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Adenosine 5′-Triphosphate at the Active Site Accelerates Binding of Calcium to Calcium Adenosinetriphosphatase†

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ABSTRACT: The complex of Mg·ATP and the calcium adenosinetriphosphatase of sarcoplasmic reticulum (E·ATP) reacts with 50–300 μM Ca^{2+} to form phosphoenzyme (E-P·Ca_2) with a rate constant of 70 s^{-1} (pH 7.0, 100 mM KCl, 5 mM $MgSO_4$, 25 °C, and SR vesicles passively loaded with Ca^{2+}). This rate constant is independent of Ca^{2+} concentration above 50 μM. It is 4–6 times faster than the rate constants of 11–15 s^{-1} for the conformational change associated with Ca^{2+} binding in the absence of activation by ATP. The reaction of 200 μM Ca^{2+} with enzyme preincubated in 0.9 μM [γ-^{32}P]ATP·Mg shows a burst of [^{32}P]E-P·Ca_2 formation. This result indicates that Mg·ATP bound to the active site, and not a regulatory site, can accelerate the conformational change associated with Ca^{2+} binding because this concentration of Mg·ATP is well below the K_d of 160–500 μM for the putative regulatory site. When an unlabeled ATP chase is added with the Ca^{2+} to enzyme preincubated with [γ-^{32}P]ATP·Mg, the amount of [^{32}P]E-P·Ca_2 that is formed increases with the concentration of ATP in the preincubation solution and is consistent with a maximum fraction trapped of 0.55 and K_d = 4.5 μM for the dissociation of Mg·ATP from the active site. The fact that labeled E·ATP can be trapped by added Ca^{2+} confirms the conclusion that dissociation of ATP from E·ATP·Ca_2 is slow relative to phosphorylation.

While there is good evidence that ATP accelerates the conformational change associated with Ca^{2+} binding to the calcium adenosinetriphosphatase (Ca-ATPase) of SR[1] (Sumida et al., 1978; Takisawa & Tonomura, 1978; Scofano et al., 1979; Inesi et al., 1980; Guillain et al., 1981; Pickart & Jencks, 1984), there is disagreement as to the mechanism of this effect. One proposal is that binding of ATP at the active site before the conformational change provides an alternate reaction pathway with a faster rate of Ca^{2+} binding (Boyer & Ariki, 1980; Inesi et al., 1980; Pick, 1981). Another possibility is that there is a regulatory site at which ATP acts to increase the rate of the conformational change (de Meis & Boyer, 1978; Scofano et al., 1979; Pick & Bassilian, 1981). Studies of ATP binding at equilibrium have indicated the existence of a second site to which ATP binds with a K_d between 160 and 500 μM (Yates & Duance, 1976; Dupont, 1977; Nakamura & Tonomura, 1982; Clore et al., 1982). We report here experiments which show directly that ATP bound to the active site accelerates the conformational change associated with Ca^{2+} binding.

Materials and Methods

Materials. Reagents were generally of the highest purity available and were used without further purification. Na_2ATP was obtained from Boehringer Mannheim ("Sonderqualitat"), and [γ-^{32}P]ATP (>98% purity) was purchased from New England Nuclear.

Tightly sealed sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by a slight modification of the MacLennan procedure, as described previously (Pickart &

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[1] Abbreviations: SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicle(s); E, calcium adenosinetriphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid.

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Jencks, 1982). The preparations hydrolyzed ATP at 3–5 μmol (mg of total protein)$^{-1}$ min^{-1} when the vesicles were made permeable with the calcium ionophore A23187. The total amount of phosphoenzyme, E_{tot}, that was observed for intact vesicles with saturating [Ca^{2+}] and [ATP] is 2.24 nmol/mg of total protein.

Methods. Ca-ATPase activity was assayed spectrophotometrically by coupling ADP production to NADH oxidation using pyruvate kinase and lactate dehydrogenase (Rossi et al., 1979). Standard conditions were 40 mM MOPS, 100 mM KCl, 5 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.40 mM EGTA (23 μM free calcium), 1.5 mM ATP, pH 7.0, and 25 °C.

Concentrations of free calcium were calculated from a dissociation constant of 7.4 × 10^{-7} M for Ca·EGTA (Godt, 1974). For experiments in which high concentrations of Ca^{2+} and EGTA were mixed, the release of protons from EGTA upon the formation of the Ca·EGTA complex was neutralized with 1.47 equiv of KOH added with the Ca^{2+}. Protein concentrations were determined by the procedure of Lowry et al. (1951), using bovine serum albumin as standard.

The formation of phosphoenzyme was followed on a rapid mixing apparatus that can be used with either three or four syringes. It consists of a series of mixing blocks that are connected to each other with narrow bore Teflon tubing secured to the mixing block by modified Omnifit connectors from Rainin. Each mixing block contains a Durrum jet mixer fitted into a Teflon slab through which 1-mm flow channels have been cut. Reaction times shorter than 10 ms are obtained with a mixing block containing two Durrum mixers separated by a short pathway cut into the Teflon slabs which hold them. For three-syringe mixing, a nitrogen-driven ram pushes the temperature-equilibrated contents of syringes A and B into a first mixing block and the reaction is quenched in a second mixing block with HCl from syringe C. Another mixing block is added in four-syringe experiments, a second reactant is placed in syringe C, and the quench solution is pushed from syringe D. Reaction times of 2.5–300 ms can be obtained by varying the tubing length or changing the nitrogen pressure to vary the flow rate. The reaction times were calibrated from measurements of 2,4-dinitrophenyl acetate hydrolysis by hydroxide ion (Barman & Gutfreund, 1964).

Passively loaded SRV were used in order to inhibit the hydrolysis of phosphoenzyme and permit accurate end point determinations in the presence of a nonradioactive ATP chase. SRV were passively loaded with Ca^{2+} by incubation for 4–16 h at 4 °C in a solution containing 14.5 mg/mL SRV, 0.1 M KCl, 5 mM $MgSO_4$, 5 mM MOPS, pH 7.0, 0.32 M sucrose, and 20 mM $CaCl_2$. For each reaction, 10 μL of this stock SRV solution was mixed with 0.89 mL of a solution containing 5 mM EGTA (resulting in free [Ca^{2+}] <30 nM), followed by 100 μL of the radioactive ATP solution. This solution was loaded into syringe A of the rapid mixer, and the reaction was started within 15 s. Under these conditions, control experiments showed that the radioactive phosphoenzyme formed upon the addition of calcium and excess unlabeled ATP decayed with a half-time of 5 s. Thus, less than 2% of the phosphoenzyme has decayed at times shorter than 100 ms. The amount of [^{32}P]*E*-P in quenched reaction mixtures was determined essentially as described by Verjovski-Almeida et al. (1978).

Results and Discussion

Reaction of E·ATP with Ca^{2+}. Figure 1 shows that the complex of passively loaded SRV and [γ-^{32}P]ATP·Mg, formed in a 15-s preincubation of *E* with 300 μM [γ-^{32}P]ATP in the presence of 5 mM EGTA and 5 mM $MgSO_4$, reacts with 50

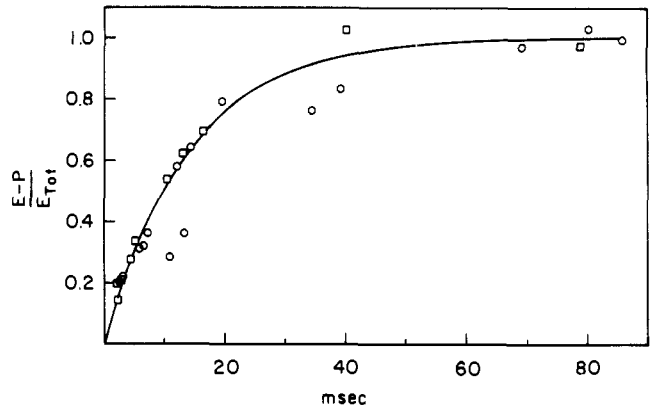

FIGURE 1: Reaction of *E*·ATP·Mg with Ca^{2+}. Final conditions were 40 mM MOPS (pH 7.0), 100 mM KCl, 5 mM $MgSO_4$, 2.5 mM EGTA, 2.41 (○) or 2.8 mM (□) $CaCl_2$ (resulting in 50 and 300 μM free calcium, respectively), 150 (○) or 100 μM (□) [γ-^{32}P]ATP, 0.065 mg/mL SR protein, and 25 °C. Syringe A contained 5.0 mM EGTA, 300 (○) or 200 μM (□) [γ-^{32}P]ATP, and 0.13 mg/mL SR protein; syringe B contained 4.82 (○) or 5.6 mM (□) $CaCl_2$; syringe C contained 1.5 N HCl and 40 mM KH_2PO_4. Other components were present in all syringes except C at their final concentrations. A zero time point was measured by reversing the order of addition of syringes B and C. Bovine serum albumin was added to the quenched reaction mixtures (0.30 mg/mL final concentration), followed by trichloroacetic acid at a final concentration of 12%, and the amount of [^{32}P]*E*-P was determined as described under Methods. The line is calculated for a first-order reaction with a rate constant of 70 s^{-1}.

μM free Ca^{2+} to form [^{32}P]*E*-P·Ca_2 with a rate constant of 70 s^{-1}. The reaction involves the ATP-activated binding of calcium, any associated conformational changes, and phosphorylation by bound ATP. The rate constant of 70 s^{-1} cannot represent second-order binding of Ca^{2+} because increasing the Ca^{2+} concentration to 300 μM results in identical behavior (Figure 1, squares). It does not represent phosphorylation, according to the model of eq 1, because phosphorylation of

$$E\cdot ATP + 2Ca^{2+} \xrightarrow{k_1} E\cdot ATP\cdot Ca_2 \xrightarrow{k_a} E^a\cdot ATP\cdot Ca_2 \xrightarrow{k_p} E\text{-}P\cdot Ca_2 + ADP \quad (1)$$

$E\cdot Ca_2$ has been shown to occur with a rate constant of k_p >150 s^{-1} under similar or identical conditions (Froehlich & Taylor, 1975; Pickart & Jencks, 1982; J. Petithory and W. P. Jencks, unpublished results). It is consistent with a rate-limiting conformational change, with k_a = 70 s^{-1} (eq 1),[2] to give an activated species that undergoes rapid phosphorylation. It is 4–6-fold faster than rate constants of 11–15 s^{-1} that have been assigned to the conformational change associated with Ca^{2+} binding under similar conditions in the absence of ATP (Guillain et al., 1980; Fernandez-Belda et al., 1984). Although ATP binding provides a way to bypass one slow conformational change associated with Ca^{2+} binding (e.g., "$E_2 + 2Ca^{2+} \rightarrow E_1\cdot Ca_2$"), *E*·ATP may still undergo a conformational change before phosphorylation occurs. An alternative, but less likely, hypothesis is that the rate constant for the phosphorylation step in the species *E*·ATP·Ca_2 is different depending on the order in which ATP and calcium bind to the enzyme.

The observation of good first-order kinetics (Figure 1) shows that all of the *E*·ATP·Mg behaves as either a single form of the enzyme or two forms that interconvert rapidly. Furthermore, the absence of a lag in the phosphorylation time

[2] A value of 115 s^{-1} for the rate constant of the conformational change in the presence of ATP was derived previously from manual fitting of a process with two exponentials (Pickart & Jencks, 1984). However, the data are also consistent with a rate constant of 70 s^{-1} for the conformational change based on a nonlinear least-squares fit.

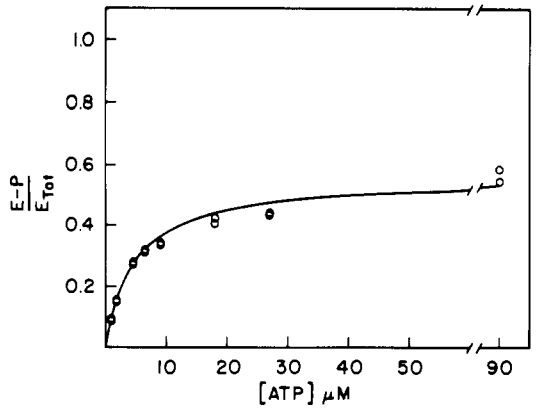

FIGURE 2: Trapping of E·ATP by 200 μM Ca^{2+}. Conditions were the same as for Figure 1 (□) except that syringe A contained the indicated concentration of [γ-^{32}P]ATP and syringe B contained 2.7 mM $CaCl_2$ and 2.0 mM nonradioactive ATP. The reactions were quenched after 60 ms. The amount of E_{tot} was measured with 90 μM [γ-^{32}P]ATP in syringe A by omitting the nonradioactive ATP from syringe B. The line is calculated for a K_d of 4.5 μM and a maximum fraction trapped of 0.55.

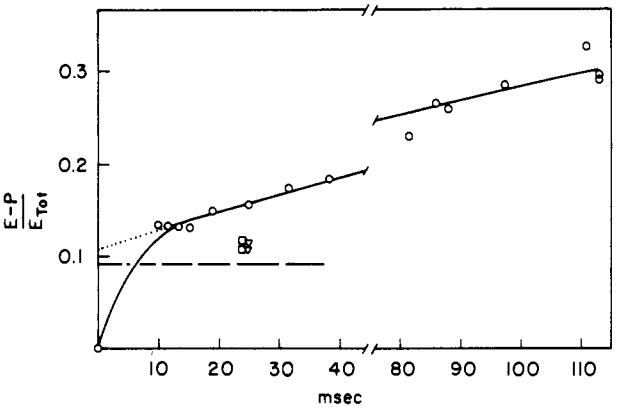

FIGURE 3: Formation of E-P·Ca_2 upon addition of 200 μM Ca^{2+} to enzyme preincubated with 0.9 μM [γ-^{32}P]ATP. Conditions were the same as for Figure 2 except that syringe A contained 0.9 μM [γ-^{32}P]ATP and syringe B contained 0 (○), 0.2 (▽), or 2.0 mM (□) nonradioactive ATP. The end point for the reaction was 0.67 of E_{tot}. The broken line indicates the amount of [^{32}P]E-P·Ca_2 predicted from trapping with 0.9 μM [γ-^{32}P]ATP preincubation, a K_d of 4.5 μM, and maximum fraction trapped of 0.55. The solid line following the burst is calculated for a first-order reaction with a rate constant of 3.5 s^{-1}.

course shows that for the model of eq 1 the rate constant for phosphoryl transfer, k_p, must be much larger than 70 s^{-1}. This rate constant must have a value of $k_p \geq 500$ s^{-1}, if phosphorylation occurs according to the mechanism of eq 1; a smaller value would cause a detectable deviation from first-order behavior. The phosphorylation step is responsible for the vectorial component of coupled calcium transport because it changes the direction in which calcium dissociates, from dissociation to the outside from E^a·ATP·Ca_2, with $k \sim 50$ s^{-1}, to dissociation inside the SRV, with $k \sim 17$ s^{-1} (Pickart & Jencks, 1982, 1984).

Trapping E·ATP with Ca^{2+}. When passively loaded SRV are preincubated with [γ-^{32}P]ATP·Mg and the reaction is initiated by adding 200 μM Ca^{2+} with a chase of excess unlabeled ATP, [^{32}P]E-P·Ca_2 is formed (Figure 2). This trapping of E·ATP by Ca^{2+} confirms the conclusion that the release of ATP is slower than phosphorylation (Pickart & Jencks, 1982; Shigekawa & Kanazawa, 1982). None of the E-P·Ca_2 would be labeled during the chase period if the dissociation of ATP were in rapid equilibrium relative to the conformational change or phosphoryl transfer. Figure 2 shows that the amount of labeled E-P·Ca_2 formed increases with the concentration of [γ-^{32}P]ATP·Mg in the preincubation mixture, leveling off at a maximum of 55%. The data fit a scheme in which a constant 55% of E·ATP·Mg is trapped and the amount of E·ATP·Mg depends on the binding of [γ-^{32}P]ATP·Mg to a single site with an apparent K_d of 4.5 μM. The good fit to a model involving a single site rules out any simple scheme involving negative cooperativity of ATP binding to the active sites of two interacting subunits (Yates & Duance, 1976). A K_d of 4.5 μM is similar to values of K_d = 3–5 μM measured by other techniques (Meissner, 1973; Dupont, 1977; Dupont et al., 1982). A similar trapping of [γ-^{32}P]ATP by Na^+ has been observed for the Na^+,K^+-ATPase by Mardh & Post (1977).

Formation of E-P·Ca_2 following Preincubation with 0.9 μM Mg·ATP. Figure 3 shows that passively loaded SRV preincubated with 0.9 μM [γ-^{32}P]ATP·Mg give a burst of [^{32}P]E-P·Ca_2 formation followed by a slow rate of labeling when the reaction is initiated with 200 μM Ca^{2+}. Extrapolation of the slow phase to zero time corresponds to an 11% burst of [^{32}P]E-P·Ca_2 formation (dotted line, Figure 3). A K_d of 4.5 μM predicts that 17% of the total enzyme should exist as E·ATP at equilibrium in 0.9 μM Mg·ATP, but dissociation of some [γ-^{32}P]ATP from the active site upon dilution into the Ca^{2+} solution is expected to decrease the amount that is trapped. The rate constant for the appearance of [^{32}P]E-P·Ca_2 in the burst is ≥ 70 s^{-1}; it is much faster than the rate constants of 11–15 s^{-1} reported for the conformational change in the absence of ATP (Guillain et al., 1980; Fernandez-Belda et al., 1984).[3] This result shows directly that Mg·ATP bound to the active site of a small fraction of enzyme molecules, at [Mg·ATP] far below that required to fill a regulatory site with K_d = 160–500 μM (Yates & Duance, 1976; Dupont, 1977; Nakamura & Tonomura, 1982; Clore et al., 1982), activates the conformational change for those molecules to which it is bound when Ca^{2+} is added.

When a nonradioactive chase of 0.1 or 1.0 mM ATP is included with the Ca^{2+} (Figure 3, triangles and squares, respectively), the amount of [^{32}P]E-P·Ca_2 observed is close to the amount predicted from a K_d of 4.5 μM for Mg·ATP and a fraction trapped of 0.55 (Figure 3, broken line). The mitochondrial F_1 ATPase shows an increase in the rate constant for nucleotide dissociation with increasing ATP concentration (Hutton & Boyer, 1979; Cross et al., 1982). There does not appear to be a similar effect for the Ca-ATPase because the fraction of [γ-^{32}P]ATP trapped is independent of the concentration of ATP in the chase up to 1 mM (Figure 3), indicating that the rate constant for dissociation of ATP does not change with ATP concentration.

The dependence of the steady-state ATPase reaction rate on ATP concentration shows nonhyperbolic behavior, with three discernible regions in double-reciprocal plots (Møller et al., 1980; A. L. Bodley and W. P. Jencks, unpublished experiments). In addition to a K_m in the range of 1–4 μM for the lowest region, several investigators have observed a K_x of 10–100 μM (Møller et al., 1980; Vianna, 1975; Neet & Green, 1977; Yamamoto & Tonomura, 1967; A. L. Bodley and W. P. Jencks, unpublished experiments) and K_y of 0.3–3 mM (Taylor & Hattan, 1979; Møller et al., 1980; Anderson &

[3] The rate constant for the burst is expected to be faster than 70 s^{-1} because the 2-fold decrease in the concentration of [γ-^{32}P]ATP upon mixing syringes A and B will lead to net dissociation of [γ-^{32}P]ATP from the enzyme. In this case the observed first-order rate constant approaches the sum of the rate constants for the conformational change and the dissociation of [γ-^{32}P]ATP.

Murphy, 1983; A. L. Bodley and W. P. Jencks, unpublished results).[4] It is likely that the K_x region is caused by acceleration of a conformational change associated with Ca^{2+} binding by the mechanism described here because the steady-state E-P·Ca_2 level increases with ATP concentrations near the K_x (Inesi et al., 1970; A. L. Bodley and W. P. Jencks, unpublished results) and concentrations of ATP in the region 5–10 μM accelerate this process when added together with Ca^{2+} (Sumida et al., 1978; Takisawa & Tonomura, 1978; Scofano et al., 1979; Inesi et al., 1980; Guillain et al., 1981). This is consistent with an ATP-dependent increase in the rate of the steps that involve interconversion of dephosphorylated enzyme forms. It has been shown previously that mechanistic schemes in which the binding of a substrate affects the rate constant for a slow conformational change can show a non-hyperbolic dependence of rate on substrate concentration (Smith et al., 1980; Moczydlowski & Fortes, 1981; N. Stahl and W. P. Jencks, unpublished results). It has not been unambiguously shown whether the K_y region, which may be caused by an increase in the rate constant for Ca^{2+} release from E-P·Ca_2, is caused by ATP binding to a separate effector site or, with lowered affinity, to the active site.

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[4] K_x and K_y represent the values obtained from extrapolation to the abscissa of the second and third linear regions in double-reciprocal plots. These may not be equal to the K_m for the process (Klotz & Hunston, 1971).