Reaction of a Carbodiimide Adduct of ATP at the Active Site of Sarcoplasmic Reticulum Calcium ATPase†

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ABSTRACT: An adduct of a carbodiimide and ATP was synthesized from 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) and the nucleotide. Despite its limited stability ($t_{1/2}$ for hydrolysis of about 5 min at 25 °C), it was shown to react with and inactivate the calcium ATPase of sarcoplasmic reticulum in its vesicular, nonionic detergent-solubilized and purified forms. Saturation kinetics, with an ATP–EDC concentration dependence midpoint in the 10 μM range, were observed, suggesting an active-site affinity which is similar to ATP. The reaction was specific in that inactivation required reaction of about one adduct per ATPase. The modified enzyme could no longer be phosphorylated by ATP or P_i or hydrolyze *p*-nitrophenyl phosphate, but retained the ability to undergo the high-affinity calcium-dependent fluorescence change. It also bound trinitrophenyl-ADP and other nucleotides at least 10-fold more weakly than the unmodified ATPase. The inactivation reaction required the presence of Mg^{2+} and Ca^{2+} and was prevented by nucleotides such as ATP and ADP. For magnesium, the inactivation-enabling effect occurred with a midpoint of 3 mM. In the case of calcium, the transition resembled high-affinity binding in that it occurred cooperatively with a midpoint in the micromolar range. Higher [Mg^{2+}] shifted this transition to higher [Ca^{2+}]. Polyacrylamide gel electrophoresis (PAGE) demonstrated that the reaction converted the ATPase ($M_r = 1.1 \times 10^5$) to a species with an apparent $M_r = (1.7–1.8) \times 10^5$. Since nonionic detergent-solubilized ATPase and purified ATPase gave similar results, intramolecular cross-linking is implicated. Comparison with control ATPase of the effect of limited trypsin proteolysis, which normally produces three fragments (A_1, A_2, and B), showed only the A_2 fragment was obtained from the cross-linked ATPase; a cross-link between the A_1 and B fragments is thereby suggested. Trypsinization before and after ATP–EDC treatment gave similar PAGE patterns, suggesting that the chemical modification does not appreciably alter the environments of the tryptic cleavage sites.

Relaxation of striated muscle depends upon rapid active transport of calcium by the ATPase of sarcoplasmic reticulum (SR)[1] (Inesi, 1985). With amino acid sequences of two of its forms having been deduced from cDNA clones (Brandl et al., 1986), the functional structure of this ATPase is a subject of intense investigations. Although it is capable of forming crystalline arrays (Dux & Martonosi, 1983; Pikula et al., 1988; Stokes & Green, 1990), nondiffraction approaches will continue to be important for gaining additional structural and mechanistic insights. Among these are efforts aimed at identifying essential side chains. For example, a series of site-directed mutagenesis experiments has implicated a number of anionic side chains located in hypothetical transmembrane segments as being part of one or both calcium binding sites (Clarke et al., 1989, 1990). The ATP active site contains an aspartate (351 in the sequence of the fast-twitch enzyme) to which the substrate's γ-phosphoryl group is transfered during the transport cycle (Degani & Boyer, 1973). On the basis of the behavior of fluorescein isothiocyanate as an affinity label (Pick & Bassilian, 1981; Mitchinson et al., 1982; Murphy, 1988), a specific lysine (515) has been located there also. Other lysines have been implicated as a result of reactivity toward pyridoxal phosphate (Murphy, 1977) or adenosine triphosphopyridoxal (Yamamoto et al., 1988), while reaction with dicarbonyls suggests the presence of arginine (Murphy, 1976; Bishop, 1989).

Carbodiimides have long been used to activate terminal phosphoryl groups of nucleotides (Khorana, 1953; Kurzer & Douraghi-Zadeh, 1967). In this study, the synthesis and characterization of a reactive ATP analogue, ATP–EDC, are described. Made by reacting ATP and the water-soluble carbodiimide EDC, this adduct reacts rapidly with the SR and related ATPases (Murphy, 1990), probably at the nucleotide active site. The reaction causes inactivation and cross-linking, producing an inactive ATPase protein with an apparent molecular weight of $(1.7–1.8) \times 10^5$. Tryptic digestion experiments suggest that the cross-linking joins amino acids which are part of the A_1 and B tryptic fragments.

Materials and Methods

SR vesicles were prepared from rabbit hind leg muscle (Eletr & Inesi, 1972). Solubilized SR was prepared as previously described (Coll & Murphy, 1984); its stability was improved by routinely using $C_{12}E_9$ stock solutions which had been brought to 100 °C for at least 30 min and cooled before using (Coll & Murphy, 1986). Sigma Chemical Co. was the source of Na_2ATP, NaADP, *p*-nitrophenyl phosphate (ditris salt), EDC, lactic dehydrogenase, PK (both type II), Sepharose

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[1] Abbreviations: SR, sarcoplasmic reticulum; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; TEOA, triethanolamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PEP, phosphoenolpyruvate; PK, pyruvate kinase; AMP-PNP, adenyl-5′-yl imidodiphosphate; AMP-PCP, adenyl-5′-yl methylenediphosphonate; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate (isomer I); TNP-ADP, 2′,3′-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-diphosphate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; DPC, *N*,*N*′-diisopropylcarbodiimide; EP, phosphorylated enzyme intermediate; ATP–EDC or –DPC, γ-P adducts of ATP and EDC or DPC, respectively; $C_{12}E_9$, poly(oxyethylene) 9-lauryl ether; A, B, A_1, and A_2, tryptic fragments of the SR CaATPase as defined by Thorley-Lawson and Green (1977).

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4B-CL, reactive red 120, and the molecular weight standards; tetrabutylammonium phosphate was obtained from Aldrich.

Because of variability in the yield and quality of purified ATPase depending on the lot of purchased reactive red–agarose, the original purification procedure (Coll & Murphy, 1984) was modified by using material prepared from agarose and reactive red 120. The preparation, adapted from Atkinson et al. (1981), was carried out as follows: to 5 mL of packed Sepharose 4B-CL were added with gentle stirring 9 mL of H_2O, 4 mL of 25 mg/mL reactive red 120, 2 mL of 4 M NaCl, and 0.27 mL of 30% NaOH. This mixture was heated without stirring for 16 h at 58 ± 2 °C, cooled, poured into a column, and washed successively with 10–15 column volumes of H_2O, 2 M NaCl, H_2O, and the column buffer used previously (Coll & Murphy, 1984).

Calcium-dependent ATPase activities were measured by coupled assay (Anderson & Murphy, 1983) at 37 °C and pH 7.0 in a solution containing 80 mM KCl, 50 mM Mops, 5 mM $MgCl_2$, 1.1 mM $CaCl_2$, 1 mM EGTA, 3 mM MgATP, 0.5 μg/mL calcium ionophore, and 10 μg/mL SR protein. Control activities were in the range of 15–20 μmol min^{-1} mg^{-1}. *p*-Nitrophenylphosphatase activities were measured spectrophotometrically at 400 nm under the same conditions except that the SR protein and ionophore concentrations were 10-fold greater, and $CaCl_2$ was added after tracing the "calcium-independent" activity. The initial concentration of substrate was 10 mM.

Steady-state EP levels from ATP were determined as described previously (Coll & Murphy, 1984) except that SR protein concentration was 0.2 mg/mL, [γ-^{32}P]ATP concentration was 25 μM, and calcium ionophore was omitted. Phosphorylation by P_i [adapted from de Meis and Inesi (1982)] was measured at 25 °C in 50 mM Mops, 10 mM $MgCl_2$, 1 mM EGTA, 0.93 mM [^{32}P]P_i, and 20% (by volume) DMSO, pH 7.0; SR protein concentration was 5 mg/mL and control experiments contained 1.1 mM $CaCl_2$ in addition to the components stated above; 180 μL of incubation mixture was added to 1 mL of cold 5% TCA. Centrifugation, decantation, and homogenization in 1 mL of 5% TCA/1 mM P_i were done 3 times before the pellet was counted for ^{32}P using Ecolume (ICN) scintillation fluid.

HPLC was done at 22–24 °C on a reverse-phase C_{18} Ultrasphere (Beckman) column (46 × 250 mm main column and 46 × 45 mm guard column) at a flow rate of 1 mL/min with a UV absorbance detector set at 259 nm; the solvent used was 30% CH_3CN/70% (30 mM KH_2PO_4/10 mM tetrabutylammonium phosphate in H_2O) v/v adjusted to pH 6.0 with 5 M KOH. PAGE was carried out according to Laemmli (1971) on 0.75-mm slab gels (10%) which were then stained with Coomassie blue R250. Bands containing fluorescein from FITC labeling were visualized with a UV lamp before staining. Protein loaded was 5 μg per lane. Apparent molecular weights were estimated graphically by using the mobility of proteins of a high molecular weight standard (Sigma).

ATP–EDC was synthesized by adding 1 mL of CH_3CN and then 134 mg (0.7 mmol) of EDC to 1 mL of 0.2 M Na_2ATP (0.2 mmol) in H_2O. After 15 min at 25 °C, an aliquot was injected into the above HPLC system (flow rate 1 mL/min); the main peak was collected in an ice-cold tube and added within 30 s to SR incubation mixtures or frozen and stored at −50 °C. ATP–DPC was made similarly using DPC. Prepared from NaADP in the same way as ATP–EDC, ADP–EDC had a retention time of 2.5 min (compared to 5.2 min for ADP). Its time course of formation and hydrolysis was quite similar to that of ATP–EDC: between 6 and 30 min,

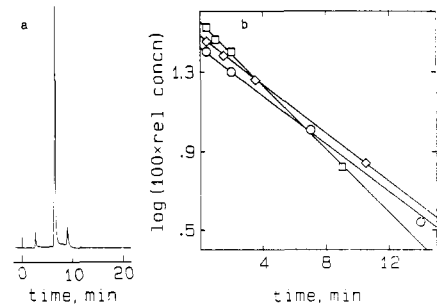


FIGURE 1: (a) HPLC chromatogram of the ATP–EDC reaction mixture. From standards, the peak at 9.2 min corresponds to ATP. (b) Semilog plot of the hydrolysis of ATP–EDC. (○) pH 5.8; (□) pH 6.8; (◇) pH 8.0. Reaction conditions are described under Materials and Methods.

the ADP–EDC fraction was close to 0.8 and reached a value of 0.4 at about 80 min, (cf. Figure 2). The kinetics of ATP–EDC hydrolysis were measured in 100 mM buffer (Mes, Mops, or TEOA for pH 5.8, 6.8, and 8.0, respectively) by adding ATP–EDC to an initial concentration of 1–2 mM and using HPLC to separate components and estimate concentrations.

Testing for the incorporation of nucleotide upon ATP–EDC modification was done at 37 °C and pH 7.4 in incubation mixtures containing 100 mM Mops, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mg/mL SR vesicles, and 20 μM ATP–EDC. After 30 min, mixtures were centrifuged at 30000*g* for 1 h. As controls, an identical set was made up so that ATP–EDC was allowed to hydrolyze for 30 min before SR was added. Bound nucleotide was measured by difference from absorbance readings at 259 nm of the supernatants; controls had absorbances of 0.28 ± 0.02.

Calcium-dependent fluorescence changes were measured at 25 °C on a Perkin-Elmer MPF-44B spectrofluorometer (excitation and emission wavelengths of 295 and 340 nm, respectively). Conditions: 0.17 mg/mL SR vesicles, 20 mM TEOA, 20 mM $MgCl_2$, and 0.1 mM $CaCl_2$, pH 7.2. The intensity was recorded before and after addition of EGTA to 1 mM.

Binding of nucleotides to SR was estimated spectrofluorometrically by adaptation of a previously described method (Coll & Murphy, 1986) developed for solubilized SR. Exciting at 440 nm and detecting emission at 550 nm, we measured the fluorescence intensity of SR (control and ATP–EDC modified) as TNP-ADP was added and then displaced by AMP-PCP. The titration mixture contained 0.1 mg/mL SR protein, 50 mM Mops, and 1 mM EGTA, pH 7.0.

Tryptic digestions were performed at 25 °C, pH 7.4, in 50 mM TEOA (chloride), 50 mM KCl, and 5 mM $CaCl_2$ (KCl and $CaCl_2$ were added to minimize digestion of the A_1, A_2, and B fragments; Imamura et al., 1984); the concentrations of SR protein and trypsin were respectively 1 and 0.05 mg/mL.

Results

Formation and Properties of ATP–EDC. ATP reacted with the water-soluble carbodiimide EDC to form an adduct (retention time = 5.8 min) which was separable by HPLC (Figure 1a) from the parent nucleotide (9.2 min). These two components made up more than 95% of the 259-nm-absorbing material in the reaction mixture. HPLC provided a convenient method to measure the time course of the reaction. As shown in Figure 2, the relative concentration of ATP–EDC rose rapidly, reaching a maximum of about 0.8 in 10–15 min, after which it decreased toward zero. In accordance with its being respectively a precursor and product of the formation and

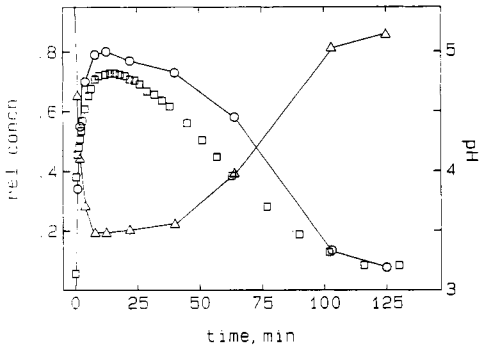
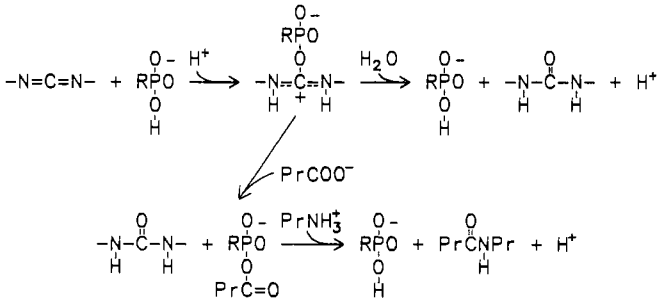


FIGURE 2: Time course of the formation and decomposition of ATP–EDC. Relative concentrations (left ordinate) of ATP (△) and ATP–EDC (○) were calculated from the areas of their HPLC peaks divided by the total area. (□) pH of reaction mixture. Reaction conditions are described under Materials and Methods.

Scheme I



hydrolysis of ATP–EDC, ATP over the same time course decreased and subsequently increased in concentration, returning to its initial level after about 2 h. Simultaneous measurement of the pH revealed that it first rose and then fell, consistent with H^+ being a reactant in ATP–EDC formation and a product of its breakdown.

On the basis of these results and the known reactivity of phosphomonoesters (anhydrides) toward carbodiimides (Smith & Khorana, 1958), Scheme I is a plausible description of the reactions involved. There are additional reasons for postulating that ATP–EDC is the γ-P adduct of ATP and EDC. Compared to one of the nonbridging oxygens of the terminal phosphorus, other functional groups on ATP are much less nucleophilic. Lack of modification of the adenine moiety was indicated by a UV absorption spectrum which was essentially identical with that of adenosine. Addition of ATP–EDC to methanol produced a stable product (ATPMe) which had an HPLC retention time of 9.0 min compared to ATP with 11.2 min (solvent as described under Materials and Methods except CH_3CN was 25% and the pH was 7.0). Reaction of ATPMe with periodate showed the presence of 2′- and 3′-hydroxyls (Dixon & Lipkin, 1954); it bound Mg^{2+} at pH 7 with a K_d of 1 mM, and was not a substrate for but bound to the SR CaATPase with a K_d of 3 μM. ATPMe is thus most likely the γ-methyl ester of ATP.

To assess the stability of ATP–EDC under conditions which would be used for reaction with proteins, the kinetics of hydrolysis between pH 6 and 8 and at 25 °C were measured (Figure 1b). Using HPLC, we observed that the concentration of ATP–EDC decreased as a single exponential with rate constants at 25 °C and pH 5.8, 6.5, and 7.9 of 0.14, 0.18, and 0.14 min^{-1}, respectively, indicating that the ATP–EDC hydrolysis rate constant is essentially independent of pH in the neutral range.

Effect of ATP–EDC on SR ATPase Activity. Incubation of micromolar ATP–EDC with SR ATPase resulted in rapid

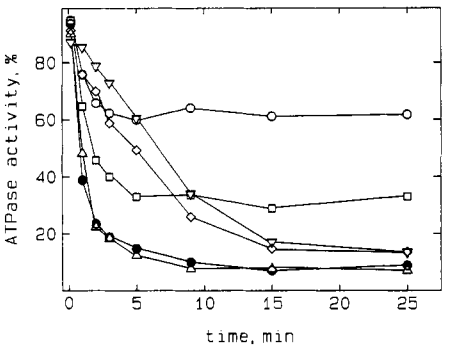


FIGURE 3: Time course of the ATP–EDC-produced loss of calcium ATPase activity of SR vesicles. Incubation mixtures contained 100 mM TEOA (chloride), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mg/mL SR vesicles, pH 7.2, 25 °C. At times indicated on the abscissa, aliquots were quenched by 100-fold dilution into ATPase assay solution at 0 °C. Initial ATP–EDC concentrations (in micromolar) were 2.5 (○), 5 (□), 10 (△), 20 (●), 40 (◇), and 80 (▽).

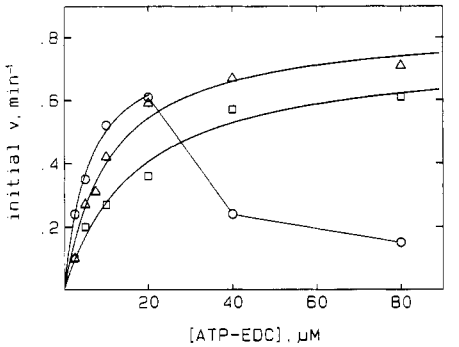


FIGURE 4: ATP–EDC concentration dependence of the initial velocity of calcium ATPase inactivation. Data for vesicles are from Figure 3. (○) SR vesicles; (□) purified ATPase; (△) solubilized SR. The lines are drawn (except for those connecting the 20–80 μM points for SR vesicles) by using parameters obtained by nonlinear regression of the data to a function derived from a model of rapid reversible binding which precedes irreversible inactivation.

loss of activity (Figure 3). The change did not follow a single-exponential time course (pseudo-first-order kinetics) inasmuch as the inactivator changes concentration, being converted to ATP, a substrate, and protector from inactivation. Under the same conditions, equivalent concentrations of EDC produced no detectable inactivation. Consistent with inactivation being due to a substance which also decomposes in H_2O, delaying addition of SR until 30 min after adding ATP–EDC resulted in no detectable loss of ATPase activity (not shown).

At ATP–EDC concentrations which were about the same or less than the ATPase concentration of about 5 μM, inactivation was incomplete (Figure 3). The rate of inactivation was maximal at 10 and 20 μM ATP–EDC and actually decreased when the concentration was raised to 40 or 80 μM. A parallel set of experiments on purified ATPase did not show this complex behavior, the rate increasing until a concentration of 40 μM ATP–EDC; at 80 μM, the rate was insignificantly faster (not shown). To test the possibility that the complex behavior exhibited by SR vesicles was due to a component which is removed by the purification procedure, the experiments were repeated on solubilized but not purified SR. The results resembled those for purified ATPase, implying that the complex behavior of SR vesicles (Figure 3) is attributable to intermolecular interactions within the vesicular membrane or to ATP generated from ATP–EDC hydrolysis.

The initial inactivation rate at various initial concentrations of ATP–EDC is plotted in Figure 4. For both solubilized and purified ATPase, a hyperbolic dependence on [ATP–EDC] was observed. This appeared to be the case for SR vesicles

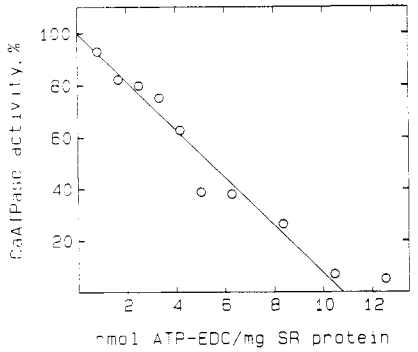


FIGURE 5: Measurement of the amount of ATP-EDC required to inactivate the SR calcium ATPase. Incubations were done at 37 °C, pH 7.4, and contained 100 mM TEOA (chloride), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mg/mL SR vesicles, and ATP-EDC at initial concentrations to give the nanomoles of ATP-EDC per milligram of SR protein indicated on the abscissa. After 30 min, ATPase activities were measured as described under Materials and Methods.

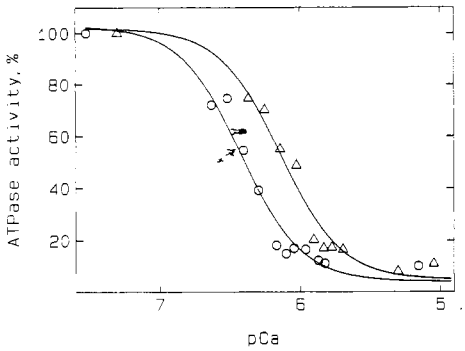


FIGURE 6: Calcium concentration dependence of the extent of SR ATPase inactivation by ATP-EDC. SR vesicles were incubated with 20 μM ATP-EDC for 30 min as described in Figure 3 except that pCa was varied as indicated on the abscissa and [$MgCl_2$] was 10 (○) or 30 (△) mM.

also up to 20 μM ATP-EDC; above this concentration, the initial rate was anomalously low. A fit of these data to a simple scheme wherein reversible binding precedes irreversible inactivation yielded $[ATP\text{-}EDC]_{1/2}$ values of 6, 11, and 17 μM for vesicular, solubilized, and purified ATPase, respectively. On the assumption that binding and dissociation are fast relative to the inactivation reaction, these values provide an estimate of the affinity of the reagent for the ATPase.

An estimate of the specificity of the reaction was obtained by comparing the extent of inactivation to the concentration of ATP-EDC initially present in the incubation mixture (Figure 5). With an intercept corresponding to a value of 10–11 nmol/mg of SR protein required for complete inactivation, the data imply a considerable degree of specificity. In fact, taking into account ATP-EDC hydrolysis, the rate of which is similar to the rate of inactivation and the product of which protects the enzyme (see below), these results suggest that the effects of ATP-EDC are due to the interaction of roughly one ATP-EDC per ATPase.

Dependence of the ATP-EDC Reaction on the Concentrations of Calcium and Magnesium. In the absence of Ca^{2+} or Mg^{2+} or both, ATP-EDC does not inactivate the SR ATPase (Murphy, 1990). The dependence of ATP-EDC inactivation on the concentration of Ca^{2+} is shown in Figure 6. Consistent with cooperative Ca^{2+} binding, the extent of inactivation increased steeply as the concentration was increased in the micromolar range ($pCa_{1/2}$ = 6.5 with 10 mM Mg^{2+}). Consistent with a competitive Mg^{2+} effect, the transition was shifted to lower pCa in the presence of 30 mM Mg^{2+}, so that $pCa_{1/2}$ was about 6.2.

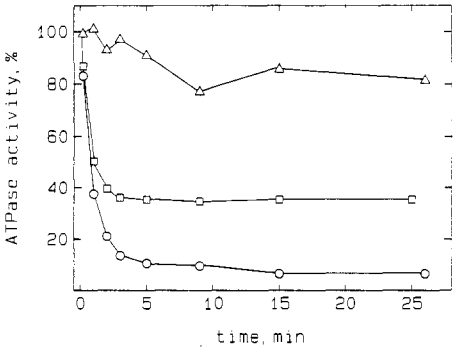


FIGURE 7: Protective effect of ATP toward the inactivation of SR ATPase by ATP-EDC. SR vesicles (0.2 mg/mL) were incubated with 20 μM ATP-EDC as described in Figure 3. Additional components in incubation mixtures: none (○); 4 mM PEP and 4 units/mL PK (□); 4 mM PEP, 4 units/mL PK, and 40 μM ATP (△).

Table I: Properties of SR CaATPase Modified by ATP-EDC[a]

	control	ATP-EDC modified[a]
EP formation from ATP (nmol mg^{-1})	3.5 ± 0.4	0.4 ± 0.2
EP formation from P_i (nmol mg^{-1})	1.8 ± 0.3	0.1 ± 0.1
Ca^{2+}-dependent fluorescence change (%)	4.3 ± 1	4.8 ± 1
p-nitrophenylphosphatase activity (μmol min^{-1} mg^{-1})	0.50 ± 0.06	0.07 ± 0.05
K_d for TNP-ADP binding (μM)	0.02 ± 0.007	0.12 ± 0.04
K_d for AMP-PCP binding (μM)	12 ± 3	200 ± 60

[a] The reaction was done at 80 μM ATP-EDC as described in Figure 3; the resulting ATPase activity was less than 10% that of the control.

The extent of inactivation by ATP-EDC increased with increasing concentrations of Mg^{2+}. This effect showed saturation behavior, the data being adequately described by a noncooperative transition with a midpoint of about 3 mM Mg^{2+} (not shown).

Protection by ATP and ADP. The protective effect of ATP is illustrated in Figure 7. The inclusion in the incubation mixture of PEP and PK to regenerate ATP resulted in significant protection. Including additional ATP (40 μM) resulted in an inactivation rate which was more than an order of magnitude less than the unprotected rate and an activity loss of about 20%. Because the concentration of ATP was not constant during the incubations, an accurate quantitation of a protection constant is not possible, but the results are consistent with a value in the micromolar range. ADP also protected, exhibiting a midpoint of 280 μM in a series of inactivation experiments done at 10 μM ATP-EDC (not shown).

Properties of SR ATPase Modified by ATP-EDC. The effect of ATP-EDC modification on the ability of the ATPase to be phosphorylated by ATP (EP formation) was measured (Table I). For SR ATPase treated with ATP-EDC so that it had a residual CaATPase activity which was 6% of control activity, the phosphorylatable enzyme was 0.4 nmol/mg under conditions where untreated SR gave 3.5 nmol/mg. Measurements of EP formation from P_i after ATP-EDC modification showed that the enzyme's ability to be phosphorylated by this route also was also essentially lost (Table I). To determine whether ATP-EDC modification prevented high-affinity calcium binding, the calcium-dependent tryptophan fluorescence change was measured. As shown in Table I, the modification did not affect the change produced by binding to the enzyme's high-affinity calcium sites.

Since some nucleotide site modifiers, e.g., FITC, do not eliminate the ATPase's activity toward pseudosubstrates, the nitrophenylphosphatase activity of the ATPase modified by ATP-EDC was measured to see if it was retained; as shown

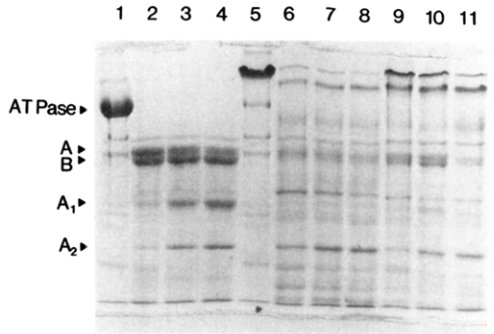


FIGURE 8: Electrophoretogram of SR vesicles reacted with ATP–EDC. SR vesicles were incubated with 20 μM ATP–EDC for 30 min under conditions stated in Figure 3. Digestion with trypsin is described under Materials and Methods. Lane 1, SR vesicles; lanes 2–4, SR vesicles digested with trypsin for 1, 10, and 40 min, respectively; lane 5, ATP–EDC-treated SR vesicles; lanes 6–8, ATP–EDC-treated SR vesicles digested with trypsin for 1, 10, and 40 min, respectively; lanes 9–11, SR vesicles digested with trypsin for 1, 10, and 40 min, respectively, and then treated with ATP–EDC.

in Table I, however, this activity was decreased to about the same extent as the ATPase activity. The affinity of the SR ATPase modified by ATP–EDC for nucleotides was decreased but not eliminated. Thus, the K_d for TNP-ADP was found to be greater by a factor of about 6 compared to the control enzyme (Table I). As measured by the displacement of this nucleotide, the affinity for AMP-PCP was more markedly attenuated.

Modification of SR ATPase by ATP–EDC resulted in no measurable amount of incorporation of nucleotide into the enzyme. This was indicated by the lack of a significant difference in the absorbance at 259 nm of the supernatant of ATP–EDC-inactivated SR compared to controls in which the ATP–EDC was allowed to hydrolyze prior to addition of SR. Thus, under conditions where incorporation of 5 nmol/mg would have given an absorbance difference of 0.15, the observed difference range was −0.04 to 0.02.

Other Nucleotide–Carbodiimide Adducts. The inactivating effects of related nucleotide–carbodiimide adducts were measured. ADP–EDC, the adduct of ADP and EDC, was ineffective as an inactivator; there was insignificant change in ATPase activity in the presence and absence of Ca^{2+} and/or Mg^{2+} at concentrations comparable to those which were effective for ATP–EPC inactivation (not shown). On the other hand, an ATP adduct with a different carbodiimide, ATP–DPC, was also found to inactivate the SR ATPase; as found with ATP–EDC, this effect was dependent on the presence of both divalent cations (not shown).

Effect of ATP–EDC on the PAGE Mobility of the SR ATPase. SR which had been modified by ATP–EDC was subjected to PAGE. The decrease in ATPase activity which occurred as a result of incubation of SR vesicles with ATP–EDC was correlated with loss of the principal protein (ATPase) band corresponding to $M_r = 1.1 \times 10^5$ (Figure 8, lane 1) and appearance of a new band with of M_r apparent $(1.7–1.8) \times 10^5$ (Figure 8, lane 5), suggesting that reaction with ATP–EDC involves cross-linking. Incubation of solubilized vesicles with ATP–EDC gave a similar PAGE pattern (not shown), consistent with cross-linking which is intramolecular. This was also supported by PAGE of purified ATPase inactivated by ATP–EDC, which gave a cross-linked protein with essentially the same mobility (not shown).

In Figure 8 is shown a comparison of tryptic digestion patterns of ATP–EDC-cross-linked ATPase and its normal counterpart. At digestion times by which the unmodified ATPase was largely converted to its A_1, A_2, and B fragments (lanes 3 and 4), the A_2 fragment was the only one of these three which appeared from the digestion of the cross-linked enzyme (lanes 7 and 8). This result suggests that the A_1 and B fragments are the ones which are linked by the action of ATP–EDC. Tryptic digestion of ATP–EDC-treated SR also produces a band with of apparent M_r 140000 (Figure 8, lanes 6–8), which is likely to correspond to the fragment remaining after the removal of A_2, i.e., A_1 and B cross-linked to one another.

Experiments were also done in which the proteolysis preceded incubation with ATP–EDC (Figure 8, lanes 9–11). Although some differences were observed, the patterns are similar to reverse order ones, with fragment A_2 being relatively unaffected by ATP–EDC. These results demonstrate that neither of these treatments perturbs the protein's structure so as to grossly alter its response to the other treatment. Purified ATPase subjected to ATP–EDC and trypsin followed by PAGE gave results (not shown) similar to SR vesicles.

Implication of the B fragment in the cross-link was supported by experiments on SR which was first modified by ATP–EDC and then FITC. Gels of SR modified with only the latter reagent and then trypsinized were illuminated by UV light; fluorescence was emitted from the B fragment only, so that FITC is a marker for this fragment (Pick & Karlish, 1980). Gels of doubly modified SR emitted FITC fluorescence from the $(1.7–1.8) \times 10^5$ dalton band and after trypsinization from the 1.4×10^5 dalton band (not shown).

Discussion

This work reveals the presence at the SR ATPase active site of a group which is reactive to an adduct of ATP and EDC. The method described here for making the terminal phosphoryl group of ATP reactive is straightforward, requiring no elaborate apparatus or purification. Preparative HPLC may even be omitted since similar results were obtained using the unpurified ATP–EDC reaction mixture; for other enzymes or conditions, however, the presence of EDC might present problems. Although its instability may be a drawback, the large number of ATP-utilizing enzymes make ATP–EDC likely to be useful for structural investigation of at least some of them. In fact, testing on a small sample of such enzymes showed that ATP–EDC had no significant effect on the activity of myosin S1 ATPase, pyruvate kinase, or hexokinase while it did inactivate two additional ion pumps, the Na,K-ATPase and the H,K-ATPase (Murphy, 1990).

Inactivation of the SR CaATPase by the substrate analogue ATP–EDC suggests that reaction at the nucleotide active site (affinity labeling) is occurring. This is supported by inactivation kinetics with a hyperbolic dependence on ATP–EDC concentration (Figure 4), the low ratio of reagent to ATPase required (Figure 5), the protective effect exhibited by ATP (Figure 7) and ADP, the alteration of the enzyme's ability to bind nucleotides, and elimination of its ability to be phosphorylated (Table I). Finding that the ATPase's affinity for ATP–EDC is similar to that for ATP implies that the active site accommodates bulky additions to the terminal phosphoryl of the substrate. On the other hand, the failure of ADP–EDC to react suggests the importance of positioning the EDC moiety.

The low number of nanomoles of ATP–EDC per milligram of SR protein required for inactivation indicates the reaction is relatively specific, with little or no reaction occurring at sites other than the active site. Although some have suggested the ATPase content of SR vesicles is 7–8 nmol/mg, stoichiometry measurements of enzyme phosphorylation by ATP (Froehlich

& Taylor, 1975) or P_i (Inesi et al., 1984), reaction with FITC (Andersen et al., 1982; Mitchinson et al., 1982; Highsmith & Murphy, 1984; Murphy, 1988), binding of trinitrophenyl nucleotides (Dupont et al., 1982; Coll & Murphy, 1986), and purification of the ATPase (Coll & Murphy, 1984) give values in the range of 4–5 nmol/mg. In the present work, ATPase inactivation is found to require an initial concentration of ATP–EDC corresponding to 10–11 nmol/mg. Given an ATP–EDC hydrolysis rate which is in the same range as the inactivation rate and a hydrolysis product which protects the enzyme, about half of this 10–11 nmol/mg is probably the amount that actually reacts with the ATPase. Reaction of a single site on the enzyme is therefore implied.

The calcium concentration dependence in the micromolar range of ATP–EDC inactivation clearly indicates that the reaction requires occupancy of the high-affinity sites by calcium. From a shift of the [Ca] dependence curve at higher [Mg], a competitive effect at these sites by the latter cation is inferred. At the same time, this effect shows that magnesium binding there does not enable the ATP–EDC reaction. This calcium-specific response is the same as activation of ATPase activity and transport and suggests that the ATP–EDC reaction traps a conformation which is an essential part of the pumping cycle.

Magnesium has a variety of effects on the CaATPase. These include accelerating EP hydrolysis (Inesi et al., 1970), changing its intrinsic fluorescence (Guillian et al., 1982), and competing with calcium at the high-affinity transport sites (Guillian et al., 1984). At least some of these effects have been attributed to the existence of a magnesium site which is distinct from the substrate site (Takakuwa & Kanazawa, 1982). Because of this, it is not possible to conclude that the magnesium concentration dependence of the ATP–EDC reaction is due to MgATP–EDC being the reactive species. It is clear, however, that the reaction requires magnesium.

The appearance on gels of a single major product [M_r = (1.7–1.8) × 10^5] from the reaction of ATP–EDC with the ATPase also supports reaction at a specific site. Since this is also the product when the reaction is done with solubilized and purified ATPase, it is probably a result of the formation of an intramolecular cross-link. Decreased mobility (higher apparent molecular weight) seems to be a common consequence of an induced intramolecular cross-link. Recent examples include myosin S1 (Mornet et al., 1985; Ue, 1987; Bonet et al., 1988) and the SR ATPase (Ross & McIntosh, 1987a,b; Bill et al., 1988). In the one case in which the linked residues have been identified (Ue, 1987), a loop of 184 residues in a M_r 95 000 polypeptide changes the apparent molecular weight to 1.05 × 10^5. If this is representative, the loop induced by ATP–EDC would be quite large.

As noted above, progress is being made in revealing which amino acids are essential for the function of the CaATPase. A related major challenge is to determine which side chains, though far apart in the sequence, are near one another in the folded functional protein, or move near to one another during one or more steps of the catalytic cycle. The question of whether the side chains joined by ATP–EDC are very far apart in the primary sequence will only be answered definitively by peptide isolation and sequencing. Nevertheless, a partial answer is provided by the tryptic digestion results. Since trypsinization produced a normal A_2 fragment (residues 1–198) while A_1 and B were absent, a cross-link between the latter two fragments (residues 199–505 and 506–1001, respectively) is suggested. These are the same two fragments which are cross-linked by reagents such as glutaraldehyde (Ross & McIntosh, 1987a). The order in which trypsinization and ATP–EDC modification were done made little difference in the resulting PAGE patterns, indicating that neither modification alters the sites at which the other acts. The cross-link produced by ATP–EDC, because it occurs only under conditions required for EP formation and subsequent calcium translocation, probably links residues which move close to one another during some part of the pumping cycle. Since phosphorylation by P_i is blocked, one of these residues may well be the one which is phosphorylated during turnover, Asp-351.

The chemical nature of the cross-link also remains to be revealed, but a plausible scenario for its formation would be that ATP–EDC first activates a carboxyl group which is then attacked by a nearby nucleophile (most likely an amino group) to form a stable product such as an amide (Scheme I). A similar hypothesis has been put forth to account for the reaction of SR with ATP imidazolidate (Bill et al., 1988), the effects of which resemble those of ATP–EDC. They differ, however, in that with the former inactivation is biphasic and the ATPase is converted to a polypeptide with an apparent molecular weight of 1.3 × 10^5. In both cases, identification of the linked side chains will likely be revealing of the enzyme's active-site structure.

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Registry No. ATPase, 9000-83-3; ATP-EDC, 130096-08-1; ATP, 56-65-5; EDC, 1892-57-5; NaADP, 1172-42-5; DPC, 693-13-0; ADP, 58-64-0; TNP-ADP, 97902-37-9; AMP-PCP, 3469-78-1; ATP-DPC, 130096-09-2; P_i, 14265-44-2; Ca, 7440-70-2; Mg, 7439-95-4.

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Purification and Characterization of Calmodulin-Dependent Multifunctional Protein Kinase from Smooth Muscle: Isolation of Caldesmon Kinase†

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ABSTRACT: Previously, it was reported that smooth muscle caldesmon is a protein kinase and is autophosphorylated [Scott-Woo, G. C., & Walsh, M. P. (1988) *Biochem. J. 252*, 463-472]. We separated a Ca^{2+}/calmodulin-dependent protein kinase from caldesmon in the presence of 15 mM $MgCl_2$. The Ca^{2+}/calmodulin-dependent caldesmon kinase was purified by using a series of liquid chromatography steps and was characterized. The subunit molecular weight (MW) of the kinase was 56K by SDS gel electrophoresis and was autophosphorylated. After the autophosphorylation, the kinase became active even in the absence of Ca^{2+}/calmodulin. The substrate specificity of caldesmon kinase was similar to the rat brain calmodulin-dependent multifunctional protein kinase II (CaM PK-II) and phosphorylated brain synapsin and smooth muscle 20-kDa myosin light chain. The purified kinase bound to caldesmon, and the binding was abolished in the presence of high $MgCl_2$. Enzymological parameters were measured for smooth muscle caldesmon kinase, and these were K_{CaM} = 32 nM, K_{ATP} = 12 μM, $K_{caldesmon}$ = 4.9 μM, and $K_{Mg^{2+}}$ = 1.1 mM. Optimum pH was 7.5-9.5. The observed properties were similar to brain CaM PK-II, and, therefore, it was concluded that smooth muscle caldesmon kinase is the isozyme of CaM PK-II in smooth muscle.

It is well-known that the change in the intracellular Ca^{2+} concentration affects many physiological processes including muscle contraction. One of the major systems which transmits this signal to a change in the cell function is the major intracellular Ca^{2+} binding protein calmodulin and calmodulin-dependent enzymes.

In smooth muscle, the increase in the intracellular Ca^{2+} concentration causes the activation of a calmodulin-dependent protein kinase, myosin light chain kinase (MLC kinase). MLC kinase phosphorylates 20-kDa myosin light chain and activates the actomyosin ATPase activity. This is followed by the initiation of contraction (Hartshorne, 1987; Adelstein & Eisenberg, 1980). Although phosphorylation of myosin is thought to be a key event in the initiation of smooth muscle contraction, several recent results using intact smooth muscle strips have suggested that other control mechanisms may exist which modulate smooth muscle contraction. Murphy and his

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