Modulation of the Binding Characteristics of a Fluorescent Nucleotide Derivative to the Sarcoplasmic Reticulum Adenosinetriphosphatase†

James E. Bishop,* Robert K. Nakamoto, and Giuseppe Inesi

Department of Biological Chemistry, School of Medicine, University of Maryland at Baltimore, Baltimore, Maryland 21201

Received May 21, 1985

ABSTRACT: Trinitrophenyladenosine monophosphate (TNP-AMP) binding to the phosphorylated Ca^{2+}-ATPase of sarcoplasmic reticulum results in manyfold higher fluorescence intensity and longer lifetimes of the nucleotide analogue, as compared to TNP-AMP binding to the nonphosphorylated enzyme. This is observed when the phosphoenzyme intermediate is formed either from ATP or from inorganic phosphate (P_i). An important question is whether the TNP-AMP fluorescence properties can also reflect the kinetically defined interconversions of different phosphoenzyme species during catalysis. We have approached this question by manipulating the phosphorylation conditions in a manner which is known to result in accumulation of different species of the phosphoenzyme, i.e., by variations in pH, substrates, and K^+ and Ca^{2+} concentrations. Decreasing pH or increasing [K^+] caused large decreases in fluorescence intensity at a given concentration of TNP-AMP under conditions of phosphorylation with either ATP or P_i. In contrast, low to high intravesicular Ca^{2+} concentrations had no effect on fluorescence during steady-state turnover. TNP-AMP titrations of the phosphorylated enzyme stabilized in different states revealed that H^+ and K^+ caused a shift in TNP-AMP binding affinity to the site responsible for high fluorescence enhancement, while maintaining approximately the same maximal fluorescence yield at saturation. The fluorescence lifetimes of TNP-AMP bound to phosphoenzyme did not change with variations in pH, [K^+], and substrates. We conclude that the environment of that part of the TNP-AMP binding site which binds the trinitrophenyl moiety undergoes a change upon enzyme phosphorylation resulting in enhanced fluorescence yield; this change is invariant between different phosphoenzyme species. Changes in affinity for the nucleotide analogue induced by H^+ and K^+ are responsible for the variable increase in total fluorescence intensity observed upon enzyme phosphorylation at subsaturating TNP-AMP concentration.

The fluorescent nucleotide derivative 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)-nucleotide (TNP-nucleotide)[1] is an environmentally sensitive probe of the nucleotide binding site and has been used to study the myosin ATPase (Hiratsuka & Uchida, 1973), the (Na^+, K^+)-ATPase (Moczydlowski & Fortes, 1981a,b), the F_1-ATPase (Grubmeyer & Penefsky, 1981a,b), and aspartokinase I (Broglie & Takahashi, 1983). TNP-nucleotides were introduced to the study of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase by Dupont et al. (1982) and Watanabe & Inesi (1982b) and were found to bind to the extent of about 7 nmol/mg of protein to the Ca^{2+}-ATPase with higher affinity than that of ATP. It was the original findings of Watanabe & Inesi (1982b) that the fluorescence intensity of bound TNP-ATP is enhanced severalfold upon formation of the phosphoenzyme intermediate from ATP in the presence of Ca^{2+}. Subsequently, a large fluorescence enhancement was also found upon phosphoenzyme formation from P_i in the absence of Ca^{2+} (Dupont & Pougeois, 1983). The three nucleotide analogues TNP-AMP, TNP-ADP, and TNP-ATP were shown to respond in a qualitatively similar fashion under both of these conditions (Nakamoto & Inesi, 1984). At least two types of TNP-nucleotide binding on the Ca^{2+}-ATPase have been differentiated (Nakamoto & Inesi, 1984; Bishop et al., 1984; Dupont et al., 1985). TNP-nucleotide binds the catalytic site of the nonphosphorylated enzyme as a slowly dissociating dead-end complex forming in competition with both ATP and P_i and resulting in a low fluorescence and a short fluorescence lifetime. The second type of binding, whose exact location is a subject of controversy, occurs only after phosphorylation of the enzyme. TNP-nucleotide is in rapid equilibrium with this site, binding to which results in a high fluorescence and a longer lifetime due to phosphorylation-induced conversion of the site to a more hydrophobic and restrictive character. The high fluorescence enhancement of TNP-nucleotides was found (Nakamoto & Inesi, 1984; Bishop et al., 1984) to parallel total phosphoenzyme levels (measured by ^{32}P incorporation into the enzyme) in both the transient and steady states under conditions of fixed pH and [KCl].

An important and outstanding question is whether the fluorescence enhancement of bound TNP-nucleotides is different for different kinetically defined phosphoenzyme species, in that this fluorescence may monitor changes in the content (de Meis et al., 1980; Dupont & Pougeois, 1983) or structure (Wiggins, 1982) of water at the catalytic site associated with the energy transduction process. This possibility must be carefully evaluated, since a higher fluorescence intensity was in fact observed (Dupont & Pougeois, 1983; Nakamoto & Inesi, 1984) when the enzyme was phosphorylated with P_i rather than with ATP. On the other hand, Bishop et al. (1984) suggested that different fluorescence intensities of the TNP-nucleotide bound to phosphoenzyme are more simply related to the [KCl] and/or other variables in the reaction medium. In this regard, a shortcoming of previous studies is that changes in total fluorescence were taken as such, often without cor-

† This research was supported by U.S. Public Health Service Grant HL27687 and by the Muscular Dystrophy Association of America.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; P_i, inorganic phosphate; TNP-AMP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; SR, sarcoplasmic reticulum.

0006-2960/86/0425-0696$01.50/0

rection for changes in the phosphoenzyme level and in the amount of bound TNP-nucleotide.

In order to clarify these uncertainties, we have performed a series of experiments in which enhancement of TNP-AMP fluorescence intensity was studied by using identical media for enzyme phosphorylation with P_i or with ATP *plus* Ca^{2+}. In these studies, we have related the total fluorescence change to measured levels of phosphoenzyme and bound TNP-nucleotide. We have also studied systematically the effects of H^+, K^+, and Ca^{2+} on fluorescence and evaluated whether these effects could be meaningfully related to parallel modulation of enzyme catalytic intermediates by these electrolytes. Finally, we have also evaluated independently by fluorescence lifetime measurements the influence of the enzyme on the fluorescence properties of TNP-AMP bound to phosphoenzyme in conditions favoring one phosphoenzyme species over the other.

Materials and Methods

Materials. Creatine phosphate was obtained as a Tris salt and creatine phosphokinase as a salt-free powder, both from Sigma Chemical Co. (St. Louis, MO). ATP, P_i, and EGTA were pH-adjusted with either Tris or triethanolamine. TNP-AMP was synthesized and purified as previously described (Hiratsuka & Uchida, 1973; Watanabe & Inesi, 1982b). [^{32}P]P_i was purified by the method of Kanazawa & Boyer (1973).

Native SR vesicles were prepared from rabbit leg muscle (Eletr & Inesi, 1972). Leaky vesicles of partially purified Ca^{2+}-ATPase were obtained by extraction of native SR vesicles with deoxycholate as described by Meissner et al. (1973).

Assays. Protein concentrations were determined by the method of Lowry et al. (1951). Ca^{2+}-dependent ATPase activity was measured by colorimetric determination of the P_i released (Lin & Morales, 1977).

Phosphoenzyme formed from [γ-^{32}P]ATP and from [^{32}P]P_i (both at 2000–3000 cpm/nmol) was acid-quenched and isolated by Millipore filtration, essentially as described by Nakamura (1984).

Fluorescence Measurements. Steady-state TNP-AMP fluorescence intensity measurements were done on an Amin-co-Bowman fluorometer with the excitation monochromator set at 408 nm and emission at 540 nm. Slit widths were 22 nm each. The fluorescence intensity of added TNP-AMP increased linearly with concentrations up to 2.5 μM utilizing a standard 1 × 1 cm cuvette. For titrations of TNP-AMP up to 20 μM, a 0.5 × 1 cm cuvette was used with the narrow edge toward the emission window to minimize inner filter effects. This arrangement allowed a linear fluorescence response up to 5 μM TNP-AMP. Above this concentration, excitation light was attenuated by approximately 10% at 10 μM TNP-AMP and by 30% at 20 μM, and appropriate correction factors were applied (Lakowicz, 1983).

For the TNP-AMP titrations of Figure 7, the fluorescence intensities arising specifically from TNP-AMP bound to the phosphorylated enzyme were calculated as follows. The total fluorescence intensities were first corrected for inner filter effects as described in the preceding paragraph and then corrected for dilution caused by the additions of TNP-AMP which was a maximum of 1.7%. The appropriate blank intensity which was obtained by a sample consisting of Ca^{2+}-ATPase, the appropriate TNP-AMP concentration, and either ATP *plus* 1 mM EGTA or P_i *plus* 5 mM $CaCl_2$ was then subtracted. These blank values included scattering due to the protein and small components of fluorescence both from free TNP-AMP and from TNP-AMP bound to the nonphosphorylated enzyme and were 10–15% of the fluorescence intensity value obtained at saturating TNP-AMP concentration. Another method of blank subtraction assumed a mutually exclusive binding of the TNP-AMP bound either to the nonphosphorylated enzyme (low fluorescence enhancement) or to the phosphorylated enzyme (high fluorescence enhancement) up to a total maximum of 4 nmol/mg of protein and included an estimation of the free TNP-AMP and its associated fluorescence intensity. Both methods gave similar results. Minimization of inner filtering effects was the major factor affecting the data presented in Figure 7. Lack of attention to this phenomenon in a recent study (Davidson & Berman, 1985) may account for their different conclusion regarding binding of TNP-nucleotide to the Ca^{2+}-ATPase.

Fluorescence lifetimes of TNP-AMP were measured on a variable-frequency phase-modulation fluorometer in the laboratory of Dr. J. Lakowicz [see Lakowicz & Maliwal (1985)]. The excitation light was the 442-nm line obtained from a He–Cd laser, and the emission light was measured after first passing through a Corning 3-70 cutoff filter and then a Corning 4-96 filter. Emission spectra taken with 442-nm excitation of TNP-AMP in the presence of SR vesicles and substrate (identical with the conditions of the lifetime measurements) showed that the combination of the Corning 3-70 and 4-96 filters reduced light scatter to maximally 2% of the total steady-state intensity. Polarization artifacts were eliminated by setting the emission polarizer to 54.7° relative to the excitation light. The frequency of modulation of the excitation light was incremented between 10 and 210 MHz. Phase shift and demodulation due to the TNP-AMP sample were measured against a light-scattering reference made of dimyristoylphosphatidylcholine vesicles. Data analysis was done as previously described (Gratton et al., 1984; Lakowicz et al., 1984).

All experiments were performed at 25 °C. Presented data are the averages of two to five determinations with standard deviations of less than ±15%.

Results

Fluorescence of TNP-AMP Bound to Ca^{2+}-ATPase Assembled in "Leaky" Vesicles and Phosphorylated with ATP in the Presence of Ca^{2+}. Fluorescence was measured under several conditions which have been reported to alter the relative levels of the different catalytic intermediates of the Ca^{2+}-ATPase during steady-state turnover. Large differences in fluorescence of TNP-AMP were found in the absence of K^+ at different pH values (Figure 1A, closed circles). The enzyme used in these experiments was a preparation of partially purified Ca^{2+}-ATPase (Meissner et al., 1973) which leaves the vesicles permeable; therefore, no net calcium accumulation and no "back-inhibition" by accumulated calcium were produced. It should also be noted that the substrate concentrations used, i.e., 300 μM ATP and 100 μM Ca^{2+}, resulted in the maximal or nearly maximal levels (>90%) of fluorescence that could be obtained in each condition. It can be seen in Figure 1A that a low fluorescence is observed at low pH, as compared to the very high levels seen at high pH. It is also shown in Figure 1A (open triangles) that addition of 120 mM KCl caused a large decrease in the fluorescence of TNP-AMP at all pH values tested. This effect of K^+ is a saturable function with a $K_{1/2}$ of approximately 50 mM. Similar variations of K^+ concentrations did not affect the fluorescence of nonbound TNP-AMP (i.e., in the absence of enzyme), either in aqueous or in low polarity (dimethylformamide) media (data not shown). Also, the fluorescence of free TNP-AMP in aqueous media was not affected by pH changes between 6 and 9.

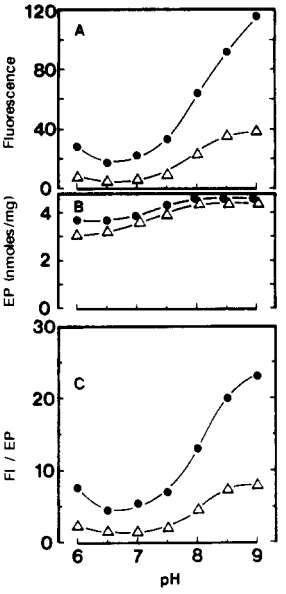


FIGURE 1: Fluorescence of TNP-AMP bound to leaky vesicles of Ca^{2+}-ATPase in the presence of ATP *plus* Ca^{2+}. (A) Fluorescence was measured and blanks were subtracted as given under Materials and Methods, in the following medium: 50 mM Tris–maleate at the indicated pH, 20% glycerol, 10 mM $MgCl_2$, 0.1 mg of protein/mL Ca^{2+}-ATPase (leaky vesicles), 1 μM TNP-AMP, 100 μM $CaCl_2$, and 300 μM ATP with 1 mM creatine phosphate *plus* 0.05–0.4 mg/mL creatine phosphokinase to maintain ATP levels in the absence (●) and in the presence (△) of 120 mM KCl. Temperature was 25 °C. (B) ^{32}P-Labeled phosphoenzyme was measured as given under Materials and Methods under the same conditions given in fluorescence measurements in the absence of the ATP-regenerating system and using 300 μM [γ-^{32}P]ATP. (C) Phosphoenzyme-normalized fluorescence values (Fl/EP) were calculated by dividing the data of (A) by those of (B).

Therefore, the observed effects of H^+ and K^+ on the fluorescence of TNP-AMP are mediated by the Ca^{2+}-ATPase.

Since previous work has shown that only the phosphorylated enzyme produces the high TNP-nucleotide fluorescence and not the nonphosphorylated species (see above), we determined the phosphoenzyme levels under the same conditions and found them to increase somewhat with higher pH values and to be slightly diminished by KCl at lower pH values (Figure 1B). Normalization of the fluorescence (Figure 1A) to these phosphoenzyme levels to yield specifically the phosphoenzyme-dependent fluorescence also shows marked changes as functions of H^+ and K^+ concentrations (Figure 1C).

Fluorescence of TNP-AMP Bound to the Ca^{2+}-ATPase of "Native" SR Vesicles and Phosphorylated with ATP in the Presence of Ca^{2+}. In the presence of low (100 μM) Ca^{2+} in the outside medium, accumulation of high internal Ca^{2+} concentration under turnover conditions in native SR vesicles is expected to produce high levels of calcium-bound phosphoenzyme over calcium-free phosphoenzyme, as compared to the permeable Ca^{2+}-ATPase preparation which does not accumulate Ca^{2+}. The occurrence of a back-inhibition by accumulated Ca^{2+} in our preparation of native SR vesicles was validated in assays of Ca^{2+}-ATPase activity which showed a 2–5-fold stimulation of activity at pH values from 6.0 to 8.5 when either of the divalent cation ionophores A23187 (2 μM) or X537A (20 μM) was included to render the native vesicles leaky. This stimulation of activity was not observed with the permeable Ca^{2+}-ATPase preparation which displayed high activity even in the absence of ionophores.

The pH profile of the fluorescence of TNP-AMP bound to native SR vesicles phosphorylated with ATP in the presence of Ca^{2+} was similar to that seen with the permeable Ca^{2+}-ATPase preparation in Figure 1 and virtually identical upon inclusion of 2 μM A23187. Representative values are shown in Table I. Further studies (not shown) revealed that inclusion of 100 mM KCl diminished the fluorescence similarly whether or not ionophore was present.

Table I: Phosphoenzyme-Dependent Fluorescence of TNP-AMP under Steady-State Conditions with SR Vesicles with and without Ionophore[a]

pH	A23187	fluorescence	[^{32}P]E–P (nmol/mg)	fluorescence/E–P
6.0	–	20.1 ± 0.8	3.17 ± 0.09	6.34
6.0	+	23.5 ± 1.1	3.06 ± 0.14	7.68
6.5	–	19.0 ± 0.6	3.45 ± 0.14	5.51
6.5	+	15.1 ± 1.1	2.79 ± 0.11	5.41
8.5	–	74.7 ± 0.3	4.04 ± 0.10	18.5
8.5	+	80.0 ± 1.3	4.06 ± 0.26	19.7

[a] Fluorescence and phosphoenzyme levels were measured as described in Figure 1 using 0.1 mg/mL native SR vesicles in the absence or presence of 2 μM A23187. Average ± SD of three to six determinations is reported.

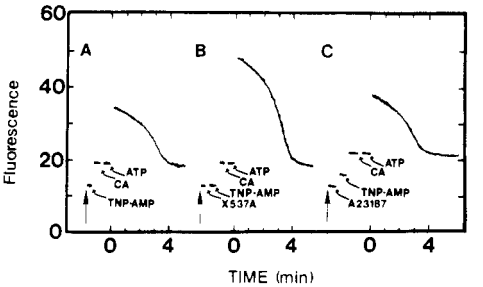


FIGURE 2: Effect of ionophores on fluorescence of TNP-AMP bound to leaky vesicles of Ca^{2+}-ATPase. Fluorescence measurements were initiated (large arrows) by addition of 0.1 mg/mL Ca^{2+}-ATPase in a medium consisting of 50 mM Tris–maleate (pH 7.0), 20% glycerol, 10 mM $MgCl_2$, and 500 μM EGTA. This initial apparent fluorescence was due to light scattering by the enzyme preparation. Further additions where indicated were 2 μM A23187, 20 μM X537A, 1 μM TNP-AMP, 600 μM $CaCl_2$, and 200 μM ATP. Time after the addition of ATP is as indicated. The total dilution by these additions was about 3%. The small fluorescence enhancement seen upon addition of the Ca^{2+} ionophores was due to the slight overlap of their excitation/emission spectra (Pfeiffer et al., 1974; Verjovski-Almeida, 1981) with that of TNP-AMP.

In experiments in which we used the ionophore X537A (instead of A23187), we found (Figure 2) an enhancement of the fluorescence of TNP-nucleotide bound to phosphoenzyme similar to that reported by Dupont & Pougeois (1983). However, in experiments in which we used the permeable Ca^{2+}-ATPase preparation (Figure 2), we found that A23187 produced no effect other than a small additive fluorescence of the ionophore itself while X537A specifically enhanced the high phosphoenzyme-dependent TNP-AMP fluorescence. Furthermore, a similar enhancement by X537A of the fluorescence of TNP-AMP bound to phosphoenzyme made with P_i at pH 7.0 was also observed (data not shown). Therefore, this effect of X537A appears due to a direct effect of the ionophore on the fluorescence of the analogue–phosphoenzyme complex, an enhancement unrelated to permeabilization of the SR membrane and shift of calcium-bound to calcium-free phosphoenzyme. It is of interest to note that valinomycin was shown in a recent study (Davidson & Berman, 1985) to produce a similar enhancement of TNP-nucleotide fluorescence.

Steady-state Ca^{2+}-ATPase activity had a pH profile and a [K^+] dependence roughly opposite to the fluorescence pattern shown in Figure 1A (data not shown), suggesting a functional

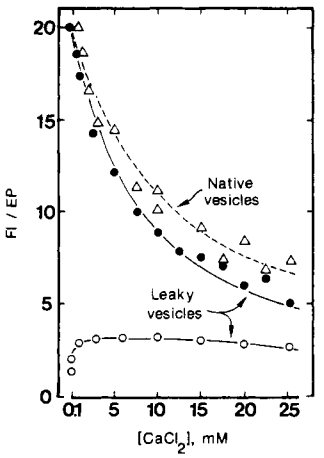


FIGURE 3: Inhibition of the high fluorescence of TNP-AMP under steady-state conditions by high levels of Ca^{2+}. Fluorescence was monitored at pH 8.5 under the conditions of Figure 1A in the absence (○) and in the presence (●, △) of 10 mM $MgCl_2$, using 0.1 mg of protein/mL of the indicated Ca^{2+}-ATPase preparation. The initial fluorescence values were measured with 100 μM $CaCl_2$. Fluorescence values were normalized to phosphoenzyme levels which were about 4.2 nmol/mg under all conditions. To prevent any significant accumulation of Ca^{2+} into SR vesicles in that experiment (△), the medium used originally contained 400 μM EGTA and no Ca^{2+}. Fluorescence increases were initiated by addition of $CaCl_2$ in excess of the EGTA to give the indicated levels and were recorded 4 s after this addition. It should be noted that the turnover time in the presence of low [Ca^{2+}] at pH 8.5 was measured to be approximately 8 s. The two upper curves are theoretical fits to the data to a single binding site with K_d values for Ca^{2+} of 12 mM (native vesicles) and 8 mM (leaky vesicles).

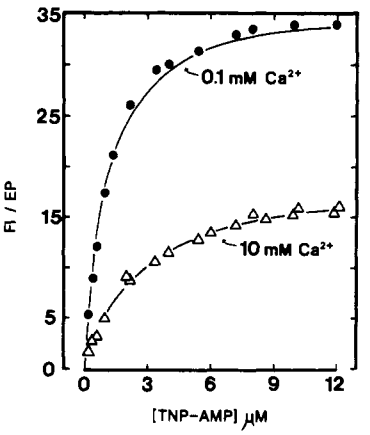


FIGURE 4: Binding of TNP-AMP to Ca^{2+}-ATPase in the presence of ATP at low and high [Ca^{2+}]. Fluorescence titrations of TNP-AMP were conducted at pH 8.5 under the conditions of Figure 1A, using either 100 μM or 10 mM $CaCl_2$. Phosphoenzyme levels were decreased somewhat by high levels of TNP-AMP in a similar fashion to that shown in Figure 7A, and the data are presented as phosphoenzyme-normalized fluorescence (Fl/EP). The solid lines are theoretical fits of the data to single binding sites with Fl/EP_{max} and K_d values of, respectively, 35 and 0.7 μM (0.1 mM $CaCl_2$) and 18 and 2.1 μM (10 mM $CaCl_2$).

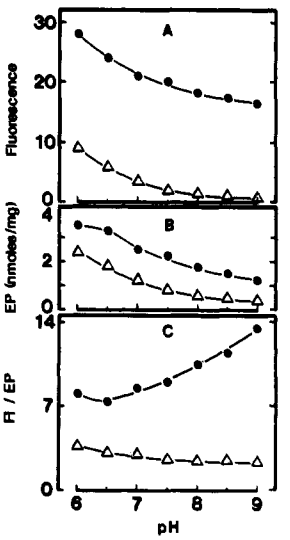


FIGURE 5: Fluorescence of TNP-AMP bound to leaky vesicles of Ca^{2+}-ATPase under conditions of equilibrium with P_i. (A) Fluorescence was measured under the same conditions as for Figure 1A in the absence (●) or presence (△) of 100 mM KCl, except that 1 mM EGTA was substituted for $CaCl_2$ and 12 mM P_i was substituted for the ATP *plus* ATP-regenerating system. The fluorescence scale is the same as that used in Figure 1A. (B) ^{32}P-Labeled phosphoenzyme was measured under the same conditions as for fluorescence, except that 12 mM [^{32}P]P_i was used. (C) Phosphoenzyme-normalized fluorescence (Fl/EP) was calculated from the data of panels A and B.

inverse correlation between the two phenomena. However, the small changes in the fluorescence of SR vesicles upon addition of ionophore (Table I) do not correlate at all with the large increase in ionophore-stimulated phosphoenzyme turnover.

Inhibition of Steady-State TNP-AMP Fluorescence by High [Ca^{2+}]. We further investigated the relative fluorescence levels of different phosphoenzyme species by addition of millimolar levels of Ca^{2+}, which is expected to bind to the low-affinity inward-oriented calcium sites of the Ca^{2+}-ATPase reassembled into leaky vesicles to produce high levels of Ca^{2+}-bound phosphoenzyme. It was found that millimolar Ca^{2+} levels reduced the fluorescence of bound TNP-AMP (Figure 3) as reported previously by Dupont & Pougeois (1983). The $K_{1/2}$ for this effect at pH 8.5 was 8 mM (Figure 3). A similar inhibition constant of 12 mM Ca^{2+} was found when native SR vesicles under conditions in which the internal calcium sites are not accessible to the medium Ca^{2+} (Figure 3) were used. Similar constants of approximately 10 mM Ca^{2+} were found at pH 6.0 for both leaky and native SR vesicles (not shown). In order to reveal whether the fluorescence reduction by high Ca^{2+} (Figure 3) was due to decreased levels of total phosphoenzyme, we checked these levels and found them unchanged. It would appear that this effect of millimolar Ca^{2+} concentrations is mediated by external cation sites of the Ca^{2+}-ATPase in that little effect was noted where only internal binding sites were exposed to high [Ca^{2+}] (Table I).

The possible alteration in binding affinity of highly fluorescent TNP-AMP to the steady-state Ca^{2+}-ATPase by high [Ca^{2+}] was then investigated by fluorescence titrations with TNP-AMP (Figure 4). Addition of 10 mM $CaCl_2$ was indeed found to decrease the affinity of the enzyme for TNP-AMP about 3-fold. Yet, the 50% decrease in fluorescence produced by 10 mM Ca^{2+} in the presence of saturating TNP-AMP represents a real decrease in the fluorescence of the TNP-AMP–phosphoenzyme complex.

It is noteworthy that where the enzyme was phosphorylated with ATP in the absence of Mg^{2+}, the observed fluorescence was very low at all Ca^{2+} concentrations while phosphoenzyme levels were about 4.2 nmol/mg throughout (Figure 3). This indicates that the enzyme state yielding the high fluorescence of bound TNP-AMP requires Mg^{2+} in addition to phosphorylation.

Fluorescence of TNP-AMP Bound to Ca^{2+}-ATPase under Conditions of Enzyme Phosphorylation with P_i at Equilibrium. In contrast to the steady state obtained with ATP and Ca^{2+}, in which changes in pH and [KCl] may alter the relative levels of various phosphoenzyme species, the phosphoenzyme

formed with P_i in the absence of Ca^{2+} results in a single species at equilibrium. In these experiments, we included glycerol (20% v/v) to favor the formation of measurable levels of phosphoenzyme under all conditions of pH and [KCl] studied (de Meis et al., 1980). We found that both fluorescence (Figure 5A) and phosphoenzyme levels (Figure 5B) decrease with higher pH values. Inclusion of 100 mM KCl caused a decrease in phosphoenzyme levels at all pH values (Figure 5B) similar to that described in previously published work (Masuda & de Meis, 1973; Chaloub & de Meis, 1980) and a more marked decrease in the fluorescence of TNP-AMP (Figure 5A). However, where the total fluorescence was normalized to the phosphoenzyme values, the fluorescence yield of the TNP-AMP phosphoenzyme complex was found to increase with pH in the absence of K^+ (Figure 5C) similar to the pattern seen under steady-state conditions (Figure 1C). Furthermore, inclusion of 100 mM KCl caused a reduction of this parameter at all pH values (Figure 5C) as was found also under steady-state conditions (Figure 1C), though the profile is different. It should be noted that the $K_{1/2}$ values for P_i for both phosphorylation and fluorescence increase with both pH and [KCl] such that the 12 mM P_i employed becomes increasingly subsaturating. However, in separate experiments (not shown), it was found that the phosphoenzyme-dependent fluorescence was independent of the fractional saturation of the enzyme with P_i at a given pH and KCl concentration. It is concluded from the experiments of Figure 5 that the phosphoenzyme-normalized fluorescence is not constant when either the H^+ or the K^+ concentrations are changed. Since in all these conditions only one phosphoenzyme species is expected, the observed differences in TNP-AMP fluorescence must be due to factors other than shifts in the type of phosphoenzyme species present.

Fluorescence of TNP-AMP Bound to Phosphoenzyme in the Presence of Organic Solvents. A most interesting observation regarding phosphorylation of SR ATPase with P_i is that this reaction is greatly favored by the inclusion of certain organic solvents in the medium (de Meis et al., 1980). Such an effect of organic solvents has been attributed to exclusion of water from the catalytic site, and the TNP-nucleotide fluorescence enhancement has been related to such an effect of organic solvents (Dupont & Pougeois, 1983). However, we observed that the "basal" fluorescence (the small fluorescence of TNP-AMP bound to the *non*phosphorylated enzyme) is not increased significantly under the less polar conditions which favor enzyme phosphorylation by P_i, i.e., the presence of organic solvents and the absence of K^+ (Figure 6). In fact, it can be seen that inclusion of the organic solvents glycerol or dimethyl sulfoxide increases the basal fluorescence a maximum of 1.6-fold (Figure 6), whereas the $K_{1/2}$ for phosphorylation by P_i was decreased 80-fold by organic solvents, from a value of 8 mM in aqueous media to 3 mM in glycerol and to 0.1 mM in dimethyl sulfoxide at pH 7.0. The inclusion of 100 mM KCl, which increased the $K_{1/2}$ for P_i by 3–5-fold under the conditions of Figure 6, had no effect on basal fluorescence for a given solvent media (Figure 6). Furthermore, Ca^{2+} dissociation from the enzyme, which is required to render the P_i reaction possible, does not change the basal fluorescence of bound TNP-AMP (Watanabe & Inesi, 1982b; see also Figure 2). Finally, measurements of the basal fluorescence as a function of pH showed no changes from pH 6.0 to 9.0 in either the absence or the presence of 100 mM KCl while it is known that pH has a profound influence on the phosphorylation of SR ATPase with P_i. It is clear that large changes of fluorescence intensity are obtained only *after* addition of P_i and enzyme phosphorylation (Figure 6). Therefore, even though the effect of organic solvents may well be related to the perturbation of water structure on P_i and the phosphorylation site, the large enhancement of TNP-AMP fluorescence yield reports directly the conformational change produced by enzyme phosphorylation rather than the effect of experimental perturbations to favor the phosphorylation reaction.

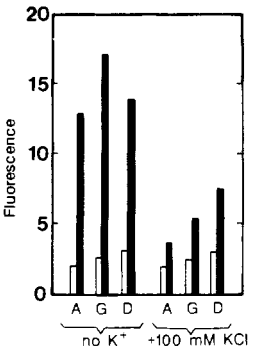


FIGURE 6: Effect of different solvents on the fluorescence of TNP-AMP bound to Ca^{2+}-ATPase under conditions of equilibrium with P_i. Fluorescence was measured at pH 7.0 in 50 mM Tris–maleate, 10 mM $MgCl_2$, 0.1 mg/mL Ca^{2+}-ATPase (leaky vesicles), and 1 mM EGTA, with the indicated additions of KCl, in aqueous medium (A) or with the inclusion of 20% glycerol (G) or 20% dimethyl sulfoxide (D). TNP-AMP was added to 1 μM to obtain the basal fluorescence (open bars). The high fluorescence (solid rectangles) was obtained by adding saturating amounts of P_i. These levels, from left to right, were 45, 20, 4, 50, 30, and 8 mM P_i, respectively.

Binding of TNP-AMP to the Phosphorylated Enzyme under Different Conditions of pH and [KCl]. Fluorescence titrations with TNP-AMP were conducted to explore the possibility that the changes in phosphoenzyme-dependent fluorescence (documented in Figures 1C and 5C) were due to changes in the amount of TNP-AMP bound to phosphoenzyme. Conditions of both high fluorescence and high phosphoenzyme levels were chosen to ensure the most accurate data, namely, the steady-state system at pH 8.5 and the equilibrium condition at pH 6.0. Addition of increasing amounts of TNP-AMP caused small to large reductions in phosphoenzyme levels under all conditions (Figure 7A,B), in accordance with the known competition between the TNP-nucleotide and ATP or P_i for the catalytic site (Nakamoto & Inesi, 1984; Bishop et al., 1984). The phosphoenzyme-normalized fluorescence titrations (Figure 7C) show a K_d of 0.4 μM for the TNP-AMP–phosphoenzyme complex at pH 8.5 and 3.3 μM at pH 6.0. Furthermore, our experiments show that K^+ decreased the affinity of TNP-AMP for the site, resulting in high fluorescence enhancement both for the steady-state phosphoenzyme at pH 8.5 (curves a vs. b) and for the phosphoenzyme from P_i at pH 6.0 (curves c vs. d). Further, it can be seen that in the absence of K^+, TNP-AMP binds with much higher affinity at pH 8.5 (curve a) than at pH 6.0 (curve c). To elucidate whether this latter shift in K_d was due to the change in pH or to the type of phosphoenzyme, experiments were conducted at pH 6.0 using native SR vesicles. Titrations of TNP-AMP under conditions of phosphorylation with P_i and with ATP in the absence or presence of 2 μM A23187 all gave K_d's and maximal values which were similar to those of curve c in Figure 7C (not shown). It is apparent from these data that the fluorescence yield of the TNP-AMP–phosphoenzyme complex is approximately the same for both phosphorylation conditions at a given pH and [K^+]. The large differences in fluorescence originally noted in Figures 1C and 5C using 1 μM TNP-AMP can therefore be accounted

Table II: Fluorescence Lifetime Parameters for TNP-AMP in Propylene Glycol and Bound to SR ATPase in Different Conditions

conditions	short component[c]			long component[c]		
	τ_1 (ns)	α_1 fraction	f_1 fraction	τ_2 (ns)	α_2 fraction	f_2 fraction
propylene glycol[a]						
0 °C	0.17	0.87	0.66	0.58	0.13	0.34
−20 °C	0.56	0.74	0.48	1.71	0.26	0.52
pH 8.5, ATP, 25 °C[b]						
0.1 mM Ca^{2+}, 0 KCl	0.23	0.77	0.38	1.24	0.23	0.62
0.1 mM Ca^{2+}, 60 mM KCl	0.21	0.81	0.41	1.27	0.19	0.59
0.1 mM Ca^{2+}, 120 mM KCl	0.22	0.83	0.44	1.30	0.17	0.56
10 mM Ca^{2+}, 0 KCl	0.33	0.91	0.65	1.79	0.09	0.35
pH 6.0, P_i, EGTA, 25 °C[b]						
0 KCl	0.35	0.75	0.43	1.37	0.25	0.57
60 mM KCl	0.28	0.82	0.47	1.43	0.18	0.53
120 mM KCl	0.30	0.83	0.49	1.49	0.17	0.51

[a] TNP-AMP was 10 μM in propylene glycol at the indicated temperature. [b] Conditions were identical with those in Figure 7 except 5 μM TNP-AMP and 0.28 mg/mL SR protein were used. The phosphenzyme level from ATP was 4.1 nmol/mg at all K^+ concentrations. For pH 6.0 + P_i, 50 mM P_i was used, and the phosphoenzyme level was 4.2 nmol/mg for 0 KCl, 3.8 nmol/mg for 60 mM KCl, and 3.6 nmol/mg for 120 mM KCl. Each experinent was done within 60 min. The stability of the enzyme system was checked independently by measuring total fluorescence intensity over an equal time period. [c] Phase (ϕ_ω) and modulation (m_ω) data were taken as described under Materials and Methods. ϕ_ω and m_ω were related to time fluorescence decay as described in detail by Lakowicz (1983). Data were fit to multiple exponential decays by using the nonlinear least-squares method as described in detail by Gratton et al. (1984) and Lakowicz et al. (1984). Expected values of $\phi_{c\omega}$ and $m_{c\omega}$ at each modulation frequency can be predicted for an assumed impulse function, $I(t)$: $I(t) = \sum_i^n \alpha_i e^{-t/\tau_i}$. $I(t)$ is the sum of the exponential decays of a mixture of fluorophores each with a single lifetime, τ_i, and a preexponential proportionality constant, α_i. The fractional steady-state intensity of each component, f_i, is given by $f_i = \alpha_i\tau_i/\sum_i \alpha_i\tau_i$. A goodness of fit between measured values and an assumed model was judged by the value of χ^2. A reduced χ^2 value near 1 was taken as a good fit. In all cases above, two components gave a good fit of data. Invoking three components did not significantly improve the reduced χ^2 but did give a third component with a short τ_3 and an f_3 equal to 0–0.1 which is consistent with the 5–10% of steady-state intensity resultant from scatter and free TNP-AMP. The standard error for each value above as given by the fit of the data was 0.01–0.05 ns for the τ components and 0.01–0.07 for α. Data were reproduced for certain conditions and did not vary by more than 10%.

for by differences in the amount of complex present as a result of pH- and K^+-induced shifts in affinity for TNP-AMP.

Fluorescence Lifetime Measurements. The experiments described above (Figure 7) demonstrate that the different degree of total fluorescence enhancement exhibited by the TNP-nucleotide following enzyme phosphorylation in various reaction mixtures does not reflect necessarily a different fluorescence yield of the TNP-AMP–phosphoenzyme complex. The complexity of measurements required to relate the fluorescence intensity to the stoichiometric fluorescence yield of the TNP-AMP–phosphoenzyme complex is apparent in the previous sections of this paper. Alternatively, we obtained an independent evaluation of whether the environment of bound TNP-nucleotide differs in various phosphoenzyme forms by measuring fluorescence lifetimes (τ_{fl}) which are very sensitive to the microenvironment of the fluorescent probe, but not to its concentration.

Lifetime measurements of TNP-AMP nucleotide bound to phosphoenzyme formed from ATP at pH 8.5 showed a complex decay that could be resolved by a biexponential with τ values of, respectively, 0.2–0.3 and 1.2–1.5 ns, with α values (preexponential factor representing the fractional contribution to the time-resolved decay of each lifetime component) of 0.75–0.83 and 0.17–0.25, respectively (Table II). It should be noted that in these experiments the fractional intensity contribution of unbound TNP-AMP and of TNP-AMP bound to nonphosphorylated enzyme is approximately 5% of the total fluorescence. Due to the very short ($\tau < 0.1$ ns) lifetimes (Nakamoto & Inesi, 1984), these low-intensity forms are not resolved as a separate component. Therefore, the biexponential decay is a result of only the TNP-AMP bound to the site(s) responsible for the enhanced fluorescence intensity of phosphoenzyme. Considering the two lifetime components, it is tempting to speculate that the probe may reside in two different sites of the phosphoenzyme or reflects a distribution of two states of phosphoenzyme. However, we obtained decays that were similarly fit to biexponentials when TNP-AMP was dissolved in propylene glycol in the absence of enzyme (Table II). It is apparent that TNP-AMP has a nonexponential decay

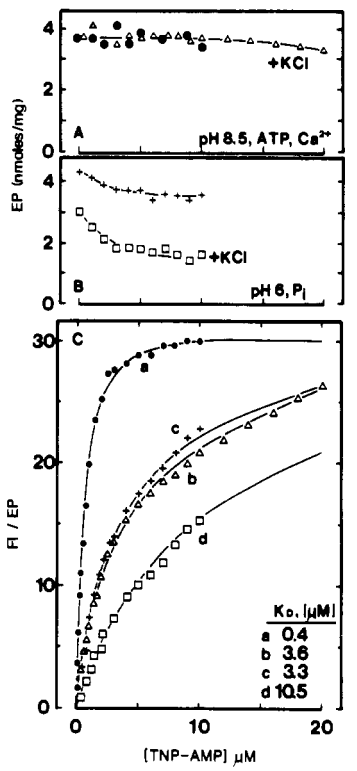
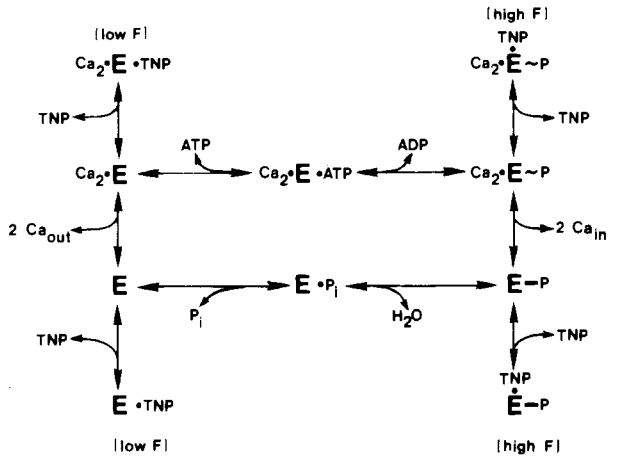


FIGURE 7: TNP-AMP binding to the Ca^{2+}-ATPase under steady-state conditions with ATP at pH 8.5 and under equilibrium conditions with P_i at pH 6.0. Measurements of ^{32}P-labeled phosphoenzyme as a function of [TNP-AMP] were conducted for the steady-state experiments (A) under the conditions of Figure 1B and for the equilibrium experiments under the conditions described in Figure 5B, both in the absence and in the presence of 100 mM KCl. Phosphoenzyme-normalized fluorescence values were calculated by dividing fluorescence (not shown) by the respective phosphoenzyme values in (A) and (B) and plotted against the total [TNP-AMP]. Steady state, in the absence (●) and presence (△) of 100 mM KCl. Equilibrium condition, in the absence (+) and presence (□) of 100 mM KCl. The solid lines are the theoretical fits to the data for single binding sites, using a binding equation utilizing the total ligand concentration (Gutfreund, 1972), with the indicated K_d values and the same maximal value of 30 units.

Scheme I



of excited states which may be due to excited-state reactions, complex solvent relaxation, or specific solvent effects [see Lakowicz (1984) for a discussion of these behaviors] and is an intrinsic property of the fluorophore because the complex decay is observed either in bulk solvent or in the microenvironment of the enzyme binding site. However, it is important to emphasize that TNP-AMP fluorescence lifetimes are sensitive to environmental conditions which is apparent by the sensitivity to the lowering of temperature when in propylene glycol (Table II). In addition, the sensitivity of TNP-AMP lifetimes to the enzyme binding site is evident by the obvious difference between phosphorylated and nonphosphorylated enzyme (Nakamoto & Inesi, 1984).

It is indicated in Table II that the lifetime characteristics of TNP-AMP bound to phosphoenzyme are not significantly changed when the enzyme is phosphorylated with P_i at pH 6 in the absence of Ca^{2+}, as compared to phosphorylation with ATP at pH 8.5 in the presence of Ca^{2+}, and are independent of the presence or the absence of K^+. Since average lifetime values can vary from a minimum of 0.03 ns for the probe in aqueous medium to a maximum of 3.4 ns, which is the intrinsic lifetime in the absence of quenching (Nakamoto & Inesi, 1984), the ±7% variation between the τ_2 values measured under the different conditions of phosphorylation, pH, and $[K^+]$ indicate a largely invariant environment for the probe on the phosphoenzyme.

A special case is that observed in the presence of high $[Ca^{2+}]$ which produced an obvious change in the environment of the probe, manifested by longer lifetimes and a shift of the fractional intensity in favor of the shorter lifetime component (Table II). This accounts for the reduction of the total fluorescence intensity by 10 mM Ca^{2+} shown in Figure 4.

Discussion

A major goal of this study was to systematically determine the response of TNP-nucleotide fluorescence intensity to variations in enzymatic states of the Ca^{2+}-ATPase. Our findings as well as those of previous studies are depicted in Scheme I and matched with the lowest number of ATPase partial reactions required to explain the findings ["TNP" represents the TNP-nucleotide and "F" fluorescence; Ca_2·E~P and E–P represent phosphoenzyme species that are capable or uncapable of reacting with ADP to form ATP; see Inesi (1985) for further details of the catalytic cycle]. An obvious and large increase in the fluorescence of TNP-nucleotide bound to SR ATPase is produced by phosphorylation and formation of the phosphorylated enzyme intermediate (Watanabe & Inesi, 1982b). The extent of this fluorescence enhancement is apparently different, depending on the conditions used for enzyme phosphorylation (Dupont & Pougeois, 1983; Nakamoto & Inesi, 1984; Bishop et al., 1984), which raised the possibility that the TNP-nucleotide may report environmental changes in the catalytic site for different phosphoenzyme species.

We report here that while the large fluorescence effect produced by phosphorylation is in fact due to a change in the environment of the probe, the variable intensities of the phosphoenzyme-dependent fluorescence reported both in this study and in previous studies are not due to the particular phosphoenzyme species present but rather to H^+- or K^+-induced dissociation of the probe, resulting in the presence of differing amounts of the highly fluorescent phosphoenzyme–TNP-nucleotide complex. This was shown by the following experiments. The equilibrium phosphoenzyme E–P (see Scheme I) formed from P_i in the absence of K^+ showed a marked change in TNP-AMP fluorescence with pH (Figure 5C) where only a single phosphoenzyme species is present, and consequently, a constant level of fluorescence would have been expected. Very similar fluorescence levels with pH were observed for the steady-state enzyme (Figure 1C) where the calcium-bound phosphoenzyme (Ca_2·E~P) predominates under most pH conditions (McIntosh & Boyer, 1983) with perhaps significant levels of E–P accumulating only at pH 6.0 (Inesi & Hill, 1983). Use of native SR vesicles, which accumulate internal Ca^{2+} to produce high amounts of Ca_2·E~P (Pickart & Jencks, 1982; Froehlich & Heller, 1985), did not produce any marked changes in TNP-AMP fluorescence, even at pH 6.0 (Table I) where the largest shift from E–P to Ca_2·E~P would be expected. Inclusion of K^+ diminished the fluorescence of the equilibrium phosphoenzyme E–P (Figure 5C), again, where only a single species is present. A similar inhibition by K^+ of fluorescence under steady-state conditions at all pH values (Figure 1C), an effect that was not altered by high intravesicular $[Ca^{2+}]$ in native vesicles (see Results), would speak against explanations for the inhibition by shifts in phosphoenzyme species. Though E–P levels would be diminished by the K^+-induced acceleration of the hydrolysis step (Chaloub & deMeis, 1980), the effects of K^+ on the translocation and internal Ca^{2+} release steps are not clear (Shigekawa & Akowitz, 1979; Bishop et al., 1984).

Though addition of millimolar Ca^{2+} concentrations to permeabilized SR vesicles has classically been used to drive the pump in the reverse direction by binding to the internal Ca^{2+} sites to form high levels of the ADP-sensitive Ca_2·E~P, the observed inhibition of TNP-AMP fluorescence under these conditions (Figure 3) cannot be explained by a decreased quantum yield of the Ca_2·E~P species. A similar inhibition was observed by high $[Ca^{2+}]$ added to sealed vesicles under conditions in which the internal Ca^{2+} sites were inaccessible to this medium $[Ca^{2+}]$ (Figure 3). Furthermore, accumulation of high intravesicular $[Ca^{2+}]$ during steady-state transport with the probable parallel increase in Ca_2·E~P in a medium of low extravesicular $[Ca^{2+}]$ did not diminish the high fluorescence (Table I). The low fluorescence observed in the absence of Mg^{2+} (Figure 3), a condition reported to cause accumulation of Ca_2·E~P (Shigekawa & Dougherty, 1978), also cannot be explained by a low quantum yield of Ca_2·E~P, for the preceding reason. Rather, we would suggest that the externally oriented TNP-nucleotide binding site requires Mg^{2+} to obtain "normal" fluorescence upon enzyme phosphorylation and that high $[Ca^{2+}]$ displaces this Mg^{2+}. The lowered quantum yield at saturating [TNP-AMP] (Figure 4) and the altered α and τ values for the fluorescence decay (Table II) might then be

a measure of the properties of this Mg^{2+}-deprived site.

The above discussion makes it clear that the observed fluorescence changes with pH and $[K^+]$ (Figures 1C and 5C) cannot be explained by shifts in the relative amounts of the different phosphoenzyme species. Rather, we have found that binding of TNP-AMP to the phosphorylated enzyme is pH and $[K^+]$ dependent, binding with highest affinity at high pH and low $[K^+]$ (Figure 7C). The maximal fluorescence at saturating [TNP-AMP] was found to be about the same under all conditions, suggesting that, if it were possible to repeat the experiments of Figures 1C and 5C at saturating [TNP-AMP] rather than at the utilized 1 μM level, no differences in fluorescence levels would be observed. The similarities in the α and τ values for fluorescence decay of the bound TNP-AMP (Table II) also support the contention that the degree of hydrophobicity/hydrophilicity of the enzyme in the region of the trinitrophenyl ring is nearly identical for the different phosphoenzyme species and is not altered by pH or $[K^+]$.

It can be noted that though changes in pH and $[K^+]$ alter the binding affinity of TNP-AMP for the phosphorylated enzyme, the fluorescence properties are the same once bound. It seems, therefore, that K^+ and H^+ must alter that portion of the binding site on the enzyme which interacts with the adenine, ribose, and/or phosphate moieties of the TNP-AMP molecule, but not that portion interacting with the trinitrophenyl group. It is of interest that pH (Yates & Duance, 1976) and $[K^+]$ (Nakamura & Tonomura, 1982) have similar effects to that of TNP-AMP on the binding affinity of ATP.

We conclude that TNP-nucleotide fluorescence does not detect phosphoenzyme interconversions, though changes undetected by the bound analogue may well occur, nor does it reflect the facility with which the enzyme may be phosphorylated (Figure 6 and Results), nor does it reflect the rate of flux through the various catalytic intermediates (Results). Rather, the fluorescence enhancement is specific for a conformation unique to, and shared by, all phosphoenzyme species (see Scheme I). Previous experiments showing a marked enhancement of solubilization of SR Ca^{2+}-ATPase upon formation of the phosphoenzyme intermediate in the presence of low levels of detergent (Watanabe & Inesi, 1982a) indicate that the enzyme transition associated with phosphorylation is rather extensive and not localized only to the TNP-AMP binding site. It may be that this major transition is involved in the allosteric coupling of phosphorylation and calcium sites during the catalytic and transport cycle.

ACKNOWLEDGMENTS

We thank Dr. Joseph Lakowicz for the use of the variable-frequency fluorometer and for his invaluable advice.

Registry No. G, 56-81-5; D, 67-68-5; TNP-AMP, 97902-38-0; ATPase, 9000-83-3; ATP, 56-65-5; X537A, 25999-31-9; Ca, 7440-70-2; K, 7440-09-7; P_i, 14265-44-2.

REFERENCES

Bishop, J. E., Johnson, J. D., & Berman, M. C. (1984) *J. Biol. Chem. 259*, 15163–15171.

Broglie, K. E., & Takahashi, M. (1983) *J. Biol. Chem. 258*, 12940–12946.

Chaloub, R. M., & de Meis, L. (1980) *J. Biol. Chem. 255*, 6168–6172.

Davidson, G. A., & Berman, M. C. (1985) *J. Biol. Chem. 260*, 7325–7329.

de Meis, L., Martins, O., & Alves, E. (1980) *Biochemistry 19*, 4252–4261.

Dupont, Y., & Pougeois, R. (1983) *FEBS Lett. 156*, 93–98.

Dupont, Y., Chapron, Y., & Pougeois, R. (1982) *Biochem. Biophys. Res. Commun. 10*, 1272–1279.

Dupont, Y., Pougeois, R., Ronjat, M., & Verjovsky-Almeida, S. (1985) *J. Biol. Chem. 260*, 7241–7249.

Eletr, S., & Inesi, G. (1972) *Biochim. Biophys. Acta 282*, 174–179.

Froehlich, J. P., & Heller, P. F. (1985) *Biochemistry 24*, 126–136.

Gratton, E., Lakowicz, J. R., Maliwal, B., Cherek, H., Laczko, G., & Limkeman, M. (1984) *Biophys. J. 46*, 479–486.

Grubmeyer, C., & Penefsky, H. S. (1981a) *J. Biol. Chem. 256*, 3728–3734.

Grubmeyer, C., & Penefsky, H. S. (1981b) *J. Biol. Chem. 256*, 3718–3727.

Gutfreund, H. (1972) in *Enzymes, Physical Principles*, p 71, Wiley-Interscience, London.

Hiratsuka, T., & Uchida, K. (1973) *Biochim. Biophys. Acta 320*, 635–647.

Inesi, G. (1985) *Annu. Rev. Physiol. 47*, 573–601.

Inesi, G., & Hill, T. L. (1983) *Biophys. J. 44*, 271–280.

Kanazawa, T., & Boyer, P. (1973) *J. Biol. Chem. 248*, 3163–3172.

Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.

Lakowicz, J. R., & Maliwal, B. (1985) *Biophys. Chem. 21*, 61–78.

Lakowicz, J. R., Gratton, E., Laczko, G., Cherek, H., & Limkeman, M. (1984) *Biophys. J. 46*, 463–477.

Lin, T., & Morales, M. (1977) *Anal. Biochem. 77*, 10–17.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McIntosh, D. B., & Boyer, P. D. (1983) *Biochemistry 22*, 2867–2875.

Meissner, G., Conner, G., & Fleischer, S. (1973) *Biochim. Biophys. Acta 298*, 246–269.

Moczydlowski, E. G., & Fortes, P. A. G. (1981a) *J. Biol. Chem. 256*, 2346–2356.

Moczydlowski, E. G., & Fortes, P. A. G. (1981b) *J. Biol. Chem. 256*, 2357–2366.

Nakamoto, R. K., & Inesi, G. (1984) *J. Biol. Chem. 259*, 2961–2970.

Nakamura, Y. (1984) *J. Biol. Chem. 259*, 8183–8189.

Nakamura, Y., & Tonomura, Y. (1982) *J. Bioenerg. Biomembr. 14*, 307–318.

Pfeiffer, D. R., Reed, P. W., & Lardy, H. A. (1974) *Biochemistry 13*, 4007–4014.

Pickart, C. M., & Jencks, W. P. (1982) *J. Biol. Chem. 257*, 5319–5322.

Shigekawa, M., & Dougherty, J. P. (1978) *J. Biol. Chem. 253*, 1458–1464.

Shigekawa, M., & Akowitz, A. A. (1979) *J. Biol. Chem. 254*, 4726–4730.

Verjovski-Almeida, S. (1981) *J. Biol. Chem. 256*, 2662–2668.

Watanabe, T., & Inesi, G. (1982a) *Biochemistry 21*, 3254–3259.

Watanabe, T., & Inesi, G. (1982b) *J. Biol. Chem. 257*, 11510–11516.

Wiggins, P. M. (1982) *J. Theor. Biol. 99*, 645–664.

Yates, D. W., & Duance, V. C. (1976) *Biochem. J. 159*, 719–728.