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Inactivation of Ca^{2+}-, Na^+K^+-, and H^+K^+-ATPases with a carbodiimide derivative of ATP

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The γ-P adduct of ATP with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (ATP-EDC) was synthesized and incubated with the Ca-ATPase of sarcoplasmic reticulum with the result that time-dependent complete loss of the enzyme's activity occurred. The inactivation required calcium and magnesium while ATP had a protective effect. ATP-EDC incubation with the NaK-ATPase and HK-ATPase produced partial (> 50%) inactivation, but had no effect on myosin S1, pyruvate kinase and hexokinase, suggesting that this ATP analog is a specific inactivator of the so-called 'P-type' ATPases.

ATPase; Affinity label; Carbodiimide; ATP derivative

1. INTRODUCTION

ATP-utilizing enzymes constitute one of the largest groups of proteins. Among these catalysts, ATPases are an important subgroup which couple ATP hydrolysis to such endergonic processes as ion transport and motility. With the publication of the amino acid sequences of many of these proteins [1–6] has come the realization that they have varying degrees of structural relatedness, to a first approximation correlated with functional and mechanistic similarity [7–10]. While proposed models have been valuable in summarizing structural data and suggesting directions for further studies, much additional information is required to map specific sites and the proximity of side chains distant in the primary structure. For example, studies have implicated fluorescein isothiocyanate as an affinity label of the nucleotide site of the SR Ca-ATPase [11–14] (analogous results have been reported for the NaK-ATPase [15,16] and HK-ATPase [17]), and clarification of the site's structure will benefit from development of additional labels. This would include the synthesis and application of substrate derivatives capable of reacting at a specific part of the active site. We report here the synthesis of a gamma phosphoryl carbodiimide derivative of ATP which reacts with and inactivates the Ca^{2+}-, Na^+K^+-, and H^+K^+-ATPases of rabbit muscle sarcoplasmic reticulum, canine kidney and porcine stomach, respectively.

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Abbreviations: SR, sarcoplasmic reticulum; Mops, 3-(*N*-morpholino)propanesulfonic acid; TEOA, triethanolamine; EGTA, ethylene glycol bis(2-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; AMP-PNP, adenyl-5'-ylimidodiphosphate; AMP-PCP, adenyl-5'-ylmethylenediphosphonate; TNP-ADP, 2',3'-(2,4,6-trinitrocyclohexadienylidene) adenosine 5'-diphosphate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; EP, phosphorylated enzyme intermediate; ATP-EDC, γ-P adduct of ATP and EDC; PK, pyruvate kinase; LDH, lactate dehydrogenase; PEP, phosphoenol pyruvate

2. MATERIALS AND METHODS

SR vesicles were prepared from rabbit hindleg muscle [18]. HK-ATPase from hog stomach was kindly supplied by Dr William Reenstra of UC Berkeley. NaK-ATPase (grade 4 from canine kidney), LDH (type 2), PK (type 2), hexokinase (type C-300), glucose-6-phosphate dehydrogenase (type 7), EDC and ATP were obtained from Sigma. TNP-ADP was from Molecular Probes.

ATP-EDC was prepared by adding 1 ml of acetonitrile and 0.8 mmol of EDC to 1 ml of 0.2 M Na_2ATP at 25°C. After 10–12 min, aliquots were frozen and stored at −50°C. Reverse-phase HPLC showed more than 80% of the ATP was converted to ATP-EDC. The ADP analog was prepared the same way from Na-ADP. Proteins were incubated with ATP-EDC or ADP-EDC at pH 7.4 in 50 mM TEOA (chloride) at 25°C; other components are described in the figures and table.

Enzyme activities at 37°C were measured spectrophotometrically at 340 nm by coupling one of the enzyme's products to oxidation of NADH or reduction of $NADP^+$. For the SR enzyme, this was done as previously described [19]. The NaK-ATPase assays were done in solutions containing 40 mM TEOA, pH 7.4, 120 mM NaCl, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 2 mM MgATP, 1 mM PEP, 0.3 mM NADH, 0.02 mg/ml PK and 0.02 mg/ml LDH. HK-ATPase activity was measured in 100 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM ADP, 2 mM PEP, 0.3 mM NADH, 0.02 mg/ml PK and 0.02 mg/ml LDH. Assay solutions for PK contained 100 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM ADP, 2 mM NADH and 0.02 mg/ml LDH. Hexokinase assays were carried out in 40 mM TEOA, pH 7.4, 250 mM glucose, 7 mM $MgCl_2$, 1 mM ATP, 1 mM $NADP^+$ and 20 μg/ml glucose-6-phosphate dehydrogenase.

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3. RESULTS

As shown in Fig. 1, incubation of SR vesicles isolated from rabbit muscle with ATP-EDC resulted in a rapid loss of Ca-ATPase activity. If either Mg^{2+} or Ca^{2+} or both were omitted from the incubation mixture, the ATPase activity remained unchanged (± 5%) from control (ATP-EDC omitted) mixtures. Fig. 1 also shows that the inactivation rate was much slower in the presence of 1 mM ATP. The possibility that ATP conferred protection by reacting with ATP-EDC was ruled out by the lack of effect of ATP at higher concentrations on the rate of hydrolysis of ATP-EDC as followed by HPLC (not shown). The inactivation effect of ATP-EDC was not produced by an equivalent concentration of EDC. Incubation of ADP-EDC with SR vesicles under the same conditions caused no significant change in Ca-ATPase activity.

The effect of incubating the same concentration of ATP-EDC with NaK-ATPase was little (<20%) inactivation (not shown). At a 5-fold higher concentration, however, ATP-EDC caused the activity to decrease to about 30% in 10 min (Fig. 2); a second addition of ATP-EDC reduced the activity by an equivalent additional factor. Since ATP-EDC hydrolyzed with a half-time of about 5 min under these conditions (not shown), partial inactivation is likely not to be due to the presence of a refractory enzyme but to the relative rates of inactivation and reagent hydrolysis. Omission of Na^+, K^+, or Mg^{2+} decreased the effectiveness of ATP-EDC as an inactivator (Table I), and addition of Mg-ATP or TNP-ADP just prior to ATP-EDC addition resulted in preservation of most of the enzyme's activity.

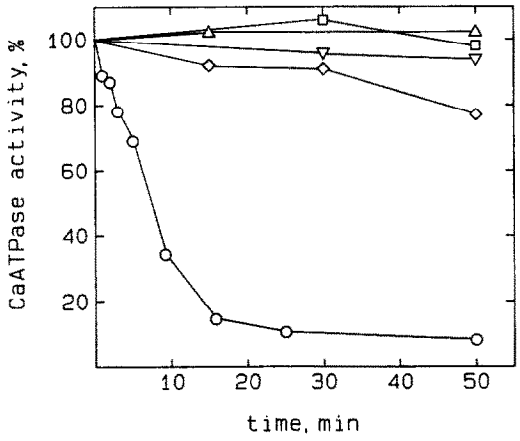

Fig. 1. Time course of the inactivation of SR Ca-ATPase by ATP-EDC. Incubation mixtures contained 100 mM TEOA (chloride), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mg/ml SR vesicles, pH 7.2, 25°C. The reaction was started by adding a freshly defrosted solution of ATP-EDC to 80 μM. At times indicated on the abscissa, aliquots were quenched by 100-fold dilution into the assay mixture at 0°C. The assay procedure is described in section 2. (○) No additional components; (◇) 1 mM ATP included; (▽) $MgCl_2$ deleted; (□) $CaCl_2$ deleted, 1 mM EGTA included; (△) $MgCl_2$ and $CaCl_2$ deleted, 1 μM EGTA included. The control activity was 14–16 μmol/min per mg.

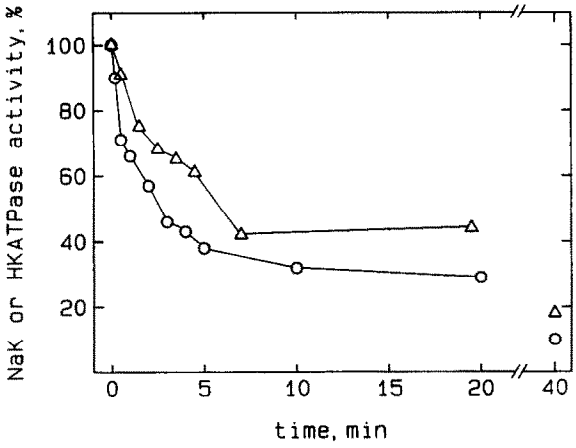

Fig. 2. Time course of the inactivation of NaK-ATPase and HK-ATPase by ATP-EDC. Incubation mixtures for NaK-ATPase contained 50 mM TEOA (chloride), 5 mM $MgCl_2$, 0.1 mM EGTA, 20 mM KCl, 120 mM NaCl and 0.5 mg/ml NaK-ATPase, pH 7.2, 25°C. For HK-ATPase they contained 50 mM TEOA (chloride), 5 mM $MgCl_2$, 10 mM KCl and 0.3 mg/ml HK-ATPase. For both enzymes, the reaction was started by adding a freshly defrosted solution of ATP-EDC to 0.4 mM. A second addition of ATP-EDC equivalent to the first was added at 20 min. At times indicated on the abscissa, aliquots were quenched by 100-fold dilution into the assay mixture at 0°C. The assay procedures are described in section 2. (○) NaK-ATPase; (△) HK-ATPase. Control activities were 1.0–1.2 and 0.6–0.7 μmol/min per mg, respectively.

The time course of HKATPase inactivation by ATP-EDC is shown in Fig. 2. As observed for NaK-ATPase, addition of a second dose of ATP-EDC after enzymatic activity had leveled off produced a roughly equivalent additional degree of inactivation. Unlike the other two ATPases, the extent of inactivation was somewhat greater if one of the enzyme's ligands, potassium, was omitted from incubation mixtures (Table I). ATP and TNP-ADP only partly protected this ATPase. EDC itself produced some (about 20%) activity loss (not shown), suggesting that not all of the inactivation shown in Fig. 2 is attributable to ATP-EDC.

Table I

Effect of various ligands on the inactivation of NaK-ATPase and HK-ATPase by ATP-EDC[a]

Ligand omitted (−) or added (+)	Activity (% of control)	
	NaK-ATPase	HK-ATPase
No additions or omissions	36	54
$-K^+$	63	26
$-Na^+$	70	–
$-Mg^{2+}$	95	74
+Mg-ATP	86	66
+TNP-ADP	85	73

[a] Incubations were carried out for 20 min as described in Fig. 2. When present, the concentrations of Mg-ATP and TNP-ADP were 1 mM and 100 μM, respectively

In contrast to these enzymes, the ATPase activity of the S1 fragment of myosin was not affected by incubation with ATP-EDC (S. Highsmith, personal communication). Similarly, the enzymatic activities of pyruvate kinase and hexokinase were not appreciably changed by ATP-EDC incubation under a variety of conditions (the presence of K^+, Mg^{2+}, pyruvate or PEP for the former and Mg^{2+} or glucose for the latter enzyme).

4. DISCUSSION

ATP-EDC is an easily made adduct of ATP and the water-soluble carbodiimide EDC. Its synthesis exploits the nucleophilicity of monosubstituted phosphoryl groups [19,20]. It rapidly inactivated the Ca-ATPase of SR, doing so only in the presence of both Mg^{2+} and Ca^{2+}. These are the divalent cations required for optimal phosphorylation and turnover of the enzyme [21,22], so the results imply that in addition to nucleotide binding, which occurs in the absence of Ca^{2+} and/or Mg^{2+}, ATP-EDC modification requires a conformation change associated with EP formation [23–25]. This conformation change presumably includes movement of a side chain into a position where it can react with the reagent. The protective effect of ATP provides additional support for the suggestion that site-directed (affinity) labeling occurred. That ADP-EDC failed as an inactivator suggests that positioning at the active site is critical for reaction to occur.

ATP-EDC was similarly effective as an inactivator of the NaK-ATPase. As found for the Ca-ATPase, the reaction worked best if ions required for optimal turnover (Na^+, K^+ and Mg^{2+}) were present, again suggesting that a turnover-required conformation change following substrate binding is necessary for positioning of the reactive side chain.

The results indicate that the HK-ATPase should also be included as an ATP-EDC inactivatable enzyme. On the other hand, testing under a variety of conditions failed to show that myosin S1, pyruvate kinase or hexokinase are susceptible to inhibition by this adduct, allowing the tentative conclusion that ATP-EDC is a specific inactivator of ATPases which form a covalent phosphorylated intermediate during their catalytic cycle; these have been termed P-type ATPases [7]. Given the large number of nucleotide-utilizing enzymes in nature, however, it may be premature to rule out ATP-EDC or other nucleotide-carbodiimide adducts as useful site-specific reagents in the study of other enzymes. In any case, these results give additional support for structural similarity among these ATPases.

Reaction with ATP-EDC may provide a way to identify additional active site residues of these ATPases. It may be significant that for all 3 proteins the amino acid side chain group which is phosphorylated is a carboxyl group. This is a side chain type capable of reacting with a nucleotide-carbodiimide adduct [20]. Other nucleophilic side chains are of course also plausible candidates.

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