The Binding of ATP and Mg^{2+} to the Calcium Adenosinetriphosphatase of Sarcoplasmic Reticulum Follows a Random Mechanism†

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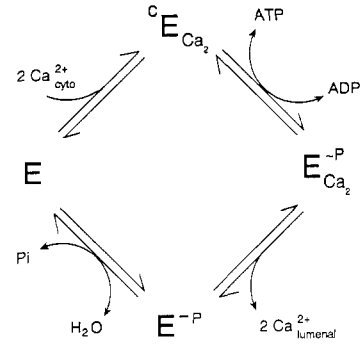
ABSTRACT: The enzyme form of the calcium adenosinetriphosphatase of sarcoplasmic reticulum (CaATPase) that is stable in the presence of calcium, $^{c}E{\cdot}Ca_2$, has a binding site for the catalytic Mg^{2+} ion with a dissociation constant of 0.94 ± 0.15 mM at 25 °C, pH 7.0, and 100 mM KCl. This is ≈10 times smaller than that reported for the free enzyme, E, (8.8 mM) under similar conditions [Punzengruber, C., Prager, R., Kolassa, N., Winkler, F., & Suko, J. (1978) *Eur. J. Biochem. 92,* 349–359]. This difference shows that the sites for the catalytic and the transported ions interact in the absence of ATP. The addition of ATP and EDTA to enzyme that had been incubated with Ca^{2+} and Mg^{2+} resulted in the formation of 61% phosphoenzyme. The addition of unlabeled ATP and Mg^{2+} to enzyme that had been incubated with 3.5 μM free Ca^{2+} and labeled ATP gave 39% labeled phosphoenzyme. This shows that the binding of ATP and Mg^{2+} to $^{c}E{\cdot}Ca_2$ follows a random mechanism. The rate constants for dissociation of ATP and Mg^{2+} from $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ are different: $k_{diss}(ATP) = 120\ s^{-1}$ and $k_{diss}(Mg^{2+}) = 60\ s^{-1}$. This shows that Mg^{2+} and ATP can bind and dissociate independently; they do not have to associate or dissociate from ^{c}E as a Mg·ATP complex. Calcium-free enzyme binds metal-free ATP at the active site with a dissociation constant of 44 ± 4 μM, $k_{diss} = 130 \pm 7\ s^{-1}$, and a calculated association rate constant of $3 \times 10^6\ M^{-1}\ s^{-1}$. Calcium-free enzyme that was incubated with [γ-^{32}P]ATP gave 38% labeled phosphoenzyme when chased with unlabeled ATP, Mg^{2+}, and Ca^{2+}. An increase of the Mg^{2+} concentration did not increase the amount of $E^{32}P$ formed. This shows that the binding of Mg^{2+} and ATP to free E also follows a random mechanism. The Mg^{2+} ion is not buried under ATP, and ATP is not under a Mg^{2+} ion. Incubation of free E with Mg^{2+} and ATP causes a conformational change that activates the enzyme for phosphorylation and decreases the rate constant for the dissociation of ATP from $k_{diss} = 120\ s^{-1}$ to $k_{diss} = 47\ s^{-1}$.

The calcium adenosinetriphosphatase (CaATPase)[1] from sarcoplasmic reticulum (SR) transports Ca^{2+} ions from muscle tissue into the lumen of the SR. The energy for pumping Ca^{2+} against an electrochemical gradient is provided by the free energy of hydrolysis of ATP to ADP and inorganic phosphate in the presence of Mg^{2+} as the catalytic ion (Hasselbach & Makinose 1961, 1963; Ebashi & Lipmann, 1962). The transport cycle shown in Scheme I (Makinose, 1973) includes the binding of two calcium ions and ATP from the cytosolic side of the SR (Yamamoto & Tonumura, 1967), transfer of the terminal phosphate group of ATP to aspartate 351 (Allen & Green, 1976) to form acid-stable phosphoenzyme (Degani & Boyer, 1973), and dissociation of Ca^{2+} to the lumen of the SR, which allows hydrolysis of the phosphate anhydride bond to complete the cycle. The formation and breakdown of the phosphoenzyme occur with inversion, which leads to the retention of configuration that is observed for the overall reaction (Webb & Trentham, 1981).

Scheme I



It has been shown that Ca^{2+} and ATP can bind randomly to the CaATPase in the presence of Mg^{2+} to form the catalytically active enzyme, $^{a}E{\cdot}Ca_2{\cdot}ATP$, which is phosphorylated very rapidly with a rate constant of $k_p \geq 1000\ s^{-1}$ (Scheme II; Petithory & Jencks, 1986; Stahl & Jencks, 1987). The properties of the complexes of enzyme with bound Ca^{2+} and ATP, however, are different for the two pathways: enzyme incubated with Ca^{2+} first forms the enzyme species $^{c}E{\cdot}Ca_2$, and the addition of ATP induces a structural change to form $^{a}E{\cdot}Ca_2{\cdot}ATP$ with $k_d = 220\ s^{-1}$ (Petithory & Jencks, 1986); addition of Ca^{2+} to E·ATP, on the other hand, results in a structural change with $k_b = 70\ s^{-1}$ to give $^{a}E{\cdot}Ca_2{\cdot}ATP$ (Stahl & Jencks, 1987).

We were interested to learn what are the contributions of the catalytic Mg^{2+} ion and the substrate ATP to the conformational change of $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ to $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$. The overall rate of catalysis (ATPase activity) is slowed with

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[1] Abbreviations: SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles; E, calcium adenosinetriphosphatase (CaATPase); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; ATP*, [γ-^{32}P]ATP; PEP, phosphoenolpyruvate; TRIS, tris(hydroxymethyl)aminomethane; $^{c}E{\cdot}Ca_2$, the enzyme form that is stable in the presence of Ca^{2+}; $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, the enzyme form that is activated for phosphorylation by ATP; $^{m}E{\cdot}ATP{\cdot}Mg$, the enzyme form that is stable in the presence of Mg^{2+} and ATP; E_{tot}, maximal amount of phosphoenzyme.

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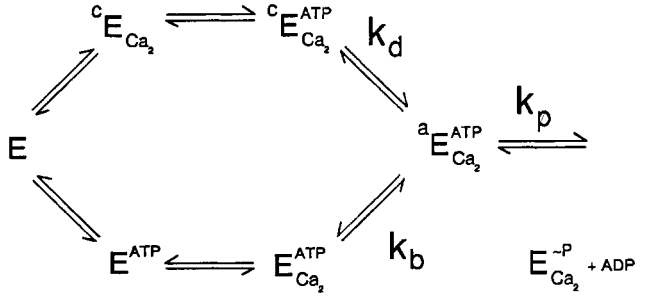
Scheme II



Ca^{2+}, La^{3+}, Cr^{3+}, or Co^{2+} as the catalytic ion (Vianna, 1975; Garrahan et al., 1976; Yamada & Ikemoto, 1980; Wakabayashi & Shigekawa, 1984; Yamada et al., 1986; Vilsen & Andersen, 1987; Fujimori & Jencks, 1990; Hanel & Jencks, 1990), whereas Mn^{2+} or Zn^{2+} allow turnover of ATP at a rate similar to that observed with Mg^{2+} (Yamada & Ikemoto, 1980; Chiesi & Inesi, 1981; Henao & Gutierrez-Merino, 1989; Ogurusu et al., 1991). The rates of phosphoenzyme formation from ATP and its hydrolytic breakdown are decreased to a similar extent if Mg^{2+} is replaced by Ca^{2+} or La^{3+} as the catalytic ion (Garrahan et al., 1976; Domonkos et al., 1985; Fujimori & Jencks, 1990).

It was shown by Hanel and Jencks (1990) that La^{3+} as the catalytic ion decreases the phosphorylation rate because the conformational change from $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}La$ to $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}La$ is slowed 40-fold, to 6.5 s^{-1}. This indicates that the catalytic ion not only plays a role in the chemical step of phosphoryl transfer but also controls a conformational change. There is also evidence that the Ca^{2+} transport sites and the catalytic site interact in the presence of ATP, because the affinity of Ca^{2+} for the transport sites is increased when Ca^{2+} or La^{3+} is the catalytic ion (Hanel & Jencks, 1990).

In order to improve our understanding of the contributions of Mg^{2+} and ATP to the formation of a catalytically active enzyme species, the following questions have been addressed: (1) What is the binding mechanism of ATP and Mg^{2+}? (2) If Mg^{2+} and metal-free ATP are able to bind independently, what are the properties of E·Mg and E·ATP, compared to the complex with Mg *and* ATP? (3) How many Mg^{2+} ions are involved in the catalytic reaction?

The results of transient kinetic experiments show the following: (1) The binding of ATP and Mg^{2+} follows a random mechanism: Mg^{2+} and ATP can bind and dissociate independently or as Mg·ATP in order to form a productive complex with the enzyme. (2) Contrary to a report in the literature (Makinose & Boll, 1979), there is no evidence that more than one catalytic Mg^{2+} ion is involved in the phosphoryl transfer reaction or that metal-free ATP cannot serve as substrate if Mg^{2+} is already bound to the enzyme. (3) The affinity of Mg^{2+} for the enzyme form of CaATPase that is stable in the presence of Ca^{2+} ($^{c}E{\cdot}Ca_2$) is ten times higher than for the free enzyme form (E). This indicates that the catalytic and the transport ions interact in both the absence and the presence of nucleotide. (4) Binding of Mg^{2+} induces a conformational change; the enzyme that is stable in the presence of ATP and Mg^{2+}, $^{m}E{\cdot}ATP{\cdot}Mg$, is different from the enzyme that is stable in the presence of metal-free ATP, as indicated by the different rate constants for dissociation of ATP from these two species after Ca^{2+} (and Mg^{2+}, to E·ATP) is added.

MATERIALS AND METHODS

Materials. The disodium salt of ATP, Tris (ultrapure), NADH (grade I), phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were purchased from Boehringer Mannheim; $CaCl_2{\cdot}2H_2O$, the calcium-sensitive dye Calmagite, and bovine serum albumin (BSA), from Sigma; MOPS (Ultrex grade) and the calcium ionophore A23187, from Calbiochem; $MgCl_2{\cdot}6H_2O$, from Aldrich; EGTA, EDTA, KCl, and a standard calcium solution (1 g of calcium/L), from Fluka; [γ-^{32}P]ATP, from New England Nuclear; and sucrose (enzyme grade), trichloroacetic acid, perchloric acid, and KH_2PO_4, from Fisher. All solutions were prepared with Milli-Q grade water (Millipore Co.).

Sarcoplasmic reticulum vesicles from rabbit muscle were prepared as described previously (Khananshvili & Jencks, 1988). Enzyme solutions (20 mg/mL) were dialyzed at 4 °C overnight against 0.5 L of 100 mM KCl, 40 mM MOPS, pH 7.0, 0.2 M sucrose, and 20 mM $CaCl_2$ in order to obtain passively loaded, intact vesicles. The preparations were frozen in dry ice and stored in aliquots at −80 °C until they were used. The steady-state ATPase activity of the preparations used in this work was 6–7 μmol min^{-1} (mg of protein)$^{-1}$. The amount of CaATPase in the preparation was estimated to be approximately 50% of the total protein, according to denaturing sodium dodecylsulfate–polyacrylamide gel electrophoresis (Laemmli, 1970).

Methods. The concentrations of calcium chloride in stock solutions were measured as described by Brittain (1979). Steady-state ATP hydrolysis was measured spectrophotometrically by coupling the formation of ADP to oxidation of NADH with pyruvate kinase and lactate dehydrogenase (Rossi et al., 1979). Standard conditions for the assay were 25 °C, 100 mM KCl, 40 mM MOPS, pH 7.0, 1.5 mM ATP, 5 mM $MgSO_4$, 400 μM EGTA, 410 μM $CaCl_2$ (18 μM free Ca^{2+}), 1.5 mM phosphoenolpyruvate, 0.15 mM NADH, 0.05 mg of pyruvate kinase, 0.05 mg of lactate dehydrogenase, and 2 μM A23187 in a total volume of 2 mL. The ATPase activity without the ionophore was 4% of the activity in the presence of A23187. Protein concentrations were measured by using the method of Lowry et al. (1951) with bovine serum albumin (BSA) as standard.

The concentrations of free metal ions and metal–chelator complexes were calculated with the Newton–Raphson algorithm (Press et al., 1989, pp 305 ff), taking into account the chelating properties of EGTA, ATP, and EDTA when applicable. The affinity constants were taken from Fabiato and Fabiato (1979) and are valid for pH 7.0: Ca·EGTA, 2.57×10^6 M^{-1}; Mg·EGTA, 3.3×10^1 M^{-1}; Ca·EDTA, 1.76×10^7 M^{-1}; Mg·EDTA, 1.72×10^5 M^{-1}; Ca·ATP, 4.35×10^3 M^{-1}; Mg·ATP, 1.06×10^4 M^{-1}. Tris base was added to solutions that did not contain enzyme in order to neutralize the protons that are released upon chelation of the metals.

The kinetic equations were derived according to Cleland (1975).

Rapid Mix–Quench Experiments. The formation of phosphoenzyme was measured at 25 °C by using a thermostated rapid-mixing chemical-quench apparatus, as described previously (Stahl & Jencks, 1984). For three-syringe experiments, the temperature-equilibrated contents of syringes A and B were forced through a mixing block connected to Teflon tubing for a reaction time t_1 before being quenched with perchloric acid from syringe C in a second mixing block. For four-syringe experiments, a second reactant was allowed to react for a time t_2 before the quenching acid was added from

syringe D in an additional mixing block. All syringes had equal volumes and contained 40 mM MOPS, pH 7.0, and 100 mM KCl except the final syringe, which contained the quench solution and gave a final concentration of 0.125 M $HClO_4$ and 13 mM phosphate. Chase solutions that contained high concentrations of unlabeled ATP were incubated for 30 min with 0.5–1.5 mM phosphoenolpyruvate and 50 μg/mL pyruvate kinase to reduce the amount of contaminating ADP. The amount of phosphoenzyme formed with intact, passively loaded vesicles at saturating concentrations of Ca^{2+} and [γ-^{32}P]·ATP (E_{tot}) was 4.4–4.5 nmol/mg of total protein.

Determination of [^{32}P]EP. The amount of radiolabeled phosphoenzyme was measured essentially as described by Verjovski-Almeida et al. (1978). Bovine serum albumin and ATP were added to quenched reaction mixtures to give a final concentration of 0.18–0.26 mg/mL BSA and 75–100 μM ATP, and the samples were put on ice for at least 30–150 minutes. The precipitated protein was centrifuged at 1500*g* for 15 min at 4 °C, and the supernatant solution was decanted. The pellets were resuspended in 5% ice-cold trichloroacetic acid containing 40 mM KH_2PO_4, and the protein was collected on Whatman GF/C glass fiber filters by vacuum filtration and rinsed with ≈30 mL of resuspension solution. The radioactivity was measured by liquid scintillation counting in glass vials containing 7 mL of Aquasol-2.

RESULTS

Phosphorylation with Ca^{2+} as the Catalytic Ion. The rate-determining step for the phosphorylation of enzyme that has been incubated with Ca^{2+} ($^{c}E{\cdot}Ca_2$) is a conformational change that is induced by the binding of ATP and Mg^{2+} to form the activated enzyme, $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$; this is followed by fast phosphorylation, with $k \geq 1000\ s^{-1}$ (Petithory & Jencks, 1986). We were interested to determine if the catalytic Mg^{2+} ion is necessary to induce this conformational change, or if metal-free ATP is sufficient. If metal-free ATP induces the conformational change to $^{a}E{\cdot}Ca_2{\cdot}ATP$ and the binding of Mg^{2+} to form $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ is fast, rapid phosphorylation of the enzyme with $k \geq 1000\ s^{-1}$ would be expected after the addition of Mg^{2+} to $^{a}E{\cdot}Ca_2{\cdot}ATP$. The initial phase of Figure 1 shows that, in the absence of Mg^{2+}, 4.4 nmol/mg passively loaded SRV incubated with 3.5 or 28 μM free Ca^{2+} and 100 μM [γ-^{32}P]ATP form 0.8 ± 0.2 nmol/mg EP* after t_1 = 58 ms (Scheme III); this corresponds to a rate constant for phosphorylation of k_3 = 3.5 ± 0.5 s^{-1}, according to $k = -\ln(1 - EP(t)/E_{tot})/t_1$.

This is similar to the rate constant of 3 s^{-1} under similar conditions reported by Hanel and Jencks (1990) in the presence of 1 mM Ca^{2+}. It was shown previously that Ca^{2+} can replace Mg^{2+} as the catalytic ion (Wakabayashi & Shigekawa, 1984, 1987). Since the amounts of EP* formed in 58 ms are virtually the same for 3.5 and 28 μM free Ca^{2+}, it appears that 3.5 μM calcium nearly saturates the transport and catalytic binding sites to form the species $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$.

Exchange of Mg^{2+} for Ca^{2+} as the Catalytic Ion in the $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Mg$ Complex. Addition of magnesium and calcium to $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$ after t_1 = 58 ms results in phosphorylation with a rate constant of 23 s^{-1}; there is no initial burst of phosphorylation (○ in Figure 1). This phosphorylation can be explained by exchange of Mg^{2+} for Ca^{2+} as the catalytic ion (k_1 in Scheme III). The $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ complex is phosphorylated rapidly, with a rate constant of k_2 = 220 s^{-1} (Petithory & Jencks, 1986), so that the exchange of Mg^{2+} for Ca^{2+} is rate limiting with $k_1 = k_{obs}$ – k_3 (23 – 3.5) $s^{-1} \simeq 20\ s^{-1}$. The concentration of free Ca^{2+} was increased during the chase in order to prevent competition of Mg^{2+} with Ca^{2+} for the transport sites at very low calcium concentrations. Ogurusu et al. (1991) showed recently with filter binding experiments that one of the three $^{45}Ca^{2+}$ ions that are bound to Mg^{2+}-free CaATPase in the presence of ATP, but in the absence of Mg^{2+}, is replaced by low concentrations of $^{54}Mn^{2+}$, which is believed to act as an analogue for Mg^{2+} at the catalytic site.

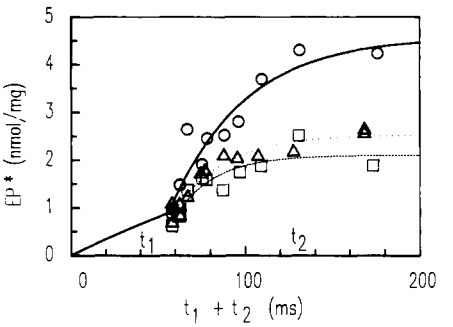
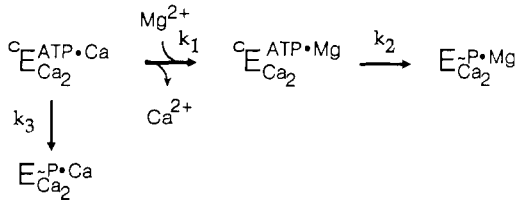


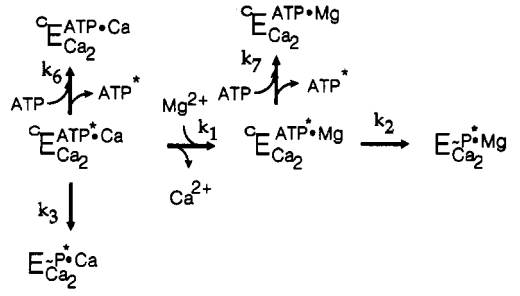
FIGURE 1: Exchange of Mg^{2+} for Ca^{2+} as the catalytic ion in the $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Ca$ complex. $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Ca$ was formed by mixing 0.085 mg/mL passively loaded SRV (4.5 nmol/mg, 20 mM $CaCl_2$) with 150 μM [γ-^{32}P]ATP and either 3.5 μM free Ca^{2+} (○, □) or 28 μM free Ca^{2+} (△) (final concentrations). The labeled phosphoenzyme (EP*) is formed slowly during the first 58 ms (0.8 nmol/mg) with a rate constant of 3.5 s^{-1}; this results from the activity of Ca^{2+} as the catalytic ion. The remaining $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Ca$ was then chased with $MgCl_2$ and $CaCl_2$ to give 5 mM free Mg^{2+} and 50 μM free Ca^{2+} (○); $MgCl_2$, $CaCl_2$, and unlabeled ATP to give 3 mM free Mg^{2+}, 43 μM free Ca^{2+}, and 2 mM unlabeled ATP (□); or Mg^{2+} and EGTA to give 4.3 mM free Mg^{2+} and 0.07 μM free Ca^{2+} (△). The lines drawn correspond to single-exponential functions with phosphorylation rate constants and amounts of EP* formed during t_2 according to a nonlinear fitting procedure: 24 ± 8 s^{-1}, 3.5 ± 0.5 nmol/mg (○; —); 45 ± 20 s^{-1}, 1.4 ± 0.2 nmol/mg (□; – –); 35 ± 8 s^{-1}, 1.7 ± 0.1 nmol/mg (△; ···). All syringes except D contained 100 mM KCl and 40 mM MOPS/Tris, pH 7.0, at 25 °C. Syringes A and B contained 1 mM EGTA and either 0.9 mM $CaCl_2$ (○, □) or 1.03 mM $CaCl_2$ (△). Additionally, syringe B contained 300 μM ATP*, and syringe C contained 15 mM $MgCl_2$ and either 5.8 mM $CaCl_2$ and 5.5 mM EGTA (○), 5.8 mM $CaCl_2$, 5.5 mM EGTA, 6 mM unlabeled ATP, 1.5 mM PEP, and 5 μg/mL pyruvate kinase (□), or 13 mM EGTA (△). Syringe D contained 0.5 M $HClO_4$ and 50 mM KH_2PO_4 as quench solution.

Scheme III

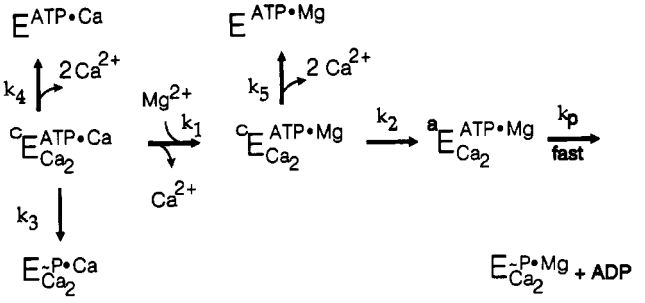


Exchange of ATP for ATP in the $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Ca$ Complex.* The rate constant of k_1 = 20 s^{-1} for the exchange of Mg^{2+} for Ca^{2+} could represent either the dissociation of Ca^{2+} from the catalytic site of $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Ca$ or the dissociation of ATP*, followed by fast dissociation of Ca^{2+} that was buried under ATP* in the catalytic site. In order to address this question, the $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Ca$ complex was formed as described above and then chased with 5 mM free Mg^{2+}, 50 μM free Ca^{2+}, and 2 mM unlabeled ATP (□ in Figure 1). The observed rate constant for phosphorylation is 45 ± 20 s^{-1} with a yield of 1.4 ± 0.2 nmol/mg EP*, which corresponds to 39% of the $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Ca$ remaining after t_1. This result is consistent with the mechanism shown in Scheme IV, in which ATP* can dissociate either from $^{c}E{\cdot}Ca_2{\cdot}ATP^{*}{\cdot}Ca$ with the dissociation rate constant k_6 or from

Scheme IV



Scheme V



$^cE{\cdot}Ca_2{\cdot}ATP^*{\cdot}Mg$ with the dissociation rate constant k_7 = 120 s^{-1} and the enzyme phosphorylates with a rate constant of 220 s^{-1} (Petithory & Jencks, 1986, 1988).

The analysis of the experimental data is simplified if k_1 is assumed to be the rate-determining step for the formation of $EP{\cdot}Mg{\cdot}Ca_2$. The error introduced by this simplification does not exceed 10%, which is below the experimental error because $k_2 = 10k_1$. The relationship $k_{obs} \approx 45\ s^{-1} = k_1 + k_3 + k_6 = (20 + 3.5)\ s^{-1} + k_6$ gives an approximate value of $k_6 = 22\ s^{-1}$ for the dissociation of ATP* from $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$. The expected yield of 36% EP*, from eq 1, is in agreement with

$$\frac{k_3}{k_1+k_3+k_6} + \frac{k_1}{k_1+k_3+k_6}\frac{k_2}{k_2+k_7}E_{tot} = \frac{3.5\ s^{-1}}{(20+3.5+22)\ s^{-1}} + \frac{20\ s^{-1}}{(20+3.5+22)\ s^{-1}} \times \frac{220\ s^{-1}}{(220+120)\ s^{-1}}(4.4)\ \text{nmol/mg} = (0.36 \times 4.4)\ \text{nmol/mg} = 1.67\ \text{nmol/mg} \quad (1)$$

the 39% observed yield. If ATP* were required to dissociate before Mg^{2+} could bind, labeled phosphoenzyme would be formed only from $^cE{\cdot}Ca_2{\cdot}ATP^*{\cdot}Ca$ with k_3 = 3.5 s^{-1}, which would give 3.5 s^{-1}/45 s^{-1} = 8% EP* and does not account for the 39% yield of phosphoenzyme. This shows that the catalytic Ca^{2+} ion is not buried under the ATP* and, more important, that the Mg^{2+} ion binds to $^cE{\cdot}Ca_2{\cdot}ATP^*$ without dissociation of ATP*. This means that the binding of ATP and Mg^{2+} to form $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ does not follow a compulsory ordered mechanism with Mg^{2+} binding first.

Trapping of Ca^{2+} in the $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$ Complex. To determine the rate constant for dissociation of Ca^{2+} bound to the transport sites in the $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$ complex, passively loaded vesicles were incubated with 28 μM free Ca^{2+} and 150 μM ATP* for t_1 = 58 ms and then chased with $MgCl_2$ and EGTA to give 0.07 μM free Ca^{2+} after the chase (Δ in Figure 1). The observed rate constant for phosphorylation is 35 ± 8 s^{-1} with a yield of 1.7 ± 0.1 nmol/mg of EP*, which corresponds to 1.7/(4.4 − 0.8) = 47% of $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$ remaining after t_1. This result is consistent with the mechanism of Scheme V, in which Ca^{2+} can dissociate from the catalytic site of $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$ and be exchanged for Mg^{2+} as the catalytic ion with the dissociation rate constant k_1, from the transport sites of $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$ with the rate constant k_4 = 11 s^{-1}, and from the $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ complex with k_5 = 80 s^{-1} (Petithory & Jencks, 1986). The phosphoenzyme is formed rapidly (k_p) after a rate-limiting conformational change (k_2). The observed yield of 47% phosphoenzyme formed during t_2 is, within experimental error, in accordance with the expected yield of 53%, according to the mechanism of Scheme V and the assigned rate constants in eq 2.

$$\frac{k_3}{k_1+k_3+k_4} + \frac{k_1}{k_1+k_3+k_4}\frac{k_2}{k_2+k_5}E_{tot} = \frac{3.5\ s^{-1}}{(20+3.5+11)\ s^{-1}} + \frac{20\ s^{-1}}{(20+3.5+11)\ s^{-1}} \times \frac{20\ s^{-1}}{220\ s^{-1}+80\ s^{-1}}(4.4)\ \text{nmol/mg} = 2.3\ \text{nmol/mg} \quad (2)$$

The rate-limiting conformational change for the phosphorylation of $^cE{\cdot}Ca_2$ with ATP and Mg^{2+} as the catalytic ion converts $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ to the active enzyme species $^aE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ with the rate constant k_2 (Scheme V). This species catalyzes rapid phosphoryl transfer to form EP, with a rate constant of $k_p \geq 1000\ s^{-1}$, followed by rapid dissociation of ADP. A rate-limiting conformational change, preceding the chemical step, is indicated because there is an immediate burst in the decay of phosphoenzyme after a chase with ADP when EP is formed from ATP·Mg as the substrate, which shows that the phosphoryl-transfer step itself is very fast. Furthermore, phosphorylation by ATP follows the same first-order rate constant for approach to equilibrium in the presence of ADP and for the complete reaction in the absence of ADP, which shows that a step prior to the phosphorylation itself is rate-limiting for phosphorylation (Pickart & Jencks, 1982; Stahl & Jencks, 1984, 1987; Petithory & Jencks, 1986; Ogawa & Harafuji, 1986). This burst was also observed with ATP·La as the substrate for phosphorylation, which indicates that the rate-limiting step of 6.5 s^{-1} to form EP with La^{3+} as the catalytic ion also is a conformational change (Hanel & Jencks, 1990).

However, the decay of EP formed from ATP·Ca as substrate shows no significant burst with ADP (Hanel & Jencks, 1990), so that the rate-limiting step with ATP·Ca as substrate is not certain. If the rate-limiting step of 3.5 s^{-1} for phosphorylation with ATP·Ca were the chemical step, the active enzyme species $^aE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$ would be formed rapidly and a chase with Mg^{2+} and EGTA should give 70% EP/E_{tot} (at t_2 = 58 ms), because Mg^{2+} exchanges to replace Ca^{2+} as the catalytic ion (O in Figure 1), and phosphorylation with $k_p \geq 1000\ s^{-1}$ is much faster than the dissociation of Ca from the transport sites of $^aE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, with k = 20–45 s^{-1} (Stahl & Jencks, 1987). Therefore, virtually no Ca^{2+} would dissociate from $^aE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, the only loss of transport Ca^{2+} ions would occur from $^aE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$ with the rate constant k_4 = 11 s^{-1} (Scheme V), and the yield of phosphoenzyme would be 70%. The observed yield of 47% phosphoenzyme is significantly lower than 70%. This suggests that the rate-limiting step for phosphorylation with ATP·Ca as substrate also is a slow conformational change to form $^aE{\cdot}Ca_2{\cdot}ATP{\cdot}Ca$, which is phosphorylated rapidly.

Does Mg^{2+} Bind to $^cE{\cdot}Ca_2$ in the Absence of ATP? The degree to which the catalytic site of $^cE{\cdot}Ca_2$ is saturated with

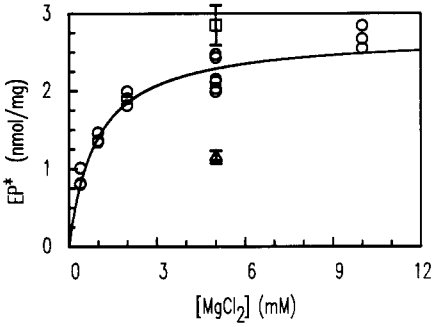


FIGURE 2: Affinity of Mg^{2+} for the catalytic site in the $^cE{\cdot}Ca_2$ complex. The dependence on [Mg^{2+}] of phosphoenzyme formation by trapping $^cE{\cdot}Ca_2{\cdot}Mg$ with [γ-^{32}P]ATP and EDTA was determined. All syringes except syringe C contained 40 mM MOPS buffer, pH 7.0, and 100 mM KCl. Passively loaded SRV (20 mM $CaCl_2$) were incubated at 25 °C with 1 mM EGTA, 1.1 mM $CaCl_2$, and various concentrations of $MgCl_2$ (0.4, 1, 2, 5, and 10 mM) for 15 s. The concentration of free Ca^{2+} in syringe A was 100 μM. The phosphorylation reaction was started by simultaneously adding 250 μM [or 50 μM (△)] [γ-^{32}P]-ATP and 15 mM [or 7.5 mM (□)] EDTA (final concentrations) after mixing the contents of syringes A and B, and quenched after a reaction time of 11 ms, which gives 98% completion of the phosphorylation reaction. The concentrations of free Mg^{2+} and Ca^{2+} after the quench did not exceed 3 μM Mg^{2+} and 0.003 μM Ca^{2+}. The solid line is drawn for a hyperbolic saturation function for the binding of Mg^{2+} to $^cE{\cdot}Ca_2$ with $K_d = 0.94 \pm 0.15$ mM and maximal formation of 2.7 ± 0.1 nmol/mg EP, which is 61% of the total enzyme (4.4 nmol/mg). Syringe C contained 39 mM KH_2PO_4 and 0.37 M $HClO_4$ as the quench solution.

Mg^{2+} to form $^cE{\cdot}Ca_2{\cdot}Mg$ was assayed by incubating SRV with 100 μM free Ca^{2+} and 0.4–10 mM $MgCl_2$ for 15 s and then chasing with [γ-^{32}P]ATP and EDTA for a time period that is sufficient to give complete phosphorylation (○ in Figure 2). Since only enzyme that had Mg^{2+} bound before the chase with EDTA can form phosphoenzyme, the affinity of Mg^{2+} for $^cE{\cdot}Ca_2$ can be calculated by plotting EP* against the concentration of Mg^{2+} in syringe A, as shown in Figure 2. The data follow a hyperbolic saturation curve with $K_d = 0.94 \pm 0.15$ mM and a maximal amplitude for EP* of 2.7 ± 0.1 nmol/mg. This experiment shows that there is a catalytic binding site for Mg^{2+} in $^cE{\cdot}Ca_2$ and that $^cE{\cdot}Ca_2{\cdot}Mg$ is a productive complex that allows the formation of phosphoenzyme when metal-free ATP is added. The affinity of Mg^{2+} for the free enzyme has been reported to be much weaker, with $K_d = 8.8$ mM under identical conditions except at 20 °C (Punzengruber et al., 1978).

Rate Constant for Dissociation of Mg^{2+} from $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$. Phosphoenzyme was formed with an observed rate constant of 360 s^{-1} when passively loaded SRV (20 mM $CaCl_2$) were incubated with 5 mM $MgCl_2$ and 100 μM free Ca^{2+} for 15 s and then chased with EDTA and [γ-^{32}P]ATP (Figure 3). As shown in Scheme VI, the observed rate constant for phosphorylation under these conditions is the sum of the first-order rate constants for phosphorylation of $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, $k_6 = 220$ s^{-1}, dissociation of Ca^{2+} from $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, $k_5 = 80$ s^{-1}, and dissociation of Mg^{2+} from $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, k_4. Therefore, the rate constant for dissociation of Mg^{2+} from $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ is $k_4 = [360 - (220 + 80)]$ $s^{-1} = 60$ s^{-1}. The pseudo-first-order rate constant for binding of 250 μM ATP* must be ≥1000 s^{-1} because there is no detectable lag for phosphorylation (<1 ms). This is consistent with the observed second-order rate constant for binding of ATP* to $^cE{\cdot}Ca_2$ of 10×10^6 M^{-1} s^{-1} (Stahl & Jencks, 1987). The observed formation of 2.3 nmol EP*/mg is consistent with a rate constant of 60 s^{-1} for the dissociation of Mg^{2+} from the $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ complex if the incomplete saturation of the catalytic site in the presence of 5 mM Mg^{2+} is taken into account. From $K_d = 0.94$ mM for Mg^{2+} (see above) the corrected amplitude according to Scheme VI is

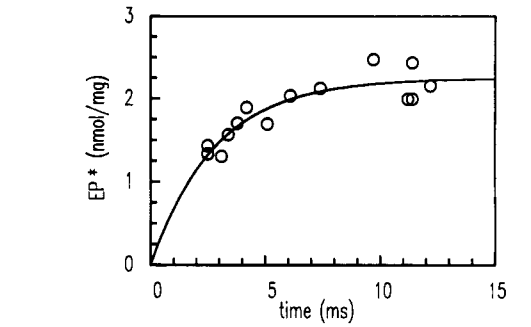
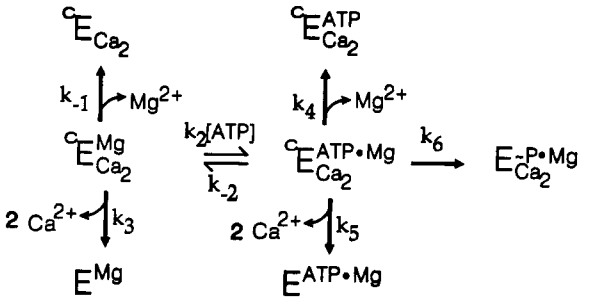


FIGURE 3: Rate constant for dissociation of Mg^{2+} from the $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ complex. All syringes except syringe C contained 40 mM MOPS buffer, pH 7.0, and 100 mM KCl. Passively loaded SRV (0.17 mg/mL, 20 mM $CaCl_2$) were incubated at 25 °C with 1 mM EGTA, 1.1 mM $CaCl_2$ (100 μM free Ca^{2+}), and 5 mM $MgCl_2$ for 15 s in syringe A. The phosphorylation reaction was started by simultaneously adding [γ-^{32}P]ATP and excess EDTA (250 μM [γ-^{32}P]-ATP and 15 mM EDTA after mixing the contents of syringes A and B). The line follows a single-exponential curve for a rate constant of 360 ± 45 s^{-1} and an amplitude of 2.2 ± 0.1 nmol/mg EP*. Syringe C contained 39 mM KH_2PO_4 and 0.37 M $HClO_4$ as the quench solution.

Scheme VI



$$\frac{k_6}{k_6 + k_5 + k_4}\frac{[Mg]}{K_{dMg} + [Mg]}E_{tot} = \frac{220\ s^{-1}}{(220 + 80 + 60)\ s^{-1}}\frac{5\ mM}{(0.94 + 5)\ mM}(4.4)\ nmol/mg = 2.3\ nmol/mg \quad (3)$$

The consistency of the observed and expected yields according to the partitioning in Scheme VI and the assumption that $k_2[ATP] \gg k_{-1}$ and k_3 indicates that only a small fraction of the bound Ca^{2+} and Mg^{2+} dissociates from $^cE{\cdot}Ca_2{\cdot}Mg$ before ATP* binds and, therefore, that the trapping by ATP* is fast. How fast the trapping by ATP* actually is was investigated further.

Rate Constant for Dissociation of Mg^{2+} from $^cE{\cdot}Ca_2{\cdot}Mg$. When $^cE{\cdot}Ca_2{\cdot}Mg$ was chased with EDTA and ATP* under the conditions described above, but with 50 μM ATP*, only 1.1 nmol/mg of EP* was formed, compared to 2.2 nmol/mg with 250 μM ATP* (△ in Figure 2). This shows that the trapping by 250 μM ATP* is very fast but not instantaneous. The amount of EP* formed is reduced by one-half when the net forward rate constant (which includes all partitioning steps from $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$) to form EP* is equal to the sum of the rate constants that do not lead to $^cE{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ (Cleland, 1975). This gives an estimate of the rate constant k_{-1} for dissociation of Mg^{2+} from the $^cE{\cdot}Ca_2{\cdot}Mg$ complex. Equation 4 describes the condition in which the net forward rate constant

equals the sum of the preceding partitioning steps:

$$\frac{k_2[ATP](k_4 + k_5 + k_6)}{k_{-2} + k_4 + k_5 + k_6} = k_{-1} + k_3 \qquad (4)$$

and therefore

$$\frac{(10 \times 10^6\ M^{-1}\ s^{-1})\ (50 \times 10^{-6}\ M)\ (60 + 80 + 220)\ s^{-1}}{(120 + 60 + 80 + 220)\ s^{-1}} = k_{-1} + 80\ s^{-1}$$

This gives $k_{-1} = 300\ s^{-1}$ for the dissociation of Mg^{2+} from $^cE{\cdot}Ca_2{\cdot}Mg$ and, therefore, an estimate of the rate constant for binding of Mg^{2+} to $^cE{\cdot}Ca_2$ of $k_1 \simeq 300\ s^{-1}/0.94\ mM \simeq 3 \times 10^5\ M^{-1}\ s^{-1}$. This is well below the diffusion-controlled limit and the rate constants of 8.7×10^6 and $3.8 \times 10^6\ M^{-1}\ s^{-1}$ for Mg^{2+} binding to ATP and ADP, respectively (Frey & Stuehr, 1974); it indicates that there is a significant barrier for Mg^{2+} binding.

How Fast is the Trapping of Free Mg^{2+} by ATP Compared to the Trapping by EDTA?* When ATP* and EDTA are added simultaneously to Mg^{2+}, the amounts of Mg·ATP and Mg·EDTA that initially form are functions of the respective dissociation and association rate constants. The system will then relax to equilibrium with a rate constant that is mainly determined by the slowest dissociation rate constant. This means that the amount of Mg·ATP that forms initially may exceed by far that present at equilibrium. If this happens, it might be the Mg·ATP* complex that phosphorylates the enzyme, and not ATP* that binds to the $^cE{\cdot}Ca_2{\cdot}Mg$ complex. To exclude this possibility, the amount of chelating EDTA was lowered from 15 to 7.5 mM, which is still in excess over the total amount of magnesium present (5 mM). The amount of EP* generated after 11.5 ms was 2.9 ± 0.3 nmol/mg (□ in Figure 2), which is only ≈10% higher than that formed when 15 mM EDTA was used. This shows that the chase with 15 mM EDTA is virtually instantaneous, and it is not a transiently high concentration of free Mg^{2+} or Mg·ATP* that is responsible for phosphoenzyme formation.

In conclusion, Mg^{2+} is initially bound to $^cE{\cdot}Ca_2$ with $K_d \simeq 1$ mM, and 250 μM ATP* then traps $^cE{\cdot}Ca_2{\cdot}Mg$ faster than Ca^{2+} or Mg^{2+} dissociates from $^cE{\cdot}Ca_2{\cdot}Mg$. The $^cE{\cdot}Ca_2{\cdot}ATP^*{\cdot}Mg$ complex is then phosphorylated with a rate constant of $220\ s^{-1}$, Ca^{2+} dissociates with a rate constant of $80\ s^{-1}$, and Mg^{2+} dissociates with a rate constant of $k_4 = 60\ s^{-1}$. Thus, the pathway through $^cE{\cdot}Ca_2{\cdot}Mg$ plus metal-free ATP is productive. The reaction does not follow an ordered pathway in which ATP must bind before Mg^{2+}. It follows from microscopic reversibility that the ATP is not held in a pocket under a Mg^{2+} ion that must dissociate before ATP can dissociate.

Interaction of Metal-Free ATP with Free Enzyme. To determine the affinity of the enzyme for metal-free ATP, passively loaded vesicles (20 mM $CaCl_2$) were incubated in syringe A with EGTA and concentrations of [γ-^{32}P]ATP in the range 20–400 μM for 15 s. The phosphorylation reaction was then started by the addition of Mg^{2+}, Ca^{2+}, and excess unlabeled ATP. The amount of EP* formed after 22 ms, which is sufficient to complete the phosphorylation reaction, is an indicator of how much ATP* was initially bound. Figure 4 shows that the amount of EP* formed from $^cE{\cdot}Ca_2{\cdot}ATP^*{\cdot}Mg$ depends on the concentration of metal-free ATP* in syringe A and follows a hyperbolic curve with a dissociation constant of $K_d = 44 \pm 4$ μM and a maximal yield of 1.66 ± 0.04 nmol/mg EP*, which is 38% of the total enzyme concentration (4.4 nmol/mg).

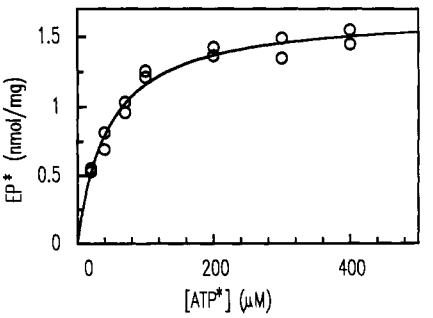


FIGURE 4: Affinity of metal-free ATP for the free enzyme. In a three-syringe experiment, E was incubated with 20–400 μM ATP* for 15 s and then reacted with excess unlabeled ATP, $MgCl_2$, and $CaCl_2$. The reaction was quenched after 22 ms, and the amount of EP* formed is indicated on the *y*-axis. All syringes except syringe C contained 40 mM MOPS (pH 7.0), 0.1 M KCl, and 1 mM EGTA at 25 °C. In addition, syringe A contained 0.17 mg/mL intact SRV passively loaded with 20 mM $CaCl_2$, 200 μM $CaCl_2$, and 20–400 μM ATP*; syringe B contained 1.86 mM $CaCl_2$, 3.6 mM unlabeled ATP, 10 mM $MgCl_2$, 0.5 mM PEP, and 0.05 mg/mL pyruvate kinase. The solution in syringe B was incubated for 30 min prior to the experiment in order to remove ADP; syringe C contained 39 mM P_i and 0.38 M $HClO_4$. The final concentrations after syringes A and B are mixed were 34 μM free Ca^{2+} and 3.2 mM free Mg^{2+}. The line drawn in the figure follows a hyperbolic saturation curve with $K_d = 44 \pm 4$ μM and an end point of 1.66 ± 0.04 nmol/mg, which is 38% of E_{tot} (4.4 nmol/mg).

The fact that 38% EP* is formed shows that (a) ATP* does not have to dissociate before the catalytic Mg^{2+} ion can bind and (b) Mg^{2+} does not have to bind as an ATP·Mg complex. This excludes an ordered binding mechanism in which Mg^{2+} must bind to E before ATP and shows that the Mg^{2+} is not buried under the ATP.

A dissociation constant of 20–25 μM for metal-free ATP at pH 7.2 in the absence of KCl has been reported by Lacapère et al. (1990). Under conditions identical to this work and in the presence of Mg^{2+}, the affinity of E for ATP is ~5-fold higher, with $K_d = 4.5$ μM (Stahl & Jencks, 1984), or 2–6 μM at pH 7.2 in the absence of KCl (Lacapère et al., 1990). The dissociation constant of Mg^{2+} from the E·Mg·ATP complex (K_{dA}) can be calculated from the affinity of Mg^{2+} for ATP in solution (K_{dC}), the affinity of metal-free ATP for the free enzyme (K_{dB}) and the affinity of ATP·Mg for the free enzyme (K_{dD}). The definitions of the corresponding dissociation constants are

$$K_{dA} = \frac{[E{\cdot}ATP][Mg]}{[E{\cdot}ATP{\cdot}Mg]};\quad K_{dB} = \frac{[E][ATP]}{[E{\cdot}ATP]};$$

$$K_{dC} = \frac{[ATP][Mg]}{[ATP{\cdot}Mg]};\quad K_{dD} = \frac{[E][ATP{\cdot}Mg]}{[E{\cdot}ATP{\cdot}Mg]}$$

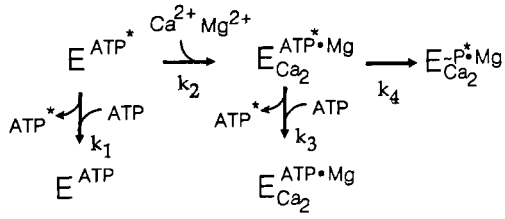
and therefore

$$K_{dA} = \frac{K_{dC}K_{dD}}{K_{dB}} = \frac{(100 \times 4.5)\ \mu M^2}{45\ \mu M} = 10\ \mu M \qquad (5)$$

Thus, the interaction with bound ATP increases the affinity of the enzyme for Mg^{2+} by ~100-fold. It is, however, impossible to decide whether this high affinity of the E·ATP complex for Mg^{2+} arises from the combined interactions of Mg^{2+} with ATP and functional groups of the enzyme or from ATP bound to the enzyme adopting a conformation that is similar to the structure of ATP·Mg in solution and, therefore, is favorable for the binding of Mg^{2+}.

The yield of 38% EP* in the experiment described above, in which the free enzyme was incubated with metal-free ATP*, is smaller than the yield of 56% EP* observed when enzyme

Scheme VII



was incubated with ATP* and Mg^{2+} and then chased with Ca^{2+} and excess unlabeled ATP (Stahl & Jencks, 1987). The yield of 56% EP* is consistent with the rate constants of 70 s^{-1} (k_4 in Scheme VII) for phosphorylation and 47 s^{-1} for dissociation of ATP* from E·Ca_2·ATP*·Mg (k_3 in Scheme VII) to give 70 s^{-1}/(70 + 47) s^{-1} = 60% EP*.

The reduced yield of EP* when the enzyme was incubated with ATP* in the absence of Mg^{2+} could be caused by one or more of the following: (1) The binding of Ca^{2+} or Mg^{2+} at 5 mM $MgCl_2$ and 34 μM free Ca^{2+} (k_2 in Scheme VII) is not fast compared to the dissociation of ATP* from E·ATP* (k_1 in Scheme VII). (2) The rate constant for phosphorylation of E·Ca_2·ATP*·Mg (k_4) is slower than 70 s^{-1} if the enzyme is initially incubated with ATP* instead of ATP*·Mg^{2+}. (3) The rate constant for dissociation of ATP* from E·ATP*·Mg (k_3) is faster than 47 s^{-1}.

These possibilities for the decreased yield of EP* were tested in the following experiments.

Rate Constant for Dissociation of Metal-Free ATP from E. Passively loaded SRV (20 mM $CaCl_2$) were incubated with 1 mM EGTA, 0.2 mM $CaCl_2$, and 100 μM ATP* for 15 s. The E·ATP* complex was then chased with 750 μM unlabeled ATP during t_1 = 4.6–104 ms. During t_1 the labeled, metal-free ATP (ATP*) dissociates with the rate constant k_1 and is irreversibly displaced by unlabeled ATP (Scheme VII). The amount of E·ATP* remaining after t_1 was assayed by initiating phosphorylation with the addition of $MgCl_2$ and $CaCl_2$ from syringe C to give final concentrations of 5 mM $MgCl_2$ and 50 μM free Ca^{2+} in the reaction mixture. The phosphorylation reaction was allowed to proceed to completion for t_2 = 32–48 ms. Figure 5 shows that the dissociation of ATP* from E·ATP* follows a single-exponential decay with a rate constant of 131 ± 7 s^{-1}. The amount of EP* at t_1 = 0 ms, 1.2 nmol/mg EP*, is 27% of E_{tot} = 4.4 nmol/mg and is close to the 1.1 nmol/mg EP* that was obtained in the experiment with 100 μM ATP* (Figure 4). The amount of EP* at t_1 = 0 was measured by adding unlabeled ATP, Ca^{2+}, and Mg^{2+} directly from syringe B to syringe A. The endpoint for EP* at t_1 = ∞ is not 0 because the radioactivity is diluted 15-fold; this accounts for 1/15 × 1 = 0.07 nmol EP*/mg.

In conclusion, the rate constant for the dissociation of metal-free ATP from E·ATP is 130 s^{-1} and, therefore, the rate constant for the binding of ATP to E is 130 s^{-1}/44 μM ≈ 3 × 10^6 M^{-1} s^{-1}.

Time Course for Formation of EP from E·ATP* after a Chase with Mg^{2+}, Ca^{2+}, and Excess Unlabeled ATP.* Passively loaded, intact vesicles (20 mM $CaCl_2$) were incubated with 100 μM ATP* for 15 s in syringe A. The E·ATP* complex was then chased with 1.2 mM unlabeled ATP, 5 mM $MgCl_2$, and 40 μM free Ca^{2+}, and the amount of EP* formed after different time periods was measured. Figure 6 shows that the enzyme is phosphorylated with a rate constant of 190 ± 11 s^{-1} to give 0.93 nmol/mg EP* under these conditions. This corresponds to 21% of the total enzyme (4.4 nmol/mg).

The amount of EP* formed did not increase significantly if the concentration of free Ca^{2+} was increased 5-fold (□ in Figure 6) or the concentration of Mg^{2+} was increased 4-fold (△ in Figure 6). This result indicates that the binding of Ca^{2+} and Mg^{2+} are fast compared to the dissociation of metal-free ATP*. The binding of Ca^{2+} has been shown to precede the rate-limiting step for phosphorylation, which is a conformational change with a rate constant of 70 s^{-1} under these conditions (Stahl & Jencks, 1987). This result also shows that the binding of ATP and Mg^{2+} is not ordered with ATP binding first because an increase in Mg^{2+} concentration would inhibit the dissociation of ATP* and increase the yield of EP* if Mg^{2+} were required to dissociate before ATP.

To rule out the possibility that contaminating Mg^{2+} in syringe A forms an E·ATP*·Mg complex, so that binding of Mg^{2+} after the chase is not required, 2 mM EDTA was added

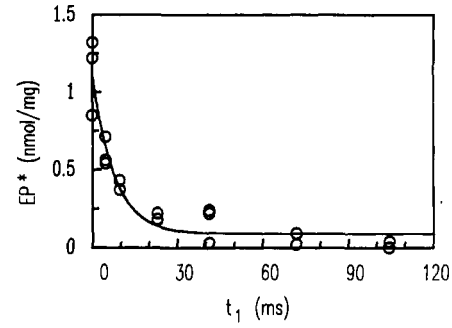


FIGURE 5: Dissociation of metal-free ATP from the E·ATP complex. In a four-syringe experiment passively loaded SRV (20 mM $CaCl_2$) were incubated for 15 s with 100 μM [γ-^{32}P]ATP in syringe A. After mixing syringes A and B the E·ATP* complex was chased with 750 μM unlabeled ATP for t_1 = 4.6–104 ms. Phosphorylation was then initiated by adding $CaCl_2$ and $MgCl_2$ to give final concentrations of 50 μM free Ca^{2+} and 4.5 mM free Mg^{2+} and allowed to go to completion during t_2 = 32–48 ms. The line drawn follows an exponential function with k = 130 s^{-1} and an amplitude of 1 nmol/mg EP*. All syringes except D contained 100 mM KCl, 40 mM MOPS/Tris, pH 7.0, and 1 mM EGTA at 25 °C. In addition, syringe A contained 0.17 mg/mL SRV, 200 μM $CaCl_2$, and 100 μM ATP*; syringe B contained 1.5 mM unlabeled ATP; syringe C contained 15 mM $MgCl_2$ and 2.92 mM $CaCl_2$; and syringe D contained 0.5 M $HClO_4$ and 52 mM KH_2PO_4.

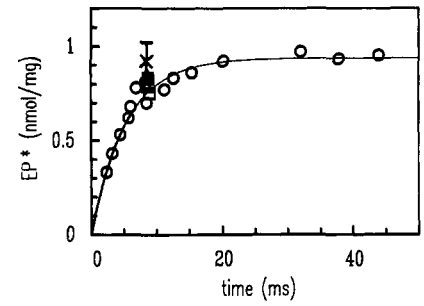


FIGURE 6: Phosphorylation of E·Ca_2·ATP*·Mg to give EP* in the presence of an ATP chase. In a three-syringe experiment, E was mixed with 100 μM [γ-^{32}P]ATP for 15 s and then chased with excess unlabeled ATP, $MgCl_2$, and $CaCl_2$. The concentrations of free Mg^{2+} and free Ca^{2+} after mixing syringes A and B were 37 μM Ca^{2+} and 3.7 mM Mg^{2+} (○), 200 μM Ca^{2+} and 3.7 mM Mg^{2+} (□), 41 μM Ca^{2+} and 13.7 mM Mg^{2+} (△) or 52 μM Ca^{2+} and 2.7 mM Mg^{2+} (×). The amount of EP* formed after different times follows a single-exponential curve with an amplitude of 0.93 ± 0.02 nmol/mg EP* and a rate constant of 190 ± 11 s^{-1}. All syringes except syringe C contained 40 mM MOPS (pH 7.0), 0.1 M KCl, and 1 mM [or 600 μM (×)] EGTA at 25 °C. (×) Syringe A contained 600 μM EGTA and 2000 μM EDTA to chelate contaminating Mg^{2+} which could be present. In addition, syringe A contained 100 μM [γ-^{32}P]ATP, 200 μM $CaCl_2$, and 0.17 mg/mL intact SRV passively loaded with 20 mM $CaCl_2$, and syringe B contained 2.4 mM unlabeled ATP, 0.5 mM PEP, 0.05 mg/mL pyruvate kinase, and either 1.86 mM $CaCl_2$ and 10 mM $MgCl_2$ (○, ×), 2.2 mM $CaCl_2$ and 10 mM $MgCl_2$ (□), 1.86 mM $CaCl_2$ and 30 mM $MgCl_2$ (△). Syringe C contained 39 mM P_i and 0.38 M $HClO_4$.

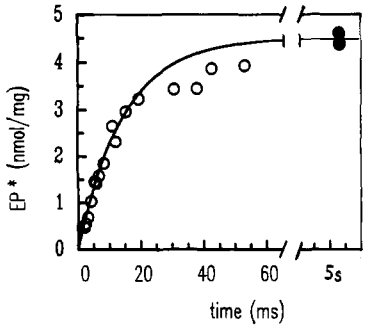


FIGURE 7: Formation of EP* by reaction of E·ATP* with 40 μM free Ca^{2+} and 4.9 mM free Mg^{2+}. The solid line is drawn for a rate constant of $k = 70\ s^{-1}$ and an amplitude of 4.5 nmol/mg. All syringes except syringe C contained 100 mM KCl, 40 mM MOPS/Tris, pH 7.0, and 1 mM EGTA at 25 °C. In addition syringe A contained 0.17 mg passively loaded SRV (20 mM $CaCl_2$), 200 μM $CaCl_2$ to give 0.1 μM free Ca^{2+}, and 200 μM labeled ATP; syringe B contained 1.86 mM $CaCl_2$ and 10 mM $MgCl_2$. Syringe C contained 39 mM KH_2PO_4 and 0.38 M $HClO_4$.

in syringe A (× in Figure 6), which would reduce the concentration of free Mg^{2+} to 0.03 μM if the concentration of contaminating Mg^{2+} were 10 μM. The yield of 0.9 ± 0.1 nmol/mg EP* after 8.5 ms does not differ significantly from the yield of 0.8 ± 0.1 nmol/mg observed with only EGTA in syringe A. This shows that contaminating Mg^{2+} in syringe A is not the source of the catalytic ion to form E·ATP*·Mg.

*Rate Constant for Phosphorylation of E That Was Incubated with Metal-Free ATP**. Passively loaded SRV (20 mM $CaCl_2$) were incubated with 200 μM ATP* and EGTA for 15 s, and phosphorylation was initiated by the addition of $MgCl_2$ and $CaCl_2$ to give final concentrations of 40 μM free Ca^{2+} and 5 mM Mg^{2+}. The formation of EP* follows a single-exponential curve with a rate constant of 70 ± 10 s^{-1} for the formation of 4.5 ± 0.1 nmol/mg EP* (Figure 7). This is the same as the rate constant of 70 s^{-1} for phosphorylation of E that was incubated with ATP* and Mg^{2+} (Stahl & Jencks, 1984) and shows that there is no difference in the rate constants for the phosphorylation of Ca^{2+}-free enzyme that was initially incubated with ATP* in the presence or absence of Mg^{2+}.

The rate constant for the dissociation of ATP* from E·Ca_2·ATP*·Mg (k_3 in Scheme VII) is equal to the difference between the observed rate constant for phosphorylation of E·ATP* that was chased with unlabeled ATP, Mg^{2+}, and Ca^{2+} (190 s^{-1}) and the rate constant for phosphorylation (k_4 = 70 s^{-1}), i.e., k_3 = (190 − 70) s^{-1} = 120 s^{-1} if the binding of Mg^{2+} and Ca^{2+} are fast compared to the partitioning of E·Ca_2·ATP*·Mg to EP* and E·Ca_2·ATP·Mg. The expected yield of EP* would then be $[k_4/(k_3 + k_4)]E_{tot}$ = [70 s^{-1}/(120 + 70) s^{-1}]4.4 nmol/mg = 1.6 nmol/mg EP*. The amount of EP* formed when E was saturated with ATP* and then chased with Ca^{2+}, Mg^{2+}, and unlabeled ATP was 1.66 nmol/mg. This shows that (1) the rate constants of k_3 = 120 s^{-1} for the dissociation of ATP* from E·Ca_2·ATP*·Mg and k_4 = 70 s^{-1} for phosphorylation are consistent with the observed yield of phosphoenzyme: EP^*/E_{tot} = 1.66/4.4 nmol/mg = 38% of E_{tot}, and 70/(120 + 70) = 37%; (2) the binding of Ca^{2+} and Mg^{2+} to E·ATP* (k_2 in Scheme VII) is fast compared with the dissociation of ATP* from E·ATP* (k_3 in Scheme VII); if it were not fast, the yield of phosphoenzyme would be <1.66 nmol/mg; and (3) ATP* dissociates faster from E·Ca_2·ATP*·Mg when the enzyme is initially incubated with metal-free ATP* (k_3 = 120 s^{-1}) than when E is incubated with Mg^{2+} and ATP* (k_3 = 47 s^{-1}; Stahl & Jencks, 1987). This shows that the Mg^{2+} ion induces a conformational change in E·ATP

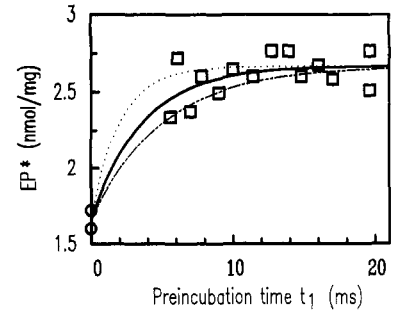


FIGURE 8: Yield of EP* after enzyme was preincubated with 250 μM ATP* and 5 mM $MgCl_2$ for different times t_1 and E·ATP*·Mg was chased with 85 μM free Ca^{2+} and 2 mM unlabeled ATP. The solid line is drawn for a rate constant of k = 280 s^{-1} and a maximal formation of EP* = 2.7 ± 0.05 nmol/mg, which is 60% of E_{tot} = 4.5 nmol/mg. Also indicated are lines for k = 200 s^{-1} (- - - -) and k = 500 s^{-1} (···). All syringes except D contained 100 mM KCl, 40 mM MOPS/Tris, pH 7.0, and 1 mM EGTA at 25 °C. In addition syringe A contained 0.17 mg/mL passively loaded SRV (20 mM $CaCl_2$) and 200 μM $CaCl_2$; syringe B contained 10 mM $MgCl_2$ and 500 μM ATP*; and syringe C contained 7 mM $MgCl_2$, 3.1 mM $CaCl_2$, 6 mM ATP, 50 μg/mL pyruvate kinase, and 1.5 mM phosphoenolpyruvate. Syringe D contained 50 mM KH_2PO and 0.5 M $HClO_4$.

that decreases that rate of ATP dissociation. However, the rate constant for phosphorylation of E·Ca_2·ATP*·Mg is 70 s^{-1} in both cases.

In order to measure the time dependence of this decrease in the rate constant for ATP* dissociation from the E·Ca_2·ATP*·Mg complex, E was incubated with 250 μM ATP* and 5 mM $MgCl_2$ for t_1 = 5–20 ms. The E·ATP*·Mg complex was then chased with $CaCl_2$ and unlabeled ATP to give 85 μM free Ca^{2+} and 2 mM unlabeled ATP, and the reaction was allowed to go to completion at t_2 = 30–55 ms (Figure 8). The amount of EP* formed as a function of t_1 is consistent with a simple exponential curve with a rate constant of 280 ± 80 s^{-1}, an initial amplitude of 1.65 nmol/mg EP* (36% of E_{tot} = 4.4 nmol/mg), and a final yield of 2.7 ± 0.1 nmol/mg EP* (61% of E_{tot}) when the enzyme was incubated with ATP and Mg^{2+} for >20 ms. This agrees with the 56% yield of EP* that was observed after incubation of E with Mg^{2+} and ATP* for 5 s in a similar experiment (Stahl & Jencks, 1987). However, the quality of the experimental data only allows us to draw a firm conclusion for a lower limit of $k \geq 200\ s^{-1}$ (- - - in Figure 8) for the conformational change that decreases the rate constant for the dissociation of ATP.

In conclusion, the Ca^{2+}-free enzyme changes its properties with respect to the dissociation of ATP when it is incubated with ATP and Mg^{2+}. This change causes the rate constant for the dissociation of ATP from E·Ca_2·ATP*·Mg to decrease from 120 s^{-1} to 47 s^{-1}; the change occurs with a rate constant of ≥200 s^{-1}.

DISCUSSION

Binding Mechanism of Mg^{2+} and ATP. It has generally been believed that magnesium activates the phosphorylation of CaATPase with ATP by forming the Mg·ATP complex, which is called the true substrate (Vianna, 1975; Ogawa & Kurebayashi, 1982), or that Mg·ATP is the true substrate and that an additional Mg^{2+} ion activates the enzyme by binding to a secondary binding site (Makinose & Boll, 1979). It is important to know whether Mg^{2+} and ATP can bind independently and what are the properties of the corresponding enzyme forms because this will provide information about the nature of the active site.

We show here that the binding mechanism of Mg^{2+} and ATP to E or $^cE{\cdot}Ca_2$ is random: Mg^{2+} and ATP can bind independently, and the binding of ATP and Mg^{2+} as the Mg·ATP complex may be productive, but is not essential. It is important to compare the species E and $^cE{\cdot}Ca_2$ since they differ in a crucial property: $^cE{\cdot}Ca_2$ allows the formation of covalent phosphoenzyme, EP, from ATP and Mg^{2+}, while E does not allow phosphorylation by ATP. Therefore the interactions of ATP and Mg^{2+} with those enzyme species are different.

Mg^{2+} Can Bind to $^cE{\cdot}Ca_2{\cdot}$ATP without a Requirement for ATP to Dissociate. Attempts to form the enzyme species $^cE{\cdot}Ca_2{\cdot}$ATP with no catalytic ion bound failed because Ca^{2+} has a very high affinity for the catalytic site in the presence of ATP. Even at 3.5 μM free Ca^{2+}, the enzyme seems to be nearly saturated with ATP·Ca (Figure 1). However, Figure 1 (□) shows that this catalytic Ca^{2+} ion can exchange with Mg^{2+} without a requirement for dissociation of ATP. This shows that the binding mechanism is not compulsory ordered with Mg^{2+} binding first and that ATP and Mg^{2+} do not have to bind as the ATP·Mg complex. The Mg^{2+} is not buried under the ATP.

Mg^{2+} Can Bind to $^cE{\cdot}Ca_2$ before ATP, and Addition of Metal-Free ATP to $^cE{\cdot}Ca_2{\cdot}$Mg Is Productive. It has not been shown before that the enzyme which is stable in the presence of Ca^{2+}, $^cE{\cdot}Ca_2$, has a binding site for the catalytic Mg^{2+} ion in the absence of ATP. Moreover, it has been suggested not only that ATP·Mg is the true substrate but also that metal-free ATP cannot be productive (Makinose & Boll, 1979). We show here directly that $^cE{\cdot}Ca_2$ has a binding site for the catalytic Mg^{2+} ion, with $K_d \approx 1$ mM, and that the binding of metal-free ATP to $^cE{\cdot}Ca_2{\cdot}$Mg is productive to form phosphoenzyme (Figure 2). This excludes mechanisms in which the binding is compulsory ordered, with ATP binding first, and, again, a mechanism in which the binding of ATP and Mg^{2+} can only occur as the ATP·Mg complex. The ATP is not buried under the Mg^{2+}.

Mg^{2+} Can Bind to E after ATP. The addition of unlabeled ATP, Ca^{2+}, and Mg^{2+} to enzyme that was incubated with metal-free [γ-^{32}P]ATP gave 38% labeled phosphoenzyme (Figure 4). This confirms that ATP* does not have to dissociate from the free enzyme, E, before Mg^{2+} can bind and that Mg^{2+} is not required to bind as the ATP·Mg complex. Metal-free ATP binds to E with $K_d = 44$ μM and dissociates with $k = 130$ s^{-1}, which gives a calculated rate constant for association of metal-free ATP with E of 3×10^6 M^{-1} s^{-1}. This is very similar to the rate constant for association of ATP·Mg with the enzyme of 5.3×10^6 M^{-1} s^{-1} (Stahl & Jencks, 1987). It was noted that such a low rate constant is indicative of a two-step binding mechanism. It now appears that the presence or absence of Mg^{2+} has no dramatic influence on the rate constant for association of ATP with E and that the 10-fold higher affinity of ATP·Mg, with $K_d = 4.5$ μM (Stahl & Jencks, 1984), is the result of a decreased rate constant for ATP dissociation.

ATP Can Bind to E after Mg^{2+}. If ATP binds first, followed by the binding of Mg^{2+} in a compulsory ordered mechanism, then high concentrations of Mg^{2+} should prevent the dissociation of ATP. The data in Figure 6 show that this is not the case: a 4-fold increase in Mg^{2+} concentration has virtually no influence on the rate constant for dissociation of ATP from $E{\cdot}Ca_2{\cdot}$ATP. This shows again that ATP does not bind first in a compulsory ordered mechanism.

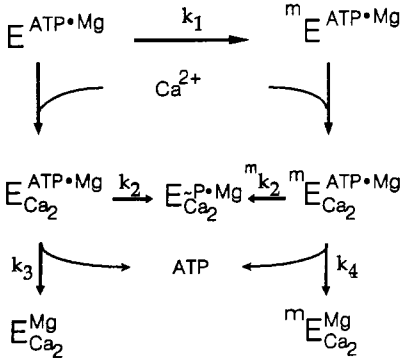
The exclusion of all possible compulsory ordered binding mechanisms and the exclusion of a mechanism in which only the binding of ATP·Mg is productive leads to the conclusion that the binding of ATP and Mg^{2+} follows a random mechanism.

The existence of independent accessible binding sites for Mg^{2+} and ATP favors a model in which both ATP and Mg^{2+} interact with the enzyme in the ternary complex, but disfavors a substrate-bridge or metal-bridge configuration on the enzyme in which Mg^{2+} is trapped under the ATP or the phosphates of ATP are held in a pocket by a Mg^{2+} ion that must dissociate before ATP.

Binding Mechanism of Phosphate and Mg^{2+}. The dissociation constant of the catalytically active magnesium ion from the free enzyne, E·Mg, was reported to be 8.8 mM at pH 7.0 and 20 °C (Punzengruber et al., 1978) or 7.8 mM at 25 °C (Martin & Tanford, 1981), which is larger than $K_d \approx$ 1 mM in the presence of Ca^{2+} (Figure 2). The signal for the binding of Mg^{2+} was the amount of EP* formed from P_i and Mg^{2+} at equilibrium. Similar equilibrium measurements led Kolassa et al. (1979) to conclude that the binding of P_i and Mg^{2+} to give $E{\cdot}P_i{\cdot}$Mg follows a random mechanism, in which P_i and Mg^{2+} can bind independently but the binding of the $P_i{\cdot}$Mg complex is unproductive. However, Champeil et al. (1985) have pointed out that it is not possible to exclude $P_i{\cdot}$Mg as a substrate because equilibrium measurements for the formation of a product cannot distinguish between different pathways, but only between different species, and there is no direct evidence that the pathways $E{\cdot}P_i + Mg^{2+}$ or $E{\cdot}Mg + P_i$ are productive. The conclusion that Mg^{2+} and P_i can bind independently, however, is not affected, and therefore we conclude that the catalytic ion and the substrates for the forward and backward reactions (ATP and P_i, respectively) can bind independently to the enzyme.

How Many Catalytic Mg^{2+} Sites are Required for Catalysis? The binding of Mg^{2+} to $^cE{\cdot}Ca_2$ follows simple hyperbolic behavior (Figure 2) and gives no indication for the involvement of two Mg^{2+} ions in catalysis. However, a Mg^{2+} ion that binds with an affinity of $K_d \leq 50$ μM would not be observed. Makinose and Boll (1979) concluded from isotope exchange experiments that two Mg^{2+} ions are involved in the phosphorylation of CaATPase by ATP. The binding of two "low-affinity" Tb^{3+} ions was also believed to support the binding of two Mg^{2+} ions, but only one low-affinity Tb^{3+} ion seemed to be displaced by Mg^{2+}, with K_d for $Mg{\cdot}E = 10.6 \pm 0.9$ mM at 25 °C and pH 7.0 and in the absence of KCl (Highsmith & Head, 1983). The authors concluded that they may have measured the binding of Mg^{2+} to the catalytic site because their K_d is similar to that of Punzengruber et al. (1978). Guillain et al. (1982) measured the binding of Mg^{2+} to the CaATPase with intrinsic fluorescence and obtained $K_d \approx 5$ mM at pH 7.0. However, this may represent binding to the transport sites (Loomis et al., 1982). Dupont (1980) showed that phosphoenzyme that was formed from ATP and Mg^{2+} reacts with ADP in the presence of EDTA and, therefore, that metal-free ADP is the substrate for the back reaction. The catalytic Mg^{2+} ion cannot be removed easily from $Mg{\cdot}E{\sim}P{\cdot}Ca_2$ even after incubation with EDTA (Dupont, 1980; Ogurusu et al., 1991). When phosphoenzyme was formed with ATP and $^{54}Mn^{2+}$, a radioactive analogue of Mg^{2+}, only one $^{54}Mn^{2+}$ ion remained bound on EP, but the phosphoenzyme remained reactive toward ADP (Ogurusu et al., 1991). It seems, therefore, that only one catalytic ion is involved in the reaction of EP with metal-free ADP, and microscopic reversibility requires that the formation of EP from enzyme and ATP in the forward reaction also requires only one catalytic ion.

Scheme VIII



Garrahan et al. (1976) concluded from atomic absorption measurements that 1 Mg^{2+} ion remains bound to CaATPase, even if the enzyme was treated with chelating agents for Mg^{2+} and ionophore, but Ogurusu et al. (1991) recently concluded, with the same method, that only 3% of the assayed CaATPase contains Mg^{2+}. We can only conclude firmly that one dissociable Mg^{2+} ion is involved in catalysis.

The Nature of the Active Site. The topography of the active site of the CaATPase was proposed to consist of three domains that contain the phosphorylation site, the nucleotide binding site, and a "transduction domain" that connects the catalytic and transport sites (MacLennan et al., 1985; Brandl et al., 1986). Petithory and Jencks (1986) suggested that the conformational change from $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ to the catalytically active enzyme, $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, could involve movement of the γ-phosphate of ATP toward the phosphorylation site, thereby aligning it to permit rapid phosphoryl transfer. Lacapère et al. (1990) suggested from filter binding and fluorescence measurements that ATP may interact with two domains: one domain binds the ADP moiety, and the other binds the γ-phosphate and Mg of ATP·Mg. The virtually identical affinities of ADP and ATP in the absence of Mg^{2+} indicate that the γ-phosphate of free ATP does not interact favorably with the enzyme. The activation of dephosphorylation by ATP shows that ATP can bind to the phosphorylated enzyme (McIntosh & Boyer, 1983), so that the active site is able to accommodate phosphate and ATP simultanously. There is also evidence that the binding of ATP to EP not only is possible but also may occur without energetic costs. Vanadate, an analogue of inorganic phosphate, and the γ-phosphate group of ATP do not compete for the same phosphorylation site, and Andersen and Møller (1985) measured a dissociation constant for K_d = 4–5 μM for the binding of ATP to the enzyme–vanadate–magnesium complex at pH 7.5, which is the same as K_d = 4.5 μM for the binding of ATP·Mg to the free enzyme (Stahl & Jencks, 1984). The observation that metal-free ATP, but not ATP·Mg nor free Mg^{2+}, inhibits the dissociation of vanadate (V) from the E·V·Mg complex led these workers to conclude that ATP may interact with the same Mg^{2+} ion at the catalytic site that is involved in the formation of the E·V·Mg complex and block vanadate dissociation. The binding of ATP·Mg to E·V·Mg was found to be unfavorable, with a dissociation constant of K_d = 810 μM in the presence of 10 mM $MgCl_2$.

A Structural Change is Induced by the Binding of ATP and Mg^{2+}. The properties of the enzyme form that is stable in the presence of ATP and Mg^{2+} are different from those of the enzyme with metal-free ATP. Figure 8 shows that the addition of Mg^{2+} to E·ATP triggers a conformational change that converts the enzyme complex that is formed initially, E·ATP·Mg, to the enzyme form that is stable in the presence

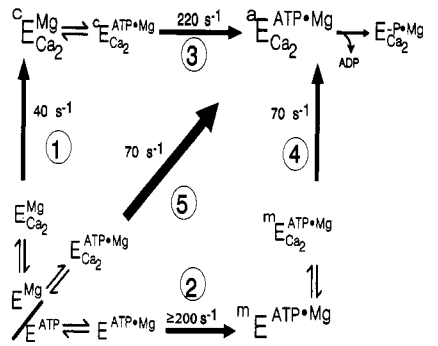


FIGURE 9: Cycle of conformational changes induced by different binding sequences of the ligands of CaATPase. The pathways that lead to the active enzyme species $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ are dependent on the sequence by which the ligands ATP, Mg^{2+}, and Ca^{2+} are added to CaATPase. The simultaneous addition of Mg^{2+} and Ca^{2+} to E·ATP gives $E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, with properties that represent a hybrid of $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ and $^{m}E{\cdot}ATP{\cdot}Mg$: ATP dissociates from $E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ with k = 120 s^{-1}, the same as from $^{c}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ (Petithory & Jencks, 1986), but forms $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ with k = 70 s^{-1}, the same as from $^{m}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ (Stahl & Jencks, 1984). It thus appears that two different conformational changes are combined into a single conformational change.

of ATP and Mg^{2+}, $^{m}E{\cdot}ATP{\cdot}Mg$, with $k_1 \geq 200$ s^{-1} (Scheme VIII). The signal for this change is a decrease in the rate constant for dissociation of ATP*, from k_3 = 120 s^{-1} to k_4 = 47 s^{-1}, which results in an increase in the yield of $E \sim P^{*}{\cdot}Ca_2$ after a chase with Ca^{2+} and unlabeled ATP. However, the rate constant for phosphorylation, $k_2 = {}^{m}k_2$ = 70 s^{-1}, is not affected (Figure 7). It is important to note that ^{m}E is not formed in the presence of Ca^{2+}, because the rate constant for dissociation of ATP from $E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$ is 80 s^{-1} (Stahl & Jencks, 1987).

Structural Changes That Lead to the Active Enzyme Species, $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$. The pathways that lead to the active enzyme species, $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, are dependent on the sequence by which the ligands ATP, Mg^{2+}, and Ca^{2+} are added to CaATPase. These pathways are summarized in Figure 9. Steps 1, 3, 4, and 5 in Figure 9 were shown to be conformational changes that are induced by the binding of the ligands Ca^{2+} and ATP (Petithory & Jencks, 1988; Petithory & Jencks, 1986; Stahl & Jencks, 1984). It now appears that step 2 also involves a conformational change. The addition of Ca^{2+} and ATP to enzyme that has been incubated with Mg^{2+} allows the enzyme to be phosphorylated with k = 67 s^{-1}, which is virtually identical to the 70 s^{-1} observed when Ca^{2+} was added to E·ATP·Mg (Stahl & Jencks, 1987). No lag above 1 ms was observed. This shows that the reaction mechanism does not follow the sequential mechanism through $^{c}E{\cdot}Ca_2{\cdot}Mg$ with two conformational changes, 1 and 3 in Figure 9. The results are consistent with a single, concerted conformational change that leads directly to $^{a}E{\cdot}Ca_2{\cdot}ATP{\cdot}Mg$, with k = 70 s^{-1}, and bypasses the formation of ^{m}E and ^{c}E. This does not exclude a stepwise mechanism, but it shows that only one step is kinetically significant.

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