Studies of the Interactions of 2',3'-O-(2,4,6-Trinitrocyclohexylidienylidine)adenosine Nucleotides with the Sarcoplasmic Reticulum (Ca²⁺ + Mg²⁺)-ATPase Active Site*

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The fluorescence of TNP-nucleotides bound to sarcoplasmic reticulum ATPase is enhanced upon formation of phosphorylated enzyme intermediate either with ATP in the presence of Ca²⁺ or, to a greater extent, with Pi in the absence of Ca²⁺. Binding of the TNP-nucleotides does not occur if the ATPase is labeled at the active site with fluorescein isothiocyanate. Addition of ADP to the TNP-nucleotide-enzyme complex phosphorylated with Pi causes dissociation of TNP-nucleotide and a proportional reduction in fluorescence. These and other kinetic observations indicate that the TNP-nucleotide exchanges with ADP following enzyme phosphorylation with ATP or occupies the ADP portion of the catalytic site following enzyme phosphorylation with Pi. This interaction with the phosphorylated site results in fluorescence enhancement of the TNP-nucleotide. Comparison of the TNP-nucleotide fluorescence features in different solvents with that of the TNP-nucleotide bound to sarcoplasmic reticulum ATPase indicates that, following phosphorylation, the binding domain excludes solvent molecules and confers restricted mobility to the TNP-nucleotide. Solvent exclusion and substrate immobilization accompany, to a greater extent, phosphorylation of the active site with Pi in the absence of Ca²⁺. TNP-nucleotides bound to the catalytic sites were also found to be acceptors of resonance energy transfer from enzyme tryptophan in the extramembranous domain of the ATPase which also contains the catalytic site.

The sarcoplasmic reticulum (Ca²⁺ + Mg²⁺)-ATPase of mammalian skeletal muscle has the primary function of vectorially transporting cytosolic Ca²⁺ to its intracellular lumen. Since the ATPase is the major protein component of the SR membranes and is easily isolated in relatively pure form, this system has provided an excellent model for study of ion transport enzymes. Extensive work has elucidated a complex enzymatic cycle which is fully reversible (see Refs. 1–3 for reviews). Conformational changes associated with certain partial reactions of the cycle have been observed by the following methods: by variation in reactive residues in various enzyme states (4–9); by changes in fluorescence of intrinsic tryptophan (10) or covalently bound labels (11, 12); by covalently bound spin labels (13–16); by circular dichroism (17); and by resonance energy transfer between differently labeled populations of ATPase chains and light scattering of subliminally solubilized SR during ATPase activation (18) (see 15 and 20 for reviews).

Most of the conformational studies have concentrated on the binding of substrates and divalent cations to the enzyme sites. Very little work has been reported on the changes occurring during the reactive portions of the cycle.

In 1982, Watanabe and Inesi (21), and Dupont and Chapron (22) introduced the use of TNP-ATP for use in the study of substrate related events. This fluorescent analogue is environmentally sensitive and has proven useful in the study of myosin ATPase (23, 24), mitochondrial F,F-,ATPase (25), and (Na⁺ + K⁺)-ATPase (26). Apparently unique to the SR Ca²⁺ ATPase is the fluorescence sensitivity to the phosphorylation state of the enzyme (21). In this paper, we have further characterized and localized TNP-nucleotide interaction with ATPase catalytic sites. Also, we have utilized the fluorescence features of the TNP-nucleotide to study the structural characteristics of the nucleotide binding domain.

MATERIALS AND METHODS

SR Vesicles—These were obtained from white rabbit hind leg muscle as previously described (27). Protein concentration was determined by the method of Lowry et al. (28) using bovine serum albumin as a standard.

TNP-Nucleotides—These were synthesized according to Hiratsuka and Uchida (23) with modifications as described by Watanabe and Inesi (21). TNP-ATP, TNP-AMP, and [³⁵S]TNP-AMP were synthesized starting from the sodium salts of ADP, AMP, and [³⁵S]AMP.

Purity of the preparations was checked by thin layer chromatography on polyethyleneimine-cellulose plates (Merk) and high performance liquid chromatography analysis utilizing a Waters C18 Radial Pak reverse phase column. Purity was at least 99% with a small amount of contamination from the unreacted adenosine nucleotide. Concentrations of TNP-nucleotides were determined by using the molar extinction coefficients reported earlier (23) i.e. ε₂₅₀ = 25,400 M⁻¹ cm⁻¹ and ε₄₅₀ = 18,500 M⁻¹ cm⁻¹ in 0.1 M Tris-Cl, pH 8.0.

Fluorescein Isothiocyanate Labeling—SR ATPase was labeled with fluorescein isothiocyanate (isomer I) as described by Andersen et al. (56).

[³⁵S]TNP-AMP Binding—TNP-AMP binding was measured in equilibrium conditions by the column chromatography method described by Hummel and Dreyer (56). Bio-Gel P-10 (50–100 mesh) columns (1 × 45 cm) were equilibrated with 20 mM Tris-Cl, pH 7.5, 80 mM KCl, 5 mM MgCl₂, 0.1 mM CaCl₂, and 0.1–5 μM [³⁵S]TNP-AMP at room temperature, 0.2–0.5 mg of SR protein was loaded and the amount bound was determined by the counts associated with the protein fractions minus base-line counts.

Spectroscopy Measurements—Absorption measurements and scans were performed on an Amino DW 2 spectrophotometer. Differential absorption measurements were done as in Ref. 21.
Fluorescence measurements were performed on an Aminco-Bowman spectrophotofluorometer for the titration and addition experiments where the wavelengths of excitation and emission remained constant. A stirring device for constant agitation of the cuvette contents was added. For all TNP-nucleotide measurements, excitation was set at 410 nm and emission at 525 nm by monochrometers. Slit widths were 11 nm. Trytophan excitation was 290 nm and emission was 340 nm with 6-nm slit widths.

For fluorescence emission and excitation spectrum scans, an SLM Series 8000 Photon-Counting spectrophotofluorometer was utilized. This instrument was also used for anisotropy measurements. Steady state anisotropy was calculated utilizing Equation 1 (50).

\[
r = \frac{I_{\text{VV}} - G_{\text{VH}}}{I_{\text{VV}} + 2G_{\text{VH}}}
\]

where the fluorescence intensity with both excitation and emission polarizers in the vertical orientation; and \( I_{\text{VV}} \) is the fluorescence intensity with the excitation polarizer vertical and the emission polarizer horizontal. \( G \) is the correction factor for instrumental polarization and is calculated according to Equation 2 (50).

\[
G = \frac{I_{\text{HH}}}{I_{\text{HH}}}
\]

The excitation polarizer is horizontal and the emission polarizer vertical for \( I_{\text{VV}} \) and horizontal for \( I_{\text{HH}} \). A Corning 3-69 filter was placed in the emission path to eliminate scattering from the SR vesicle suspensions.

Fluorescence lifetime measurements were performed on a phase-modulation instrument (SLM Instruments Inc.) utilizing 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene as a lifetime reference of 1.45 ns (29). These measurements used excitation wavelength of 410 nm by monochromator and a Corning 3-69 cutoff filter without the monochromator in the emission light path.

Alternatively, measurements of extremely short or multiple component lifetimes were made on the variable frequency phase-modulation fluorimeter in the laboratory of Dr. E. Gratton of the University of Illinois, Urbana, IL. This instrument utilized a 442-nm laser excitation source with emission detected through a Corning 3-70 cutoff filter. Glycogen scatterer was used as a lifetime reference of 1.45 ns (29). These measurements used excitation wavelength of 410 nm by monochromator and a Corning 3-69 cutoff filter without the monochromator in the emission light path.

For samples with substantial light scattering, such as those containing SR vesicles, a long lifetime component was consistently observed. This component was probably due to phosphorescence from the Corning 3-70 filter in the emission light path and was resolved only by the variable frequency fluorimeter.

Rapid kinetics of fluorescence changes were measured utilizing a Dionex D-137 stopped flow spectrometer. This device is equipped with a 75-watt xenon lamp light source with a 420 nm band pass filter for excitation and a Corning 3-69 filter for emission. Experimental curves were digitized, stored, and processed by a North Star Horizon computer equipped with 64K RAM. Generally, reported data are the average from at least five individual traces. Control traces were obtained from reaction mixtures lacking the TNP-nucleotide fluorophore and these were subtracted from the experimental curves. All of these instruments had temperature-controlled cuvette holders set at 25 °C.

ATPase Phosphorylation and P, Production—ATPase phosphorylation and P; release experiments were performed from 50 or 100 μM \([{\gamma}^{32}\text{P}]\text{ATP}\) in a reaction mixture containing 20 mM Tris-Cl, pH 7.5, 80 mM KC1, 5 mM MgCl2, 0.1 mM CaCl2, 0.1 or 0.2 mg/ml of SR vesicle protein and varying concentrations of TNP-nucleotides. For reaction times less than 10 s, a Dionex D-133 Multimixing device was utilized and standardized for reaction times as described by Verjovski-Almeida et al. (30). The reactions were stopped by quenching with 3.5% trichloroacetic acid and 0.1 mM NaHPO4. Chemical analysis of the level of phosphorylated intermediate (E-P) and P; liberation were done as described (15). For experiments in which phosphorylation was obtained from P; reaction mixtures contained 20 mM Tris-Cl, pH 7.5, 20 mM MgCl2, 30 mM [32P]P; 0.1 or 0.2 mg/ml of SR vesicle protein, and varying concentrations of TNP-nucleotides. Reactions were stopped by quenching with 0.125 N perchloric acid and 2 mM NaHPO4. E-P was then measured in the same manner as with ATP reactions. All experiments were done in temperature-controlled conditions at 25 °C.

**RESULTS**

**TNP-Nucleotide Interaction with the ATPase Phosphoenzyme**—While Ca⁴⁺- dependent hydrolysis of TNP-ATP is not catalyzed at a significant rate by the SR ATPase, TNP-nucleotides do act as reporters of ATPase activity (21). It is shown in Fig. 1 that a slight enhancement of TNP-nucleotide fluorescence intensity is observed upon TNP-ATP binding to the enzyme; a 3- to 4-fold further enhancement is then obtained upon addition of ATP in the presence of Ca⁴⁺. After depletion of ATP, the fluorescence intensity returns to the level observed in the absence of enzyme activity.

The Ca⁴⁺ requirement for the ATP-induced fluorescence

![Fig. 1. TNP-ATP fluorescence intensity changes in the presence of SR ATPase induced by various ligands.](http://www.jbc.org)
enhancement of bound TNP-ATP suggests that the effect is related to the formation of acid-stable phosphoenzyme intermediate, since transfer of the ATP \( \gamma \)-phosphate to the enzyme is also \( \text{Ca}^{2+} \)-dependent (31, 32).

We find now that TNP-nucleotide fluorescence increase (Fig. 2) is also obtained when the phosphoenzyme is formed by reacting SR ATPase with \( P_i \) (33, 34). In this case, no fluorescence increase is obtained in the presence of \( \text{Ca}^{2+} \) and/or in the absence of \( \text{Mg}^{2+} \), consistent with the requirements for phosphorylation of SR ATPase with \( P_i \) (35). Also, the amplitude of the fluorescence enhancement follows a similar pH profile to phosphoenzyme formation (33). Maximal fluorescence enhancement was obtained with a pH approaching 6.0, which is the optimal pH for phosphoenzyme formation from \( P_i \).

It is of interest that a fluorescence increase similar to that observed with TNP-ATP can be also observed with TNP-ADP and TNP-AMP. In fact, the greatest fluorescence increase upon addition of ATP is in the presence of \( \text{Ca}^{2+} \) is obtained with TNP-AMP (Fig. 3a). TNP-ADP and TNP-ATP give lower fluorescence intensities at all concentrations measured.

Since the site of TNP-nucleotide binding which results in enhanced fluorescence intensity is associated with the phosphorylation reaction, we measured phosphoenzyme levels and repeated the fluorescence increase in relation to these levels as shown in Fig. 3, 5 and c. We found that TNP-nucleotides lower the E-P levels consistent with competitive inhibition of ATP binding and ATPase activity (see Fig. 5a with respect to ATPase, and Figs. 12 and 13 with respect to inorganic phosphate reactions). Interestingly, in the plots of fluorescence changes corrected for E-P level, TNP-AMP shows the highest fluorescence enhancement upon enzyme phosphorylation with ATP.

In analogous plots of fluorescence enhancement of TNP-nucleotides by addition of \( P_i \) it is shown in Fig. 4a that the highest enhancement is with TNP-ATP. However, we also note that TNP-AMP is the strongest inhibitor of the P, reaction (Fig. 4b). In Fig. 4c, the fluorescence enhancement is corrected for phosphoenzyme level, and again, TNP-AMP yields the highest fluorescence level. The differences in strength of inhibition and fluorescence response are likely related to steric factors and/or polar contributions of the phosphate moieties in TNP-AMP, TNP-ADP, and TNP-ATP.

An important feature of these experiments is that a much greater fluorescence enhancement is obtained when the TNP-AMP enzyme complex is phosphorylated with \( P_i \) in the absence of \( \text{Ca}^{2+} \), as compared to ATP in the presence of \( \text{Ca}^{2+} \). It is shown in Fig. 5 that addition of ATP or \( P_i \) is followed by formation of similar phosphoenzyme levels which decay in the case of ATP and remain stable in the case of \( P_i \), owing to the steady state or equilibrium character of the reaction with the former or the latter substrate, respectively. The addition of substrate is accompanied by loss of approximately 50% of the bound analogue in either case, while the fluorescence intensity is increased twice in the case of ATP, and eight times in the case of \( P_i \). It is noteworthy that addition of ADP to the TNP-AMP enzyme complex phosphorylated with \( P_i \) does not change significantly the phosphoenzyme level, while causing a significant dissociation of TNP-AMP and a proportional reduction in fluorescence.

The observations described above suggest that the TNP-nucleotides bind to the ADP portion of the catalytic site. This was unambiguously proven by measuring binding of \(^{34} \text{C} \)TNP-AMP by equilibration with SR in molecular sieve chromatography columns. It was found that binding occurred with

Fig. 3. a, maximal TNP-nucleotide fluorescence change upon addition of 50 \( \mu \text{M} \) ATP. 0.1 mg/ml of SR vesicle protein was preincubated in 20 mM Tris-Cl, pH 7.5, 50 mM KCl, 5 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), and 0.5-10 \( \mu \text{M} \) TNP-nucleotide at 25 °C. ATP was added and the maximal fluorescence change recorded. Excitation wavelength was 410 nm and emission was 525 nm with 11-nm slit widths. □, TNP-AMP; △, TNP-ADP; and ×, TNP-ATP. b, the corresponding phosphoenzyme levels after 15-s reaction time with 50 \( \mu \text{M} \) \([\gamma-\text{P}]\text{ATP}\) in the presence of TNP-nucleotides. The conditions were identical to a. The reaction was quenched after 15 s with ice-cold 3.5% trichloroacetic acid and 0.1 mM NaHPO\(_4\). E-P was measured as described under "Materials and Methods." c, the fluorescence intensities of a divided by the phosphoenzyme levels in b.
SR ATPase Interaction with TNP-Nucleotides

**FIG. 4.** a, fluorescence intensity levels of TNP-nucleotides after addition of 30 mM H₃PO₄, pH 7.5, in the presence of 0.1 mg/ml of SR vesicle protein, 20 mM Tris-Cl, pH 7.5, 20 mM MgCl₂, and 1 mM EGTA at 25 °C. Excitation wavelength was 410 nm and emission was 525 nm with 11-nm slit widths. □, TNP-AMP; △, TNP-ADP; and ×, TNP-ATP. b, corresponding phosphoenzyme levels from [³²P]P, at equilibrium with conditions as in a. The reaction was stopped after 5 min with ice-cold 0.125 N perchloric acid plus 2 mM NaHPO₄. E-P was measured as described under "Materials and Methods." c, the fluorescence intensities of a divided by phosphoenzyme levels in b.

**FIG. 5.** The fluorescence intensity of 1 μM TNP-AMP, E-P level, and bound TNP-AMP upon addition of ATP (A) or P (B). In A, 0.1 mg/ml of SR vesicle protein was in 20 mM Tris-Cl, pH 6.0, 80 mM KCl, 5 mM MgCl₂, 0.1 mM CaCl₂, and 1 μM TNP-AMP at 25 °C. At the time represented by the arrow, 100 μM [γ-³²P]ATP was added. In B, conditions were the same except the buffer contained 20 mM Tris-Cl, pH 6.0, 20 mM MgCl₂, 1 mM EGTA, and 1 μM TNP-AMP; and at the arrow, 20 mM H₃PO₄, pH 6.0, was added followed by 1 mM ADP. Measurements of fluorescence and E-P were done as in Figs. 3 and 4, and bound TNP-AMP was determined by differential absorption spectroscopy as described in Ref. 21.

**FIG. 6.** TNP-ATP emission spectra. 1, 5 μM in dimethylformamide; 2, 5 μM in 20 mM Tris-Cl, pH 7.5; 3, 1 μM in 20 mM Tris-Cl, pH 7.5, 20 mM MgCl₂, and 1 mM EGTA with 0.2 mg/ml of SR protein; 4, same as 3 except 50 mM P was added. Excitation wavelength was 410 nm. Spectra were taken in absence of TNP-ATP and subtracted as blanks. Fluorescence intensity of spectrum 1 is relative to 2, and spectrum 3 is relative to 4. All spectra were taken at 25 °C.
a stoichiometry of 5.9 ± 0.4 nmol/mg of protein for analogue concentrations between 1 and 5 μM. However, if the SR ATPase was covalently labeled with fluorescein isothiocyanate at the catalytic site (57), no significant binding was detected.

Fluorescence Properties of TNP-Nucleotides Bound to ATPase—Interesting information is obtained when the fluorescence properties of TNP-nucleotide dissolved in various solvents, or bound to SR ATPase are compared. Emission spectra (Fig. 6) show a blue shift of the emission peak (from 545 to 520 nm) when TNP-nucleotide is in the organic solvent, dimethylformamide, relative to being in aqueous buffer (solid lines). The peak shift is accompanied by an order of magnitude increase in fluorescence intensity. An equivalent blue shift and increase of fluorescence intensity is observed with dioxane as the solvent. TNP-ADP and TNP-AMP show similar results.

In the presence of phosphorylated enzyme, the emission lifetime of TNP-nucleotide is expected to be short (26). In fact, the lifetime of the probe free in aqueous buffer was measured to be less than 0.03 ns (Table I). The limiting lifetime was obtained by dissolving TNP-AMP in propylene glycol at −50 °C in which no molecular reorientation occurs during the lifetime of the excited state due to the vitrified nature of the solvent. The measured lifetime was 3.4 ns and became shorter with increasing temperature along with a red shift in emission maximum and a decrease in quantum yield (not shown). Noteworthy is the lifetime of TNP-AMP in dimethylformamide or dioxane which was 0.1−0.2 ns. These results indicate that the fluorescence lifetime of TNP-nucleotide is more sensitive to the viscosity effect of the propylene glycol than the reduced polarity of dimethylformamide (ε = 36) or dioxane (ε = 2.2).

The solvent effects on the fluorescence lifetime of the TNP-nucleotide are applied to derive information from lifetime values for TNP-AMP bound to the ATPase. The measured lifetime of TNP-AMP bound to unphosphorylated enzyme was 0.1 ns and increased to 0.8 ns with phosphorylation of the enzyme by the addition of 50 mM inorganic phosphate in the presence of EGTA. These results indicate a restricted environment of the TNP-nucleotide bound to phosphoenzyme as opposed to the unphosphorylated enzyme.

Polarization measurements of TNP-AMP bound to phosphoenzyme also suggest occupancy of a restricted environment since the anisotropy at 500-nm wavelength was only slightly less than the limiting anisotropy in propylene glycol at −50 °C (Fig. 7). Considering the lifetime of TNP-AMP

**Table I**

Fluorescence lifetimes of TNP-AMP with SR ATPase or in various solvents

| Condition | τa (ns) |
|-----------|--------|
| pH 7.5, Tris-Cl buffer | <0.03 |
| pH 6.0 + SR vesicles | 0.14 ± 0.02 |
| pH 6.0 + SR vesicles + P | 0.81 ± 0.02 |
| Dimethylformamide | 0.14 ± 0.02 |
| Dioxane | 0.1−0.2 |
| Propylene glycol | 3.37 ± 0.02 |
| −50 °C | 0.32 ± 0.02 |
| 0 °C | 0.18 ± 0.02 |

* 40 mM Tris-Cl, pH 7.5, 15 μM TNP-AMP; 25 °C.
* 20 mM Tris maleate, pH 6.0, 20 mM MgCl₂, 1 mM EGTA, 0.25 mg/ml of SR vesicle protein; 5 μM TNP-AMP; 25 °C.
* Same as * except 50 mM H₃PO₄, pH 6.0, was added.
* 10 μM TNP-AMP in dimethylformamide, 25 °C.
* 10 μM TNP-AMP in dioxane, 25 °C.
* 10 μM TNP-AMP in propylene glycol.

**Fig. 7.** Anisotropy spectra of 5 μM TNP-AMP in propylene glycol (○), −50 °C, or with phosphoenzyme (×). 2 μM TNP-AMP with phosphoenzyme was 0.25 mg/ml of SR vesicle protein in 20 mM Tris maleate, pH 6.0, 20 mM MgCl₂, 1 mM EGTA, and 50 mM P, at 25 °C. Emission wavelength was 530 nm with a Corning 3-69 filter in the emission light path. Excitation slit width was 4 nm.

**Fig. 8.** Transient kinetic experiments of E-P formation (○), P release (×), and TNP-AMP fluorescence intensity (— — —) upon addition of ATP. 0.2 mg/ml of SR protein was preincubated in 20 mM Tris-Cl, pH 7.5, 80 mM KCl, 5 mM MgCl₂, 0.1 mM CaCl₂, and 1.5 μM TNP-AMP (final concentrations) at 25 °C. 50 μM [γ-³²P]ATP was rapidly mixed in either the rapid quench apparatus for E-P and P measurement, or the stopped flow apparatus for fluorescence measurements as described under "Materials and Methods." The P, release scale is reported ½ of actual value. Δ is TNP-AMP fluorescence level divided by E-P.
bound to phosphoenzyme (0.8 ns) and using the Perrin equation

$$\frac{r_0}{r} = 1 + \frac{t_0}{\phi}$$

in which \(r_0\) is the limiting anisotropy in a vitrified solution, \(t_0\) is the fluorescence lifetime of the probe, and \(\phi\) is the rotational correlation time of free probe in solution (0.15 ns at 25 °C by the Stokes-Einstein equation), the calculated anisotropy of TNP-AMP when bound to the enzyme but motionally free would be 0.05. Our measured value of 0.32 for TNP-AMP bound to phosphoenzyme, as compared to 0.34 in a vitrified solution, indicates restricted movement of TNP-AMP in the binding site.

The anisotropy of TNP-AMP free in buffer or bound to unphosphorylated enzyme shows no depolarization. However, the lifetimes in these conditions are less than the rotational correlation time so the excited state is expected to decay before substantial rotation occurs.

**Kinetic Observations**—In order to establish the time sequence of enzyme phosphorylation by ATP or Pi, and fluorescence enhancement of bound TNP-nucleotides, we performed parallel series of rapid quench and stopped flow experiments.

Fig. 8 shows the transient kinetic measurements of E-P, Pi release, and TNP-AMP fluorescence when 0.2 mg/ml of SR vesicle protein and 1.5 μM TNP-AMP (final concentrations) in the presence of 0.1 mM CaCl₂ are rapidly mixed with 50 μM [γ-32P]ATP. The TNP-AMP fluorescence increases with a \(t_0\) of 1.5 s and closely follows E-P formation. If we replot the fluorescence intensity relative to E-P level, the fluorescence quickly reaches maximal level (\(t_0 = 0.25\ s\)) and maintains that level. It is apparent that the fluorescence enhancement occurs in parallel with enzyme phosphorylation, with the exception of an initial burst of phosphorylation which is not accompanied by fluorescence changes, and is observed in the presence of non-saturating TNP-AMP concentrations. The size of the burst is inversely proportional to the TNP-AMP concentration (Fig. 9a) and is likely related to the binding competition of ATP and TNP-AMP for the enzyme (phosphorylation) sites. Assuming a relatively slow exchange of TNP-AMP among the sites (see “Discussion”), the rapid phase reflects the percentage of sites which are not occupied by the analogue and are available for ATP binding and rapid

![Fig. 9](http://www.jbc.org/)
E-P formation. P_i release also reflects this behavior with an initial fast phase ending soon after the E-P burst and followed by slower steady state rates (Fig. 9b). These observations indicate that fluorescence enhancement is due to TNP-AMP exchange with ADP, following enzyme phosphorylation by ATP.

When we attempted to measure the initial rate of fluorescence enhancement by adding TNP-AMP to phosphoenzyme previously formed by a brief preincubation with ATP, we found that the fluorescence rise occurred in less than 50 ms and then decreased to a lower steady state as the level of phosphoenzyme decayed (Fig. 10).

We also studied the behavior of TNP-AMP fluorescence under conditions of enzyme phosphorylation with P_i, in the absence of Ca^{2+}. These experiments are somewhat simpler than those with ATP owing to the equilibrium, rather than steady state, conditions which are permitted by the P_i reaction.

The time course of E-P formation and fluorescence increase following addition of 30 mM [32P]P_i to SR vesicles preincubated with 1 mM TNP-AMP is shown in Fig. 11. In analogy with ATP experiments described above, enzyme phosphorylation with P_i occurs with a rapid phase which is not accompanied by fluorescence increase, and then by a slower phase accompanied by fluorescence enhancement. The initial phosphorylation burst is evidently due to rapid reaction of the fraction of sites not occupied by the TNP-nucleotide. This pattern is related to binding competition of TNP-AMP with P_i, as independently shown by measuring the phosphoenzyme equilibrium level as a function of P_i concentration in the absence and in the presence of TNP-AMP (Fig. 12).

When TNP-AMP is rapidly mixed to SR vesicles preincubated with 30 mM P_i, the fluorescence enhancement occurs within the mixing time (1 ms), and then a rapid decay of both fluorescence and E-P levels is observed (Fig. 13), due to competition of the analogue with P_i for the binding sites and inhibition of phosphoenzyme formation.

We also attempted to obtain the TNP-AMP fluorescence increase utilizing vanadate ion in place of P_i. Vanadate is known to form at a low rate, a stable complex with Na^+ + K^+ (37-39) and Ca^{2+} (40-42) ATPase. It is shown in Fig. 14 (A) that vanadate in the presence of 1 mM TNP-AMP results in a slow increase in TNP-AMP fluorescence. The fluorescence increase by vanadate is approximately 20% of that obtained with 50 mM P_i. Owing to the strong competition of vanadate with P_i (42), addition of vanadate to SR vesicles in the presence of both TNP-AMP and 30 mM P_i results in a decay of the TNP-AMP fluorescence to a final level corresponding to that produced by vanadate (Fig. 14, (B)).

The lower fluorescence enhancement produced by vanadate, as compared to P_i, may be related to the analogy of the vanadate-ATPase complex to a pentacoordinated transition state of P_i, rather than to the actual covalent phosphorylation state. Therefore, steric differences as well as variation in the hydrophobic character of the local environment, may account for the lower vanadate enhancement of the fluorescence of bound TNP-AMP.

Sensitization of Bound TNP-Nucleotide by Energy Transfer from ATPase Tryptophans—Overlap between the fluorescences.
cense emission spectrum of ATPase tryptophans and the TNP-nucleotide absorbance spectrum (Fig. 15) raises the possibility of energy transfer from tryptophans to TNP-nucleotides. Energy transfer was in fact observed by scanning excitation wavelengths and recording 525 nm emission. In these measurements, TNP-AMP in the presence of SR vesicles shows an additional excitation peak at 290 nm, which corresponds to tryptophan excitation (Fig. 16). This peak (290 nm) also responds to phosphorylation of the enzyme showing a fluorescence enhancement.

It was also found that the ATPase tryptophan fluorescence is quenched by TNP-nucleotides (Fig. 17a). Plots of the reduction of tryptophan fluorescence as a function of TNP-nucleotide concentration (Fig. 17b) show two components. The first component is obtained at low TNP-nucleotide concentrations and is related to binding of 1 nucleotide/ATPase unit, as previously shown by direct measurements of binding (21, 22). The second component is observed at much higher TNP-nucleotide concentrations and is due to inner filtering effects.

These same results are obtained when the membranous tryptophans are quenched (approximately 70% of total tryptophan fluorescence) by energy transfer to the lipophilic Ca$^{2+}$ ionophore, X-537A (43). This indicates that the tryptophan(s) affected by TNP-nucleotide constitutes a limited population. On the other hand, no significant tryptophan quenching is observed with water-soluble agents (i.e. acrylamide) excluding their presence at the water interface.

**DISCUSSION**

We have observed interaction of TNP-nucleotides with SR Ca$^{2+}$-ATPase in two distinct conditions. The first condition is related to TNP-nucleotide binding to ATP sites of the enzyme in the absence of phosphorylating substrates and is characterized by a relatively low fluorescence intensity. This interaction also results in inhibition of substrate binding. The second condition is related to TNP-nucleotide interaction with the phosphorylated enzyme and is characterized by a large increase in fluorescence yield of the bound TNP-nucleo-
tide. Although interaction of the TNP-nucleotides with the phosphoenzyme may be due to binding of the analogue to a site external to the ATPase active site, our present experiments can be most simply explained by binding of TNP-nucleotides to the phosphorylated enzyme site in exchange for ADP. This is supported by the best fit of the monophasic (as compared to di- or triphosphate) species into the site holding phosphate, by the competitive inhibition of the TNP-nucleotides with the phosphorylating substrates (Figs. 9 and 12), and by the TNP-AMP dissociation and fluorescence reduction induced by ADP on the enzyme phosphorylated with P, (Fig. 5). Furthermore, fluorescein isothiocyanate labeling of the enzyme, while allowing phosphorylation by P, does not allow TNP-AMP binding. A similar mechanism of TNP-ATP binding has been proposed for the (Na + K+)-ATPase by Moczdykowski and Fortes (26).

Our experiments indicate unambiguously that TNP-AMP and ADP compete for binding at the same portion of the catalytic site. On the other hand, the TNP-AMP competition with P, is more complex, although it can still be overcome by increasing the P, concentration. It is therefore apparent that P, (or -P) and ADP are accepted by the catalytic site with an ideal fit, while the TNP moiety of the analogue produces a nonideal fit with Pi (Fig. 3). The fluorescence enhancement of bound TNP-nucleotides upon enzyme phosphorylation is likely due to a change of the enzyme site, as clearly reflected by the fluorescence properties showing displacement of at least the trinitrophenyl moiety into a hydrophobic pocket. As the affinities of the various TNP-nucleotides for the phosphoenzyme do not differ greatly (Figs. 3c and 4c), differences in the relative fluorescence intensities are likely due to specific "fits" of the TNP-nucleotides into the binding site.

The rapid appearance of the intensity signal upon addition of TNP-AMP to phosphoenzyme (Figs. 10 and 13) indicates that this phenomenon occurs simultaneously with binding. On the other hand, TNP-AMP binding to the nonphosphorylated enzyme does not yield the highly fluorescent signal. Therefore, the enzyme transition needed to create the hydrophobic pocket for fluorescence enhancement of the TNP moiety must occur as a consequence of phosphorylation, even in the absence of TNP-nucleotides. This transition is then revealed by the TNP-nucleotide fluorescence signal.

It is of interest that the fluorescence rise per mol of TNP-AMP enzyme complex is much greater when phosphorylation is obtained with P, in the absence of Ca++, as compared with ATP in the presence of Ca++ (Fig. 5). This reflects a specific adaptation of the catalytic site to the phosphorylation reaction obtained in the two conditions and suggests that the enzyme cycle initiated by utilization of ATP includes two sequential forms of phosphoenzyme (53) i.e. one in the presence and the other, in the presence of Ca++.

An enzyme transition (low fluorescence enhancement to high enhancement) is also induced by vanadate binding to the ATPase. As suggested by functional studies (40-42, 51) and demonstrated by Wlodaver et al. (52) for RNase A by neutron diffraction, vanadate produces a complex with the enzyme which is an equivalent of a pentacoordinate phosphate transition state.

We also note that TNP-AMP dissociation from the nonphosphorylated enzyme is rather slow. The diphasic kinetics of phosphoenzyme formation obtained upon addition of ATP or P, to the enzyme in the presence of nonsaturating concentrations of TNP-nucleotides (Figs. 9 and 11) suggest a slow exchange of TNP-nucleotides among the sites relative to the phosphorylation rate (150 s−1). The exchange should be limited essentially by the TNP-nucleotides off constant. These observations are possibly in parallel with the slow dissociation of ATP (37 s−1) estimated by Pickart and Jencks (44). Information about the environment of the nucleotide binding site may be inferred from studies on solvent effects on the fluorescence properties of TNP-nucleotides in solution. The polarity of the solvent should be considered in this regard, since increases in quantum yield and blue shifts in the emission spectra are produced by a decrease in dielectric constant. Hiratsuka (36) has reported the correlation of the TNP-nucleotides' quantum yield and emission maxima to a solvent polarity index as proposed by Kosower (45). However, propylene glycol or glycerol as solvents do not follow the polarity correlation. Solvent viscosity is another factor which may affect the fluorescence properties of TNP-nucleotides (26, 36). Hiratsuka (36) also reported a shift of the fluorescence emission peak and an increase in quantum yield with increasing viscosity.

In our studies, we found that a parameter differentiating solvent polarity and viscosity effects is the fluorescence lifetimes of the TNP-nucleotide excited states. In fact, we found that this parameter is affected much more by viscosity than by changes in solvent polarity (Table I).

These observations suggest the TNP-nucleotide bound to the phosphoenzyme site of the TNP-enzyme which excludes polar solvent molecules since the fluorescence yield is greatly increased and the emission peak is blue shifted to almost the same extent as when TNP-nucleotides are dissolved in dimethylformamide. Furthermore, the increased lifetime indicates that the phosphoenzyme site restricts molecular rearrangement of both the probe and polar groups (including amino acid side chains) within the site in the same manner that increased viscosity restricts rearrangement. The restriction in mobility is also suggested by the anisotropy of the fluorophore bound to the phosphorylated enzyme.

As opposed to the restricted environment of the TNP-nucleotide bound to phosphoenzyme, only a small fluorescence increase and emission maximum blue shift and only a slight increase in fluorescence lifetime are observed when the TNP-nucleotide is bound to the nonphosphorylated enzyme. Clearly, the nucleotide site in the absence of phosphorylation is in a much more relaxed conformation, much more open to solvent molecules. These conclusions are supportive of current ideas on substrate fit and solvent exclusion as a mechanism for efficient catalysis of phosphoryl transfer reactions (46). Specifically, changes in solvent polarity have been found to influence phosphorylation of SR ATPase with P, (54).

We also attempted to relate intrinsic protein markers (i.e. tryptophans) to the changes of the nucleotide binding site. The fluorescence energy transfer between ATPase tryptophans and bound TNP-nucleotide is of particular interest, owing to the possible localization (at least in part) of this effect to the single tryptophan of fragment 3 (47) in the sequenced (or extramembranous) portion of the ATPase. This tryptophan is within the same polar segment of the amphiphilic ATPase molecule in which the phosphorylation site is contained and is 37 residues away from the reactive lysine which is known to block ATPase activity after linkage to fluorescein isothiocyanate (11, 48).

At this time, we cannot say that this tryptophan is the only contributor to these fluorescence changes. However, the transfer effect is retained following 70% quenching of intrinsic fluorescence by action of the ionophore X-557A on tryptophan residues in the membranous portions of the protein. This heterogeneity of the tryptophan residues, with respect to their susceptibility to quenching, and the relative specificity of their involvement in energy transfer with TNP-nucleotides suggest that fluorescence measurements may be very useful in further
characterization of changes occurring during the catalytic and transport cycle of SR ATPase.

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