Phosphoenzyme Conformational States and Nucleotide-binding Site Hydrophobicity following Thiol Modification of the Ca\textsuperscript{2+}-ATPase of Sarcoplasmic Reticulum from Skeletal Muscle*  

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Enhanced fluorescence of the ATP analogue 2',3'-O-(2,4,6-trinitrocyclohexyldienylidine)adenosine 5'-triphosphate (TNP-ATP), bound to the Ca\textsuperscript{2+}-ATPase of skeletal muscle sarcoplasmic reticulum, is closely related to phosphoenzyme levels (Bishop, J. E., Johnson, J. D., and Berman, M. C. (1984) J. Biol. Chem. 259, 15163–15171) and has an emission maximum consistent with decreased polarity of the TNP-ATP-binding site. The phosphoenzyme conformation responsible for increased nucleotide-binding site hydrophobicity has been studied by redistribution of phosphoenzyme intermediates following specific thiol group modification. N-Ethylmaleimide, in the presence of 50 mM Ca\textsuperscript{2+}, 1 mM adenyI-5'-yI imidodiphosphate, pH 7.0, at 25°C for 30 min, selectively modified the SH group essential for phosphoenzyme decomposition, which resulted in decreased ATPase activity, Ca\textsuperscript{2+} uptake, and a decrease in ATP-induced TNP-ATP fluorescence. Phosphorylated (Ca\textsuperscript{2+}, Mg\textsuperscript{2+})-ATPase levels from [γ-\textsuperscript{32P}]ATP remained relatively unaffected (3.1 nmol/mg), but the ADP-insensitive fraction decreased from 56 to 15%. Phosphoenzyme levels from \textsuperscript{32P} were also decreased to the same extent as turnover, with equivalent loss of P\textsubscript{i}-induced TNP-ATP fluorescence. The E\textsubscript{1} to E\textsubscript{2} transition, as monitored by the change in intrinsic tryptophan fluorescence, was unaffected. Modification of thiol groups of unknown function did not modify turnover-induced TNP-ATP fluorescence. It is concluded that the ADP-insensitive phosphoenzyme, E\textsubscript{2}-P, is responsible for enhanced TNP-ATP fluorescence. This suggests that the conformational transition, 2Ca\textsuperscript{2+}E\textsubscript{1}-P = 2Ca\textsuperscript{2+}E\textsubscript{2}-P, is associated with altered properties of the noncatalytic, or regulatory, nucleotide-binding site.

Energy transfer between the scalar reaction of ATP hydrolysis and vectorial transfer of Ca\textsuperscript{2+} across sarcoplasmic reticulum membranes is believed to occur while Ca\textsuperscript{2+} ions are bound to phosphorylated intermediates of the catalytic cycle. The mechanism of the energy-transducing step is, as yet, unknown. However, it is believed that at least two conformational states, E\textsubscript{1} and E\textsubscript{2}, are involved. The former reacts with ATP, but not with P\textsubscript{i} and H\textsubscript{2}O, while the latter reacts with P\textsubscript{i} and H\textsubscript{2}O, but not with ATP. Isomerization is accompanied by a change in affinity and orientation of Ca\textsuperscript{2+} transport sites (for review, see Tanford (1)). A catalytic cycle compatible with the above is shown in Scheme I.

Catalytic activity of vesicular and purified Ca\textsuperscript{2+}-ATPase shows a complex dependence on substrate concentration. Following saturation of the catalytic site in the micromolar range, ATP at millimolar concentrations further accelerates turnover, an effect postulated to be due to acceleration of the rate-limiting E\textsubscript{2} to E\textsubscript{1} transition (2, 3). ATP may also affect other partial reactions of the catalytic cycle, in addition to its role as a true substrate. McIntosh and Boyer (4) have shown that ATP modulates the P\textsubscript{i} ⇌ H\textsuperscript{3+}OH exchange reaction, involving steps 6 and 7 of Scheme I. It is uncertain whether this modulation is caused by binding of ATP to an allosteric regulatory site or to the phosphorylated catalytic site following release of ADP from E\textsubscript{2}-P ADP.†

The ATP analogue TNP-ATP is a useful probe of nucleotide-binding sites and of changes in the environment of these sites during catalysis by the Ca\textsuperscript{2+}-ATPase. TNP-ATP binds to both catalytic and noncatalytic sites (5). Binding to nonphosphorylated SR vesicles in the absence of substrate is associated with a moderate increase in fluorescence. However, TNP-ATP fluorescence is increased 10-fold following E-P formation, caused by the addition of either ATP plus Ca\textsuperscript{2+} or P\textsubscript{i} in the absence of Ca\textsuperscript{2+}. The fluorescence level is related quantitatively and dynamically to E-P levels (5, 6). It is uncertain which of the ATP-binding sites is responsible for enhanced TNP-ATP fluorescence. The probe has been suggested to bind to the adenine nucleotide-binding site of the phosphorylated species after ADP has been released in the forward reaction (6). Dupont and Pougeois (7) have also suggested that the increase in fluorescence indicates that H\textsubscript{2}O molecules are expelled from the nucleotide-binding site as part of the catalytic cycle (7).

The abbreviations used are: E\textsubscript{1}−P and E\textsubscript{2}−P, phosphorylated intermediate forms of E\textsubscript{1} and E\textsubscript{2}, conformations, respectively; TNP-ATP, 2',3'-O-(2,4,6-trinitrocyclohexyldienylidine)adenosine 5'-triphosphate; NEM, N-ethylmaleimide; EGTA, [ethylene bis(oxyethyl)enitrilo]tetraacetic acid; E-P, phosphorylated (Ca\textsuperscript{2+}, Mg\textsuperscript{2+})-ATPase; AMP-P\textsuperscript{(NH)}\textsubscript{2}H, adenyI-5'-yI imidodiphosphate; Mops, 4-morpholinoethanesulfonic acid; SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles.

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Bishop et al. (8) suggested that all phosphoenzyme intermediates are related to enhanced TNP-ATP fluorescence based on conditions that result in the accumulation of different E-P species and on TNP-ATP fluorescence lifetime studies. Alternatively, the rate of increase of TNP-ADP fluorescence is closely associated with the rate of formation of the ADP-insensitive phosphoenzyme (E2-P) at low temperatures (9). TNP-ATP fluorescence is sensitive to K+. Increases in the concentration of monovalent cation salts decrease fluorescence in a manner that has similar specificity and affinity to that of the monovalent cation salt that accelerates ATP hydrolysis and enhances E-P decomposition (10). Potassium binding enhances E2-P to E1-P conversion (11) and accelerates E2-P hydrolysis, the latter step being rate-limiting for catalysis (12). Both of these actions favor a decrease in E2-P with constant levels of total E-P (13). An alternative explanation, suggested by Bishop et al. (8), is that K+ decreases the affinity of the noncatalytic site for trinitrophénylated ATP derivatives.

Kawakita et al. (14) have characterized discrete classes of thiol groups related to formation (SH2) and decomposition (SH3) of E-P species. These groups may be specifically labeled by treatment of SRV with N-ethylmaleimide (NEM) in the presence or absence of a nonhydrolyzable analogue of ATP and Ca2+. In the absence of the ATP analogue, those thiol groups necessary for E-P formation from ATP (SHP) are also blocked. The presence of an ATP analogue is necessary for protection of the SH5 group and to specifically label SHO (14, 15). Modification of SH5 thiol groups inhibits the ADP-sensitive (E1-P) to ADP-insensitive (E2-P) transition, with a net accumulation of E1-P in the forward reaction (14, 15). Nakamura and Tonomidou (16) have recently shown that, following SHO modification, Ca2+ is occluded in the ADP-sensitive conformation and that E2-P formation, in the forward reaction, is inhibited, suggesting that this is a key event of the translocation process.

The aim of the present experiments was to favor accumulation of E1-P by blocking those thiol groups (SH5) that are specifically involved in E-P decomposition and to relate the effects of this blockade to enhanced TNP-ATP fluorescence. In this study, derivatization of SH5 thiol groups with NEM was found to inhibit both ATP- and P4-induced TNP-ATP fluorescence. The significance of this phenomenon is explored with respect to the mechanism of catalysis and energy transduction by the Ca2+-ATPase.

EXPERIMENTAL PROCEDURES

N-Ethylmaleimide was a product of Sigma; and N-ethyl-2-3H) maleimide (50 Ci/mmol; 95–96% radiochemical purity), 3CaCl2, 3P (3,000 Ci/mmol), and [γ-32P]ATP were from New England Nuclear. 3P was purified by the method of de Meis and Tume (17). AMP-P(NH)P was from Boehringer Mannheim. TNP-ATP was synthesized and purified by the method of Hiraizuka (18). Concentrations of the nucleotide were determined by absorbance measurements at 408 nm in 0.1 M Tris -Cl, pH 8.0, using ε280 = 26,400 M−1 cm−1 (18).

SRV were prepared from rabbit hind leg muscle according to the method previously described by Guillaud et al. (21). Each volume each from reactant syringes (A and B), containing SRV and [γ-32P]ATP (3,000–4,000 dpm/nmol), was mixed and aged in a 450-µl coil for 4 s. The third syringe (C), containing 2 volumes (450 µl) of 10 mM MgADP and 1 mM EGTA, was mixed with aged E-32P; and approximately 500 µl of ADP-reacted E-32P was collected in the sample collect syringe, containing 500 µl of quench solution. The dead volume between syringes C and the collect syringe was 100 µl, and sample flow rate was 5 ml/s.

RESULTS

In this study, we have examined the effects of modification of sulphydryl groups of the Ca2+-ATPase on various functional activities of the enzyme. Kawakita et al. (14) have shown that relatively specific derivatization of those thiol groups involved in phosphoenzyme decomposition (SH5) can be achieved in the presence of Ca2+ and of the nonhydrolyzable analogue, AMP-P(NH)P. The latter protects those thiol groups involved in phosphoenzyme formation following nucleotide binding.

The extent of modification with the reagent NEM depends on conditions that result in the accumulation of different E-P species and on TNP-ATP fluorescence lifetime studies. Alternatively, the rate of increase of TNP-ADP fluorescence is closely associated with the rate of formation of the ADP-insensitive phosphoenzyme (E2-P) at low temperatures (9). TNP-ATP fluorescence is sensitive to K+. Increases in the concentration of monovalent cation salts decrease fluorescence in a manner that has similar specificity and affinity to that of the monovalent cation salt that accelerates ATP hydrolysis and enhances E-P decomposition (10). Potassium binding enhances E2-P to E1-P conversion (11) and accelerates E2-P hydrolysis, the latter step being rate-limiting for catalysis (12). Both of these actions favor a decrease in E2-P with constant levels of total E-P (13). An alternative explanation, suggested by Bishop et al. (8), is that K+ decreases the affinity of the noncatalytic site for trinitrophénylated ATP derivatives.

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The extent of modification with the reagent NEM depends...
on the time of the reaction and the concentration of the reagent (Fig. 1). Low concentrations (40 μM) of NEM rapidly labeled four to five SH groups in 15–20 min, followed by a slower phase, such that six to seven groups were modified by 1 h. At a high (400 μM) concentration, approximately 14 nmol/mg NEM was bound by the enzyme. These data are essentially in agreement with those of Kawakita et al. (14) and Yamada and Ikemoto (15). The ATPase, modified by 400 μM NEM for up to 50 min, has been used for further functional studies.

NEM modification resulted in a parallel decline of Ca²⁺ uptake and Ca²⁺-dependent ATPase activity (Fig. 2), consistent with inhibition of the rate-limiting step for phosphoenzyme decomposition. Derivatization thus had no apparent effect on the ratio of Ca²⁺ uptake to ATPase activity, which is a measure of coupling of transport to ATP hydrolysis. In previous studies, data on thiol group modification and its effects on transport activity were obtained in the absence of Ca²⁺. EGTA, which causes irreversible uncoupling of the Ca²⁺ pump (28), complicates interpretation of these data.

Phosphoenzyme formation, either in the forward direction from ATP plus Ca²⁺ or from Pᵢ in the presence of EGTA, enhances fluorescence of bound TNP-ATP (29). Original studies, performed at pH 8.0 in 20% (v/v) glycerol, showed Pᵢ-induced fluorescence to be half of that induced by ATP (5). In the present study (Fig. 3), Pᵢ-induced fluorescence was assayed at pH 7.0 in 10% (v/v) dimethyl sulfoxide, which maximizes E-P levels. Under these conditions, ATP- and Pᵢ-
induced fluorescence are equivalent. NEM modification diminished fluorescence enhancement by either ATP or P, to approximately similar extents (compare Fig. 3, A and B, at 16 and 40 min with Fig. 3C). NEM modification did not alter the relatively low TNP-ATP fluorescence from the nonphosphorylated enzyme prior to the addition of ATP or Pi (Fig. 3, A and B).

Fluorescence enhancement was accompanied by a blue shift in the emission spectrum of bound TNP-ATP (645 to 530 nm) (Fig. 4, traces b and c), consistent with previous suggestions of increased hydrophobicity of the TNP-ATP-binding site during turnover (6). NEM modifications that decreased fluorescence had no effect on the λmax of emission (Fig. 4, traces d and e). The alteration in fluorescence does not appear to be due to a change in light scattering as a result of flocculation of vesicles since fluorescence emission in the ranges 450–475 and 650–700 nm was unaltered. NEM modification also had little effect on the absorbance at 410 and 530 nm, causing negligible inner filter effects (data not shown). Enhanced TNP-ATP fluorescence was unchanged under conditions (40 μM NEM) that have been reported to result in the modification of those thiol groups of unknown function (SHn) (Fig. 3C).

TNP-ATP fluorescence has been related to E-P levels (5, 6) under static and dynamic conditions. Kawakita et al. (14) have reported that inclusion of AMP-P(NH)P protects a group of sulfhydryls (SH1) that are related to E-P formation; and thus, NEM modification had no effect on ATP-dependent E-P levels. These findings are confirmed in Fig. 5. However, P1-induced E-P levels were diminished by approximately 50% after derivatization. This suggests that the thiol groups that are modified (SH1) are involved in both the hydrolysis of E-P to P, and, in the reverse reaction, of the formation of covalent E-P from Pn, with the exclusion of water. Thus, under these conditions, the decline in P1-dependent TNP-ATP fluorescence is readily explained by decreased E-P formation. However, the decrease in TNP-ATP fluorescence from ATP plus Ca2+ occurs when total E-P levels are unaltered.

Total E-P levels include both E1-P and E2-P intermediates. P1-induced E-P, in the absence of Ca2+, is assumed to be predominantly E2-P; while during turnover, both E1-P and

![Figure 4](image-url)

**Figure 4.** Effect of NEM modification on TNP-ATP emission spectra. The emission spectra for Fig. 3 were scanned at an excitation wavelength of 418 nm with 10-nm band passes. Additions were 0.1 mg/ml SRV (trace a), 2 μM TNP-ATP (trace b) 100 μM ATP plus 50 μM Ca2+ at time 0 (trace c) and 16 (trace d) and 40 (trace e) min of NEM-derivatization.

**Figure 5.** Effects of NEM modification on E-P levels from ATP and P. Phosphoenzyme levels were determined from [γ-32P]ATP (O) and [32P]Pi (●) (see “Experimental Procedures”) under identical conditions as for fluorescence assays described for Fig. 3 following derivatization for varying times with 400 μM NEM. 100% values at time 0 for ATP- and P1-induced E-P levels were 3.2 and 3.1 nmol/mg, respectively.

E1-P are present with a ratio of approximately 2:1 (30, 31).

The relative proportions of ADP-sensitive and ADP-insensitive E-P species have been determined following rapid quenching by millimolar concentrations of ADP and EGTA (Fig. 6A). This shows a rapid decay in E-P from 3.1 nmol/mg, complete within the mixing time, which was followed by a slower decay in the ensuing 5 s. The data yield a value of the ADP-sensitive fraction of 44%, which is consistent with previous data (13). Similar experiments performed on NEM-modified vesicles showed that the ADP-sensitive E-P species was increased to 85% (Fig. 6B) (16, 32, 33). Exponential fits of the slow phases of E-P decay (Fig. 6, A and B) following addition of ADP gave similar values of 0.71 and 0.69 s−1 in the control and NEM-modified vesicles, respectively.

A possible explanation of the inhibition of P1-induced enzyme phosphorylation by modification of SH1 is that in addition to its effects on the transition of E1-P to E1-P, there is a concomitant block in interconversion of the free enzyme species, E1 and E2. However, the effects of Ca2+ and EGTA on intrinsic tryptophan fluorescence, which has been shown to report the E1 = E2 conformational transition (24), were unaffected by NEM modification, (data not shown).

**DISCUSSION**

TNP-ATP has been shown to bind with high affinity (Kd = 0.1–0.4 μM) to the catalytic site on the nonphosphorylated enzyme (34) and to the noncatalytic site of the phosphorylated enzyme (6, 29). The latter site shows a relatively higher hydrophobicity that results in a 7–10-fold increase in bound TNP-ATP fluorescence. TNP-AMP competitively inhibits stimulation of catalysis by millimolar concentrations of ATP, implicating this site as the regulatory site (35). However, the precise nature of the phosphoenzyme species responsible for enhanced fluorescence of bound TNP-nucleotides is controversial. It has been proposed that the E2-P intermediate alone (7, 9) or, alternatively, that all phosphorylated intermediates are involved (8). We present evidence in this study that the E2-P conformation is the most likely subspecies that results
The transition of E, to E,, whether phosphorylated or not, is both forward and reverse phosphorylation processes or the phosphorylated in the reverse direction by Pi suggests two possibilities for their conclusion that Ca2+ ions are occluded by the El-P distribution of phosphorylated intermediates. The decrease of the fitted curve intercepted the rapid decay phase in E-P at 32P]ATP for ADP sensitivity. The finding that the SHD-modified enzyme cannot be phosphorylated under conditions that do not alter total E-P levels during catalysis, TNP-ATP fluorescence was inhibited from both ATP plus Ca2+ and from P, in the absence of Ca2+. This would indicate that E,~P, the predominant species under these conditions, is unaltered by SHD modification. Furthermore, this species does not appear to be responsible for enhanced fluorescence. Two possible mechanisms were considered. The first is that E1-P formation and its level determine enhanced TNP-ATP fluorescence. The decreased fluorescence during turnover (approximately 50% following SHD modification) correlates well with the decrease of ADP-insensitive E-P from 1.3 to 0.7 nmol/mg.

A second mechanism that requires consideration is related to the possibility that catalytic and regulatory nucleotide sites may, in fact, be nonidentical, in which case events at the catalytic site would be transmitted to the noncatalytic site by a conformational transition. Fluorescence enhancement may be related to energy coupling since EGTA pretreatment that uncouples transport causes a parallel decrease in fluorescence enhancement. Berman (37, 38) has shown dissociation of enhanced TNP-ATP fluorescence from E-P formation under conditions that uncouple transport from catalytic activity and E-P levels and has proposed that intermediate conformational events of a strictly ordered type couple catalytic intermediates to the transport cycle. SHD modification would then block conformational changes originating at the catalytic site from being transmitted to the regulatory site, thus preventing enhanced fluorescence of the E,~P conformation. This mechanism would appear unlikely in view of the parallel block in E2-P formation and of TNP-ATP fluorescence from the forward and reverse directions. The E2-P species is therefore the most probable intermediate responsible for enhanced TNP-ATP fluorescence, in support of a recent study showing a close correlation between enhanced TNP-ATP fluorescence and the ADP-insensitive phosphoenzyme at varying [KCl] and pH (39).

The significance of the increased hydrophobicity of the noncatalytic, or regulatory, site in the E2-P conformation may be to prevent access of water to the acyl phosphate, prior to Ca2+ release to the lumen of the vesicle, in compliance with rules for coupling, formulated by Pickart and Jencks (40). These require that the 2Ca2+-E2-P conformation not be dephosphorylated in the Ca2+-bound conformation.

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