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Specificity of the Sarcoplasmic Reticulum Calcium ATPase at the Hydrolysis Step†

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ABSTRACT: The coupling of Ca^{2+} transport to ATP hydrolysis by the SR ATPase requires that the enzyme operate with considerable specificity, which is different at different steps. The limits of specificity of the calcium-free phosphorylated enzyme for transfer of its phosphoryl group to water have been examined. The rate of transfer of the phosphoryl group to the simple nucleophile methanol was compared to its transfer to water by following the formation of methyl phosphate from inorganic phosphate. The reverse reaction, hydrolysis of methyl phosphate, was compared to phosphate–water oxygen exchange. The reactions involving methanol as nucleophile or leaving group are at least 2–3 orders of magnitude slower than those involving water. This result indicates that the transition state for this reaction involves strong and specific interactions of the H_2O molecule with the enzyme. These interactions may also involve the bound Mg^{2+} ion. The results also suggest that the difference in specificity between Ca^{2+} free and Ca^{2+} bound states of the enzyme involves significant differences in the structure of the catalytic site.

The Ca^{2+}-ATPase of sarcoplasmic reticulum is one of the most thoroughly studied coupled membrane ion pumps; considerable progress has been made toward an understanding of the mechanism by which it mediates the interconversion of chemical energy and osmotic work (de Meis & Vianna, 1979; Ikemoto, 1982; Martonosi & Beeler, 1983; Inesi, 1985). A large body of experimental evidence has been gathered in support of a stepwise reaction cycle for the enzyme (Scheme I) that can explain the stoichiometric coupling of the vectorial transport of calcium ions across the SR[1] membrane to the hydrolysis of ATP. This reaction scheme (like other related

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[1] Abbreviations: SR, sarcoplasmic reticulum; E, calcium ATPase; EP, phosphorylated calcium ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MeOP, methyl phosphate; BSA, bovine serum albumin.

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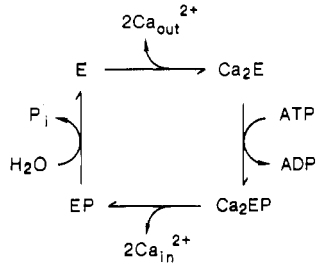
Scheme I



schemes) implicitly invokes "coupling rules"; that is, for each intermediate in the scheme, certain reactions are assumed to be allowed while others are "forbidden" (de Meis & Vianna, 1979; Jencks, 1983). These rules define the chemical and/or vectorial specificity of the enzme, which is *different* at different steps in the cycle.

The most remarkable chemical reaction catalyzed by the Ca^{2+}-ATPase of sarcoplasmic reticulum is surely the rapid, spontaneous formation of an acyl phosphate from an aspartyl carboxylate group and inorganic phosphate at the active site of the enzyme in the absence of Ca^{2+} (Kanazawa & Boyer, 1973; Masuda & de Meis, 1973; Knowles & Racker, 1975). The driving force for this reaction is some 12.5 kcal mol^{-1} of binding energy for the phosphoryl group of the acyl phosphate that must arise from a snug, exact fit in the binding site. Synthesis of the acyl phosphate requires removal of a hydroxyl group from inorganic phosphate, a difficult reaction, and is accelerated at the active site by a factor of 10^{15} compared with the nonenzymic reaction (Pickart & Jencks, 1984). The enzyme also reacts in the absence of calcium with strongly activated chemical phosphorylating agents, such as acyl phosphates and phosphate esters with acidic leaving groups, but these reactions are far slower than the reaction with inorganic phosphate (Pucell & Martonosi, 1971; Rossi et al., 1979; Inesi et al., 1980; Liguri et al., 1980; Kurzmack et al., 1981; Bodley & Jencks, 1987).

We were curious about the mechanism of this catalysis of the extraction and addition of water and would like to understand what changes in the structure of the active site cause EP to prefer to transfer its phosphoryl group to water, while Ca_2EP prefers to transfer this group to ADP. As a first step toward characterizing the mechanism, we have examined the reaction in which methanol replaces water as the acceptor of phosphate from EP. The pK of methanol, 15.4, is close to that of water at the same standard state, 15.7, and the methyl group provides a minimal increase in bulk over the proton of water. We find that the enzyme shows little or no catalysis of the reaction with methyl phosphate or methanol, so that the active site is highly specific for catalysis of the addition and expulsion of water. The very large preference for water that we have observed has significant implications for the mechanism and the active-site structure.

Materials and Methods

Materials. [^{32}P]H_3PO_4 in 0.02 N HCl was obtained from New England Nuclear Corp. (Boston, MA). To ensure its freedom from oligophosphates, it was brought to 1.3 N in HCl, heated to 100 °C for ~30 min, neutralized with KOH, and diluted with unlabeled K_2HPO_4. Terbium(III) hexahydrate and sodium orthovanadate were obtained from Aldrich (Milwaukee, WI). The ionophore A23187, pyruvate kinase, lactate dehydrogenase, *Escherichia coli* alkaline phosphatase (type III), NADH, phosphoenolpyruvate, and buffer components were obtained from Sigma Chemical Co. (St. Louis, MO).

Sarcoplasmic reticulum vesicles were prepared from rabbit back and hind leg white muscle by an adaptation (Pickart & Jencks, 1982) of the method of MacLennan (1970) and stored at −70 °C in 0.4 M sucrose–5 mM Tris-SO_4 at pH 8.0. The preparations used had a Ca^{2+}-ATPase activity at pH 7.0 and 25 °C of 3.5 μmol (mg of protein)$^{-1}$ min^{-1}, determined by using the coupled enzyme assay (Pickart & Jencks, 1982). Their Mg^{2+}-ATPase activity was only about 1% of the Ca^{2+}- and Mg^{2+}-dependent activity. The active-site stoichiometry of the preparations determined by phosphorylation from $^{32}P_i$ at pH 6.0 in 40% DMSO (Barabin et al., 1984) was 2.0 nmol (mg of protein)$^{-1}$.

Methyl [^{32}P]phosphate was prepared by the *E. coli* alkaline phosphatase catalyzed reaction of [^{32}P]phosphate with methanol (Wolfenden & Williams, 1983). A reaction mixture containing 2 units/mL alkaline phosphatase and 64 mM [^{32}P]K_2HPO_4 (1.7×10^4 cpm/nmol) in a total volume of 0.5 mL of 33% (v/v) methanol was incubated 3 days at room temperature and separated by chromatography on Whatmann 3MM paper using butanol–acetic acid–water (4:1:1 v/v) as eluent. The labeled methyl phosphate was eluted with water and stored at −15 °C. Analysis (see below) showed that it was 95% methyl phosphate, at a concentration of 2.4 mM. The concentration of methyl phosphate in samples was determined by counting of Cerenkov radiation, assuming a specific activity equal to that of the inorganic phosphate used, corrected for decay.

Assays. Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as standard.

Malate was used as chelating agent to buffer free Tb^{3+} concentrations. The free Tb^{3+} concentration was calculated by assuming the binding of Tb^{3+} to EGTA is stoichiometric and complete and that the association constant for the Tb^{3+}–malate 1:1 complex is 5.9×10^4 M^{-1} (Martell & Smith, 1977).

ATPase activities were assayed by the coupled enzyme method (Pickart & Jencks, 1982), in a 100 mM MOPS buffer (pH 7.0) containing 100 mM KCl, 5 mM $MgCl_2$, 0.3 mM EGTA, 0.3 mM $CaCl_2$, 1.5 mM ATP, and 2 μg/mL A23187. The permeability of the SR to Ca^{2+} was estimated by comparing the activity without and with the ionophore A23187.

The hydrolysis of methyl phosphate was followed in a 350-μL reaction mixture containing SR vesicles (2.0 mg/mL protein) in 20 mM $MgSO_4$, 50 mM KCl, 0.50 mM EGTA, 5 mM sodium malate, and 50 mM MES at pH 6.5 and 24 °C. Methyl [^{32}P]phosphate (80–250 μM) was added last. At times up to 4 h, 60-μL aliquots were removed and quenched in 1 mL of 14% trichloroacetic acid containing 0.1 mM phosphate. Bovine serum albumin (0.1 mg in 0.1 mL) was added, and after at least 15 min at 0 °C, the samples were centrifuged to remove precipitated protein. The supernatant (0.9 mL) was transferred to a test tube containing 1 mL of 4.2% ammonium molybdate in 4 N HCl and 1.1 mL of water saturated with 2-methyl-1-propanol and xylene. To this was added 3 mL of a 1:1 mixture of 2-methyl-1-propanol and xylene (saturated with water), and the mixture was Vortex-mixed vigorously and allowed to separate. Aliquots of each phase (2 mL) were added to vials containing 4 mL of 10% ammonia and taken for counting (Cerenkov radiation). Separate experiments demonstrated that there was no difference in counting efficiency for the two phases as long as ammonia was present. The fraction of the substrate remaining at time t was calculated from f_t/f_0, where f_t is the fraction of the total counts found in the aqueous phase upon analysis of an aliquot of the reaction mixture at time t and f_0 is the fraction found for analysis of

Table I: SR Ca^{2+}-ATPase Catalyzed Transfer of Phosphate to Methanol[a]

[phosphate] (mM)	5	5
[methanol] (M)	2.5	2.5
[Ca^{2+}] (mM)	0	0.5
incorporation of ^{32}P [mol (mol of E)$^{-1}$ min^{-1}]	0.033 ± 0.013[b]	0.064 ± 0.021[b]
"Ca^{2+}-inhibitable" incorporation [mol (mol of E)$^{-1}$ min^{-1}]	<0.015[c]	
steady-state EP/E_{total}	0.19[d]	<0.002[d]
k_{MeOH} rate of transfer to methanol (M^{-1} min^{-1})	<0.032[e]	

[a] SR vesicles were incubated in reaction mixtures containing [^{32}P]-phosphate, 10% methanol (v/v), and free Ca^{2+} at the indicated concentrations in 50 mM MES buffer–20 mM $MgSO_4$–50 mM KCl–0.5 mM EGTA at pH 6.5 and 25 °C. [b] Rate of incorporation of ^{32}P into acid-stable organic phosphate (see Materials and Methods). The enzyme stoichiometry (2.0 nmol/mg of protein) for the SR vesicle preparation used was determined by the method of Barabin et al. (1984). [c] Maximum limit of Ca^{2+} inhibitable rate within 85% confidence limits. The difference between incorporation in the absence and presence of free Ca^{2+} is –0.031 mol (mol of E)$^{-1}$ min^{-1}, with a standard deviation of 0.025. Within 85% confidence limits, the difference is between –0.072 and +0.015 mol (mol of E)$^{-1}$ min^{-1}. [d] Experimental EP/E_{total} in the presence of methanol. [e] Rate of incorporation divided by fraction of enzyme phosphorylated and methanol concentration.

the substrate itself. For aliquots of the reaction mixture taken immediately after addition of substrate, f_t/f_0 was always 0.997–1.002.

The formation of methyl phosphate was followed in a reaction mixture containing 5 mM [^{32}P]phosphate ($\sim 2 \times 10^3$ cpm/nmol), 10% methanol (v/v), and the buffer and salts used for methyl phosphate hydrolysis experiments, at pH 6.5 and 25 °C. Aliquots (0.2 mL) taken in triplicate at time 0 and 45 min were quenched by addition to 1 mL of 14% trichloroacetic acid in a conical test tube. The test tubes were immersed in boiling water for 20 min and then 0.25 mL of BSA in 0.25 mL of H_2O was added, and the tubes were cooled and centrifuged. An aliquot of the supernatant (0.75 mL) was analyzed by extraction as described above. The fraction of the phosphate transferred into acid-stable compounds other than inorganic phosphate was calculated from the ratio of counts in the aqueous phase to total counts.

Enzyme phosphorylation was followed in a pH 6.5 MES–malate buffer identical with that used for the methyl phosphate hydrolysis, with or without added methanol or inhibitors. SR vesicles (1 mg/mL final) were added to 0.5 mL of the solution, containing 5 mM [^{32}P]phosphate (1×10^4 cpm/nmol). After 15 s, the reaction was quenched and worked up as previously described (Verjovski-Almeida et al., 1978). For the determination of total phosphorylation sites, the reaction was carried out in a pH 6.0 30 mM MES–8.3 mM Tris buffer containing 20 mM $MgSO_4$, 1 mM EGTA, and 40% dimethyl sulfoxide (v/v) (Barabin et al., 1984).

Results

The formation of methyl phosphate from inorganic phosphate and methanol, catalyzed by the SR Ca^{2+}-ATPase, was studied under the conditions used by McIntosh and Boyer (1983) to study the enzyme-catalyzed phosphate–water oxygen exchange (pH 6.5, 20 mM Mg^{2+}, 50 mM K^+). The observed formation of acid-stable organic phosphate catalyzed by SR vesicles in the presence of 10% methanol is extremely slow (Table I). In addition, it appears that at least part of the observed reaction is *not* due to transfer of a phosphoryl group from phosphorylated Ca^{2+}-ATPase to methanol: It is known that external Ca^{2+} inhibits the formation of the covalent phosphate complex of this enzyme (EP) from inorganic phosphate (Masuda & de Meis, 1973; Pickart & Jencks, 1984; Inesi, 1985), presumably by forming Ca_2E, which is both kinetically and thermodynamically unreactive toward phosphate. Under the conditions of our experiments, 0.5 mM Ca^{2+} decreases the steady-state phosphorylation level nearly 100-fold (Table I). However, the observed rates of formation of acid-stable organic phosphate in the presence of 0.5 mM Ca^{2+} are actually higher than (but within experimental error of) those in the absence of Ca^{2+}. We can make a conservative estimate of the upper limit for the "Ca-inhibitable" (i.e., relevant) rate of methyl phosphate formation by taking the 85% confidence interval for the difference between the rates in the absence and in the presence of Ca^{2+}; it is less than 0.015 mol (mol of enzyme)$^{-1}$ min^{-1} (Table I).

The second-order rate constant for phosphate transfer from EP to methanol (k_{MeOH}) can be estimated by dividing the methyl phosphate formation rate by the fraction of the enzyme phosphorylated (EP/E_{total}) and the methanol concentration. Under the conditions used here, EP/E_{total} in the presence of 5 mM phosphate is 0.19, close to the value of 0.15 calculated from the parameters of McIntosh and Boyer (1983). The presence of 10% methanol does not affect EP/E_{total} (Table I), while 15% methanol increases it to about 0.25. The derived second-order rate constant for phosphate transfer to methanol, k_{MeOH}, is less than 0.032 M^{-1} min^{-1}.

Several additional controls demonstrate that the SR Ca^{2+}-ATPase is active in solutions containing up to 12% methanol and that its kinetics are essentially unaffected. After incubation for 4 h in a reaction mixture containing 12% methanol, similar to that used to follow methyl phosphate formation, and dilution into the ATPase assay mixture containing ionophore, >95% of the Ca^{2+}-dependent ATPase activity of the SR preparation remains. The rate of hydrolysis of 2,4-dinitrophenyl phosphate by the vesicles in the absence of Ca^{2+} is also unaffected by 15% methanol. Incubation in the presence of methanol does, however, accelerate the loss of integrity of the vesicles as barriers to Ca^{2+} diffusion.

The rather large experimental error in the observed rate of methyl phosphate formation (which leads in turn to uncertainty in the determination of k_{MeOH}) is due in part to a high background formation of a phosphate-containing material that is not methyl phosphate. This material was eliminated in our experiments by heating the quenched reaction mixture (12% in TCA) to 95 °C for 20 min before analysis; authentic methyl phosphate, on the other hand, is recovered in 97% yield from the overall analytical scheme used here (see Materials and Methods). The acid-labile material has a half-life for hydrolysis at 75 °C in the quenched mixture of about 4 min. Its formation requires the presence of SR vesicles and an organic solvent, but dimethyl sulfoxide can replace methanol. It can appear in quantities more than an order of magnitude greater than the enzyme site concentration, and its production is not dependent on the presence of a vesicular calcium gradient. The nature and origin of this material have not been further clarified.

Methyl phosphate hydrolysis by the Ca^{2+}-ATPase, which might proceed by the reverse of phosphoryl transfer from EP to methanol, was also studied. There are fewer experimental difficulties in following this reaction, and the reaction rates are more easily determined. Figure 1 shows a typical enzyme-catalyzed hydrolysis of methyl phosphate under pseudo-first-order conditions in the buffer system used above. The rate of disappearance of methyl phosphate in this experiment is equivalent to a second-order rate constant for the enzyme reaction, k_{cat}/K_M, of 3.0 M^{-1} s^{-1}. From the dependence of the

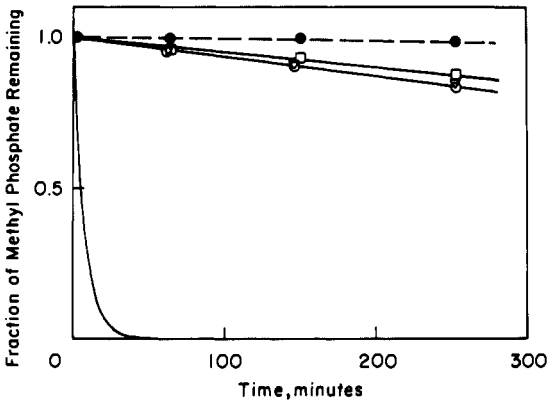


FIGURE 1: SR vesicle catalyzed hydrolysis of methyl phosphate. (○) SR vesicles (2 mg/mL, 3.84×10^{-6} M in phosphorylation sites) were added to reaction mixtures containing 130 μM methyl [^{32}P]phosphate in a pH 6.5 buffer (20 mM $MgSO_4$, 0.5 mM EGTA, 50 mM KCl, 50 mM MES, 5 mM malate). At the indicated times, aliquots were removed, quenched and analyzed as described under Materials and Methods. The apparent pseudo-first-order rate constant observed is 1.15×10^{-5} s^{-1}, from which the second-order constant $k_{cat}/K_M = 3.0$ M^{-1} s^{-1} can be calculated. For comparison, reactions were also carried out in the presence of 0.5 mM free Ca^{2+} (▽), 2 μM free Tb^{3+} (□), or 0.33 mM Vanadate (●). The rapidly descending curve is calculated from the second-order rate constant for catalysis by the enzyme of the removal of a given oxygen from inorganic phosphate, 580 M^{-1} s^{-1} (McIntosh & Boyer, 1983).

reaction rates on the initial methyl phosphate concentration (data not shown), we estimate that K_M must be 0.2 mM or greater.

Once again, however, it appears that a significant fraction of the observed activity may not be due to the Ca^{2+}-ATPase, since it is not inhibited as expected. For instance, 0.5 mM Ca^{2+} inhibits the methyl phosphatase activity by less than 10% (Figure 1). Similarly, 2 μM free Tb^{3+}, which inhibits phosphorylation by 90% (data not shown), inhibits methyl phosphatase activity by only 15–20% (Figure 1). Thermal denaturation of the SR vesicles by incubation at 65 °C for 2 min, which leaves about 3% of its Ca^{2+}-ATPase activity extant, leaves 30% of the methyl phosphatase activity intact (data not shown). The only effective inhibitor we have found for the latter activity is vanadate (Figure 1), which is a good inhibitor of many nonspecific phosphatases (Kustin & Macara, 1982). It is possible, then, that only 10–30% (or less) of the observed rate of methyl phosphate hydrolysis by SR vesicles occurs via phosphorylation of the Ca^{2+}-ATPase.

Discussion

In the experiments with the SR Ca^{2+}-ATPase we have reported here, we have compared the reactions of the Ca^{2+}-free phosphorylated intermediate, EP, with water and with methanol (eq 1 and 2, where P_i and MeOP are inorganic phosphate

$$EP \xrightarrow{H_2O} E{\cdot}P_i \rightleftharpoons E + P_i \quad (1)$$

$$EP \xrightarrow{CH_3OH} E{\cdot}MeOP \rightleftharpoons E + MeOP \quad (2)$$

and methyl phosphate, respectively). Since the latter reaction is at least 2 orders of magnitude slower than the former, it is not practical to study it by following the partitioning of the intermediate E·P formed during turnover of ATP. Instead, we have compared the two processes by comparing the rates of incorporation of labeled phosphate from P_i into methyl phosphate, as well as the rate of hydrolysis of methyl phosphate, with the rates of the phosphate–water exchange process

Table II: Kinetic Parameters for the Formation and Breakdown of EP

parameter	value	origin
k_1	2.8×10^4 M^{-1} s^{-1}	McIntosh and Boyer (1983)
k_{-1}	378 s^{-1}	McIntosh and Boyer (1983)
k_2	32 s^{-1}	McIntosh and Boyer (1983)
k_{-2}	51 s^{-1}	McIntosh and Boyer (1983)
k_{-2}'	0.93 M^{-1} s^{-1}	$k_{-2}/[H_2O]$
k_{H2O}	0.85 M^{-1} s^{-1}	$k_{-2}'k_{-1}/(k_{-1} + k_2)$
k_{MeOH}	1.16×10^{-3} M^{-1} s^{-1}	from MeOP formation
	($<5.3 \times 10^{-4}$ M^{-1} s^{-1})	(inhibitable rate)
$(k_{cat}/K_m)_{P_i}$	550 M^{-1} s^{-1}	$k_1k_2/4(k_{-1} + k_2)$, for a given oxygen
$(k_{cat}/K_m)_{MeOP}$	3.0 M^{-1} s^{-1}	MeOP hydrolysis
	(<1 M^{-1} s^{-1})	(inhibitable rate)

catalyzed by Ca^{2+}-ATPase. All of these processes can be summarized by eq 3. McIntosh and Boyer (1983) have

$$P_i + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}H\overset{*}{O}PO_3H^- \underset{H_2O^*}{\overset{k_2}{\rightleftharpoons}} EPO_3H^- \underset{CH_3OH}{\rightleftharpoons} E{\cdot}MeOP \rightleftharpoons E + MeOP \quad (3)$$

$$k_{-2} = k_{-2}'[H_2O]$$

demonstrated that the phosphate–water exchange process proceeds via the two reversible steps on the left-hand side of this equation and have determined the values of k_1, k_{-1}, k_2, and k_{-2} under conditions that we have adopted for our experiments.

From the rate of formation of methyl phosphate catalyzed by the enzyme and the steady-state concentration of EP, we have calculated the second-order rate constant for methanolysis of EP, k_{MeOH}. Unfortunately, we were faced with the common experimental problem when measuring slow "side reactions" of an enzyme preparation: Reactions catalyzed by very minor components of the preparation may be quite significant compared to the minor reaction of the enzyme of interest. If we assume that the reactions of the free enzyme (E_2) form of the Ca^{2+}-ATPase should be inhibited by Ca^{2+}, Tb^{3+}, and so on, then the correct rates of the processes of interest are the "inhibitable rates", the difference between the observed rates in the presence and absence of inhibitors. Such an estimate of the upper limit for k_{MeOH} is given in parentheses in Table II.

The estimated values of k_{MeOH} can be compared with the second-order rate constant for hydrolysis of EP, k_{H_2O}, derived from the data of McIntosh and Boyer (1983) (Table II). On the basis of this comparison, methanolysis of EP is at least 700 times slower than hydrolysis. It is more than 1500 times slower if we consider only the inhibitable rate of methyl phosphate formation to be due to methanolysis of the phosphorylated ATPase.

A similar comparison can be made of the second-order rate constants for the reverse processes. We have determined upper limits for k_{cat}/K_M for hydrolysis of methyl phosphate (Table II). From the kinetic parameters of McIntosh and Boyer, one can calculate k_{cat}/K_M for exchange of an oxygen atom of inorganic phosphate by the enzyme, 2190 M^{-1} s^{-1}. Since phosphate has four oxygens, however, the relevant rate for catalysis of exchange of a *given* oxygen is only one-fourth of this, 550 M^{-1} s^{-1}. The second-order rate constant for catalysis of the removal of methanol from methyl phosphate is from 200 to more than 600 times slower than that for removal of water from inorganic phosphate.

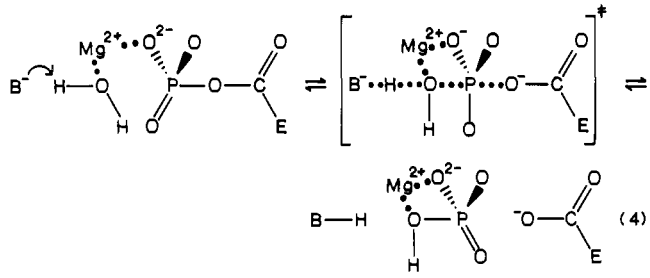
The transition state for the process in eq 2 is thus at least 3–4 kcal/mol less stable with respect to its ground state than

that of eq 1 for the calcium-free form of the SR ATPase. On the other hand, the intrinsic nucleophilic reactivity of methanol is generally at least as great as that of water in a wide variety of organic reactions, including attack on phosphoryl phosphorus mono- and dianions (Kirby & Varvoglis, 1967). Methanol competes effectively with water in many enzyme-catalyzed hydrolytic reactions as well, including those catalyzed by serine proteases (Bender et al., 1964; Seydoux & Yon, 1967), thiol proteases [e.g., Fink and Bender (1969)], and various esterases [e.g., Hellenbrand and Krupka (1970) and Greenzaid and Jencks (1971)], glycosidases [e.g., Sinnott and Viratelle (1973), Wilson and Fox (1974), and Dale et al. (1986)], and so on.

However, there *are* enzymes that are apparently highly specific for water as nucleophile and that discriminate against methanol and other simple nucleophiles. These include carboxypeptidase A (Breslow & Wernick, 1976; Breslow et al., 1983), the aspartyl proteases pepsin (Cornish-Bowden et al., 1969) and penicillopepsin (Hofmann et al., 1984), and staphylococcal nuclease (Dunn et al., 1973). In the case of carboxypeptidase, Breslow and his co-workers have estimated lower limits for k_{H_2O}/k_{MeOH} of 16–3000 for different substrates (Breslow & Wernick, 1976; Breslow et al., 1983). They have suggested that the preference for water is due to a requirement for transfer of the second proton of the attacking H_2O molecule to the leaving group in order to drive the breakdown of the tetrahedral intermediate to the product complex. According to this proposal, the tetrahedral intermediate formed upon addition of methanol would tend to break down to starting materials rather than to a methyl ester–zinc complex and leaving amine (Breslow et al., 1983). It is unlikely that this sort of mechanism is responsible for specificity for water as the nucleophile in the reaction we have studied; if a hydroxyl or methoxyl group were to add to the phosphoryl phosphorus of EP, the aspartyl carboxylate would certainly be the group of greatest intrinsic leaving tendency in the pentacovalent phosphorus intermediate thus formed.

An examination of the mechanism proposed for hydrolysis of peptide bonds by thermolysin, a zinc protease whose active-site structure shows striking similarities to carboxypeptidase (Monzingo & Matthews, 1984), suggests a number of additional reasons why such an enzyme might show high specificity for water (B. W. Matthews, personal communication). Studies based on the crystal structures of several complexes of thermolysin with transition-state analogues make it possible to describe the position and bonding in the active site of the attacking water molecule (Hangauer et al., 1984; Holden et al., 1987). This molecule is coordinated to the zinc ion, and its alignment and nucleophilicty may require that both protons be hydrogen bonded. In addition, the complementarity between bound substrate and water and the active site seems to be so great that accommodation of, e.g., the additional methyl group of methanol would lead to distortion of the active-site geometry (B. W. Matthews, personal communication).

A possible mechanism for catalysis of the hydrolysis and synthesis of the EP form of the Ca^{2+}-ATPase is shown in eq 4; this mechanism is a modification of a previous proposal (Pickart & Jencks, 1984). An examination of the hydrolysis of phosphate esters and phosphorylated pyridines has shown that Mg^{2+} is an effective catalyst for the expulsion of oxyanions by stabilizing the developing negative charge in the transition state (Herschlag & Jencks, 1987). In the reverse direction it can catalyze the reaction by increasing the concentration of oxygen anion in a position to attack the phosphoryl group.



(4)

Mg^{2+} can increase the acidity of the attacking water molecule or stabilize the developing OH^- group by a factor of $10^{4.3}$; it decreases the pK of water from 15.7 to 11.4 in the hydrate (Baes & Mesmer, 1976). Interaction of Mg^{2+} with the phosphoryl oxygen atoms is slightly inhibitory because of the dissociative, metaphosphate-like nature of the transition state (Herschlag & Jencks, 1987).

The dependence on pH of the rate and equilibrium constants for formation and cleavage of the phosphoenzyme is complex, but it is clear that the *equilibrium* formation of phosphoenzyme from inorganic phosphate is favored at low pH and that the *rate* of reaction of the phosphoenzyme is increased at high pH (MacIntosh & Boyer, 1983; Inesi et al., 1984). This means that a proton must be removed in order to go from the stable state of the phosphoenzyme to the transition state of the reaction. Since the acyl phosphate at the active site is presumably not protonated, the simplest explanation of this is that a catalyzing base is protonated in the range of pH examined and that this proton must be removed in order for hydrolysis to occur. In the reverse direction a proton is removed from $H_2PO_4^-$, and BH^+ catalyzes expulsion of the leaving HO^-. The Mg^{2+} ion acts as an electrophilic catalyst to stabilize the developing HO^- in the transition state.

If the hydrogen bonding of the *second* proton of the attacking water is important in this reaction, methanol would be a poor replacement for water. Alterantively, the mechanism might involve one less proton than shown in eq 4. The ionization responsible for the pH dependence of the rate might then be that of hydrated Mg^{2+}, the nucleophile would be $MgOH^+$, with general base assistance, and the immediate product of the hydrolysis reaction would be $MgPO_4^-$. This mechanism is obviously not possible for methanol, so that it could account for the specificity toward water. The activity of $MgOH^+$ as a nucleophile toward the phosphoryl group of phosphorylated pyridines in the presence of a second Mg^{2+} ion (Herschlag and Jencks, unpublished experiments) provides some precedent for the involvement of $MgOH^+$. In the enzymic reaction a cationic binding group for phosphate at the active site, such as the guanidinium group of an arginine residue, may serve the function of the second Mg^{2+} ion.

The low reactivity of methanol and methyl phosphate could also imply that these compounds cannot fit into the active site in a productive way, i.e., that the methyl group causes distortion of the active site in such a way that the catalytic groups are not correctly aligned for effective catalysis. This is not surprising for this enzyme in view of the very strong specific binding interaction that is responsible for stabilization of the phosphoenzyme. A major contributing factor for the rapid, spontaneous formation of the aspartyl phosphate from inorganic phosphate is certainly the decrease in entropy of the bound phosphate, which arises from an exact, snug fit of this molecule in the active site (Pickart & Jencks, 1984). Acetyl phosphate and activated phosphate esters cannot achieve the optimal structure either, but they are able to react because of their high, nonspecific chemical reactivity. The fact that they do react indicates that steric *exclusion* is not the reason

for the low reactivity of phosphate esters and anhydrides compared to phosphate itself.

Whatever may be the basis for the specificity for water as nucleophile in the dephosphorylation of the calcium-free EP form of the Ca^{2+}-ATPase, it is clear that the difference in specificity between the calcium-free and calcium-bound form of the enzyme must involve more than changes in the structure of the "nucleoside site" or its movement relative to the "phosphorylation site". The specificity difference, so crucial to the coupling of ATP hydrolysis to calcium transport, must involve significant reorganization of the catalytic site itself.

Registry No. ATPase, 9000-83-3; MeOH, 67-56-1; MeOP, 512-56-1.

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