–SR were biexponential under all of the labeling conditions tested. Best fits were obtained with a major component having a lifetime of 18.4–19.0 ns and a minor component of 2.5–4.0 ns, with the preexponential factors depending on reaction conditions. The most monoexponential decays, having the largest mole fraction of the 19-ns component, were obtained by reacting 3.0 mg of SR protein/mL (20 μM ATPase) with 30 μM IAEDANS for 3 h at pH 7.0 (at 25 °C in 30 mM MOPS, 5 mM $MgCl_2$, and 0.1 M KCl), and these conditions were used to obtain the data in Figure 2 and in the studies described below. Prelabeling with NEM prior to reaction with IAEDANS resulted in a slight (probably insignificant) decrease in the mole fraction of the short lifetime (to 0.20 ± 0.03), so unless otherwise stated below, further studies were done without NEM prelabeling. In order to determine whether the apparent heterogeneity in lifetimes originates from labeling of proteins other than the ATPase, SR purified with deoxycholate (Meissner et al., 1973) was labeled with IAEDANS under the standard conditions described above. No change in the fluorescence lifetime heterogeneity was observed.

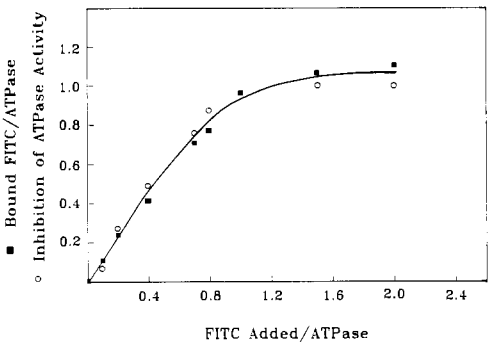

FIGURE 3: Covalent modification of AEDANS–SR with FITC. Samples containing 3.2 mg of SR protein/mL, with 1.0 ± 0.15 AEDANS bound per ATPase, were reacted at the designated total FITC/ATPase ratios (abscissa), as described under Materials and Methods. Values on the ordinate are either moles of FITC bound per mole of ATPase (■) or fractional inhibition of ATPase activity (○).

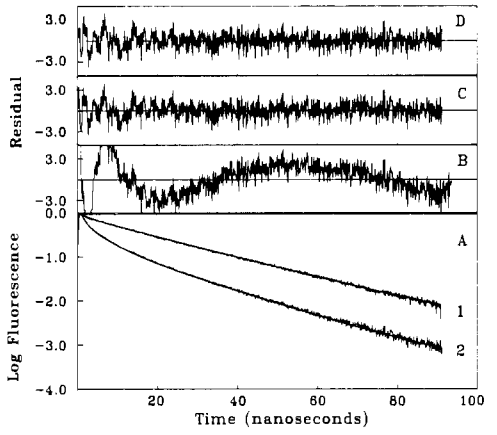

FIGURE 4: Fluorescence decays of AEDANS–SR in the presence of FITC. AEDANS–SR was labeled with FITC as described in Figure 3. (A) The fluorescence decays of AEDANS in the absence (curve 1) and presence (curve 2) of 0.94 mol of FITC/mol of ATPase are superimposed on the best fits to two-exponential and three-exponential functions, respectively. (B), (C), and (D) show the residuals for the fits to two-, three-, and four-exponential functions of AEDANS fluorescence decays in the presence of FITC.

Resonance Energy Transfer from AEDANS to FITC. The reaction of AEDANS–SR with FITC shows saturation at a stoichiometry of one bound FITC per ATPase (Figure 3). The inhibiton of enzymatic activity parallels the incorporation of FITC (Figure 3) and indicates the specificity of the FITC reaction. Figure 4 shows a typical fluorescence decay of AEDANS–SR (the same sample used in Figure 2) in the presence of 0.94 ± 0.1 mol of FITC bound per mole of ATPase, along with the results of fits to eq 1. Fit parameters, averaged over all data sets, are summarized in the last three rows of Table I. Figure 4A (decay 2) shows the data superimposed on the best fit to a three-exponential function. The fluorescence decay of AEDANS–SR in the absence of FITC

Table II: Fluorescence Energy Transfer between IAEDANS and FITC

expt	E^a $(1 - \tau_2/\tau_1)$	R^b (Å)	δR^c (Å)	$a_2{}^d$	E^e $(1 - I_{DA}/I_D)$
control (imidazole)[f]	0.40 (0.08)	52.4 (3.0)		0.27 (0.03)	0.46 (0.08)
calcium	0.31 (0.06)	55.9 (3.7)	3.5 (3.3)	0.27 (0.02)	0.47 (0.08)
monovanadate	0.30 (0.12)	56.4 (5.4)	4.0 (4.0)	0.26 (0.02)	0.47 (0.06)
control (MES[g])	0.45 (0.15)	50.7 (5.3)		0.23 (0.07)	0.41 (0.17)
P_i	0.46 (0.16)	50.3 (5.4)	0.7 (5.4)	0.19 (0.03)	0.44 (0.16)
DMSO[h]	0.52 (0.06)	48.4 (2.0)		0.22 (0.02)	0.60 (0.06)
DMSO/P_i	0.73 (0.05)	41.2 (1.8)	−6.4 (3.2)	0.20 (0.02)	0.60 (0.16)

[a] E was calculated from eq 2, using $\tau_D = \tau_1$ (from two-exponential fits without FITC, row 2 of Table I) and $\tau_{DA} = \tau_2$ (from four-exponential fits with FITC, row 6 of Table I). [b] R is the distance calculated with equ 2. [c] δR is $R' - R$, where R' was obtained in the presence of a perturbation and R is the control value. [d] a_2 is the fraction corresponding to τ_2 in the presence of FITC (see eq 1). [e] E (steady state) was calculated from eq 2 by using total intensity I_D and I_{DA} in the absence and presence of FITC (eq 4). [f] Row 1 (25 mM imidazole, 5 mM $MgCl_2$, 80 mM KCl, and 0.5 mM EGTA, pH 7.1) is the control for rows 2 and 3. [g] Row 4 (30 mM MES, 5 mM $MgCl_2$, and 0.5 mM EGTA, pH 6.2) is the control for row 5. [h] Row 6 (the same MES solution plus 40% DMSO) is the control for row 7. Results were independent of $[Ca^{2+}]$ from 0.2 to 400 μM, [monovanadate] from 10 μM to 1 mM, and $[P_i]$ from 5 to 10 mM. Values in parentheses are SEM for 3–10 experiments.

(decay 1) is also shown for comparison. The residuals and χ^2 values show clearly that the three-exponential fit (Figure 4C, $\chi^2 = 1.43 \pm 0.15$) is superior to the two-exponential fit (Figure 4B, $\chi^2 = 3.7 \pm 1$). A small improvement was obtained when the decays were fit to a four-exponential function (Figure 4D, $\chi^2 = 1.26 \pm 0.16$). It is clear that FITC causes a dramatic decrease in a_1, the amplitude (mole fraction) of the long-lifetime component, and there is at least one new (shorter) lifetime, as expected in the case of energy transfer.

We varied the stoichiometry of FITC labeling in order to gain some insight into the quantitative interpretation of energy transfer. When FITC is added, energy transfer should produce one or more new lifetimes, and variation of the FITC/ATPase ratio should produce variation in the mole fractions of the components but not in the lifetimes themselves. The original 18–19-ns component (τ_1) should be present as long as the FITC/ATPase ratio is less than 1, but its amplitude (a_1) should decrease as other amplitudes (corresponding to shorter lifetimes) increase. These expectations are met only by the four-exponential fits. As the amount of FITC increases, the three-exponential fits yield lifetimes that change gradually (see Table I for the end points), while the lifetimes from four-exponential fits remain essentially constant (see Table I and Figure 5). Therefore, our further analysis of energy transfer concerns the comparison of the two-exponential fit in the absence of FITC (e.g., row 2 in Table I) with the four-exponential fit in the presence of FITC (row 6 in Table I).

As shown in Table I, FITC decreases the mole fraction (a_1) of the 19-ns lifetime (τ_1) from 0.76 to 0.13, indicating that this major component participates in energy transfer. The clearest evidence for a new lifetime corresponding to FITC quenching is the 11.1-ns component (τ_2). If we let $\tau_1 = \tau_D$ and $\tau_2 = \tau_{DA}$, eq 2 yields $E = 0.40 \pm 0.08$, corresponding to a donor–acceptor distance $R = 52.4 \pm 3.0$ Å. However, the small value of a_2 (0.21) can only account for part of the decrease in a_1 (from 0.76 to 0.13), implying that a substantial portion of the donor–acceptor pairs have lifetimes in the range of τ_3 (4.2 ns) and τ_4 (1.2 ns). These lifetimes are not sufficiently different from those obtained in the absence of FITC (Table I) to justify the unambiguous assignment of E or R values using eq 2 and 3. Nevertheless, it is clear that a substantial fraction of the donors have E values in the range of 0.77–0.94, corresponding to R values in the range of 31–40 Å, substantially shorter than the (52.4 ± 3)-Å distance assigned to τ_2. Prelabeling with NEM has no significant effect on the lifetimes but increases the mole fractions (a_3 and a_4) of the short-lifetime components slightly. Further quantitative analysis of R values will focus on possible changes in the unambiguous distance associated with τ_2 (e.g., columns 2–4 of Table II). However, the effective steady-state transfer efficiency (eq 4), taking into account all four components, will also be tabulated (column 1 of Table II), in order to note possible changes in the shorter distance(s).

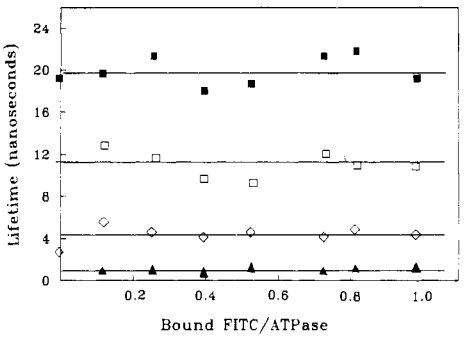

FIGURE 5: Dependence of the fluorescence lifetimes of AEDANS–SR on the amount of FITC bound. Labeling conditions were as described for Figure 3. Lifetimes were obtained by fitting the fluorescence decay to a four-exponential function (eq 1, $n = 4$).

The possibility that intermolecular energy transfer may be significant was investigated by solubilizing the ATPase with the detergent octaethylene glycol monododecyl ether ($C_{12}E_8$) at 1.5 mg of detergent/mg of SR protein (Andersen & Vilsen, 1985). Solubilization increases R by 2.6 ± 1.3 Å. Although this small (5%) change in distance may be due to a decrease in the contribution of intermolecular energy transfer or to a detergent-induced conformational change in the ATPase, it is clear that most of the observed energy transfer is intramolecular.

Ligand Effects on Energy Transfer. The effects of ligands on the fluorescence energy transfer between IAEDANS and FITC were investigated using SR samples having 1.0 ± 0.1 mol of each dye bound per ATPase, at a protein concentration of 0.2–0.5 mg/mL. Table II summarizes the effects of various ligands on the energy-transfer distance R calculated from the conversion of τ_1 (as in Table I, row 2) to τ_2 (as in Table I, row 6) by FITC. The average energy-transfer efficiency calculated from the total intensity (eq 4) is also included for comparison. Each perturbation was carefully paired with a control experiment done with the same preparation on the same day under identical conditions. This maximizes the precision of estimating the effect of the perturbation, even though it underestimates the uncertainties in comparing controls (done with different preparations at different times) with each other. Variation of $[Ca^{2+}]$, in the micromolar to millimolar range, has no significant effect on either R or the steady-state transfer efficiency. The apparent decrease in the transfer efficiency and the corresponding increase in distance calculated from τ_2

are within experimental error. In addition, no significant changes in the mole fractions (a_i) are observed. The phosphate analogue monovanadate, in concentrations ranging from 10 μM to 1 mM, also has no significant effect.

The effect of phosphorylation by inorganic phosphate was investigated under conditions that are known to stabilize the phosphoenzyme intermediate in the Ca-ATPase reaction cycle. Phosphorylation was carried out in 30 mM MES buffer (pH 6.2) containing 0.5 mM EGTA, 5 mM $MgCl_2$, and either 0, 5, or 10 mM inorganic phosphate (P_i). No significant change in energy transfer is observed due to the phosphorylation buffer (row 4) in comparison to the imidazole buffer (row 1). Phosphorylation was also carried out in the presence of 40% (v/v) dimethyl sulfoxide (DMSO), a solvent which has been found to stabilize the covalent phosphoenzyme bond (de Meis et al., 1980). No change is observed when inorganic phosphate was added in the absence of DMSO. In the presence of 40% DMSO, there is a statistically significant decrease in the distance calculated from τ_2 (rows 5 and 6 in Table II). No change in the other fit parameters is observed.

The effect of decavanadate, the oligomeric form of monovanadate (Boyd & Kustin, 1984), on the energy-transfer distance was investigated at concentrations known to inhibit the ATPase activity (Lewis & Thomas, 1986) and to form crystalline arrays of the ATPase (Dux & Martonosi, 1983; Varga et al., 1985; Maurer & Fleischer, 1984; Lewis & Thomas, 1986). At concentrations ranging from 0.01 to 5 mM, incubation of AEDANS–SR for a short period (10 min) or overnight decreases the fluorescence lifetime τ_1 of AEDANS in the absence of the acceptor. The fluorescence quenching was analyzed by using a modified Stern–Volmer analysis for quenching of a membrane-bound fluorophore by a quencher that binds to the membrane with a dissociation constant K_d (Thulborn & Sawyer, 1978). We obtained a value for K_d of 55 μM, in good agreement with the concentration previously found to inhibit 50% of Ca-ATPase activity for both unlabeled and SH-labeled SR (Lewis & Thomas, 1986), and in good agreement with the dissociation constant of 50 μM reported for the FITC-labeled enzyme (Varga et al., 1985). Since the strong quenching of the donor fluorescence by decavanadate made time-resolved analysis of energy transfer very difficult, an apparent average energy-transfer distance was calculated from the total intensity (eq 4) after correction for the effect of quenching of the donor fluorescence on R_0. No significant change in energy transfer is observed, suggesting that decavanadate has no major effect on the tertiary structure of the B-tryptic fragment of the ATPase.

Discussion

Summary of Results. We have used the fluorescence resonance energy-transfer distance between IAEDANS donors and FITC acceptors to probe proposed conformational changes in the Ca-ATPase. In order to minimize the ambiguity of data interpretation in the energy-transfer studies, we have used time-resolved fluorescence to study selectively the major population of donors and to resolve multiple donor–acceptor distances that are present. The addition of several ligands, under conditions proposed to cause changes in Ca-ATPase conformation, caused no significant changes in the donor–acceptor distance, with the exception of phosphorylation by inorganic phosphate in the presence of DMSO.

Labeling Specificity. The acceptor FITC has been shown to label lysine-515 specifically near the nucleotide binding site of the ATPase on the B tryptic fragment (Mitchinson et al., 1982; MacLennan et al., 1985), thus providing a homogeneous population of acceptor sites. Our results (Figure 3) and those of Squier et al. (1987) support this conclusion. Labeling with the donor IAEDANS is not as specific. The reaction does not show saturation after incorporation of one IAEDANS molecule per ATPase (Figure 1), and the long-lifetime component of the fluorescence decay accounts for only 76 ± 4% of the bound label (Figure 2 and Table I, row 2). This heterogeneity of fluorescence lifetimes in the absence of FITC probably reflects labeling at more than one site but could originate from a single IAEDANS site subject to different conformational states (Lewis & Thomas, 1987).

Donor–Acceptor Distance: Resolution of Distance Heterogeneity. The heterogeneity of the observed donor lifetimes in the absence of acceptor indicates the need for time resolution. Since the 19-ns lifetime is the major component, we have used time-resolved fluorescence energy transfer to resolve and selectively measure the distance(s) of this AEDANS population from FITC. This is the major advance that the present study offers over previous fluorescence energy-transfer studies in this system (Squier et al., 1987). The binding of 0.9 FITC per ATPase reduces the fraction a_1 of the 19-ns component to 0.13 (Table I), indicating that virtually all of the AEDANS molecules are quenched by (transfer energy to) FITC. However, these quenched donors are clearly resolved into at least two subpopulations. About one-third have a lifetime τ_2 = 11 ns (Table I, bottom row), corresponding to a distance of R_1 = 52 ± 3 Å from FITC (Table II, top row). The remaining two-thirds must be included in the components having lifetimes of 1.2 and 4.2 ns (Table II, bottom). These lifetimes would correspond to distances of 31 and 40 Å, respectively, using eq 2 and 3. However, because these lifetimes are quite similar to the 2.5-ns lifetime observed in the absence of FITC (Table I, row 2), we do not attempt to assign specific distances to this population. We simply conclude that the donor–acceptor distances R_2 for this more highly quenched population must fall within the range of 31–40 Å, substantially shorter than R_1.

Comparison with Previous IAEDANS–FITC Energy-Transfer Studies in SR. Since R_2 corresponds to the major population of AEDANS donors, our conclusions are significantly different from those reached in a previous energy-transfer study on similar preparations of SR labeled with IAEDANS and FITC (Squier et al., 1987). In that study, which lacked time resolution and therefore assumed a single donor–acceptor distance, the value of R was 56 Å, which is comparable to R_1 in the present study, but much longer than R_2. Although differences in the preparation cannot be ruled out, the most straightforward explanation of the difference is that Squier et al. measured only an effective lifetime by the phase-modulation method, employing a single frequency of 10 MHz. The use of this single low frequency not only would preclude the resolution of multiple lifetimes but also would skew the results toward the longest lifetime present (the lifetime of the unquenched donor), possibly even resulting in an R value longer than the longest actual distance.

Interpretation of Distance Heterogeneity. Thus, the energy-transfer measurements have resolved the major (19-ns) donor population into two subpopulations having substantially different distances from FITC. There are two possible interpretations: (a) AEDANS is bound to two distinct sites, or (b) AEDANS is bound at a single site, but the labeled enzyme has two distinct conformations having different AEDANS–FITC distances. In recent work on the sequencing of IAEDANS-labeled peptides, following essentially the same labeling procedure used in the present study, it was reported that about 75% of the label is on one site, Cys-674 (Yamashita & Ka-

wakita, 1987; Suzuki et al., 1987). Thus, we may assign the 19-ns lifetime component to AEDANS bound to Cys-674, ruling out possibility a above. However, Bishop et al. (1988) have reported that 6-(iodoacetamido)fluorescein (IAF) reacts with both Cys-670 (25%) and Cys-674 (58%) and that IAEDANS and IAF react competitively. Therefore, another plausible interpretation of our results is that AEDANS bound to one of these sites (probably Cys-670) is 52 ± 3 Å from FITC, while this distance is 40 Å or less for AEDANS bound to the other site. This is not consistent with the assumption, in previous fluorescence studies lacking time resolution, that there is a single AEDANS–FITC distance in the Ca–ATPase (Squier et al., 1987; Bishop et al., 1988).

Interpretation b above, that the two distances reflect two conformational states of the labeled protein, is consistent with EPR studies of the rotational dynamics of iodoacetamide spin-labels bound to the Ca-ATPase (Coan & Keating, 1982; Coan, 1983; Lewis & Thomas, 1987). The most recent of these studies indicates that, while the probe has uniformly high rotational mobility in the absence of nucleotides, two distinct mobilities (conformations) are resolved when nucleotides are added (Lewis and Thomas, unpublished experiments). Since FITC labels the nucleotide binding site, and inhibits nucleotide binding, the conformational state of the FITC–enzyme may be analogous to that of the nucleotide-bound enzyme. If so, the two populations resolved by energy transfer in the present study may correspond to the two conformations resolved by probe mobility in the EPR studies.

Ligand Effects on Energy Transfer. The energy-transduction cycle of the Ca-ATPase almost certainly involves the coupling of ligand binding (ATP, Ca^{2+}, phosphate, Mg^{2+}) to enzyme conformational transitions (De Meis & Viana, 1979; Eisenberg & Hill, 1985; Nakamura et al., 1986; Stahl & Jencks, 1987). Various extrinsic fluorescent probes have been used to investigate ligand-induced conformational transitions in the Ca-ATPase. The fluorescence intensity of FITC-labeled SR decreases upon high-affinity calcium binding (Pick, 1981; Pick & Karlish, 1982; Anderson et al., 1982) and upon phosphorylation with P_i (Pick, 1981), and was increased by vanadate (Pick, 1982; Highsmith et al., 1985). High-affinity calcium binding also enhances the fluorescence intensity of IAEDANS-labeled SR (Baba et al., 1986; Suzuki et al., 1987). It is clear that the heterogeneity of labeled sites in the present study complicates the interpretation of energy-transfer distances using IAEDANS and FITC. However, because we can clearly resolve at least one of the distances, we can selectively study changes in this distance that may occur upon ligand binding.

Effects of Ca^{2+} and Phosphate. We observed no significant change in the energy-transfer distances R_1 or R_2 upon binding of calcium or P_i. A significant decrease in R_1 observed upon phosphorylation of the enzyme in the presence of DMSO (Table II) may reflect the increased amount of phosphoprotein formed in the presence of DMSO, approximately 3–4 times the amount formed in the absence of DMSO (de Meis et al., 1980). Alternatively, the effect may be due to the phosphoprotein assuming a different conformation in the presence of DMSO. Our observations are consistent with the observation that P_i has an effect on the mobility of an iodoacetamide spin-label only if DMSO is present (Coan, 1983; Lewis & Thomas, 1987). The lack of large changes in distance upon binding of these ligands (calcium and P_i in the absence of DMSO) indicates that the reported changes in the quantum yield of FITC and IAEDANS fluorescence (Pick & Karlish, 1982; Anderson et al., 1982; Highsmith et al., 1985; Baba et al., 1986; Suzuki et al., 1987) are probably due to localized changes in the probe's environment that do not involve significant displacement of either the donor or the acceptor. Similarly, translocation of part of the protein mass into the bilayer, proposed to occur upon phosphorylation of the enzyme (Blasie et al., 1985), may not necessarily result in the kind of internal protein structural change required to alter the energy-transfer distance.

Effect of Vanadate. Mono- and decavanadate have been proposed to have different modes of interaction with the Ca-ATPase. The structural changes in the ATPase leading to the formation of ordered arrays of the protein have been attributed to the decavanadate species (Maurer & Fleischer 1984; Lewis & Thomas, 1986). Using a maleimide spin-label and saturation-transfer EPR, Lewis and Thomas (1986) detected a decavanadate-induced decrease in the microsecond mobility of the Ca-ATPase, whereas monovanadate had no effect. The differences in size and charge between the monomer (VO_4^{3-}) and the octahedrally coordinated decavanadate ($V_{10}O_{28}^{-6}$) (Boyd & Kustin, 1984) also predict potentially different modes of interaction with the ATPase. In the present study, we have found that monovanadate does not alter energy-transfer distances R_1 or R_2. Although decavanadate quenches the donor fluorescence directly, it also has no significant effect on the energy-transfer distance. Thus, although it is likely that structural changes occur during the formation of Ca-ATPase arrays by decavanadate, these changes do not affect the AEDANS–FITC distance.

Conclusions. Much of the evidence for conformational transitions in the Ca-ATPase comes indirectly from studies of enzyme kinetics and ligand effects on steady-state fluorescence. In the present study, we have made direct intramolecular distance measurements under conditions proposed to induce conformational transitions within the Ca-ATPase, using time-resolved spectroscopic methods to resolve the components of this complex system. The measured distance shows no effect of Ca^{2+}, despite the proposed influence of Ca^{2+} on an E_1–E_2 transition. Phosphorylation of the ATPase by P_i induces a significant change only in the presence of DMSO. Decavanadate, which forms crystalline arrays of the ATPase and decreases the microsecond mobility of the protein (Lewis & Thomas, 1986), also causes no significant change in the energy-transfer distance.

ACKNOWLEDGMENTS

We thank Robert Bennett and Dr. Steven Flom for technical assistance in the construction and maintenance of the fluorescence instrumentation. We thank Dr. Paul Barbara for the loan of photon-counting components and Dr. Frank Prendergast for the loan of the multichannel plate photomultiplier.

REFERENCES

Andersen, J. P., & Vilsen, B. (1985) *FEBS Lett. 189*, 13–17.

Andersen, J. P., Moller, J. V., & Jorgensen, P. L. (1982) *J. Biol. Chem. 257*, 8300–8307.

Baba, A., Nakamura, T., & Kawakita, M. (1986) *J. Biochem. 100*, 1137–1147.

Bigelow, D. J., Squier, T. C., & Thomas, D. D. (1986) *Biochemistry 25*, 194–202.

Bishop, J. E., Squier, T. C., Bigelow, D. J., & Inesi, G. (1988) *Biochemistry 27*, 5233–5240.

Blasie, J. K., Herbette, L. G., Pascolini, D., Skita, V., Pierce, D. H., & Scarpa, A. (1985) *Biophys. J. 48*, 9–18.

Boyd, D. W., & Kustin, K. (1984) *Adv. Inorg. Biochem. 6*, 311–365.

Coan, C. (1983) *Biochemistry 22*, 5826–5836.
Coan, C. R., & Keating, S. (1982) *Biochemistry 21*, 3214–3220.
de Meis, L., & Viena, A. L. (1979) *Annu. Rev. Biochem. 48*, 275–292.
de Meis, L., Martins, O. B., & Alves, E. W. (1980) *J. Biol. Chem. 19*, 4252–4261.
Dupont, Y. (1978) *Nature 273*, 396–398.
Dupont, Y., & Bennett, N. (1982) *FEBS Lett. 139*, 237–240.
Dux, L., & Martonosi, A. (1983) *J. Biol. Chem. 258*, 2599–2603.
Eisenberg, E., & Hill, T. L. (1985) *Science 227*, 999–1005.
Fernandez, J. L., Rosemblatt, M., & Hidalgo, C. (1980) *Biochim. Biophys. Acta 599*, 552–568.
Fernandez-Belda, F., Kurzmack, M., & Inesi, G. (1984) *J. Biol. Chem. 259*, 9687–9698.
Herrmann, T. R., Gangola, P., & Shamoo, A. (1986) *J. Biochem. 158*, 555–560.
Hidalgo, C., & Thomas, D. D. (1977) *Biochem. Biophys. Res. Commun. 78*, 1175–1181.
Highsmith, S., Barker, D., & Scales, D. J. (1985) *Biochim. Biophys. Acta 817*, 123–133.
Inesi, G., Kurzmack, M., Nakamoto, R., De Meis, L., & Bernhard, S. (1980) *J. Biol. Chem. 255*, 6040–6043.
Lewis, S. M., & Thomas, D. D. (1986) *Biochemistry 25*, 4615–4621.
Lewis, S. M., & Thomas, D. D. (1987) *Biophys. J. 51*, 403a.
Lewis, S. M., & Thomas, D. D. (1988) *Biophys. J. 53*, 132a.
MacLennan, D. H., Brandl, C. J., Korczak, B., & Green, N. M. (1985) *Nature 316*, 696–700.
Maurer, A., & Fleischer, S. (1984) *J. Bioenerg. Biomembr. 16*, 491–505.
Meissner, G., Conner, G. E., & Fleischer, S. (1973) *Biochim. Biophys. Acta 298*, 246–269.
Mitchinson, C., Wilderspin, A. F., Trinnaman, B. J., & Green, N. M. (1982) *FEBS Lett. 146*, 87–92.
Nakamura, Y., Kurzmack, M., & Inesi, G. (1986) *J. Biol. Chem. 261*, 3090–3097.
O'Neil, S. G., Rhoads, D. B., & Racker, E. (1979) *Biochem. Biophys. Res. Commun. 89*, 845–850.
Pick, U. (1981) *FEBS Lett. 123*, 131–136.
Pick, U. (1982) *J. Biol. Chem. 257*, 6111–6119.
Pick, U., & Bassilian, S. (1980) *FEBS Lett. 123*, 127.
Pick, U., & Karlish, S. J. D. (1982) *J. Biol. Chem. 257*, 6120–6126.
Scott, T. L. (1985) *J. Biol. Chem. 260*, 14421–14423.
Squier, T. C., & Thomas, D. D. (1988) *J. Biol. Chem. 263*, 9171–9177.
Squier, T. C., Bigelow, D. J., de Ancos, J. G., & Inesi, G. (1987) *J. Biol. Chem. 262*, 4748–4754.
Squier, T. C., Hughes, S. E., & Thomas, D. D. (1988) *J. Biol. Chem. 263*, 9162–9170.
Stahl, N., & Jencks, W. P. (1987) *Biochemistry 26*, 7654–7667.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–946.
Suzuki, H., Obara, M., Kuwayama, H., & Kanazawa, T. (1987) *J. Biol. Chem. 262*, 15448–15456.
Thulborn, K. R., & Sawyer, W. H. (1978) *Biochim. Biophys. Acta 511*, 125–140.
Varga, S., Csermely, P., & Martonosi, A. (1985) *Eur. J. Biochem. 148*, 119–126.
Ware, W. R. (1971) in *Creation and Detection of the Excited States* (Lamela, A. A., Ed.) Vol. 1A, Marcell Dekker, New York.
Watanabe, T., & Inesi, G. (1982) *Biochemistry 21*, 3254–3259.
Yamada, S., & Ikemoto, N. (1978) *J. Biol. Chem. 253*, 6801–6807.
Yamashita, T., & Kawakita, M. (1987) *J. Biochem. 101*, 377–385.