Adenosine 5′-Triphosphate Modulation of Catalytic Intermediates of Calcium Ion Activated Adenosinetriphosphatase of Sarcoplasmic Reticulum Subsequent to Enzyme Phosphorylation†

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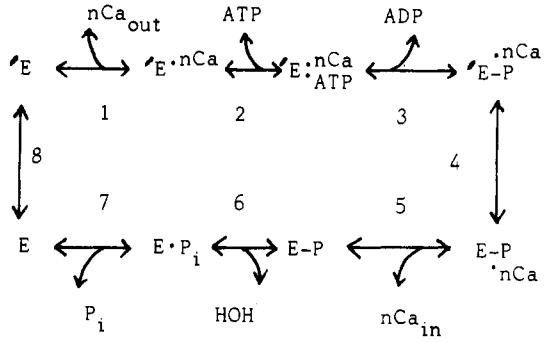
ABSTRACT: Several different catalytic intermediates of the Ca^{2+}-ATPase of sarcoplasmic reticulum which contain bound P_i and which, as a phosphoprotein, react with water exist depending on Ca^{2+} and ATP concentrations. In the absence of these ligands, phosphoenzyme (E-P) formation from P_i at fixed high Mg^{2+} concentration is governed by four rate constants, and these have been evaluated from medium $P_i \rightleftharpoons$ HOH exchange and E-P measurements. Ca^{2+} and ATP change these rate constants in a variable manner. In the presence of micromolar concentrations of Ca^{2+} the rate constant for E-P hydrolysis trebles as the ATP concentration is increased from 0 to 25 μM, decreases with more ATP to about 100 μM, and then again increases 4–5-fold over the original value as the ATP concentration is increased to 5 mM. In the higher concentration range, 50 μM to 5 mM, there are also changes in the properties of enzyme-bound P_i ($E{\cdot}P_i$) such that the rate of P_i release from the unliganded form changes from being 9 times faster than E-P formation to being only 2–3 times faster when ATP is bound. This effect can be shown in the absence of Ca^{2+} and does not depend on whether the phosphoryl group arises from medium P_i or from ATP. High concentrations of Triton X-100 do not affect this modulation of the partitioning of $E{\cdot}P_i$, suggesting that it can occur with monomeric enzyme. Another effect of millimolar concentrations of ATP is to about double the amount of E-P reactive to water. Even so the amounts of $E{\cdot}P_i$ and of E-P arising from ATP during Ca^{2+}-stimulated ATPase activity at concentrations of ATP up to 5 mM are small relative to the total amount of phosphoenzyme. This is consistent with a prior slow step in the cycle, either Ca^{2+} release to the vesicle lumen or the transposition and change in affinity of the Ca^{2+} binding sites. The results give evidence that during hydrolysis of ATP at physiological concentrations by sarcoplasmic reticulum, the ATP enters the catalytic cycle before enzyme dephosphorylation. If, as seems likely, there is only one catalytic site per ATPase, the ATP modulations at lower ATP concentrations give evidence for cooperative interactions between enzyme dimers or oligomers or, alternatively, for ATP binding at a catalytic site after ADP departure but prior to E-P hydrolysis.

The catalytic cycle of the Ca^{2+}-ATPase[1] of sarcoplasmic reticulum is commonly represented by a simplified scheme such as given in Scheme I. ′E and E represent two forms of the enzyme with high-affinity, outward orientated and low-affinity, inward orientated Ca^{2+} binding sites, respectively. The intermediates depicted here have been detected or deduced to exist largely from the many studies of the partial reactions catalyzed by this enzyme [see de Meis & Vianna (1979)]. However, the intermediates present during conditions for physiological Ca^{2+} uptake and the points of entry of the substrates into and of exit of products from the cycle may be different from those determined from individual partial reactions studied in isolation. Additional binding sites for ATP and those for K^+ and Mg^{2+} further complicate the cycle.

A prominent partial reaction is the formation of $E{\cdot}P_i$ and E-P from medium P_i in the absence of Ca^{2+} (Kanazawa & Boyer, 1973; Masuda & de Meis, 1973; Beil et al., 1977; Boyer et al., 1977; Punzengruber et al., 1978). About half of the enzyme molecules can be induced to form E-P under favorable conditions (Masuda & de Meis, 1973). One purpose of the present study was further characterization of this partial reaction. Medium $P_i \rightleftharpoons$ HOH exchange[2] and the levels of E-P formed from P_i have allowed us to evaluate four rate constants governing E-P formation in the absence of Ca^{2+} and in the presence of high Mg^{2+} concentration.

Scheme I



Previous studies have shown that in the presence of Ca^{2+} and ATP, especially if the ATP concentration is kept low,

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[1] Abbreviations: Ca^{2+}-ATPase, calcium ion activated adenosinetriphosphatase; SR, sarcoplasmic reticulum; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; P_c, partition coefficient which is defined as $k_2/(k_2 + k_{-1})$ where k_2 is the rate constant for phosphoenzyme formation from the $E{\cdot}P_i$ complex and k_{-1} is the rate constant for P_i release from the enzyme; $P^{18}O_4$, $P^{18}O_3$, $P^{18}O_2$, $P^{18}O_1$, and $P^{18}O_0$, inorganic phosphate containing four, three, two, one, and zero ^{18}O atoms per molecule, respectively; $C_{12}E_8$, dodecyl octaethylene glycol monoether.

[2] Medium $P_i \rightleftharpoons$ HOH exchange occurs when P_i binds, undergoes exchange, and is released to the medium. Intermediate $P_i \rightleftharpoons$ HOH exchange occurs when P_i formed from ATP incorporates more than one oxygen from water prior to release to the medium.

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formation of E-P from medium P_i is readily detectable (Carvalho et al., 1976; de Meis & Boyer, 1978) as is a medium $P_i \rightleftharpoons HOH$ exchange (de Meis & Boyer, 1978). It has been presumed that during ATP cleavage the same E·P_i and E-P forms are generated, but with the phosphoryl group arising from ATP, and that the same rate constants govern the reactions of these forms. However, preliminary studies (Boyer & Ariki, 1980) suggested that the presence of ATP and Ca^{2+} might change properties of E·P_i and E-P forms. Also, spin resonance studies of Coan et al. (1979) indicate that a nucleotide is bound to the enzyme at all stages of the catalytic cycle. We have thus measured the effect of ATP and of ATP plus Ca^{2+} on the properties of E-P and E·P_i formed from either P_i or ATP. Our results give evidence that the same E-P and E·P_i forms arise from either P_i or ATP but that the presence of ATP causes prominent modulations of their reaction characteristics. An important conclusion is that under physiological conditions ATP likely binds to the catalytic site prior to E-P hydrolysis.

Experimental Procedures

Materials. $H^{18}OH$ came from Norsk Hydro, New York, and $[^{18}O]P_i$ was made as described by Hackney et al. (1980). Triton X-100 was purchased from Sigma. A23187 was obtained from Calbiochem.

Sarcoplasmic Reticulum Vesicles. Sarcoplasmic reticulum (SR) vesicles were prepared from rabbit back and leg muscles by the method of Eletr & Inesi (1972). The vesicles were finally suspended in 0.3 M sucrose and 10 mM imidazole, pH 7.4, immediately frozen, lyophilized in convenient aliquots, and stored at −60 °C. Vesicles were reconstituted in the same volume of water. The Ca^{2+} transport rates of reconstituted preparations were at least 95% of fresh preparations. In some experiments freshly prepared vesicles were used.

Medium $P_i \rightleftharpoons HOH$ Exchange. Medium $P_i \rightleftharpoons HOH$ exchange was measured by following the appearance of ^{16}O into ^{18}O-enriched P_i (Ariki & Boyer, 1980). The medium and reaction conditions are given in the legends to the figures and the footnotes to the tables. The reaction was started by the addition of $[^{18}O]P_i$ and terminated by transferring aliquots into a quench medium. The latter depended on whether ATP was present in the incubation medium or not. In its absence the aliquots (100 μL) were transferred to 5 mL of ice-cold water containing 0.5 mM $CHCl_3$ and vortexed for 1 min. The supernatant was applied directly to a small anion-exchange resin column (0.7 × 3 cm, AG 1-X4) and washed with water, and the P_i was eluted with 60 mM HCl. When ATP was present, the reaction was quenched in an equal volume of 1 N perchloric acid and molybdic acid (final concentration 0.012 M) added. The phosphomolybdate complex was extracted into 2-methyl-1-propanol–benzene, 1:1, and washed with 0.5 N perchloric acid, and then the phosphate was recovered in an aqueous phase by mixing with a solution of 200 mM Tris in water. This was then diluted 6-fold and applied to a similar anion-exchange column as above. The phosphate was partially separated from the molybdate with 2.5 mL of 20 mM $MgCl_2$. Following a water wash the phosphate was eluted with 30 mM HCl. The eluate containing the phosphoric acid was lyophilized and converted to the volatile triethyl derivative with diazoethane in ether using suitable precautions. The samples were analyzed with a Hewlett-Packard 5995 A GC–mass spectrometer. The GC was operated isothermally at 156 °C with a 6- or 10-ft glass column (2 mm i.d.), packed with 3% OV 275 on 40/60 Chromosorb T support. The samples were carried with helium at a flow rate of 30 mL/min. The retention time of the phosphate was generally 0.95 min. Species with a mass range of 155–163 containing zero to four ^{18}O atoms per P_i were analyzed.

Intermediate $P_i \rightleftharpoons HOH$ Exchange. [γ-^{18}O]ATP was synthesized enzymatically by using either the glyceraldehyde-3-P dehydrogenase (Hackney et al., 1980) or the carbamoyl kinase (Mokrasch et al., 1960) method. It was purified by elution from an anion-exchange column (AG 1-X4, 1.5 × 5 cm) with HCl, and the neutralized eluate was made substantially salt free by passage through a column (2.0 × 60 cm) of Sephadex G-10. Cleavage of the γ-phosphate of the enriched ATP without intermediate $P_i \rightleftharpoons HOH$ exchange was accomplished with the glycerokinase reaction (Hayaski & Lin, 1967). The medium and conditions used for intermediate $P_i \rightleftharpoons HOH$ exchange are described in the legends to the figures and the footnotes to the tables. The reaction was terminated with an equal volume of ice-cold 1 N perchloric acid and the phosphate purified as above with molybdic acid. Usually 100 nmol of $[^{16}O]P_i$ was added to the mixture after quenching so the ATPase activity could be estimated.

ATPase Activity. ATPase activity was estimated from the distribution of phosphate species. In the case of medium exchange, since the phosphate arising from ATP did not contain ^{18}O, the amount of hydrolysis could be calculated from the relative amount of $[^{16}O]P_i$. With intermediate exchange the activity could be calculated from comparing the amount of total $[^{18}O]P_i$ species with an added amount of $[^{16}O]P_i$, usually 100 nmol.

Phosphoprotein Measurement. The medium and conditions of E-P measurement are described in the legends to the figures. The reaction was started with P_i plus $[^{32}P]P_i$ and stopped after 1 min by the addition of 10 volumes of ice-cold 0.5 N perchloric acid/100 mM orthophosphoric acid and the proteinaceous material processed as described by Punzengruber et al. (1978). Protein concentration was estimated by the Lowry procedure with 0.4% deoxycholate using bovine serum albumin as standard.

Results

Intermediate $P_i \rightleftharpoons HOH$ Exchange and the Effect of ATP Concentration. During net enzymatic hydrolysis of ATP there is an obligatory incorporation of one oxygen atom from water into P_i released from the enzyme. However, before the product P_i is released from the enzyme, it could react with the enzyme to again yield a phosphoprotein. Hydrolysis of this species would yield P_i with an additional oxygen atom from water. The incorporation of extra oxygen atoms into the released P_i when the P_i originated from ATP is defined as intermediate $P_i \rightleftharpoons HOH$ exchange. The extent of this process is dependent on the relative rate constants for phosphoprotein formation from E·P_i and that for P_i release. The probability of whether exchange or release occurs can be formally represented by the partition coefficient, P_c, which is defined as the ratio of the rate constant for E-P formation from E·P_i divided by the sum of this same rate constant and that for P_i release from E·P_i (Hackney et al., 1980). Intermediate $P_i \rightleftharpoons HOH$ exchange was measured by using [γ-^{18}O]ATP as substrate and following incorporation of ^{16}O into the released P_i. Analysis of the distribution of P_i species yields P_c.

The results obtained with Ca^{2+}-ATPase and 50 μM [γ-^{18}O]ATP is shown in Figure 1a. A relatively low amount of exchange was observed. The distribution closely fits that theoretically predicted for a reaction with a P_c of 0.1. There appears to be only one catalytic pathway operative.

Intermediate $P_i \rightleftharpoons HOH$ exchange at higher concentrations of ATP is complicated by a Ca^{2+}-independent hydrolysis of ATP by the preparation sometimes called "basal" ATPase

Table I: Effects of Ca^{2+}, ATP, and P_i on Medium and on Intermediate $P_i \rightleftharpoons HOH$ Exchanges and the Corresponding P_c Values

free $[Ca^{2+}]$ (μM)	[ATP] (mM)	$[P_i]$ (mM)	medium $P_i \rightleftharpoons HOH$ exchange[a] [ng-atom/(min·mg)]	intermediate $P_i \rightleftharpoons HOH$ exchange[b] [ng-atom/(min·mg)]	P_c[c]
~0.01		5	3060 ± 560 (6)		0.078 ± 0.016 (6)
30		5	104		0.17
30	0.05	5	4600		0.10
30	5	5	700		0.30
~0.01	0.05	5	2130		0.09
~0.01	5	5	420		0.27
~0.01	5			9 ± 3 (3)	0.063 ± 0.025 (3)
30	0.05			61 ± 28 (3)	0.096 ± 0.0053 (3)
30	5	5		700 ± 210 (4)	0.31 ± 0.074 (4)
30	5			680	0.33

[a] Medium exchange was measured at 25 °C in a medium of 20 mM Mes, pH 6.5, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM EGTA, 5 mM $[^{18}O]P_i$, with or without 0.5 mM $CaCl_2$, and 0.4 mg of SR/mL, plus ATP in some cases at the concentrations shown. The activity was calculated from $[(4 - 3P_c)/(4 - 4P_c)]4k_{av}[P_i]$/protein where P_c represents the partition coefficient for the partitioning of bound P_i between E-P formation and release and is defined below. k_{av} is the first-order rate constant for the average loss of ^{18}O for all $[^{18}O]P_i$ species (Hackney et al., 1980). [b] Intermediate exchange was carried out in essentially the same medium as for medium exchange except that when P_i was present it did not contain ^{18}O and the ATP was [γ-^{18}O]ATP. The activity was calculated as the product of the number of reversals of the hydrolysis step per P_i released and the ATPase activity. The number of reversals is given by $P_c/(1 - P_c)$ (Hackney et al., 1980). [c] The P_c or partition coefficient is calculated from $^1/_3(4 - k_4/k_{av})$, where k_4 is the first-order rate constant for the loss of $P^{18}O_4$ species and k_{av} is as above (Hackney et al., 1980). It is a measure of the probability of bound P_i going to form E-P or being released to the medium. Values close to 0 are indicative of a relatively high rate constant for P_i release compared with E-P formation, and those near 1 show the reverse; namely, the rate constant for E-P formation is large with respect to that for P_i release. In the first case there would be few reversals of the hydrolysis step and in the latter case, many reversals, before the P_i is released.

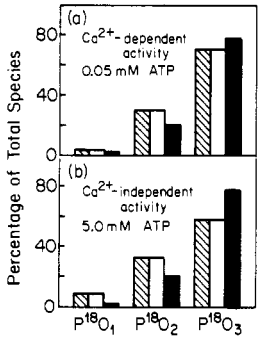

FIGURE 1: Distribution of $[^{18}O]P_i$ species on cleavage of [γ-^{18}O]ATP. (a) Ca^{2+}-dependent hydrolysis at 0.05 mM ATP and (b) Ca^{2+}-dependent hydrolysis at 5.0 mM ATP. In the latter case, the amount of each species produced from the Ca^{2+}-independent process has been subtracted. (■) Species produced on cleavage by glycerokinase with no $P_i \rightleftharpoons HOH$ exchange; (□) observed species; (hatched area) theoretical distribution assuming equivalence of all the oxygens for a P_c of 0.10 (a) and 0.34 (b). The medium consisted of 20 mM Mes, pH 6.5, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $CaCl_2$, 0.05 or 5.0 mM [γ-^{18}O]ATP, 0.4 mg of SR/mL, and 50 μM A23187.

activity (Hasselbach & Makinose, 1961). We have determined (data not shown) that this process also exhibits an intermediate $P_i \rightleftharpoons HOH$ with a slightly lower P_c (0.08) compared with the Ca^{2+}-stimulated activity. In determining the exchange at higher ATP concentrations the contribution from the basal activity was subtracted.

The distribution obtained for the Ca^{2+}-activated process at 5 mM [γ-^{18}O]ATP is shown in Figure 1b. The P_c is increased to 0.34, indicating greater incorporation of oxygen from water into P_i compared with the reaction at lower ATP concentrations. These results show that ATP binding in the millimolar range modulates reactions of the E-P and E·P_i species, where the phosphoryl group is derived from ATP.

Effect of Ca^{2+} and ATP Concentration and of pH on Oxygen Exchanges. Catalysis of the exchange of P_i oxygens during the reaction of medium P_i with the ATPase (medium $P_i \rightleftharpoons HOH$ exchange) has been characterized previously (Ariki & Boyer, 1980). These experiments have been extended to conditions more closely resembling those under which intermediate $P_i \rightleftharpoons HOH$ exchange (exchange in P_i derived from ATP) was measured in the present paper. Results are shown in Table I together with the effect of Ca^{2+} and ATP. The results obtained from intermediate exchange are also summarized. The total medium $P_i \rightleftharpoons HOH$ exchange and the P_c values obtained in the absence and in the presence of Ca^{2+} are similar to the previous published values. The low residual activity in the presence of micromolar concentrations of Ca^{2+} and the associated higher P_c value may be due to P_i reacting slowly with the 'E·nCa species (see Scheme I). Inclusion of 50 μM ATP in the medium with Ca^{2+} causes a large increase in medium exchange rate, due primarily to the generation of intermediates reactive to P_i (de Meis & Boyer, 1978). The P_c value is approximately the same as in the absence of Ca^{2+} and ATP; thus the rate constants for the release of P_i from the enzyme and for E-P formation from P_i and hence the nature of the E·P_i intermediate are essentially unchanged by the presence of Ca^{2+} and ATP together at these low ATP concentrations. A change in properties of this E·P_i intermediate is, however, observed at higher ATP concentrations as shown by the approximate 3-fold increase in P_c value. This change is similar to that observed with intermediate $P_i \rightleftharpoons HOH$ exchange when the ATP concentration is increased by the same amount. As shown in Table I it is not necessary to have both Ca^{2+} and ATP present as ATP binding at a concentration of 5 mM to the enzyme in the absence of Ca^{2+} also increased the P_c. The presence of medium P_i did not affect the modulation measured during intermediate $P_i \rightleftharpoons HOH$ exchange.

High concentrations of ATP are known to accelerate Ca^{2+} transport and ATPase activity. This is likely not by the participation of more enzyme units but rather by aiding a rate-limiting step, possibly the E to 'E transition in scheme I (Inesi et al., 1967; Yamamoto & Tonomura, 1967; Verjovski-Almeida & Inesi, 1979; de Meis & Boyer, 1978). There is a definite correlation between the modulation of the P_c by ATP and acceleration of Ca^{2+} ATPase activity (Figure 2), suggesting that the same low-affinity ATP binding site is involved in both processes. The modulating effect of ATP concentration on the P_c for intermediate $P_i \rightleftharpoons HOH$ exchange is unaffected by concentrations of Triton X-100 up to 10 mg of Triton X-100/mg of protein or 6.18 mM Triton X-100

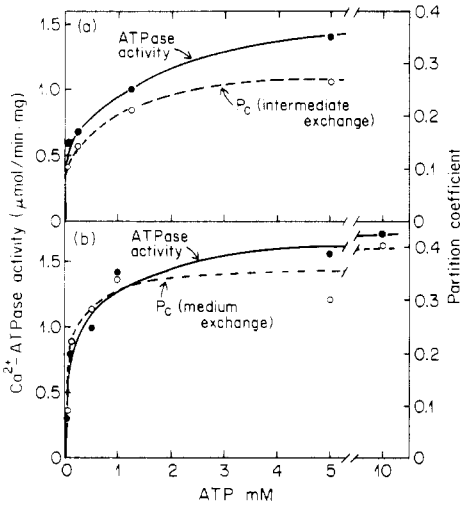

FIGURE 2: Correlation between the effect of ATP concentration on ATPase activity and the partition coefficient during intermediate (a) and during medium (b) $P_i \rightleftharpoons HOH$ exchange. Intermediate exchange was carried out in a medium similar to that of Figure 1. For medium exchange [γ-^{18}O]ATP was replaced by [γ-^{16}O]ATP, and 5 mM [^{18}O]P_i, 2.5 mM phosphoenolpyruvate, and 0.05 mg/mL pyruvate kinase were also included.

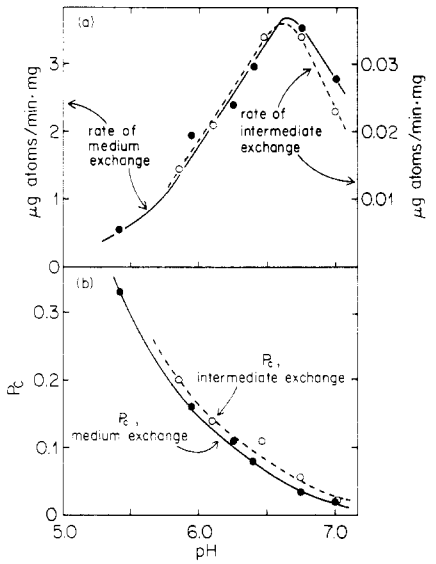

FIGURE 3: pH dependence of medium and of intermediate $P_i \rightleftharpoons HOH$ exchanges and of the associated partition coefficients. Both assays were carried out at 25 °C. The medium for intermediate $P_i \rightleftharpoons HOH$ exchange was as in Figure 1 with 5.0 μM [γ-^{18}O]ATP and the pH values shown. Medium exchange was carried out in a medium of 20 mM Mes, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM EGTA, 0.4 mg of SR/mL, and 5 mM [^{18}O]P_i at 25 °C.

(results not shown). This concentration of Triton X-100 is similar to that used to obtain monomers of the Ca^{2+}-ATPase with the detergent $C_{12}E_8$ and suggests that the low-affinity binding site coexists on the same polypeptide with the catalytic site undergoing hydrolysis.

The effect of pH on the total oxygen exchange and on the P_c value, as shown in Figure 3, is the same whether the reaction of P_i with the ATPase in the absence of Ca^{2+} or the Ca-dependent hydrolysis of ATP is followed. Both medium and intermediate $P_i \rightleftharpoons HOH$ exchanges have a pH optimum of approximately 6.6–6.7 (Figure 3a); this corresponds closely to the pH optimum of Ca^{2+} transport, but that for ATPase activity is higher. Medium $P_i \rightleftharpoons HOH$ exchange increased 2.2-fold in going from pH 6.0 to pH 6.6. This change in pH has been shown to lower the level of E-P from P_i 2.3-fold (de Meis, 1976). Since medium $P_i \rightleftharpoons HOH$ exchange = k_{-2}[E-P], where k_{-2} is the rate constant for phosphoenzyme hydrolysis, k_{-2} must increase some 5-fold from pH 6.0 to pH 6.6. Above pH 6.6, a small further increase is observed. As shown in Figure 3b, there is a sharp rise in P_c at lower pH values. Protonation of some group is either lowering the rate constant for P_i release from the enzyme or stimulating that for E-P formation from E·P_i.

Table II: Effect of Nucleotides and Pyrophosphate on Medium $P_i \rightleftharpoons HOH$ Exchange and the Partition Coefficients

additions,[a] mM	k_4[b] (s^{-1})	P_c[c]	$P_{c(5)}/P_{c(0.05)}$[d]
	0.41	0.17	
ATP, 0.05	9.3	0.10	3.0
ATP, 5.0	1.3	0.30	
ADP, 0.05	0.21	0.07	1.4
ADP, 5.0	0.25	0.10	
ITP, 0.05	10.8	0.13	0.9
ITP, 5.0	18.6	0.12	
GTP, 0.05	12.6	0.06	1.2
GTP, 5.0	12.0	0.07	
AMPPCP, 0.05	0.66	0.12	3.0
AMPPCP, 5.0	0.17	0.36	
AMPCPP, 0.05	8.4	0.07	2.7
AMPCPP, 5.0	1.8	0.19	
AMPPNP, 0.05	3.1	0.10	3.7
AMPPNP, 5.0	0.16	0.37	
PP_i, 0.05	1.32	0.07	0.9
PP_i, 1.0	2.1	0.06	

[a] The basic medium contained 20 mM Mes, pH 6.5, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $CaCl_2$, 5 mM [^{18}O]P_i, 50 μM A23187, and 0.4 mg of SR/mL. When ATP, ITP, and GTP were added, a regenerating system was included consisting of 2.5 mM phosphoenolpyruvate and 0.05 mg/mL pyruvate kinase with ATP and 0.2 mg/mL pyruvate kinase with ITP and GTP. With these nucleotides the reaction was measured over 3 min. When ADP was added, the basic medium also contained 5 units of hexokinase and 1 mM glucose to prevent possible ATP accumulation via adenylate kinase activity, and the reaction time was 20 min. In the other cases the reaction was continued for 40 min. All assays were performed at 25 °C. [b] First-order rate constant for the loss of $P^{18}O_4$. [c] See Table I. [d] Ratio of the P_c value found at 5 mM to that at 0.05 mM.

Specificity of the Low-Affinity Nucleotide Site. The effects of other nucleotides on medium $P_i \rightleftharpoons HOH$ exchange are shown in Table II. ADP, GTP, ITP, and PP_i do not exhibit the ATP effect, but the ATP analogues AMPPCP, AMPCPP, and AMPPNP are as effective as ATP. This specificity is similar to but not the same as that observed by other investigators for the low-affinity site which activates turnover. The activating site and the site which alters the P_c are similar in that ADP is without effect, and AMPPCP and AMPCPP are positive effectors (Taylor & Hattan, 1979; Dupont, 1977). Differences appear for GTP, ITP, PP_i, and AMPPNP. High concentrations of the first two nucleotides do not alter the P_c, and yet the substrate dependence of GTP and ITP hydrolysis is biphasic (Taylor & Hattan, 1979; Van Winkle et al., 1981), a phenomenon usually attributed to activation of turnover, and they both increase the rate constant for dephosphorylation (de Meis & de Mello, 1973; the results reported herein). On the other hand activation of the E to 'E transition appears to be small as the E-P levels from P_i and medium $P_i \rightleftharpoons HOH$ exchange do not change much as the concentration of these nucleotides is increased in the millimolar range (Carvalho et al., 1976; de Meis & Boyer, 1978; results reported herein). High concentrations of PP_i do not influence the P_c although they have been reported to activate ATP hydrolysis (Dupont, 1977). Conversely, AMPPNP does not appear to activate ATP hydrolysis (Dupont, 1977), although it modulates the P_c.

Table III: Values of Constants for Enzyme Phosphorylation from P_i in the Absence of Ca^{2+} and ATP

K_1 (M)	K_2	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})
1.35×10^{-2} [a]	1.6	2.8×10^5	378	32	51
1.90×10^{-3} [b]	0.93	3.4×10^7	648	55	51

[a] Enzyme phosphorylation was carried out at 25 °C in a medium of 20 mM Mes, pH 6.5, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM EGTA, 0.4 mg of SR/mL, and variable $[^{32}P]P_i$ concentrations up to 50 mM. Medium $P_i \rightleftharpoons HOH$ exchange was measured at 5 mM P_i and taken as 3060 ng-atom/(min·mg) and the P_c as 0.078 (see Table I). The level of E-P was found to be 1.0 nmol/mg under these conditions. [b] Values for K_1 and K_2 taken from Martin & Tanford (1981). The rate constants were calculated by using these values and taking k_{-2} as 51 s^{-1}. The reaction mixture (25 °C) of Martin and Tanford was 34 mM Mes-Tris (pH 6.2), 5 mM EGTA, 1 mg of SR vesicles/mL, and variable $MgCl_2$ and P_i concentrations.

Comparison of the rate constant for the loss of the $P^{18}O_4$ species at 50 μM of each substance (Table II) shows that the values are considerably larger for the compounds which are substrates due to the generation of intermediates reactive to P_i. For those which are not hydrolyzed such as ADP and AMPPCP the rate constant is approximately the same as in their absence. The result with PP_i is anomalous and is perhaps due to complexation of Ca^{2+} by PP_i, thereby relieving the Ca^{2+}-induced inhibition of the exchange at higher concentrations. Only with those compounds which increased the P_c was the rate constant smaller. The decrease could be partly explained by an activation of the step following the exchange step, thereby lowering the concentration of the intermediate reactive to P_i (de Meis & Boyer, 1978). Part of the effect might also be due to competition of effector and P_i for the same site.

Evaluation of Rate Constants for the Reaction of P_i with the ATPase in the Absence of Ca^{2+} and ATP. For the reaction

$$E + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}P$$

the following relationship holds:

$$\frac{E_t}{[E\text{-}P]} = \frac{1}{[P_i]}K_1K_2 + 1 + K_2$$

where $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$ and $E_t = [E] + [E{\cdot}P_i] + [E\text{-}P]$. If it is assumed that there is full sites reactivity in this reaction, then E_t is about 7 nmol/mg (molecular weight of the enzyme is approximately 100 000 and it makes up approximately 70% of the SR protein). From a plot of $E_t/[E\text{-}P]$ vs. $1/[P_i]$ (data not shown) we have obtained values for K_1 and K_2 (Table III). Since $P_c = k_2/(k_2 + k_{-1})$ (Hackney et al., 1980) and medium $P_i \rightleftharpoons HOH$ exchange $= k_{-2}[E\text{-}P]$, all the values for the rate constants can be evaluated and are shown in Table III. The studies by Punzengruber et al. (1978), Kolassa et al. (1979), and Martin & Tanford (1981) have shown that the binding of Mg^{2+} and P_i to the enzyme occurs in a random manner leading to the ternary complex $E{\cdot}\overset{Mg}{P_i}$ which is in equilibrium with the Mg phosphoenzyme. At the relatively high Mg^{2+} concentrations we used, the constants represent essentially those for the enzyme combined with Mg^{2+}. However, our values for K_1 and K_2 differed somewhat from these studies (largely reflected in a 7-fold lower affinity for P_i in the present work), and therefore the rate constants have also been calculated by using the values obtained by Martin & Tanford (1981) for the reaction of the E·Mg complex with P_i (Table III). Our value is in close agreement with the results obtained by de Meis (1976) and by Kanazawa (1975). The concentration at which we found half-maximal phosphorylation

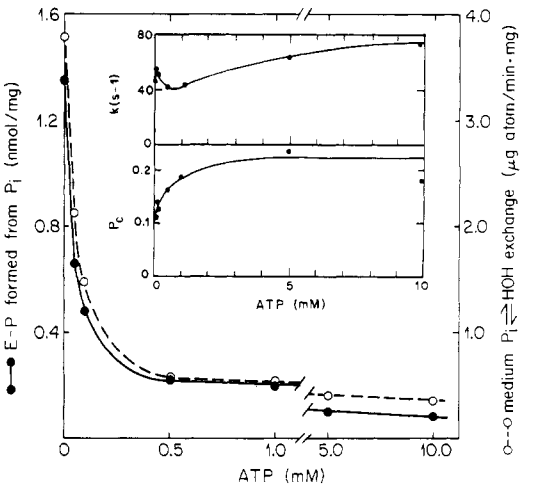

FIGURE 4: Comparison of the effect of ATP concentration on the level of E-P formed from P_i and on medium $P_i \rightleftharpoons HOH$ exchange both measured in the absence of Ca^{2+}. The medium consisted of 20 mM Mes, pH 6.5, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM EGTA, 5 mM $[^{18}O]P_i$ or $[^{32}P]P_i$, 0.4 mg of SR/mL, and variable ATP concentrations. The assays were carried out at 25 °C. (Inset) Dependence of the rate constant for phosphoenzyme hydrolysis and partition coefficient on ATP concentration.

is similar to that found by Lacapere et al. (1981) for phosphorylation-induced changes in tryptophan fluorescence although from other experiments different equilibrium constants in the presence of high Mg^{2+} were calculated from the data. Values for k_1 and k_{-1} have not previously been measured. It should be emphasized that the measurement of k_{-2}, unlike k_1, k_{-1}, and k_2, does not depend on an assumption of the number of reactive sites. For the latter three rate constants if it is eventually found that there is half-of-the-sites reactivity, then k_1 remains approximately the same and k_{-1} and k_2 are approximately 6-fold greater. Also if the equilibrium of the E and 'E forms of the enzyme is such the concentration of 'E is significant under the conditions of measurement, these values will also be affected.

Values given in Table III are for pH 6.5. From the pH dependence of medium $P_i \rightleftharpoons HOH$ exchange (Figure 3) and of E-P formation (de Meis, 1976), it can be calculated that the values for k_{-2} at pH 6.0 and at pH 7.0 are close to 17 and 112 s^{-1}, respectively.

Effect of ATP Hydrolysis and ATP Concentration on Medium $P_i \rightleftharpoons HOH$ Exchange and Phosphoenzyme Hydrolysis. The total rate of water oxygen incorporation into P_i during ATP cleavage is equal to $k_{-2}[E\text{-}P]$ where k_{-2} is the rate constant for dephosphorylation and E-P is the phosphoenzyme reactive with water (Boyer et al., 1977). If it is assumed that E·P_i and E-P have similar properties whether arising from P_i or ATP, as our results suggest, measurement of medium oxygen exchange and E-P levels from P_i provides a method for evaluating the rate constant for dephosphorylation during ATP cleavage.

The effect of ATP in the absence of Ca^{2+} is shown in Figure 4. ATP inhibited the exchange and E-P levels in a similar fashion with an approximate K_i value of 50 μM. This value is higher than the $S_{0.5}$ for ATP activation of transport or ATPase activity in the presence of Ca^{2+} (approximately 3 μM). This could be partly due to a competitive effect of P_i but could also be caused by Ca^{2+} enhancing the affinity of the catalytic site for ATP. Dupont (1980) has reported such an effect of Ca^{2+} on the ATP affinity. The parallel drop in E-P and oxygen exchange means that the rate constant for dephosphorylation is almost unaffected by ATP concentration in the 0–1.0 mM

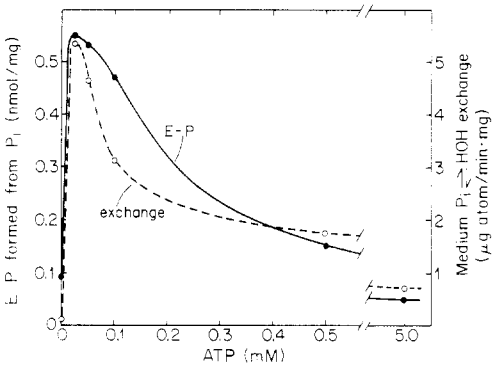

FIGURE 5: Comparison of the effect of ATP concentration on the level of E-P formed from P_i and on medium $P_i \rightleftharpoons$ HOH exchange both measured in the presence of Ca^{2+} and ATP. The reactions were done at 25 °C in 20 mM Mes, pH 6.5, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $CaCl_2$, variable ATP concentrations, 5 mM [^{18}O]P_i or [^{32}P]P_i, 2.5 mM phosphoenolpyruvate, 0.05 mg/mL pyruvate kinase, 0.4 mg of SR/mL, and 50 μM A23187.

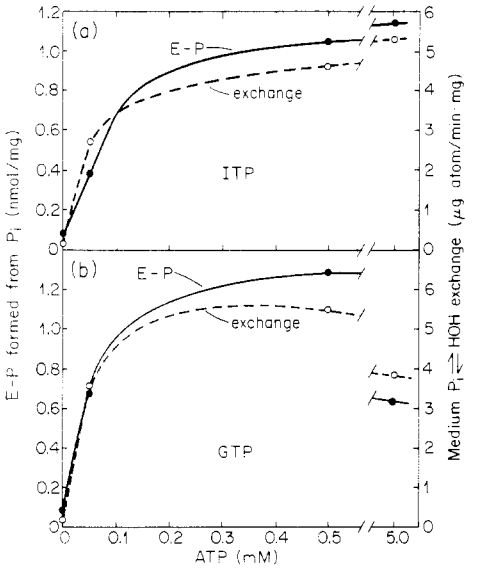

FIGURE 6: Comparison of the effect of ITP (a) and GTP (b) concentrations on the level of E-P formed from P_i and medium $P_i \rightleftharpoons$ HOH exchange both measured in the presence of Ca^{2+}. The medium was the same as in Figure 5 except the nucleotide was different and extra pyruvate kinase was included (0.2 mg/mL).

range (inset, Figure 4). At higher concentrations an activating effect is observed. As shown the P_c increased with an increase in ATP concentration. Concentrations of ATP 100-fold higher than the K_i value of 50 μM did not eliminate the medium $P_i \rightleftharpoons$ HOH exchange or E-P formation from P_i.

The effect of ATP on the exchange reaction and E-P formation from P_i with Ca^{2+} in the medium is shown in Figure 5. In the absence of ATP the enzyme was almost entirely converted into a conformation unreactive to P_i. A small amount that is reactive exhibited the same rate constant for dephosphorylation as in the absence of Ca^{2+}. Low concentrations of ATP (25 μM), about sufficient to saturate catalytic sites, resulted in a pronounced increase in levels of E-P formed from P_i and in exchange, an effect previously documented (de Meis & Boyer, 1978). Higher concentrations inhibited these parameters, an effect which may be largely attributed to activation of the E to 'E transition and thereby lowering the amount of enzyme reactive to P_i as discussed by de Meis & Boyer (1978).

Effects of ITP and GTP. These experiments made with ATP have been repeated with ITP and GTP, and results are

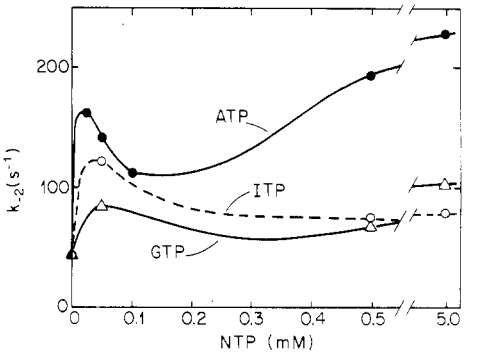

FIGURE 7: Effect of NTP concentration of the rate constant for dephosphorylation. The data were obtained from that in Figures 4 and 5 by using the relationship medium $P_i \rightleftharpoons$ HOH exchange = k[E-P], where k is the rate constant for phosphoenzyme hydrolysis and [E-P] is the concentration of E-P reactive to water which arises from medium P_i.

Table IV: Levels of E·P_i and E-P during Steady-State ATP Hydrolysis in the Presence of 5 mM P_i

[ATP] (mM)	E·P_i (from P_i)/ E·P_i (from ATP)[a]	[E-P] (nmol/mg) from P_i	[E-P] (nmol/mg) from ATP[b]
0.025	60	0.53	0.094
5.0	1	0.09	0.20

[a] Calculated from v_{ex}(medium)/v_{ex}(intermediate) = E·P_i (from P_i)/E·P_i (from ATP). [b] Calculated from ATPase activity = k_{-2}[E-P] where E-P represents the phosphoenzyme arising from ATP that reacts directly with water and k_{-2} is the rate constant for dephosphorylation.

shown in Figure 6. The maximal levels of E-P formed from P_i are approximately twice that achieved with ATP. There are two reasons for this. These nucleotides apparently generate greater amounts of the E intermediate that is reactive to P_i than does ATP, since they do not activate the E to 'E transition (Scheme I). There is an indication that GTP, at high concentrations, may provide some activation of this step as the level of E-P and the exchange activity were consistently lower at 5 mM than at 0.5 mM GTP. The higher E-P levels are also due to the slower rate constants for E-P hydrolysis observed in the presence of these ATP analogues (see below).

Comparative Effects of NTP's and Related Considerations. The rate constant for dephosphorylation at 25 μM ATP is activated approximately 3-fold over that in the absence of ATP or of both Ca^{2+} and ATP (Figure 7). Higher concentrations partially inhibited this step until in the millimolar range of ATP there was further activation, with the rate constant now some 5-fold higher than that in the absence of ATP or of Ca^{2+} and ATP.

These results show that during Ca^{2+} transport ATP can affect the rate constant for hydrolysis of E-P. Effects over at least three distinct concentration ranges are evident, very low (0–25 μM), intermediate (25–100 μM), and high (100–5000 μM).

Figure 7 shows that ITP and GTP also activate the rate constant for dephosphorylation at 50 μM concentration but not to the same extent as ATP. Some inhibition at higher nucleotide concentrations occurs, as was observed with ATP. ITP at 5 mM showed no activation of dephosphorylation over that achieved at 0.5 mM, but there was a slight effect by GTP binding. These results are in keeping with small changes in P_c values as ITP or GTP concentration is varied (Table II).

It is also possible to calculate the relative amounts of E·P_i and the absolute amounts of E-P during steady-state ATP

cleavage (Table IV). At 25 μM ATP the amount of E·P_i arising from P_i (5 mM P_i is included in the medium) is 60-fold greater than that of the same species arising from ATP. Increasing the concentration of the latter brings them to the same level. The amount of E-P from P_i is decreased considerably by this increase in ATP concentration. This change and that of the relative amounts of E·P_i can be explained by an activation of the E to 'E transition on ATP binding (de Meis & Boyer, 1978). In the same concentration range E-P arising from ATP is increased approximately 2-fold. Very little of this increase can come from E·P_i because its concentration is likely to be low. This is predicted from the large rate constant for P_i release found in the absence of Ca^{2+} and ATP and the relatively small change in P_c value. Hence the increase in E-P from ATP likely arises from a significant acceleration of the preceding steps. There are two possible explanations for this. Either reaction 5 (Scheme I) has changed so that the rate for the release of Ca^{2+} is increased or the amount of E^{P}_{Ca} is increased. The latter could arise by a change in reaction 4 such that the rate constant for the forward direction is increased or that for the backward reaction is decreased.

Discussion

The principle contribution of these studies is to show that the reactions of the Ca^{2+}-ATPase with P_i and water are modulated by ATP in the presence of Ca^{2+} under conditions likely prevailing during physiological Ca^{2+} transport. To explain the ATP modulations that occur over a wide concentration range, a capacity for at least three ATP bindings with differing K_d values is required. Also, the intermediates that participate as the concentration of ATP is varied in the presence of Ca^{2+} are different from those that participate in E-P hydrolysis and P_i binding reactions in the absence of Ca^{2+} and ATP.

Without Ca^{2+} and ATP present P_i binding and release are relatively rapid processes. The reactions involving water are slower. The rate constant we observed for the hydrolytic cleavage of the phosphoenzyme is 51 s^{-1} (25 °C, pH 6.5), a value close to the 60 s^{-1} (25 °C, pH 6.8) reported by Inesi et al. (1981), but somewhat faster than the 17–23 s^{-1} (30 °C, pH 6.3) reported by Guimaraes-Morra & de Meis (1980), both of which were obtained by diluting phosphoenzyme formed from [^{32}P]P_i with nonradioactive P_i and quenching at timed intervals. The differences could be due to the pH of the medium as we have shown that this rate constant is strongly pH dependent. These values are considerably faster than those estimated from experiments in which rephosphorylation by P_i was inhibited by addition of Ca^{2+} (de Meis & Tume, 1977; Verjovski-Almeida et al., 1978). In the latter experiments likely another rate-limiting step was being measured, possibly step 8 in scheme I in which the enzyme changes from a P_i reactive to a P_i unreactive form with a change in affinity of the Ca^{2+} binding site. The rate constant for phosphorylation, 32 s^{-1} (assuming full sites reactivity), is close to that found when intrinsic fluorescence was used to monitor phosphorylation at a lower pH, namely, 25 s^{-1} (pH 6.0, 20 °C) (Lacapere et al., 1981). The half-time for phosphorylation by [^{32}P]P_i ranges from less than 20 ms to 40 ms (Boyer et al., 1977; Chaloub et al., 1979; Beil et al., 1977; Guimaraes-Motta & de Meis, 1980; Verjovski-Almeida et al., 1978). It is not possible to obtain rate constants from these reported measurements because the reaction consists of at least two reversible steps and there is a P_i dependence; however, the rate constant we obtained is compatible with these times.

Earlier studies have shown that ATP at low concentrations behaves as a competitive inhibitor of E-P formation from P_i in the absence of Ca^{2+} (Masuda & de Meis, 1973). The inhibition of enzyme phosphorylation is accompanied by inhibition of medium $P_i \rightleftharpoons$ HOH exchange. These effects likely do not result from a direct competition as our studies suggest that ATP at low concentrations and P_i do not react with the same enzyme form. This conclusion is made from the observation that during steady-state ATP hydrolysis in the presence of Ca^{2+} and 25 μM ATP, a concentration sufficient to about saturate a catalytic site, relatively high levels of phosphoenzyme (0.55 nmol/mg) can be formed from P_i. Also an increase in ATP concentration to 100 μM has little effect on the level of E-P (0.47 nmol/mg). It is evident that ATP binding under these conditions is not competing directly with P_i binding. ATP combines preferentially with the 'E form and P_i with the E form as indicated in Scheme I.

We have shown that at higher concentrations of ATP, phosphoenzyme formation from P_i and medium $P_i \rightleftharpoons$ HOH exchange are not completely inhibited. Also the residual exchange takes place with an increased P_c, indicating a change in intermediates. Similarly, Kanazawa & Boyer (1973) found that high concentrations of ADP could not completely shut off $P_i \rightleftharpoons$ HOH exchange. It is likely that this low residual activity represents P_i binding and phosphoenzyme formation with the 'E form of the enzyme. As noted in the results Ca^{2+} is also unable to completely inhibit medium $P_i \rightleftharpoons$ HOH exchange for the same reason; P_i can combine with and form E-P with an 'E·nCa form.

Pertinent to the Ca^{2+} transport mechanism are the findings that during steady-state ATP cleavage the rate constants involving the reactions of P_i and water with the enzyme are modulated by ATP in different ways at different concentrations. The three distinct modulations of the rate constant for hydrolysis of the phosphoenzyme that have been identified over the range 0–5 mM will be discussed in order.

Modulation by 0–25 μM ATP. In this concentration range the rate constant k_{-2}, for E-P hydrolysis, is accelerated 3-fold over that measured in the absence of Ca^{2+} and ATP. This is in harmony with the acceleration of hydrolysis of phosphoenzyme formed from [γ-^{32}P]ATP by 10 μM ATP that has been reported by de Meis & de Mello (1973). Acceleration of hydrolysis deduced from an apparent transient increase in E·P_i levels was also noted by Froehlich & Taylor (1975) when the ATP concentration was increased from 5 to 100 μM. Our results show little or no acceleration of P_i departure from phosphoenzyme produced from P_i in the absence of Ca^{2+} by low concentrations of ATP. Thus ATP binding in the presence of Ca^{2+} or phosphoenzyme formation from ATP is a prerequisite for acceleration.

The concentration range in which the effect occurs indicates that the site has a high affinity for ATP and is likely a catalytic site. This could be an active site other than that which is phosphorylated, in which case a cooperative dimeric or oligomeric structure is required. Another possibility is a memory effect in which the conformational change brought about by ATP binding in the presence of Ca^{2+} or phosphoenzyme formation from ATP is slow to relax or to change so that the intermediate involved in hydrolysis is different from that formed from P_i in the absence of the foregoing steps. Alternatively there is the possibility of ATP binding to the catalytic site after the departure of ADP. The presence of the phosphoryl group already in the catalytic site area could lower the affinity for ATP some and such binding may only occur at higher ATP concentrations. In view of the evidence that the enzyme may be a functional oligomer [for review see Ikemoto (1982)], the first explanation appears attractive.

Modulation by 25–100 μM ATP. The inhibition of E-P hydrolysis occurring in this range indicates a second type of ATP binding site. The binding could be to an alternate catalytic site with lowered affinity for ATP or possibly the third alternative considered above, namely, ATP binding to the catalytic site after ADP has departed. The effects we observe could be related to the apparent negative cooperativity of ATP activation of Ca^{2+}-ATPase in the 0–100 μM range as detected by several laboratories (Yamamoto & Tonomura, 1967; Kanazawa et al., 1971; Panet et al., 1971; Vianna, 1975; Moller et al., 1980).

Modulation by 0.05–5 mM ATP. In this higher concentration range hydrolysis of E-P is again accelerated, and there is also an increase in the partition coefficient, that is, an increase in the probability of $E{\cdot}P_i$ forming E-P as compared with its release from the enzyme. These effects have been correlated with the well documented secondary acceleration of ATP hydrolysis by high ATP concentrations. This activating effect has been considered to be due to an acceleration of the E to 'E conversion (Scofano et al., 1979). We demonstrate here that the two prior steps, namely, $E \leftrightarrow E{\cdot}P_i$ and $E{\cdot}P_i \leftrightarrow E\text{-}P$, are also altered by ATP binding in this range of concentration. In addition Ca^{2+} is not required for these effects. The results with what is likely a partially monomeric preparation in Triton X-100 suggest that the catalytic site and the effector site coexist on a single polypeptide, a conclusion also reached by Taylor & Hattan (1979) from a kinetic study of the secondary acceleration of ATP hydrolysis. This site could again be an altered catalytic site after ADP has departed or possibly a control site distinct from the catalytic site.

From our data it is clear that at physiological concentrations ATP will accelerate reaction of water with phosphoenzyme in the presence of Ca^{2+} and Mg^{2+} and modulate the reactivity of 'E and $E{\cdot}P_i$ as well. Our results further suggest that the preceding steps are also accelerated in the millimolar concentration range of ATP since the steady-state level phosphoenzyme reactive with water is increased approximately 2-fold. The step which is affected could either be 4 or 5 or possibly both of Scheme I. Because the increase in level of this intermediate occurs despite a 4–5-fold increase in the rate constant for the hydrolysis step, it is apparent that a significant (up to 8–10-fold) acceleration is involved. One of these steps must still contribute considerably to rate limitation as a major proportion of the enzyme remains phosphorylated (Inesi et al., 1981), and the turnover time of the enzyme is approximately 5 s^{-1}, a value much smaller than those for hydrolysis and P_i release.

It is evident that Ca^{2+}-ATPase during Ca^{2+} uptake has ATP bound during most or all steps of the catalytic cycle. As discussed above one of the two binding sites for ATP that modulate the reactivity of $E{\cdot}P_i$ and E-P in the higher concentration range is probably the catalytic site after ADP has departed. Hence an important point is that during physiological Ca^{2+} transport, ATP likely enters the catalytic cycle prior to dephosphorylation.

Acknowledgments

We are grateful to K. Stempel for doing all the mass spectrometry and for preparing $[^{18}O]P_i$. Also, we are indebted to M. Ariki for providing us with details of his preliminary studies on ATP modulations and for help in initiation of the present studies. Discussions with F. Guillain, A. Amory, and A. Goffeau have aided in the interpretation of the data, and we thank them for their contribution.

Registry No. ATP, 56-65-5; AMPPCP, 3469-78-1; AMPCPP, 7292-42-4; AMPPNP, 25612-73-1; ATPase, 9000-83-3; ITP, 132-06-9; GTP, 86-01-1; phosphate, 14265-44-2; Ca, 7440-70-2.

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Complete Amino Acid Sequence of the Light Chain of Human Blood Coagulation Factor X: Evidence for Identification of Residue 63 as β-Hydroxyaspartic Acid†

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ABSTRACT: The complete amino acid sequence of the light chain of human blood coagulation factor X has been determined by automated Edman degradation of peptides isolated from chemical and enzymatic digests of the carboxymethylated light chain. The protein consists of 139 amino acid residues, which include 11 residues of γ-carboxyglutamic acid. The first 100 residues of the human factor X light chain exhibit approximately 80% homology when compared to the amino-terminal sequence of bovine factor X light chain. This homology decreases to approximately 50% in the remaining 39 residues of the carboxyl-terminal region of the protein. Proton nuclear magnetic resonance spectroscopy and mass spectrometry analyses of isolated residue 63 identified this residue as L-*erythro*-β-hydroxyaspartic acid, a hitherto unrecognized amino acid in proteins. Evidence is also presented for the presence of this residue in the corresponding regions of the light chains of bovine factor X and bovine protein C. The biological function of β-hydroxyaspartic acid in these proteins is unknown.

Human blood coagulation factor X is a vitamin K dependent protein that circulates in blood as a precursor of a serine protease. Factor X is a glycoprotein composed of a heavy chain (M_r 42 000) and a light chain (M_r 17 000) held together by a single disulfide bond. The amino-terminal portion of the light chain contains γ-carboxyglutamic acid residues that are instrumental in calcium and phospholipid binding. The molecule contains 15% carbohydrate associated exclusively with the heavy chain (DiScipio et al., 1977a).

During the coagulation process, factor X is converted to an enzyme, factor Xa, by either the intrinsic or extrinsic pathway of blood coagulation [see Davie et al. (1979) and Nemerson & Furie (1980) for reviews]. In the activation of factor X, a specific arginine–isoleucine bond is cleaved in the amino-terminal region of the heavy chain resulting in an activation peptide (M_r 14 000) and factor Xa (DiScipio et al., 1977b). Factor Xa participates in the common pathway of blood coagulation, converting prothrombin to thrombin in the presence of factor Va, phospholipid, and calcium ions (Davie et al., 1979).

The complete amino acid sequences have been reported for the heavy and light chains of bovine factor X (Titani et al., 1975; Enfield et al., 1975, 1980). A preliminary sequence analysis performed in this laboratory (DiScipio et al., 1977b) revealed extensive homology between human and bovine factor X in those regions compared, with the notable exception of the amino terminus of the heavy chain. This paper presents the amino acid sequence of the light chain of human factor X. In addition, evidence is presented for the existence of a previously unrecognized amino acid, β-hydroxyaspartic acid, in this peptide, as well as in the light chains of bovine factor X and bovine protein C.

Materials and Methods

Carboxypeptidases A and B, TPCK-trypsin, and α-chymotrypsin were obtained from Worthington. Before use, the TPCK-trypsin and α-chymotrypsin were purified further on benzamidine–agarose (Fujikawa & McMullen, 1983). Iodoacetic acid was purchased from Sigma and aminopeptidase from Boehringer Mannheim. L-[2,3-$^3H_2$]Aspartic acid was obtained from New England Nuclear. L-*threo*-β-Hydroxyaspartic acid and L-*erythro*-β-hydroxyaspartic acid were kindly provided by Drs. N. Izumiya and T. Kato, Faculty of Biochemistry, Kyushu University, Fukuoka, Japan (Okai et al.,

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