Phosphorylation of the Calcium-Transporting Adenosinetriphosphatase by Lanthanum ATP: Rapid Phosphoryl Transfer following a Rate-Limiting Conformational Change†

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Received December 5, 1989; Revised Manuscript Received February 21, 1990

ABSTRACT: The calcium-transport ATPase (CaATPase) of rabbit sarcoplasmic reticulum preincubated with 0.02 mM Ca^{2+} ($^cE{\cdot}Ca_2$) is phosphorylated upon the addition of 0.25 mM $LaCl_3$ and 0.3 mM [γ-^{32}P]ATP with an observed rate constant of 6.5 s^{-1} (40 mM MOPS, pH 7.0, 100 mM KCl, 25 °C). La·ATP binds to $^cE{\cdot}Ca_2$ with a rate constant of 5×10^6 M^{-1} s^{-1}, while ATP, Ca^{2+}, and La^{3+} dissociate from $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ at ≤ 1 s^{-1}. The reaction of ADP with phosphoenzyme (EP) formed from La·ATP is biphasic. An initial rapid loss of EP is followed by a slower first-order disappearance, which proceeds to an equilibrium mixture of EP·ADP and nonphosphorylated enzyme with bound ATP. The fraction of EP that reacts in the burst (α) and the first-order rate constant for the slow phase (k_b) increase proportionally with increasing concentrations of ADP to give maximum values of 0.34 and 65 s^{-1}, respectively, at saturating ADP (K_S^{ADP} = 0.22 mM). The burst represents rapid phosphoryl transfer and demonstrates that ATP synthesis and hydrolysis on the enzyme are fast. The phosphorylation of $^cE{\cdot}Ca_2$ by La·ATP at 6.5 s^{-1} and the kinetics for the reaction of EP with ADP are consistent with a rate-limiting conformational change in both directions. The conformational change converts $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ to the form of the enzyme that is activated for phosphoryl transfer, $^aE{\cdot}Ca_2{\cdot}La{\cdot}ATP$, at 6.5 s^{-1}; this is much slower than the analogous conformational change at 220 s^{-1} with Mg^{2+} as the catalytic ion [Petithory & Jencks (1986) *Biochemistry 25*, 4493]. The rate constant for the conversion of $^aE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ to $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ is 170 s^{-1}. ATP does not dissociate measurably from $^aE{\cdot}Ca_2{\cdot}La{\cdot}ATP$. Labeled EP formed from $^cE{\cdot}Ca_2$ and La·ATP with leaky vesicles undergoes hydrolysis at 0.06 s^{-1}. It is concluded that the reaction mechanism of the CaATPase is remarkably similar with Mg·ATP and La·ATP; however, the strong binding of La·ATP slows both the conformational change that is rate limiting for EP formation and the dissociation of La·ATP. An interaction between La^{3+} at the catalytic site and the calcium transport sites decreases the rate of calcium dissociation by greater than 60-fold. When $^cE{\cdot}Ca_2$ is mixed with 0.3 mM ATP and 1.0 mM $CaCl_2$, the phosphoenzyme is formed with an observed rate constant of 3 s^{-1}. The phosphoenzyme formed from Ca·ATP reacts with 2.0 mM ADP and labeled ATP with a rate constant of 30 s^{-1}; there may be a small burst ($\alpha \leq 0.05$).

The formation of the covalent phosphoenzyme of the calcium-transporting ATPase in sarcoplasmic reticulum (CaATPase)[1] is critical in changing the vectorial specificity of the enzyme for dissociation of the calcium ions bound to the transport sites. The phosphoenzyme can be formed either from inorganic phosphate, when the calcium concentration in the external medium is very low (Masuda & deMeis, 1973), or from ATP, when calcium is bound to the exterior transport sites (de Meis & Vianna, 1979; Jencks, 1980a; Inesi, 1985). Both reactions require the presence of a divalent cation that binds to a site on the enzyme that is distinct from the exterior calcium transport sites. This catalytic metal ion is usually magnesium and is reported to bind to the enzyme as part of a Mg·ATP complex (Vianna, 1975). Calcium can also facilitate phosphorylation of the enzyme by ATP (Takakuwa & Kanazawa, 1979; Dupont, 1980; Yamada & Ikemoto, 1980; Shigekawa et al., 1983).

Changing the catalytic metal ion results in significant changes in several partial reactions of the catalytic cycle. The rate of phosphoenzyme formation and steady-state turnover are slower when Ca·ATP is the substrate for phosphorylation (Shigekawa et al., 1983). There is evidence that a conformational change is rate limiting for formation of the phosphoenzyme from Mg·ATP (Petithory & Jencks, 1986; Stahl & Jencks, 1987), but it is not known whether the same conformational change is rate limiting for phosphorylation by Ca·ATP. In the reverse reaction the phosphoenzyme formed from Mg·ATP reacts rapidly with ADP, giving a burst of EP disappearance (Shigekawa & Dougherty, 1978; Sumida et al., 1980; Pickart & Jencks, 1982; Froehlich & Heller, 1985; Fernandez-Belda & Inesi, 1986; Wang, 1986), while the phosphoenzyme with Ca^{2+} at the catalytic ion binding site reacts with ADP with little or no burst (Yamada & Ikemoto, 1980). Recent work has indicated that a variety of ions can exchange with calcium bound at the catalytic site, resulting in changes in the rate of calcium dissociation from the interior transport sites of the phosphoenzyme (Wakabayashi & Shigekawa, 1988). These results demonstrate that the partial reactions of the catalytic cycle are affected by the ion that

† Publication No. 1710 from the Graduate Department of Biochemistry, Brandeis University. This research was supported in part by grants from the National Institutes of Health (GM20888) and the National Science Foundation (DMB-8715832).

[1] Abbreviations: CaATPase, calcium-transporting ATPase; SRV, sarcoplasmic reticulum vesicles; SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; EP, phosphoenzyme; P_i, inorganic phosphate.

0006-2960/90/0429-5210$02.50/0

occupies the catalytic ion binding site. However, the way in which these effects are brought about is unclear.

In this paper we report that La^{3+} bound at the catalytic ion binding site supports phosphorylation of the enzyme by ATP. These studies with La^{3+} were undertaken because we were interested in understanding the mechanism by which this ion acts as a quench reagent (Chiesi & Inesi, 1979) in assays measuring calcium binding to and dissociation from the CaATPase. The observed rate of phosphorylation with La^{3+} is slow compared with that seen with magnesium as the catalytic ion and is reversible upon the addition of ADP. Surprisingly, however, the phosphoryl-transfer step of the reaction remains rapid; it is too fast to measure. The conformational change that is rate limiting for phosphorylation of the enzyme by Mg·ATP remains rate limiting when La^{3+} is the catalytic ion. The substitution of La^{3+} for Mg^{2+} at the catalytic site causes a decrease in the forward rate constant of the conformational change from 220 s^{-1} to 6.5 s^{-1}, as well as large decreases in the rate constants for the dissociation of ATP and for hydrolysis of the phosphoenzyme. It also causes a large decrease in the rate of dissociation of Ca^{2+} from the high-affinity transport sites, which shows that there is a strong interaction between the catalytic site and the high-affinity transport sites. The slow disappearance of La·E~P·Ca_2 may account for the observed inhibitory effect of La^{3+} on the steady-state turnover of the enzyme that was observed by Yamada and Tonomura (1972).

Materials and Methods

Materials. Disodium ATP ("Sonderqualitat"), NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were purchased from Boehringer Mannheim. ADP (grade XVIII, potassium salt), phosphocreatine, creatine kinase, and $CaCl_2 \cdot 2H_2O$ were obtained from Sigma. The sodium salt of MOPS (Ultrex grade) and the calcium ionophore A23187 were purchased from Calbiochem. KCl and EGTA were obtained from Fluka and $LaCl_3 \cdot 7H_2O$ (Gold Label) was from Aldrich. [γ-^{32}P]ATP (>99% pure) was obtained from New England Nuclear. All solutions were made with Milli-Q grade water (Millipore Co.) and stored in polypropylene containers (Nalgene Co.) that had been previously boiled for 10 min in 10 mM EDTA (pH 12), repeatedly rinsed with Milli-Q water, and dried.

Sarcoplasmic reticulum vesicles were prepared as described by Khananshvili and Jencks (1988). The preparations were stored at −80 °C until they were used. Enzyme solutions (0.2–0.5 mL) were dialyzed at 4 °C overnight against 1 L of 100 mM KCl, 40 mM MOPS, pH 7.0, and 0.2 M sucrose in order to lower the concentration of calcium inside the vesicles to ambient calcium levels (~6 μM). SRV were loaded passively by dialyzing enzyme solutions against 100 mM KCl, 40 mM MOPS, pH 7.0, 0.2 M sucrose, and 20 mM $CaCl_2$ overnight at 4 °C. The steady-state ATPase activity of the preparations used in this work was 3–5 μmol (min·mg of total protein)$^{-1}$ after the vesicles were made permeable to calcium by the addition of A23187 in ethanol (2.0 μM, 0.1% ethanol final concentration). The amount of phosphoenzyme was measured with [γ-^{32}P]ATP at 5 s and was typically found to be 2–3 nmol (mg of total protein)$^{-1}$.

Methods. Steady-state ATP hydrolysis was measured spectrophotometrically by coupling the formation of ADP to the 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, 5 mM $MgSO_4$, 1.0 mM EGTA, 1.0 mM $CaCl_2$ (21 μM free Ca^{2+}),[2] 1.5 mM PEP, 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 2.0 mL. Protein concentrations were estimated by using the method of Lowry et al. (1951) with bovine serum albumin as the protein standard.

Rapid Mix–Quench Experiments. The formation and decay of the phosphoenzyme were measured at 25 °C by using a thermostated rapid-mixing chemical-quench apparatus described previously (Stahl & Jencks, 1984; Petithory & Jencks, 1988). The instrument was fit with either three or four syringes (0.89 mL/syringe) so that either a single reaction time or two consecutive reaction times could be followed. The sample syringe (syringe A) containing the starting enzyme solution could be filled from a Y-mixer that mixes equal volumes of two solutions, which then fill syringe A. All syringes 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 0.5 M HCl and 13 mM KH_2PO_4. Solutions containing La^{3+} and ATP were ≤0.6 mM and were stable for the 1-h time period that they were used. Precipitates were observed in some solutions after 2 h; however, precipitates were not observed in solutions that also contained ADP.

Reactions at times greater than 0.3 s were followed by using a pulsed quench configuration for the mixer similar to that described by Fersht (1986). The contents of syringes A, B, and C were mixed and filled an aging tube that accommodated exactly their combined volume. The contents of the aging tube were displaced following a preset time delay and mixed with an equal volume of 0.25 M $HClO_4$ and 4 mM KH_2PO_4 to quench the reaction.

Determination of [^{32}*P*]*EP.* The amount of radiolabeled phosphoenzyme was measured essentially as described by Verjovski-Almeida et al. (1978). Bovine serum albumin was added to quenched reaction mixtures to give a final concentration of 0.18–0.2 mg/mL, followed by trichloroacetic acid in water to give a final concentration of 10% (w/v). The samples were put on ice for ~1 h and then centrifuged at 1500*g* for 15 min. at 4 °C. The supernate was decanted, the pellets were resuspended in ice-cold 5% trichloroacetic acid and 10 mM KH_2PO_4, and the protein was collected by vacuum filtration over Whatman GF/C glass fiber filters. Precipitates of lanthanum phosphate did slow the rate of filtration but did not cause an increase in background counts. The sample collection tubes were rinsed with three 5-mL portions of the resuspension solution, and the labeled protein was determined by liquid scintillation counting in glass vials containing ~7 mL of Aquasol-2 (New England Nuclear).

Computer Programs. Rate constants were estimated by using a program that allows the fitting of experimental data by a nonlinear least-squares procedure, and simulations of kinetic data were carried out with an IBM personal computer as described previously (Stahl & Jencks, 1987).

Results

Phosphorylation by La·ATP. Table I shows the effects of La^{3+} on the amount of phosphoenzyme formed at 20 ms and 2 s under standard assay conditions (0.3–0.4 mM [γ-^{32}P]ATP, 40 mM MOPS, pH 7.0, 100 mM KCl, 25 °C, dialyzed intact SRV). Category A shows control reactions that measured the

[2] An apparent dissociation constant of 3.9×10^{-7} M for the calcium–EGTA complex at pH 7.0 (Allen et al., 1977) and 25 °C was used to calculate the concentration of free calcium. The equilibrium dissociation constant (K_{Ca}) of 1.27×10^{-11} M^2 for two calcium ions bound to the free enzyme (Petithory & Jencks, 1988) that was determined by using the apparent dissociation constant of 7.4×10^{-7} M for the calcium–EGTA complex (Godt, 1974) is 4.0×10^{-12} M^2 ($K_{0.5}$ = 2.0 μM) when the value of Allen and co-workers is used.

Table I: Phosphoenzyme Formation in the Presence of La^{3+} [a]

category	syringe A: SRV and	syringe B: [γ-^{32}P]ATP and	fraction EP 20 ms	fraction EP 2 s
A	0.04 mM Ca^{2+}	2.0 mM Mg^{2+}	1.0	0.91
	0.02 mM Ca^{2+}, 1.0 mM Mg^{2+}	0.02 mM Ca^{2+}, 1.0 mM Mg^{2+}		1.0
	no added metals	2.0 mM Mg^{2+}	0.70	
	no added metals	2.0 mM Mg^{2+}		0.67
B	0.5 mM La^{3+}, 0.04 mM Ca^{2+}	2.0 mM Mg^{2+}	0.04	0.82
	1.0 mM La^{3+}, 0.04 mM Ca^{2+}	2.0 mM Mg^{2+}		0.27
C	0.5 mM La^{3+}	0.5 mM La^{3+}		0.05
	0.04 mM Ca^{2+}	0.5 mM La^{3+}	0.10	0.83
	0.04 mM Ca^{2+}	1.0 mM La^{3+}		0.77

[a] The data were obtained at 25 °C with two different enzyme preparations. The final concentrations were 40 mM MOPS, pH 7.0, 100 mM KCl, and half the concentration of the metal ions specified for syringes A and B. The reactions were initiated by mixing equal volumes from syringes A and B for either 20 ms or 2 s and were then quenched by the addition of HCl and KH_2PO_4 to a final concentration of 0.5 M and 13 mM, respectively. Reactions in which EP levels were measured at 20 ms and 2 s contained a final concentration of 0.74 mg/mL dialyzed intact SRV and 0.3 mM [γ-^{32}P]ATP (10 μCi/μmol). The total EP, 1.97 nmol/mg, is given a fractional value of 1.0. Reactions in which EP levels were measured only at 2 s contained a final concentration of 0.3 mg/mL dialyzed intact SRV and 0.4 mM [γ-^{32}P]ATP (10 μCi/μmol). The total EP measured was 2.77 nmol/mg and is given a fractional value of 1.0.

total amount of EP that was formed in the presence of Mg^{2+}, ambient or added Ca^{2+}, and no La^{3+}. The amount of EP measured in the first turnover (20 ms) is higher than at 2 s because an overshoot of EP formation occurs before steady-state levels of EP are established (Froehlich & Taylor, 1976; Takisawa & Tonomura, 1978; Sumida et al., 1980; Stahl & Jencks, 1987). Omission of added Ca^{2+} from these reactions gave ~70% EP formation at 20 ms and 2 s; this indicates that the ambient Ca^{2+} levels in the reaction mixtures were sufficient to support partial phosphorylation.

Category B shows that both the rate of formation and the amount of EP that is formed in the steady-state from Mg·ATP decrease when the SRV are preincubated with 0.5 mM La^{3+} and 0.04 mM Ca^{2+}. Increasing the La^{3+} concentration to 1.0 mM resulted in a further decrease in EP at 2 s, from 82% to 27% of the control value. These results are consistent with reversible binding of La^{3+} to the high-affinity calcium binding sites (Chevallier & Butow, 1971) to form enzyme species that do not phosphorylate.

Category C describes experiments which show that La^{3+} can serve as the catalytic ion and support phosphorylation when it is added to the enzyme with the ATP, in the absence of magnesium. SRV that were preincubated with 0.5 mM La^{3+} and ambient Ca^{2+} formed only 5% of the EP of the control reactions in 2 s. However, omitting the La^{3+} from the preincubation resulted in the formation of 10% EP at 20 ms and 83% EP at 2 s when phosphorylation was initiated by the addition of 0.5 mM La^{3+} (0.25 mM final concentration) and ATP. Doubling the concentration of La^{3+} did not significantly change the levels of EP at 2 s. These results show that La·ATP is capable of supporting the formation of the phosphoenzyme.

A rate constant of $k_p = 6.5\ s^{-1}$ for the formation of EP·Ca_2 when La^{3+} and Ca^{2+} are the only multivalent ions present was measured by mixing dialyzed intact SRV and 0.02 mM Ca^{2+} with 0.3 mM [γ-^{32}P]ATP and 0.25 mM $LaCl_3$ (eq 1; Figure

$$^{c}E\cdot Ca_2 + La\cdot ATP \xrightarrow{k_p} EP\cdot Ca_2 + ADP \qquad (1)$$

1, closed circles; $^{c}E\cdot Ca_2$ refers to the stable form of the enzyme with bound Ca^{2+}). This rate constant is smaller than the rate

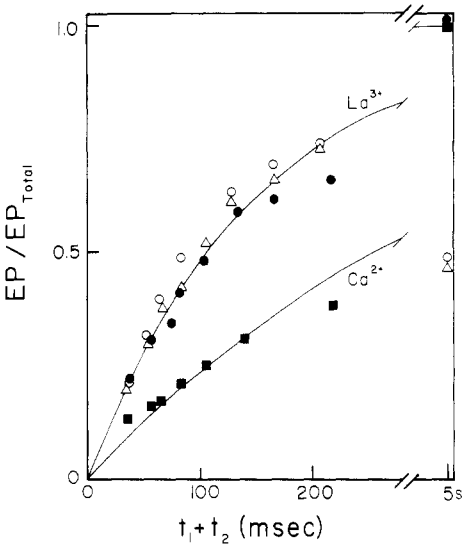

FIGURE 1: Phosphorylation of E·Ca_2 by La·ATP and Ca·ATP and slow dissociation of La·ATP and Ca^{2+} from E·Ca_2·La·ATP. The rapid-mix apparatus was used in the four-syringe configuration. All syringes except syringe D contained 40 mM MOPS, pH 7.0, and 100 mM KCl at 25 °C. Syringe A contained 0.73 mg/mL dialyzed intact SRV and 0.04 mM Ca^{2+} (●, ○, △) or 0.73 mg/mL passively loaded intact SRV (■). Syringe B contained 0.6 mM [γ-^{32}P]ATP (9.7 μCi/μmol) and either 0.5 mM $LaCl_3$ (●, ○, △) or 2 mM $CaCl_2$ (■). Syringe C contained either 30 mM unlabeled ATP (○) or 0.3 mM [γ-^{32}P]ATP (9.7 μCi/μmol) and 0.25 mM $LaCl_3$ (●), 15 mM EGTA (△), or 1.0 mM $CaCl_2$ (■). Syringe D contained 2 M HCl and 53 mM KH_2PO_4. The contents of syringes A and B were mixed and aged for 35 ms (t_1) before mixing with syringe C, marking the onset of t_2. Blanks were measured by reversing the order of mixing of syringe D and syringe B. End points taken at 5 s were measured by adding the quench solution manually. The solid lines were drawn to fit first-order rate constants of 6.5 s^{-1} (●) and 3 s^{-1} (■). The total EP formed at 5 s was 2.2 (●) and 2.5 nmol/mg (■).

constant of 220 s^{-1} that is observed for phosphorylation by Mg·ATP under similar conditions, which reflects a rate-limiting conformational change that precedes phosphoryl transfer (Petithory & Jencks, 1986). It will be shown below that the slow phosphorylation with La·ATP also represents a rate-limiting conformational change that precedes rapid phosphoryl transfer. Similar phosphorylation experiments with 1.0 mM Ca^{2+} as the only multivalent ion gave slower phosphorylation (Figure 1, squares), consistent with a rate constant of 3 s^{-1}.

The dissociation of ATP from $^{c}E\cdot Ca_2\cdot La\cdot ATP$ was investigated by mixing $^{c}E\cdot Ca_2$ with 0.3 mM [γ-^{32}P]ATP and 0.5 mM $LaCl_3$ for 35 ms, followed by the addition of a 50-fold excess of unlabeled ATP (open circles, Figure 1). The time course of EP formation during the first 165 ms of exposure to unlabeled ATP is similar to that of the phosphorylation reaction occurring at 6.5 s^{-1}, and a rate constant of $\leq 1\ s^{-1}$ was estimated for the dissociation of ATP from $^{c}E\cdot Ca_2\cdot La\cdot ATP$, based on the estimated error in the data of Figure 1; this is much slower than the dissociation of ATP from $^{c}E\cdot Ca_2\cdot Mg\cdot ATP$ at 120 s^{-1} (Petithory & Jencks, 1986). Dissociation of the labeled ATP during t_2 would result in a decrease in the amount of E^{32}P formed. A decrease in EP levels does occur over 5 s; it will be shown later that this can be accounted for by both the dissociation of ATP ($k \sim 0.6\ s^{-1}$) and slow hydrolysis of the phosphoenzyme that is formed from La·ATP. The fact that phosphorylation does not cease after t_1 when unlabeled ATP is added also demonstrates that the binding of 0.3 mM ATP is not rate limiting for the phosphorylation reaction.

A similar experimental approach was used to investigate whether Ca^{2+} bound at the transport sites or La^{3+} bound at

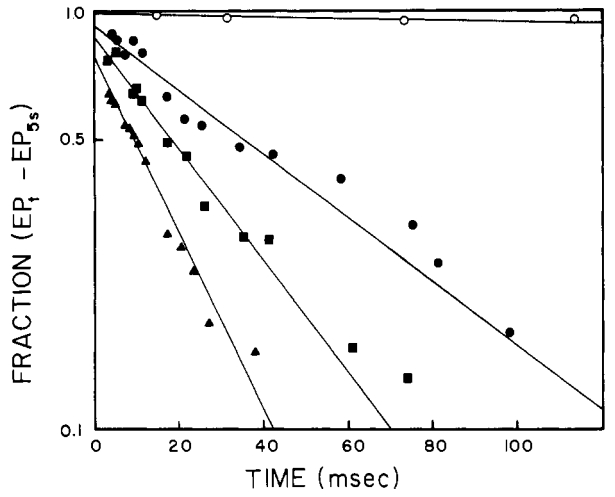

FIGURE 2: Reaction of the phosphoenzyme formed from [γ-^{32}P]-La·ATP with ADP in the presence of labeled ATP. The rapid mixer was used in the three-syringe configuration, with a Y-mixer to fill syringe A. All syringes, except syringe C, contained 40 mM MOPS, pH 7.0, and 100 mM KCl at 25 °C. The phosphoenzyme was formed in syringe A that was filled from the Y-mixer by mixing an equal volume of dialyzed intact SRV (1.5 mg/mL) and 0.04 mM $CaCl_2$ with 0.02 mM [γ-^{32}P]ATP (9.0 μCi/μmol) and 0.5 mM $LaCl_3$. After 5 s, the phosphoenzyme was mixed with syringe B containing 0.25 mM $LaCl_3$, 0.02 mM $CaCl_2$, and either 0.6 mM unlabeled ATP (○) or 0.6 mM [γ-^{32}P]ATP (9.0 μCi/μmol) and 0.066 (●), 0.2 (■), or 0.6 mM (▲) ADP. Reactions were quenched with 1.5 M HCl and 40 mM KH_2PO_4 delivered from syringe C. The final concentrations of ADP were 0 (○), 0.033 (●), 0.1 (■), or 0.3 mM (▲). Phosphoenzyme levels at t_0 were 2.36 nmol/mg (●, ■, ▲) and 4.39 nmol/mg (○). Phosphoenzyme levels at 5 s were 1.73 (○), 0.9 (●), 0.53 (■), and 0.37 (▲) nmol/mg. The solid lines were drawn to fit first-order rate constants of 0.5 (○), 15 (●), 30 (■), and 50 s^{-1} (▲).

the catalytic site of ^{c}E·Ca_2·La·ATP can dissociate and prevent phosphorylation of the enzyme. ^{c}E·Ca_2·La·ATP was formed during t_1 = 35 ms and then mixed with EGTA and 0.3 mM labeled ATP. The time course for phosphoenzyme formation under these conditions (Figure 1, triangles) shows no indication for the dissociation of either ion; there is no significant deviation from the time course for phosphorylation with 0.25 mM La^{3+} and 20 μM Ca^{2+} at 6.5 s^{-1}. An upper limit of 1 s^{-1} was set for the dissociation of Ca^{2+} and La^{3+} during the phosphorylation reaction from these results. In sharp contrast, Ca^{2+} dissociates rapidly from ^{c}E·Ca_2·Mg·ATP, with a rate constant of 80 s^{-1} (Petithory & Jencks, 1986).

Reaction of the Phosphoenzyme with ADP and Labeled ATP. The reactivity toward ADP of the phosphoenzyme with La^{3+} as the catalytic ion is shown in Figure 2. The phosphoenzyme was formed during an incubation of dialyzed intact SRV with 10 μM [γ-^{32}P]ATP and 0.25 mM $LaCl_3$ for 5 s. It was then mixed with unlabeled ATP or with different concentrations of ADP and 0.3 mM [γ-^{32}P]ATP. Figure 2 shows that mixing EP with unlabeled ATP results in the slow decay of EP with a rate constant of $\leq$0.5 s^{-1} (open circles). The phosphoenzyme reacts rapidly with ADP, and the disappearance of EP is biphasic (closed symbols). There is a rapid initial loss of EP that is followed by a slower, first-order decrease to an equlibrium mixture of phosphorylated and nonphosphorylated ATP-bound enzyme species. Extrapolation of the slow phase to zero time gives the fraction of EP (α) that reacts *and* does so during the burst. Both the size of the burst and the first-order rate constant describing the reaction of EP with ADP after the burst increase with increasing concentrations of ADP. Similar behavior has been observed previously with phosphoenzyme that was formed from Mg·ATP (Shigekawa & Dougherty, 1978; Sumida et al., 1980; Pickart & Jencks, 1982; Froehlich & Heller, 1985; Fernandez-Belda & Inesi, 1986; Wang, 1986).

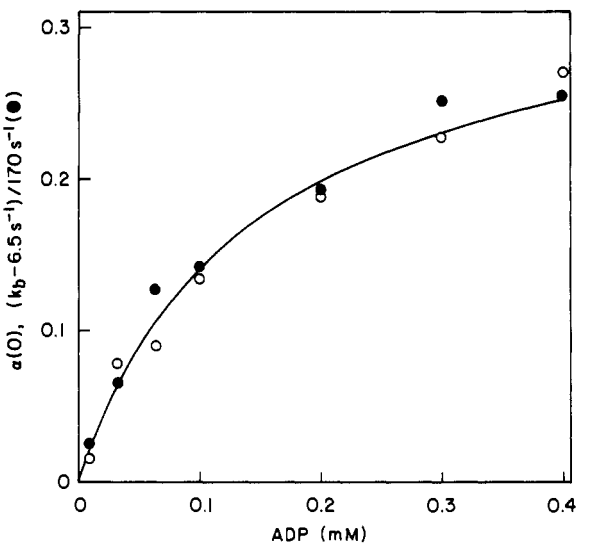

FIGURE 3: Dependence on the concentration of ADP of the burst size (α) and the rate constant of the slow phase (k_b). The observed values of α (○) and values of α calculated from the observed values of k_b and eq 5 (●) are taken from Figure 2 and similar experiments. The solid line was calculated from eq 3 with K_P = 1.94 and K_S^{ADP} = 0.22 mM.

Scheme I

$$ADP + E^{La}_{Ca_2}\text{-}P \rightleftharpoons E^{La}_{Ca_2}\text{-}P\cdot ADP \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} {}^{a}E\cdot Ca_2\cdot La\cdot ATP$$

(K_S^{ADP} for the first equilibrium; K_P for the second)

The increase in the size of the burst with increasing concentrations of ADP is consistent with binding of ADP to the phosphoenzyme in a rapid equilibrium step, with the equilibrium constant K_S^{ADP}, followed by rapid synthesis of ATP, the extent of which is governed by the equilibrium constant for phosphoryl transfer at the active site, K_P (Scheme I). The existence of a burst and the first-order time course of the slow phase indicate that the binding of ADP and the rapid phosphoryl-transfer reaction have come to equilibrium before the first measurement was made at 3 ms.

The open circles in Figure 3 show that the burst size, α, approaches a maximal value (α_{max}) when the concentration of ADP in the reaction is increased. According to Scheme I, the value of α is related to the equilibrium constant for rapid phosphoryl transfer on the enzyme, K_P, by eq 2 and by eq 3,

$$K_P = (1 - \alpha_{max})/\alpha_{max} \quad (2)$$

$$\alpha = [1 + K_P(1 + K_S^{ADP}/[ADP])]^{-1} \quad (3)$$

which describes the increase in α as a function of increasing concentration of ADP (Pickart & Jencks, 1982). The solid line in Figure 3 was calculated from values of α_{max} = 0.34, K_S^{ADP} = 0.22 mM, and K_P = 1.94 for the reaction with La^{3+} as the catalytic ion and shows satistifactory agreement with the data.

A lower limit of 700–1000 s^{-1} may be set for the approach to equilibrium for phosphoryl transfer described by the rate constants k_2 and k_{-2}. It is assumed that 3–5 half-lives of the reaction have occurred before the first measurement is made at 3 ms; the half-time for equilibration of phosphoryl transfer on the enzyme is $\leq$0.6–1 ms. This means that phosphoryl transfer in both directions is very much faster than the observed rate constants of up to $\sim$50 s^{-1} for the slow phase of phosphoenzyme disappearance in Figure 2. It is also much faster than the rate constant of 6.5 s^{-1} for phosphorylation upon

Scheme II

$$ADP + {}^{La\text{-}P}E_{Ca_2} \underset{K_S^{ADP}}{\rightleftharpoons} {}^{La\text{-}P}E_{Ca_2}\cdot ADP \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} {}^{a}E\cdot Ca_2\cdot La\cdot ATP \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} {}^{c}E\cdot Ca_2\cdot La\cdot ATP$$

K_P K_c

the addition of La·ATP to $^cE\cdot Ca_2$. We conclude that these observed rate constants cannot represent phosphoryl transfer and must represent a slow conformational change, which is described by the rate constants k_1 and k_{-1} in Scheme II. This conformational change is rate limiting for phosphorylation of $^cE\cdot Ca_2$ and the disappearance of EP that follows the burst during the dephosphorylation reaction. It was concluded previously that a conformational change is rate limiting for the phosphorylation of $^cE\cdot Ca_2$ by Mg·ATP (Petithory & Jencks, 1986).

Figure 2 also shows that the rate constant for the slow phase of the reaction, k_b, increases with increasing concentrations of ADP. This increase is expected if a nonphosphorylated species of the enzyme that is formed in the burst phase and is at equilibrium with the phosphoenzyme reacts further in the second phase. This is consistent with the mechanism shown in Scheme II, in which a slow conformational change (k_{-1}) follows the rapid transfer of the phosphoryl group from the enzyme to ADP (k_{-2}). The intermediate $^aE\cdot Ca_2\cdot La\cdot ATP$ is the enzyme species with bound ATP that is activated to catalyze the phosphoryl-transfer reaction, whereas $^cE\cdot Ca_2\cdot La\cdot ATP$ is the catalytically inactive intermediate that is formed initially when La·ATP binds to $^cE\cdot Ca_2$. The slow phase of the reaction with ADP, which occurs after the formation of $^aE\cdot Ca_2\cdot La\cdot ATP$ in the burst, represents the approach to equilibrium between these two species. The observed rate constant for approach to equilibrium is the sum of the first-order rate constants for the forward and reverse reactions (Frost & Pearson, 1953). Therefore, the observed rate constant for the slow phase, k_b, can be described by eq 4, in which

$$k_b = \alpha k_{-1} + k_1 \quad (4)$$

α is the fraction of enzyme that reacts with the rate constant k_{-1} and k_1 is the rate constant of 6.5 s^{-1} that is observed for the formation of EP from $^cE\cdot Ca_2$ and La·ATP in the absence of added ADP. Double-reciprocal plots of $(k_b - 6.5\ s^{-1})$ against the concentration of ADP give a value of $(k_b - 6.5\ s^{-1}) = 58\ s^{-1}$ at saturating concentrations of ADP when $\alpha = 0.34$. This value of α represents the fraction of enzyme reacting in the slow phase. The calculated value for k_{-1} is $(58\ s^{-1}/0.34) = 170\ s^{-1}$. The closed circles in Figure 3 show values of α that were calculated from the observed value of k_b and eq 5. The

$$\alpha = (k_b - k_1)/k_{-1} \quad (5)$$

agreement between the observed and calculated values of α shows that the change in k_b with increasing concentrations of ADP is consistent with Scheme II, in which a slow conformational change follows rapid phosphoryl transfer when EP reacts with ADP.

The equilibrium amounts of phosphoenzyme that were measured 5 s after the addition of ADP and 0.3 mM labeled ATP (legend, Figure 2) are consistent with values of $K_S^{ADP} = 0.22$ mM, $K_P = 1.9$, and $K_C = 0.05$ for the reaction described in Scheme II. These values are in good agreement with those obtained from the analysis of the kinetic data of Figure 2 (Table II).

Slow Dissociation of ATP from $^aE\cdot Ca_2\cdot La\cdot ATP$. The experiment shown in Figure 4 was carried out to determine whether ATP dissociates from the nonphosphorylated intermediate, $^aE\cdot Ca_2\cdot La\cdot ATP$, during the reaction of EP with ADP. The phosphoenzyme was formed as described in Figure 2 and then reacted with 0.2 mM ADP and either [γ-^{32}P]ATP (closed circles) or unlabeled ATP (open circles). There is no apparent difference in the disappearance of the phosphoenzyme for the two conditions during the first 75 ms of the reaction. This shows that there is no significant dissociation of ATP during this period. The decrease in the phosphoenzyme at 5 s (open circles) is consistent with either slow dissociation of the labeled ATP, with $k \leq 3\ s^{-1}$, or hydrolysis of the phosphoenzyme.

Binding of La·ATP to $^cE\cdot Ca_2$. The second-order rate constant for the binding of ATP to $^cE\cdot Ca_2$ in the presence of La^{3+} is $(5 \pm 2) \times 10^6\ M^{-1}\ s^{-1}$, as shown in Figure 5. The reactions were carried out by exposing the enzyme to 0.25 mM La^{3+} and different concentrations of ATP for a fixed period of time, $t_1 = 5.5$ ms, rather than by using a fixed concentration of

Table II: Comparison of the Rate and Equilibrium Constants for Phosphoenzyme Formation from Mg·ATP and La·ATP at pH 7.0, 100 mM KCl, and 25 °C[a]

constant	value	
	Mg·ATP	La·ATP
$^ck_{ATP}$	$1 \times 10^7\ M^{-1}\ s^{-1}$ [b]	$(0.5 \pm 0.2) \times 10^7\ M^{-1}\ s^{-1}$ [c]
$^ck_{-ATP}$	$120\ s^{-1}$ [d]	$0.6 \pm 0.4\ s^{-1}$ [c]
$K_S^{ATP} = {}^ck_{-ATP}/{}^ck_{ATP}$	1.2×10^{-5} M	1.2×10^{-7} M
k_c	$220\ s^{-1}$ [d]	$6.5\ s^{-1}$ [c]
k_{-c}	$200\ s^{-1}$ [e]	$170\ s^{-1}$ [c]
$K_c = k_c/k_{-c}$	1.1	0.04[c]
		0.05[f]
K_p	0.84[e]	1.94[c]
		1.9[f]
K_S^{ADP}	0.73 mM[g]	0.22 mM[c]
		0.22 mM[f]

[a] The rate constants k_x refer to reactions leading to the formation of the phosphoenzyme, and the rate constants k_{-x} refer to the reverse reactions. [b] Petithory & Jencks, 1988. [c] This work. [d] Petithory & Jencks, 1986. [e] This work and Pickart and Jencks (1982). [f] This work, from the 5-s end points of Figure 2 and Figure 4. [g] Pickart & Jencks, 1982.

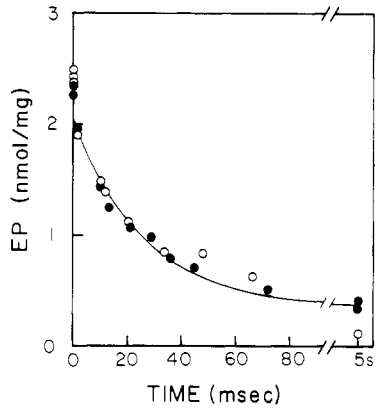

FIGURE 4: Reaction of EP with ADP in the presence of labeled or unlabeled ATP. All syringes contained 40 mM MOPS, pH 7.0, and 100 mM KCl at 25 °C, except syringe C. The phosphoenzyme was formed in syringe A by using a Y-mixer to mix an equal volume of dialyzed intact SRV (1.5 mg/mL) and 0.04 mM $CaCl_2$ with 0.020 mM [γ-^{32}P]ATP (9.0 μCi/μmol) and 0.50 mM $LaCl_3$. The reaction with ADP was initiated 5 s later by mixing the contents of syringe A with syringe B containing 0.25 mM $LaCl_3$, 0.020 mM added $CaCl_2$, 0.40 mM ADP, and either 0.60 mM [γ-^{32}P]ATP (9 μCi/μmol) (●) or 0.60 mM unlabeled ATP (○). The reactions were quenched with 1.5 M HCl and 40 mM KH_2PO_4 delivered from syringe C. Blanks were measured by reversing the order of mixing syringes B and C, and end points were measured by manually adding the quench at 5 s. The solid line is drawn for a first-order rate constant of 39 s^{-1} that follows a burst of 0.38 nmol/mg.

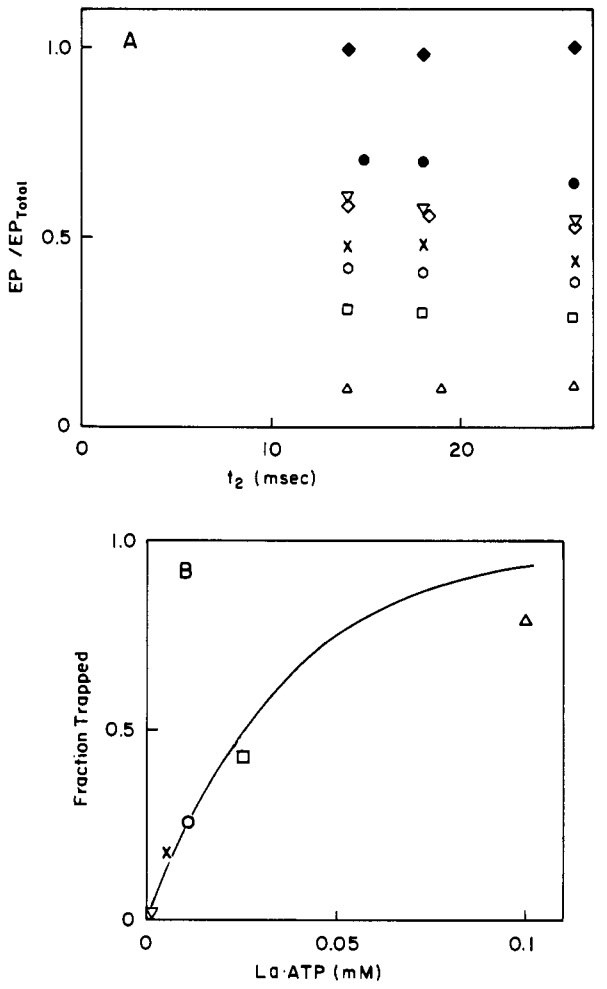

FIGURE 5: (A) Measurement of the second-order rate constant for the binding of La·ATP to E·Ca_2. The rapid-mixing apparatus was used in the four-syringe configuration. E·Ca^2 was mixed with increasing concentrations of unlabeled La·ATP for t_1 = 5.5 ms. The enzyme was then mixed for t_2 = 14–28 ms with Mg^{2+}, EGTA, and [γ-^{32}P]ATP. The decrease in the amount of E–^{32}P that is formed during t_2 is a measure of the extent of binding of unlabeled La·ATP during t_1. All syringes contained 40 mM MOPS, pH 7.0, and 100 mM KCl at 25 °C, except syringe D. Syringe A contained 0.04 mM $CaCl_2$ and 0.73 mg/mL dialyzed intact SRV. Syringe B contained buffer only (♦, ●) or 0.5 mM $LaCl_3$ and either 0.2 (▽), 2.0 (◊), 10 (×), 20 (○), 50 (□), or 200 μM (△) unlabeled ATP. Syringe C contained 0.9 mM [γ-^{32}P]ATP (10.3 μCi/μmol), 15 mM $MgSO_4$, and either 0.02 mM $CaCl_2$ (♦) or 15 mM EGTA (●, ◊, ▽, ×, ○, □, △). Syringe D contained 2 M HCl and 53 mM KH_2PO_4. Blanks were measured by reversing the order of mixing of syringes B and D. The duration of t_1 was held constant at 5.5 ms, and t_2 was varied. (B) Fraction of enzyme that binds unlabeled La·ATP during an exposure for 5.5 ms to different concentrations of La·ATP. The fraction of enzyme that was trapped due to the binding of unlabeled ATP was calculated from the observed decrease in labeled EP formed at 14 ms shown in (A); the amount of labeled EP at 14 ms with 0.2 and 2.0 μM unlabeled ATP during t_1 (▽, ◊) is the same and is taken to represent zero trapping. The decrease in EP at 14 ms represents the concentration-dependent increase in the amount of unlabeled ATP that binds during t_1 = 5.5 ms. The line shows the calculated fraction of enzyme trapped for the binding of ATP to the enzyme with a second-order rate constant of 5 × 10^6 M^{-1} s^{-1}.

La·ATP and varying the time of exposure. The amount of ^{c}E·Ca_2 remaining after t_1 was measured by adding labeled ATP and EGTA. The assay is shown in eq 6. The enzyme was

$$^{c}E\cdot Ca_2 + La\cdot ATP \xrightarrow[t_1]{k_{on}} {}^{c}E\cdot Ca_2\cdot La\cdot ATP \quad (6)$$

$$t_2 \downarrow Mg\cdot ATP^*/EGTA$$

$$E\sim P^*\cdot Ca_2$$

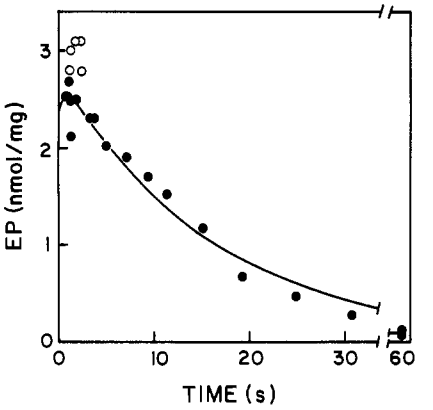

FIGURE 6: Dephosphorylation of EP formed from La·ATP. The rapid-mixing apparatus was used in the pulsed-quench configuration. All syringes, except syringe D, contained 40 mM MOPS, pH 7.0, and 100 mM KCl at 25 °C. Syringe A contained 0.58 mg/mL dialyzed SRV, 0.04 mM $CaCl_2$, and 4 μM A23187. Syringe B contained 0.5 mM $LaCl_3$ and 0.07 mM [γ-^{32}P]ATP (12 μCi/μmol). Syringe C contained either 7 mM unlabeled ATP, 1 mM $MgSO_4$, 1 mM phosphocreatine, and 0.1 mg/mL creatine kinase (●) or 0.035 mM [γ-^{32}P]ATP (12 μCi/μmol), 0.02 mM $CaCl_2$, and 0.25 mM $LaCl_3$ (○). The enzyme was mixed with labeled ATP for t_1 = 43 ms and then mixed with excess unlabeled ATP (●) or labeled ATP (○) for various times t_2. The reactions were quenched with 0.25 M $HClO_4$ and 4 mM KH_2PO_4 delivered from syringe D. Blanks were measured by reversing the order of mixing of syringe B and syringe D. The line is drawn for a first-order rate constant of 0.06 s^{-1}.

first mixed with 0.02 mM Ca^{2+}, 0.25 mM La^{3+}, and different concentrations of unlabeled ATP for t_1 = 5.5 ms, after which EGTA, Mg^{2+}, and [γ-^{32}P]ATP were added and allowed to react for t_2 = 14, 18, and 24 ms. The amount of E–^{32}P that is formed during t_2 is a measure of the amount of enzyme that has not bound La·ATP during t_1; it decreases as the concentration of unlabeled La·ATP during t_1 is increased because the binding of unlabeled La·ATP during t_1 protects the enzyme from phosphorylation by labeled Mg·ATP* during t_2 (eq 6). The slow dissociation of La·ATP ($k \leq 1$ s^{-1}) ensures that the binding of unlabeled La·ATP during t_1 is irreversible under the conditions of the experiment.

The total amount of phosphoenzyme that is formed from labeled Mg·ATP in the absence of La^{3+} is shown in Figure 5A by the solid symbols, and the inclusion of EGTA in the reaction results in the expected 30% decrease in the observed levels of EP (Petithory & Jencks, 1986). The open symbols show that when the enzyme is incubated with unlabeled ATP and La^{3+} during t_1, increasing the concentration of unlabeled La·ATP results in a decrease in the amount of labeled EP that is formed during t_2. The fraction of enzyme that is trapped by the binding of unlabeled La·ATP, which results in the observed decrease in formation of labeled phosphoenzyme, is plotted in Figure 5B as a function of the concentration of unlabeled La·ATP during t_1. The concentration of the La·ATP complex was determined from an apparent dissociation constant of 0.7 μM for La·ATP that was obtained from the observed dissociation constant of 0.33 μM at pH 8.0 (Morrison & Cleland, 1983) and adjusted to pH 7.0 with the equilibrium computer programs of Fabiato (1988). The solid line shows the predicted fraction of ^{c}E·Ca_2 that binds unlabeled La·ATP in 5.5 ms for a second-order rate constant for the binding reaction of 5 × 10^6 M^{-1} s^{-1}.

Dephosphorylation of EP Formed from La·ATP. Figure 6 shows that the hydrolysis of phosphoenzyme that was formed from La·ATP and ionophore-treated SRV follows first-order kinetics with a rate constant of 0.06 s^{-1}. The calcium ionophore A23187 was included to prevent calcium accumulation that

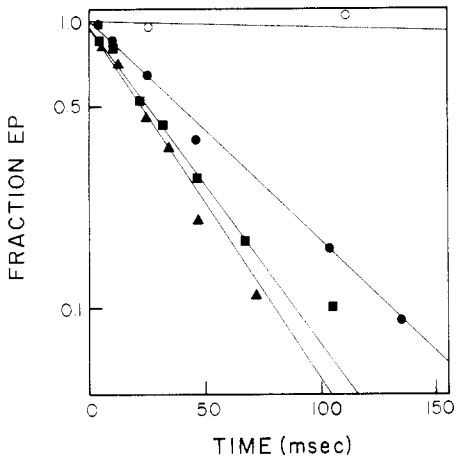

FIGURE 7: Reaction of the phosphoenzyme formed from Ca·ATP with ADP. The rapid mixer was used in the three-syringe configuration with a Y-mixer to fill syringe A. All syringes contained 40 mM MOPS, pH 7.0, and 100 mM KCl at 25 °C, except syringe C. The phosphoenzyme was formed in syringe A with the Y-mixer by mixing an equal volume of passively loaded SRV (1.5 mg/mL) and 1.0 mM $CaCl_2$ with 0.02 mM [γ-^{32}P]ATP (10 μCi/μmol) and 1.0 mM $CaCl_2$. The phosphoenzyme formed in 5 s was then mixed with syringe B, which contained 1.0 mM $CaCl_2$ and either 0.6 mM unlabeled ATP (○) or 0.6 mM [γ-^{32}P]ATP (10 μCi/μmol) and 0.2 mM (●), 1.0 mM (■), or 4.0 mM (▲) ADP. Reactions were quenched with 1.5 M HCl and 40 mM KH_2PO_4 delivered from syringe C. Reaction blanks were measured by reversing the mixing order of syringes B and C. Equilibrium end points were measured at 5 s by manual addition of the quench. Fraction EP refers to the fraction of total EP that disappears in the approach to equilibrium. The final concentrations of ADP were 0 (○), 0.1 (●), 0.5 (■), or 2.0 mM (▲). The amount of EP at t_1 = 0 was 2.05 nmol/mg. The amount of EP remaining after 5 s was 0.5 (●), 0.32 (■), 0.34 (▲), and 0.10 (○) nmol/mg. The lines are drawn to fit first-order rate constants of 0.6 (○), 18 (●), 26 (■), and 30 s^{-1} (▲) with values of α = 0 (○, ●) or 0.05 (■, ▲).

might inhibit EP hydrolysis. The labeled phosphoenzyme was formed by first mixing $^{c}E{\cdot}Ca_2$ with 0.035 mM [γ-^{32}P]ATP and 0.25 mM $LaCl_3$ for t_1 = 43 ms and then adding either labeled ATP (open circles) or 2.33 mM unlabeled ATP for t_2 (closed circles). The total phosphoenzyme present was 2.95 nmol/mg when measured by adding labeled ATP during t_2. However, addition of unlabeled ATP at 43 ms resulted in the formation of ~2.6 nmol/mg of EP at 0.6 s, which is the time required for the completion of the phosphorylation reaction, and this EP disappeared with a first-order rate constant of 0.06 s^{-1} as shown by the calculated solid line. The difference between the total EP and the maximal level of EP that is observed after the addition of unlabeled ATP can be accounted for as the amount of $^{c}E{\cdot}Ca_2{\cdot}La{\cdot}ATP$ that dissociates labeled ATP during t_2, with k = 0.6 ± 0.4 s^{-1}, while the remaining $^{c}E{\cdot}Ca_2{\cdot}La{\cdot}ATP$ phosphorylates with the rate constant k = 6.5 s^{-1} (Figure 1) and subsequently decays with the rate constant k = 0.06 s^{-1}. Separate experiments in which the disappearance of labeled phosphoenzyme was measured following the addition of ADP and unlabeled ATP gave rate constants in the range of 0.2–1 s^{-1} for the dissociation of ATP from $^{c}E{\cdot}Ca_2{\cdot}La{\cdot}ATP$ (data not shown).

Reaction with ADP of the Phosphoenzyme Formed from Ca·ATP. Figure 7 shows the kinetics of phosphoenzyme disappearance when ADP is added to the phosphoenzyme formed from Ca·ATP. The phosphoenzyme was formed by incubating calcium-loaded vesicles with 1.0 mM $CaCl_2$ and 0.01 mM [γ-^{32}P]ATP for 5 s. The addition of unlabeled ATP to the phosphoenzyme results in a very slow decrease of the labeled phosphoenzyme, consistent with a rate constant of ≤0.5 s^{-1} (Figure 7, open circles). The addition of 0.1 mM ADP and

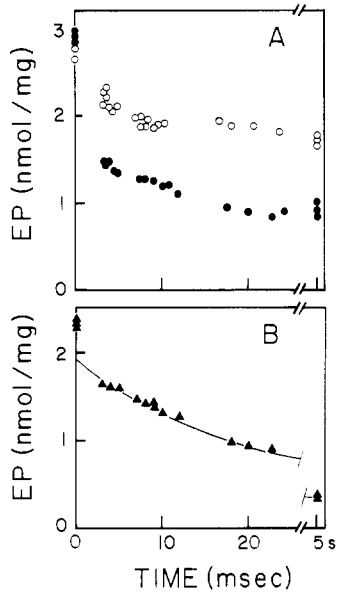

FIGURE 8: (A) Reaction of the phosphoenzyme formed from Mg·ATP with ADP. The rapid mixer was used in the three-syringe configuration with a Y-mixer to fill syringe A. All syringes contained 40 mM MOPS, pH 7.0, 100 mM KCl, 0.99 mM $CaCl_2$, 1.0 mM EGTA, and 5 mM $MgSO_4$ at 25 °C, except syringe C. The phosphoenzyme was formed in syringe A with the Y-mixer by mixing an equal volume of passively loaded SRV (1.5 mg/mL) with 0.04 mM [γ-^{32}P]ATP (12 μCi/μmol). The phosphoenzyme formed in 5 s was then mixed with syringe B containing 1.0 mM [γ-^{32}P]ATP (12 μCi/μmol) and either 0.5 (○) or 5.0 mM (●) ADP. Reactions were quenched with 1.5 M HCl and 40 mM KH_2PO_4 delivered from syringe C. Reaction blanks were measured by reversing the mixing order of syringes B and C. Equilibrium end points were measured at 5 s by manual addition of the quench. The final concentrations of ADP were 0.25 (○) and 2.5 mM (●). (B) Reaction of the phosphoenzyme formed from La-ATP with 0.3 mM ADP in the presence of labeled ATP, for comparison. The data were taken from Figure 2, and the line is drawn to fit a first-order rate constant of 50 s^{-1} and a burst size of 0.22.

0.3 mM [γ-^{32}P]ATP gave a much faster decrease in EP, with k = 18 s^{-1} (closed circles) and no significant burst. Increasing the concentration of ADP to 0.5 and 2.0 mM resulted in an increase in the rate constant for the disappearance of phosphoenzyme to 26 s^{-1} and 30 s^{-1}, respectively. The data are consistent with, but do not firmly establish, the presence of a small burst ($\alpha \leq 0.05$) at the highest concentrations of ADP.

Reaction with ADP of the Phosphoenzyme Formed from Mg·ATP. Figure 8A shows the kinetics of phosphoenzyme disappearance when the labled EP formed from Mg·ATP reacts with either 0.25 mM ADP (open circles) or 2.5 mM ADP (solid circles) in the presence of labeled ATP. The loss of labeled EP in the initial phase is rapid; within 3 ms it reaches ~70–80% of the total disappearance observed at the higher concentration of ADP. The reactions represent an approach to equilibrium, as described above for La·ATP. They are too rapid for accurate kinetic analysis, but the data obtained with 2.5 mM ADP are definitely biphasic and require a burst of EP loss followed by a slower exponential process. Equation 2 of Pickart and Jencks (1982), which describes the size of the bursts that were measured at different concentrations of ADP, accurately describes the end points at 5 s in Figure 8A when values of K_S^{ADP} = 0.73 mM and K_{Int} = 0.44 (Pickart & Jencks, 1982, 1984) are used. For comparison, the data in Figure 8B show the kinetics for the disappearance of phosphoenzyme that is formed from La·ATP over the same time period when it reacts with 0.3 mM ADP in the presence of labeled ATP.

Discussion

Inhibition and Support of Phosphoenzyme Formation by La^{3+}. The decrease in the amount of EP formation from Mg·ATP when the enzyme is preincubated with both La^{3+} and Ca^{2+} (Table I) is consistent with binding of La^{3+} to the exterior calcium transport sites (Chevallier & Butow, 1971), which inhibits formation of the phosphoenzyme. Increasing the ratio of La^{3+} to Ca^{2+} in the preincubation solution decreases the amount of EP formation at 2 s. This shows both that La^{3+} can compete with the binding of Ca^{2+} (Domonokos et al., 1985) and that La^{3+} is not a congener of Ca^{2+} for the CaATPase reaction. The observed decreases in the amount of EP formation shown in Table I are consistent with the model of eq 7 with $K_{Ca} = 4.0 \times 10^{-12}$ M^2 and $K_{0.5}$ in the range of

$$E\cdot La \underset{K_{La}}{\overset{La^{3+}}{\rightleftharpoons}} E \underset{K_{Ca}}{\overset{2Ca^{2+}}{\rightleftharpoons}} {}^{c}E\cdot Ca_2 \xrightarrow{Mg\cdot ATP} {}^{Mg\sim P}E_{Ca_2} + ADP \quad (7)$$

2–11 μM for the binding of La^{3+} to the enzyme to form a complex that will not phosphorylate.[2,3] It has also been shown that gadolinium ion, another lanthanide ion, does not replace Ca^{2+} and activate the CaATPase reaction (Itoh & Kawakita, 1984).

We were surprised to find that in the absence of Mg^{2+} the phosphoenzyme does form slowly, with a rate constant of 6.5 s^{-1}, when the reaction is initiated by the addition of 0.3 mM ATP and 0.25 mM La^{3+} (Table I and Figure 1). This is faster than the formation of EP from Ca·ATP, with a rate constant of 3 s^{-1} (Figure 1), but much slower than the reaction with Mg·ATP with $k = 220$ s^{-1} (Petithory & Jencks, 1986).

Formation of the phosphoenzyme with La·ATP and the inhibition of EP formation when La^{3+} binds to the exterior calcium transport sites can explain why Chiesi and Inesi (1979) observed 0.5 nmol/mg of EP and a burst of 2.5 nmol/mg release of P_i when phosphorylation of the enzyme by Mg·ATP was quenched with 10 mM $LaCl_3$. This result shows that La^{3+} does not stop the hydrolysis of EP, so that there is a burst of P_i release, while the 15–20% of the enzyme that is not phosphorylated in the steady state (Stahl & Jencks, 1987) binds La·ATP and is phosphorylated at 6.5 s^{-1} (Figure 1); the phosphoenzyme then undergoes hydrolysis slowly at 0.06 s^{-1} (Figure 6). Following hydrolysis, further phosphorylation of the EP formed from Mg·ATP is prevented by the binding of La^{3+} to the exterior calcium sites. Thus, it is not necessary for La^{3+} to cross the membrane of the SR to stop the reaction (Chiesi & Inesi, 1979).

Rate-Limiting Conformational Change. The results shown in Figures 2, 3, and 8B support the conclusion that a conformational change is rate limiting for the formation of the phosphoenzyme from La·ATP and for its disappearance in the reverse reaction with ADP, after the initial burst of phosphoenzyme disappearance. The burst of phosphoenzyme disappearance demonstrates that phosphoryl transfer is too fast to measure; similar behavior has been observed previously with Mg^{2+} as the catalytic ion (Shigekawa & Dougherty, 1978; Sumida et al., 1980; Pickart & Jencks, 1982; Froehlich & Heller, 1985; Fernandez-Belda & Inesi, 1986; Wang, 1986). The similar dependence of the size of the burst and of the rate constant for the ensuing slow phase on the concentration of ADP (Figure 3) is consistent with the establishment of a rapid equilibrium between EP·ADP and a nonphosphorylated enzyme species with bound ATP. This species is active for catalysis of very rapid phosphoryl transfer between ATP and the enzyme and is designated ${}^{a}E\cdot Ca_2\cdot La\cdot ATP$ (Scheme I).

Scheme IIA

$$ADP + {}^{Mg\sim P}E_{Ca_2} \underset{K_S^{ADP}}{\rightleftharpoons} {}^{Mg\sim P}E_{Ca_2}\cdot ADP \underset{K_P}{\overset{k_{-2}}{\rightleftharpoons}} {}^{a}E\cdot Ca_2\cdot Mg\cdot ATP \underset{K_c}{\overset{k_{-1}}{\rightleftharpoons}} {}^{c}E\cdot Ca_2\cdot Mg\cdot ATP \xrightarrow{k_{-ATP}} Mg\cdot ATP + {}^{c}E\cdot Ca_2$$

The slow phase that limits the rate of disappearance of EP after the burst represents the formation of an additional nonphosphorylated intermediate, ${}^{c}E\cdot Ca_2\cdot La\cdot ATP$. The slow phase is not caused by the dissociation of ATP from ${}^{a}E\cdot Ca_2\cdot La\cdot ATP$ because the reaction was carried out in the presence of a large excess of labeled ATP. The formation of ${}^{c}E\cdot Ca_2La\cdot ATP$ from ${}^{a}E\cdot Ca_2\cdot La\cdot ATP$ represents an approach to equilibrium with a rate-limiting conformational change, as shown in Scheme II. The increases in the size of the burst and the rate constant for the slow phase of the reaction with increasing concentrations of ADP are both consistent with the same value of $K_S^{ADP} = 0.22$ mM and an equilibrium constant of $K_P = 1.94$ for phosphoryl transfer from ${}^{a}E\cdot Ca_2\cdot La\cdot ATP$ to the enzyme when La^{3+} is the catalytic ion. The forward and reverse rate constants for the conformational change that forms ${}^{a}E\cdot Ca_2\cdot La\cdot ATP$ from ${}^{c}E\cdot Ca_2\cdot La\cdot ATP$ are 6.5 s^{-1} and 170 s^{-1}, respectively.

The disappearance of the phosphoenzyme formed from Mg·ATP after mixing with 2.5 mM ADP and labeled ATP is qualitatively the same as that observed for the phosphoenzyme that has La^{3+} at the catalytic site, as shown in Figure 8, panels A and B, respectively. The phosphoenzyme formed from Mg·ATP reaches equilibrium in a biphasic reaction that is complete within 15 ms; it is too fast to provide an accurate estimate of the size of the initial burst. The second phase of the reaction represents the interconversion through a conformational change of ${}^{a}E\cdot Ca_2\cdot Mg\cdot ATP$ and a second nonphosphorylated intermediate with bound ATP, ${}^{c}E\cdot Ca_2\cdot Mg\cdot ATP$. Previous evidence for this conformational change in the opposite direction was obtained from an analysis of the rate constants describing the approach to equilibrium for phosphoenzyme formation from ${}^{c}E\cdot Ca_2$ and Mg·ATP in the presence and absence of 0.25 mM ADP, and from differences in the rate constants for the dissociation of ATP and Ca^{2+} in reactions that were initiated by the addition of ATP to ${}^{c}E\cdot Ca_2$ or the addition of ADP to $Mg\cdot E\sim P\cdot Ca_2$ (Pickart & Jencks, 1982; Petithory & Jencks, 1986; Stahl & Jencks, 1987). This conformational change that follows dephosphorylation is now shown directly as the second phase of the reactions in Figure 8A.

Pickart and Jencks (1982) measured the disappearance of labeled EP at times greater than 10 ms in reactions with ADP in the presence of excess unlabeled ATP. The slow phase that they measured represents the irreversible first-order dissociation of labeled ATP after the equilibrium between $Mg\cdot E\sim P\cdot Ca_2\cdot ADP$, $Mg\cdot E\sim P\cdot Ca_2$, ${}^{a}E\cdot Ca_2\cdot Mg\cdot ATP$, and ${}^{c}E\cdot Ca_2\cdot Mg\cdot ATP$ is rapidly established (Scheme IIA). The ratio of phosphorylated to nonphosphorylated species in this equilibrium mixture at saturating concentrations of ADP ($K_S^{ADP} = 0.73$ mM), obtained by extrapolation of the slow phase to zero time, is defined as $K_{Int} = 0.44$ (Pickart & Jencks, 1982). The value of K_{Int} is related to the equilibrium constant for phosphoryl transfer from ${}^{a}E\cdot Ca_2\cdot Mg\cdot ATP$ to $Mg\cdot E\sim P\cdot Ca_2\cdot ADP$, K_p, and the equilibrium constant for the formation of ${}^{a}E\cdot Ca_2\cdot Mg\cdot ATP$ from ${}^{c}E\cdot Ca_2\cdot Mg\cdot ATP$, K_c, by eq 8. These values for K_S^{ADP} and K_{Int} accurately describe the amounts of EP at

[3] A dissociation constant of (0.9–3) $\times 10^{-9}$ M^2 for two La^{3+} ions bound to the enzyme is also consistent with the observed decreases in EP.

5 s that are shown in Figure 8A.

$$K_{Int} = K_p/(1 + 1/K_c) \quad (8)$$

The Binding of Ligands to $^cE{\cdot}Ca_2$ and Their Dissociation. The binding of La·ATP to $^cE{\cdot}Ca_2$ occurs with higher affinity than does the binding of Mg·ATP; the dissociation constant K_S^{ATP} of approximately 0.12 μM for La·ATP, calculated from the observed rate constants for binding and dissociation, is 100-fold smaller than the dissociation constant of K_S^{ATP} = 12 μM for Mg·ATP (Petithory & Jencks, 1988). The difference in affinity is the result of the much slower rate of dissociation of La·ATP, with $k \sim 0.6$ s^{-1} (Figures 1 and 6), compared to Mg·ATP, with k = 120 s^{-1} (Petithory & Jencks, 1986). The second-order rate constant of 0.5×10^7 M^{-1} s^{-1} (Figure 5) for the binding of La·ATP to $^cE{\cdot}Ca_2$ is similar to values reported for Mg·ATP in the range of $(0.45–1.0) \times 10^7$ M^{-1} s^{-1} (Sumida et al., 1980; Fernandez-Belda & Inesi, 1986; Petithory & Jencks, 1988). The added positive charge and increased charge density of the catalytic La^{3+} ion increases the stability of the $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ complex, presumably because favorable electrostatic interactions between the La^{3+} ion and negatively charged residues at the catalytic site are stronger than those with Mg^{2+}. Electrostatic interactions of this type have been proposed as an explanation for the much higher affinity for binding to hexokinase of lanthanide ion–ATP complexes compared with Mg·ATP (Morrison & Cleland, 1983).

The dissociation of La·ATP during reactions of the phosphoenzyme with ADP is preceded by a conformational change that equilibrates the reactive form of the enzyme, $^aE{\cdot}Ca_2{\cdot}ATP$, with $^cE{\cdot}Ca_2{\cdot}ATP$ (Figure 4). The rate constant for this conformational change can be measured in the reaction with ADP when La^{3+} is the catalytic ion (Figures 2, 4, and 8B), but it is too rapid for reliable measurement when Mg^{2+} occupies the catalytic site (Figure 8A). It can be shown that all of the observed dissociation of Mg·ATP that occurs during the reaction with ADP can be accounted for by dissociation from $^cE{\cdot}Ca_2Mg{\cdot}ATP$ that is formed by the conformational change.

In principle, the dissociation of labeled ATP during reactions of $Mg{\cdot}E{\sim}P{\cdot}Ca_2$ with ADP could occur from either $^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$, $^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$, or both. Dissociation of ATP from $^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ occurs with the rate constant k_{-ATP} = 120 s^{-1} (Petithory & Jencks, 1986) whereas the observed rate constant for the dissociation of ATP from the equilibrium mixture of all forms of the enzyme with bound ATP is 37 s^{-1} in reactions of the phosphoenzyme with ADP (Pickart & Jencks, 1982). All of the dissociation of ATP can be accounted for by dissociation from $^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ if the rate constant for the formation of $^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ is k_{-c} = 200 s^{-1} (eq 9).

$$^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP \underset{K_c}{\overset{k_{-c}}{\underset{220\ s^{-1}}{\rightleftharpoons}}} {}^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP \xrightarrow{120\ s^{-1}} {}^cE{\cdot}Ca_2 + Mg{\cdot}ATP \quad (9)$$

The value of 200 s^{-1} was estimated by using the method of Cleland for calculating the net rate constant in a single turnover (Cleland, 1975). The observed rate constant of k = 37 s^{-1} is less than 200 s^{-1} because only 0.52 of the nonphosphorylated enzyme that is formed after the addition of ADP is in the form $^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$, which is in rapid equilibrium with the phosphoenzyme. Furthermore, 65% of this fraction that reacts with a rate constant of k_{-c} = 200 s^{-1} to give $^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ will re-form $^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ with k = 220 s^{-1}, instead of dissociating Mg·ATP with k = 120 s^{-1} (eq 10).

The range of acceptable values for k_{-c} is constrained for two reasons: values of $k_{-c} > 200$ s^{-1} would result in faster dissociation of ATP than is observed, with k = 37 s^{-1}, and values of $k_{-c} < 200$ s^{-1} would decrease K_p and increase the size of the burst in the reactions with ADP. This is required by eq 8 and the known values of K_{Int} = 0.44 and $K_c = k_c/k_{-c}$ = 220 s$^{-1}/k_{-c}$. These increases, in turn, result in an increasingly poor fit to the data for the minimal rate constant of 220 s^{-1}, which describes the approach to equilibrium that follows phosphoryl transfer to ADP. Thus, the data in Figure 8A are consistent with dissociation of all the ATP from $^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$; there is no experimental requirement for dissociation of ATP from $^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$. Petithory and Jencks (1986) proposed that one purpose of the conformational change could be to properly align the γ-phosphate of ATP for rapid phosphoryl transfer to aspartate 351 of the enzyme. In addition, the conformational change appears to prevent the dissociation of ATP from $^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ and $^aE{\cdot}Ca_2{\cdot}La{\cdot}ATP$; in this conformation of the enzyme the bound ATP is occluded. In contrast, the dissociation of Ca^{2+} in the reaction of $Mg{\cdot}E{\sim}P{\cdot}Ca_2$ with ADP must occur from both $^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ and $^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ in order to account for the observed rate of phosphoenzyme disappearance in the presence of EGTA (Stahl & Jencks, 1987).

$$37\ s^{-1} = \frac{[^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP]}{[^aE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP + {}^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP]}\frac{k_{-c}(120\ s^{-1})}{(120\ s^{-1}) + (220\ s^{-1})} \quad (10)$$

The rate constant for the irreversible dissociation of Ca^{2+} from $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ that leads to forms of the enzyme that do not phosphorylate is ≤ 1 s^{-1} (Figure 1). This finding is surprising because similar experiments with Mg^{2+} as the catalytic ion have shown that Ca^{2+} dissociates from $^cE{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ with a rate constant of k = 80 s^{-1} (Petithory & Jencks, 1986); calcium dissociates from $^cE{\cdot}Ca_2$ with k = 60 s^{-1} in the absence of ATP (Petithory & Jencks, 1988). The dissociation of calcium from $^cE{\cdot}Ca_2$ is slowed considerably in the presence of 10 mM La^{3+} (Inesi, 1987). This observation led to the proposal that the transport sites exist in a crevice and La^{3+} can bind to the opening of the crevice and trap the calcium that is bound at the transport sites (Inesi, 1988). The very slow dissociation of calcium from $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ suggests that the slow dissociation of calcium in the presence of 10 mM La^{3+} may be caused by binding of La^{3+} at the catalytic site rather than at the opening of a crevice in which the exterior calcium transport sites reside. Large changes in the rate of calcium dissociation from the low-affinity transport sites of the phosphoenzyme have been observed previously when the catalytic ion is varied (Wakabayashi & Shigekawa, 1987). Removal of the catalytic ion from EP results in the formation of a metal-free phosphoenzyme that is unreactive toward ADP (Takakuwa & Kanazawa, 1979; Dupont, 1980) and which apparently allows outward flow of calcium from the SRV (Chiesi & Wen, 1983); the enzyme becomes uncoupled.

The phosphoenzyme that is formed from La·ATP and $^cE{\cdot}Ca_2$ undergoes hydrolysis slowly; the observed rate constant shown in Figure 6 is 0.06 s^{-1}, which is consistent with previous reports that the disappearance of EP formed in the presence of La^{3+} or when La^{3+} is substituted for Ca^{2+} at the catalytic site is slow (Inesi & Lewis, 1987; Wakabayashi & Shigekawa, 1988). Wakabayashi and Shigekawa (1988) have shown that the rate of hydrolysis of phosphoenzyme at 11 °C with La^{3+} at the catalytic site is the same as the rate of dissociation of the calcium ions bound to the transport sites. This suggests that the dissociation of calcium from the phosphoenzyme is rate limiting for turnover and that La^{3+} causes a large decrease

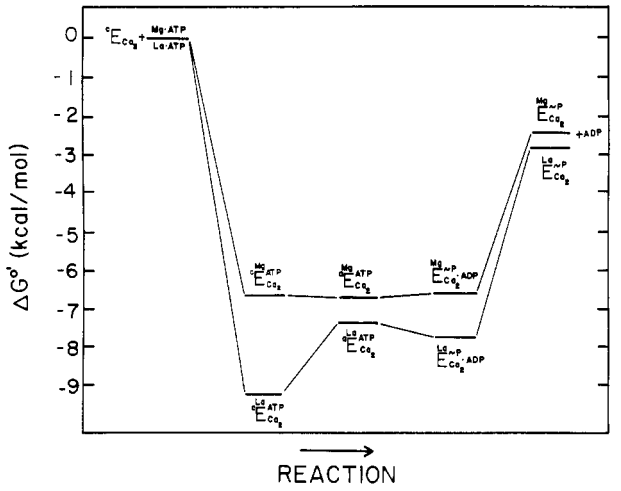

FIGURE 9: Gibbs free energy diagrams for the formation of the phosphoenzyme and ADP from $^cE{\cdot}Ca_2$ and either La·ATP or Mg·ATP. The values of $\Delta G^{\circ\prime}$ are calculated for the equilibrium constants given in Table II and are valid for the following conditions: pH 7.0, 25 °C, 100 mM KCl, and a standard state of 1 M for all reactants and products except hydrogen ion.

in this rate, while the hydrolysis reaction is relatively fast. Thus, La^{3+} decreases the observed rate constant for dissociation of Ca^{2+} from the transport sites to the inside, as well as to the outside, of the vesicle.

These observations indicate that there is a strong functional interaction between the catalytic ion and the calcium transport sites and that changes in the size or charge of the ion bound to the catalytic site can have large effects on the dissociation of calcium from the transport sites. A model for the enzyme proposed by MacLennan, Gren, and co-workers (MacLennan et al., 1985) predicts that these effects occur over a distance of 50–100 Å (Brandl et al., 1986). However, NMR (Klemens & Grisham, 1988) and energy-transfer measurements (Herrmann et al., 1986) have led to the conclusion that the binding site for the catalytic ion may be ≤10 Å from the calcium transport sites.

Free Energy Profile for Phosphorylation of the Enzyme by ATP. The Gibbs free energy profiles for reaction of $^cE{\cdot}Ca_2$ with La·ATP and with Mg·ATP to form phosphoenzyme and ADP are compared in Figure 9. The changes in the standard free energies for the species shown were calculated from the equilibrium constants listed in Table II and refer to 1 M substrates and products at pH 7.0, in 100 mM KCl at 25 °C. Inspection of the two profiles reveals that the changes in free energy for the phosphoryl-transfer step and the dissociation of ADP from the phosphoenzyme are surprisingly similar. Striking differences are apparent, however, in the free energy changes for the binding of ATP to $^cE{\cdot}Ca_2$ and for the conformational change that forms $^cE{\cdot}Ca_2{\cdot}ATP$, the species of enzyme that is activated to catalyze rapid phosphoryl transfer. This conformational change is approximately isoenergetic when Mg·ATP is bound to $^cE{\cdot}Ca_2$ but becomes unfavorable by 1.9 kcal/mol when La^{3+} is substituted for Mg^{2+}. Thus, the increase in the barrier for the formation of $^aE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ is primarily thermodynamic, not kinetic; it arises from the added ground-state stabilization of $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ that results from favorable interactions of the enzyme and the catalytic La^{3+} ion. These interactions are decreased when the enzyme undergoes the change in structure that permits rapid phosphoryl transfer from ATP to the enzyme. The stabilization that results from these interactions leads to the decrease in the observed rate constant from 220 s^{-1} to 6.5 s^{-1} for phosphoenzyme formation when La^{3+} replaces Mg^{2+}. The rate constants of k_{-c} = 200 s^{-1} and 170 s^{-1} for the reverse reaction with Mg·ATP and La·ATP, respectively, are very similar.

Comparing the two reactions illustrates that in order to bring about acceptable rates of catalysis, an enzyme must avoid the formation of overly stable intermediates (Pauling, 1946; Jencks, 1980b; Fersht, 1985). Failure to do so in the formation of $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ decreases both the equilibrium constant and the forward rate constant for the conformational change that is rate limiting for phosphoenzyme formation.

Other Matters. The disappearance of the phosphoenzyme at 0.06 s^{-1}, which is rate limiting for turnover when La·ATP is the substrate, is approximately 200-fold smaller than the turnover rate of 12–17 s^{-1} seen with Mg·ATP (Chiesi & Inesi, 1979; Pickart & Jencks, 1984). Phosphorylated forms of the enzyme will accumulate when the concentration of ADP is low because of this small rate constant and because most of the $^cE{\cdot}Ca_2{\cdot}La{\cdot}ATP$ that is formed goes on to phosphorylate at 6.5 s^{-1} (Figure 6) instead of dissociating La·ATP slowly with k = 0.6 ± 0.4 s^{-1} (Figures 1 and 6). This can explain why Yamada and Tonomura (1972) reported a decrease in the rate of steady-state turnover and an increase in the levels of phosphoenzyme when increasing amounts of La^{3+} in the range of 0.1–25 μM were added to reactions containing 30 μM Mg^{2+} and 20 μM ATP.

The observed rate constant of 3 s^{-1} for the formation of phosphoenzyme from Ca·ATP is half as large as k = 6.5 s^{-1} for La·ATP (Figure 1), but there is no definite burst of phosphoenzyme disappearance in the reactions with 2.5 mM ADP (Figure 7). Therefore, it is not known whether a conformational change or the phosphoryl-transfer step is rate limiting for phosphorylation with Ca·ATP.

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CORRECTIONS

Iron(II)–Ethylenediaminetetraacetic Acid Catalyzed Cleavage of DNA Is Highly Specific for Duplex DNA, by Maria J. Jezewska, Wlodzimierz Bujalowski, and Timothy M. Lohman*, Volume 28, Number 15, July 25, 1989, pages 6161–6164.

We have repeated a study of the Fe(II)–EDTA-catalyzed cleavage of single-stranded (ss) vs duplex DNA that was recently reported in the above mentioned paper in order to obtain a quantitative estimate of the relative preference for cleavage of duplex DNA. Upon repeating these studies, we did *not* observe the dramatic difference in cleavage of ss vs duplex DNA that we had previously reported. We do still observe that the duplex [$d(pT)_{70}$·$dA(pA)_{69}$] is cleaved preferentially compared to $d(pT)_{70}$ (pH 7.2); however, this preference is *only moderate*, rather than highly specific as we originally reported. The disappearance of the ^{32}P-labeled intact $d(pT)_{70}$ when single stranded vs when in a duplex with $dA(pA)_{69}$ was monitored after separation of the reaction products by polyacrylamide gel electrophoresis (in 7 M urea). The amount of ^{32}P-labeled $d(pT)_{70}$ was quantitated with a Betascope 603 blot analyzer (Betagen, Waltham, MA). The cleavage reaction was performed as a function of the concentration of Fe(II)–EDTA (20, 50, 100, and 200 μM) as described previously. The results indicate only a slight preference (factor of 1.3–2) for cleavage of duplex DNA, based on six repetitions of this experiment. At this time we cannot explain the basis for the different quantitative results that we now observe vs those reported previously. On the basis of these recent data, we conclude that the cleavage of DNA that is catalyzed by Fe(II)–EDTA shows only a modest preference for duplex over single-stranded nucleic acids, which is in agreement with the recent report by Celander and Cech [Celander, D. W., & Cech, T. R. (1990) *Biochemistry 29*, 1355–1361], who also reported only a slight preference for cleavage of duplex over single-stranded DNA and RNA. In any event, this small preference for cleavage of duplex DNA indicates that the mechanism of the cleavage reaction requires further study.

Glycinamide Ribonucleotide Synthetase from *Escherichia coli*: Cloning, Overproduction, Sequencing, Isolation, and Characterization, by Y. S. Cheng, J. Rudolph, M. Stern, J. Stubbe,* K. A. Flannigan, and J. M. Smith*, Volume 29, Number 1, January 9, 1990, pages 218–227.

Page 218. The first author on this paper should be Y. S. Cheng and *not* Y. Shen.