Phosphorylation of the Calcium Adenosinetriphosphatase of Sarcoplasmic Reticulum: Rate-Limiting Conformational Change Followed by Rapid Phosphoryl Transfer†

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ABSTRACT: The calcium adenosinetriphosphatase of sarcoplasmic reticulum, preincubated with Ca^{2+} on the vesicle exterior ($^{c}E{\cdot}Ca_2$), reacts with 0.3–0.5 mM Mg·ATP to form covalent phosphoenzyme ($E{\sim}P{\cdot}Ca_2$) with an observed rate constant of 220 s^{-1} (pH 7.0, 25 °C, 100 mM KCl, 5 mM $MgSO_4$, 23 μM free external Ca^{2+}, intact SR vesicles passively loaded with 20 mM Ca^{2+}). If the phosphoryl-transfer step were rate-limiting, with k_f = 220 s^{-1}, the approach to equilibrium in the presence of ADP, to give 50% EP and $k_f = k_r$, would follow $k_{obsd} = k_f + k_r$ = 440 s^{-1}. The reaction of $^{c}E{\cdot}Ca_2$ with 0.8–1.2 mM ATP plus 0.25 mM ADP proceeds to 50% completion with k_{obsd} = 270 s^{-1}. This result shows that phosphoryl transfer from bound ATP to the enzyme is not the rate-limiting step for phosphoenzyme formation from $^{c}E{\cdot}Ca_2$. The result is consistent with a rate-limiting conformational change of the $^{c}E{\cdot}Ca_2{\cdot}ATP$ intermediate followed by rapid ($\geq$1000 s^{-1}) phosphoryl transfer. Calcium dissociates from $^{c}E{\cdot}Ca_2{\cdot}ATP$ with k_{obsd} = 80 s^{-1} and ATP dissociates with k_{obsd} = 120 s^{-1} when $^{c}E{\cdot}Ca_2{\cdot}ATP$ is formed by the addition of ATP to $^{c}E{\cdot}Ca_2$. However, when $E{\cdot}Ca_2{\cdot}ATP$ is formed in the reverse direction, from the reaction of $E{\sim}P{\cdot}Ca_2$ and ADP, Ca^{2+} dissociates with k_{obsd} = 45 s^{-1} and ATP dissociates with k_{obsd} = 35 s^{-1}. This shows that different $E{\cdot}Ca_2{\cdot}ATP$ intermediates are generated in the forward and reverse directions, which are interconverted by a conformational change.

We report here kinetic experiments using the rapid mix–quench technique that demonstrate a conformational change of the $^{c}E{\cdot}Ca_2{\cdot}ATP$ complex, formed from the calcium ATPase of SR[1] following addition of ATP to $^{c}E{\cdot}Ca_2$, that is rate-limiting for phosphoenzyme formation. The fact that a kinetically significant conformational change occurs following ATP binding to $^{c}E{\cdot}Ca_2$ shows that enzyme with Ca^{2+} bound to the exterior, high-affinity sites is not yet in a form capable of reacting with ATP. This means that changes in vectorial specificity (exterior vs. interior Ca^{2+} binding/dissociation) and chemical specificity (ATP/ADP vs. P_i/H_2O reactivity) do not occur simultaneously, as implied in the classical "E_1–E_2" (or "E–E*") model for active transport (deMeis & Vianna, 1979), but occur in separate steps along the reaction pathway.

Materials and Methods

Materials. Reagents were generally of the highest available purity and were used without further purification. Na_2ATP (Sonderqualitat) and K-ADP were from Boehringer Mannheim; [γ-^{32}P]ATP (>99% pure) was from New England Nuclear.

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by a slight modification of the MacLennan procedure, as described previously (Pickart & Jencks, 1982). The preparations hydrolyzed ATP at 3.5–5.0 μmol (mg of total protein)$^{-1}$ min^{-1} when the vesicles were made permeable with the calcium ionophore A23187. SRV as isolated were ~98% sealed, as shown by a 50-fold increase in the steady-state hydrolysis rate upon addition of ionophore in the standard assay. The amount of phosphoenzyme observed with intact vesicles at saturating [Ca^{2+}] and [ATP] was 2.0–3.0 nmol (mg of total protein)$^{-1}$.

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

Concentrations of free calcium were calculated from a dissociation constant of 7.4 × 10^{-7} M for Ca·EGTA (Godt, 1974). This value was determined under conditions (pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP) similar to those used in our experiments. Protein concentration was estimated by the procedure of Lowry et al. (1951) using bovine serum albumin as a standard.

The formation of phosphoenzyme was followed by using a rapid mixing apparatus that can be used with either three or four syringes, as described previously (Stahl & Jencks, 1984). The temperature-equilibrated contents of syringes A and B are driven into a mixing block, and after flowing through a length of narrow-bore tubing made of Teflon for an amount of time t_1, the reaction is quenched in a second mixing block by hydrochloric acid in syringe C. For four-syringe experiments, another reactant is placed in syringe C and quenching by acid in syringe D takes place in an additional mixing block after an amount of time t_2. The reaction times were calibrated from measurements of 2,4-dinitrophenyl acetate hydrolysis by hydroxide ion (Barman & Gutfreund, 1964).

SRV were passively loaded with Ca^{2+} by incubation for 12–16 h at 4 °C in a solution containing 30 mg/mL SRV

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[1] Abbreviations: SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles; E, calcium adenosinetriphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; PEP, phosphoenolpyruvate; AMP-P(NH)P, adenyl-5'-yl imidodiphosphate; P_i, inorganic phosphate.

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Table I: Rate Constants and the Fraction EP/E_{tot} Formed in the Reaction of $^cE{\cdot}Ca_2$ with ATP ± ADP[a]

$[ATP]_{tot}$ (mM)	$[ADP]_{tot}$ (mM)	$[MgSO_4]$ (mM)	k_{obsd} (s^{-1})	EP/E_{tot}	[Mg·ATP][b] (mM)	[Mg·ADP] (mM)
0.20	0	5.0	190	1.0	0.20	0
0.50	0	5.0	220	1.0	0.50	0
0.50	0	0.9	240	1.0	0.49	0
0.20	0.25	5.0	128	0.50	0.20	0.24
0.20	0.25	5.0	144	0.47	0.20	0.24
0.20	0.25	0.9	206	0.40	0.19	0.20
0.50	0.25	5.0	196	0.52	0.50	0.24
0.50	0.25	1.2	270	0.49	0.48	0.20
0.80	0.25	1.4	260	0.46	0.76	0.20
0.80	0.25	5.0	270	0.51	0.79	0.24
1.20	0.25	1.8	265	0.46	1.13	0.20

[a] The reactions were carried out as described in the legend to Figure 1. Final conditions were 40 mM MOPS, pH 7.0, 100 mM KCl, 1.96 mM $CaCl_2$, 2.00 mM EGTA (23 μM free Ca^{2+}), and ATP, ADP, and $MgSO_4$ concentrations as listed above, 25 °C. [b] The concentrations of Mg·ATP and Mg·ADP were calculated from the dissociation constants given by Pecoraro et al. (1984).

protein, 0.1 M KCl, 5 mM $MgSO_4$, 40 mM MOPS, pH 7.0, 0.2 M sucrose, and 20 mM $CaCl_2$. For each reaction, 10 μL of this stock SRV solution was mixed with 0.99 mL of a Ca·EGTA-buffered solution, lowering the free exterior Ca^{2+} concentration to 23 μM. This solution was loaded into syringe A of the rapid mixing apparatus, and the reaction was started within 30 s. The amount of $[^{32}P]$E-P in the quenched reaction mixtures was determined essentially as described by Verjovski-Almeida et al. (1978). E_{tot} was taken as the amount of phosphoenzyme formed after a 5-s reaction of intact vesicles, quenched manually with acid.

Results and Discussion

Reaction of $^cE{\cdot}Ca_2$ with ATP. The reaction of passively loaded SRV preincubated with 23 μM free exterior Ca^{2+} ($^cE{\cdot}Ca_2$) with 0.3–0.5 mM $[^{32}P]$ATP·Mg results in the formation of $[^{32}P]E{\sim}P{\cdot}Ca_2$ with a first-order rate constant of 220 ± 20 s^{-1} at 25 °C (Figure 1, closed symbols). Since k_{obsd} is independent of [ATP], it is the first-order rate constant for the phosphorylation of $^cE{\cdot}Ca_2$ by bound ATP, k_f (eq 1). A

$$^cE{\cdot}Ca_2 + ATP \rightleftharpoons {}^cE{\cdot}Ca_2{\cdot}ATP \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \begin{cases} E{\sim}P{\cdot}Ca_2{\cdot}ADP \\ \uparrow\downarrow \\ E{\sim}P{\cdot}Ca_2 + ADP \end{cases} \quad (1)$$

rate constant of 150 s^{-1} was estimated by Froehlich and Taylor (1975) for phosphorylation of $^cE{\cdot}Ca_2$ by 100 μM ATP at 20 °C; we observe the same rate constant upon phosphorylation by 100 μM ATP at 25 °C (Figure 3 and unpublished results). The phosphorylation reaction proceeds to completion, because EP hydrolysis is inhibited by >95% in calcium-loaded vesicles. The back-reaction with ADP (k_r, eq 1) is not significant under these conditions because the amount of ADP produced is less than 1 μM; the dissociation constant of ADP from $E{\sim}P{\cdot}Ca_2$ is 0.73 mM (Pickart & Jencks, 1984).

Reaction of $^cE{\cdot}Ca_2$ with ATP plus ADP. The following experiments were performed in order to identify the rate-limiting step for the phosphorylation of $^cE{\cdot}Ca_2$ by bound ATP. If the rate-limiting step were phosphoryl transfer, then the observed rate constant for approach to the equilibrium concentration of phosphoenzyme in the presence of ADP would equal the sum of the first-order rate constants for the forward and the ADP concentration dependent reverse phosphoryl-transfer steps; i.e., $k_{obsd} = k_f + k_r$ (Frost & Pearson, 1953).[2]

[2] The reverse phosphoryl-transfer step follows a pseudo-first-order rate constant, k_r, which is equal to the rate constant for reaction of $E{\sim}P{\cdot}Ca_2{\cdot}ADP$ multiplied by the fraction of phosphoenzyme with ADP bound. The binding and dissociation of ADP with $E{\sim}P{\cdot}Ca_2$ is very fast, with $k_{on} > 5 \times 10^6$ M^{-1} s^{-1} and $k_{off} > 3700$ s^{-1} (Pickart & Jencks, 1984).

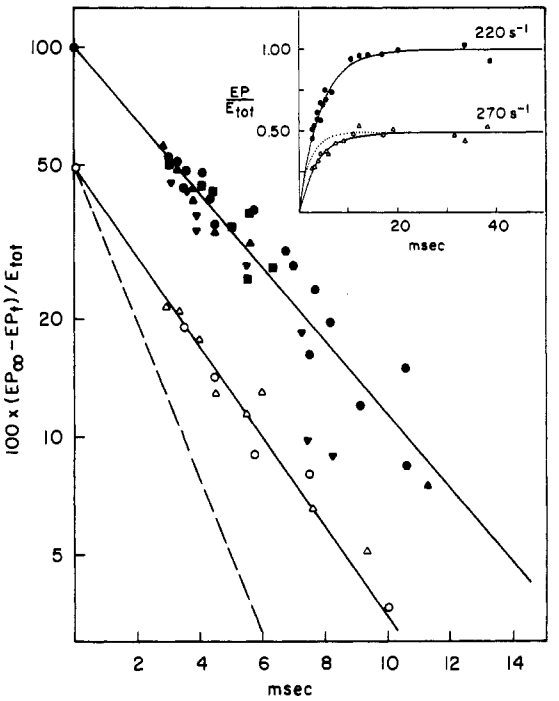

FIGURE 1: Reaction of $^cE{\cdot}Ca_2$ with ATP ± ADP. Final conditions were 40 mM MOPS, pH 7.0, 100 mM KCl, 1.96 mM $CaCl_2$, 2.00 mM EGTA (23 μM free Ca^{2+}), 5.0 mM $MgSO_4$ (unless otherwise specified), 0.30 (●), 0.40 (▲), 0.50 (▼, ■, △), or 0.80 mM (○) [γ-^{32}P]ATP, 0.25 mM ADP (○, △), and 1.2 mM $MgSO_4$ (▲, ■), 25 °C. Syringe A contained 0.3 mg/mL SRV passively loaded with Ca^{2+}. Syringe B contained [γ-^{32}P]ATP at twice the final concentrations (●, ▲, ▼, ■), plus 0.50 mM ADP (○, △). Syringe C contained 1.5 M HCl and 40 mM KH_2PO_4. All other components were present in syringes A and B at the final concentrations. The inset shows the data for final concentrations of 0.4 and 0.5 mM ATP combined (●) and for 0.5 mM ATP, 0.25 mM ADP, and 1.2 mM $MgSO_4$ (△) plotted directly. The solid lines were drawn for first-order rate constants of 220 s^{-1} (closed symbols, no ADP) and 270 s^{-1} (open symbols, +0.25 mM ADP). The dashed lines are drawn for a first-order rate constant of 440 s^{-1}.

Phosphorylating the enzyme in the presence of sufficient [ADP] to lower the equilibrium concentration of EP to 50% of E_{tot} requires that $k_f = k_r = 220$ s^{-1} and, therefore, a value of $k_{obsd} = 220 + 220 = 440$ s^{-1} according to this model. However, if some step other than phosphoryl transfer, i.e., a conformational change, were the rate-limiting step, k_{obsd} would not increase from 220 s^{-1} because the rate-limiting step would not be involved in the equilibration of phosphoryl transfer.

Figure 1 shows that the phosphorylation reaction in the presence of 0.25 mM ADP proceeds to equilibrium to give 50% of the maximum phosphoenzyme with an observed first-order rate constant of 260–270 s^{-1}. This does not approach the 440

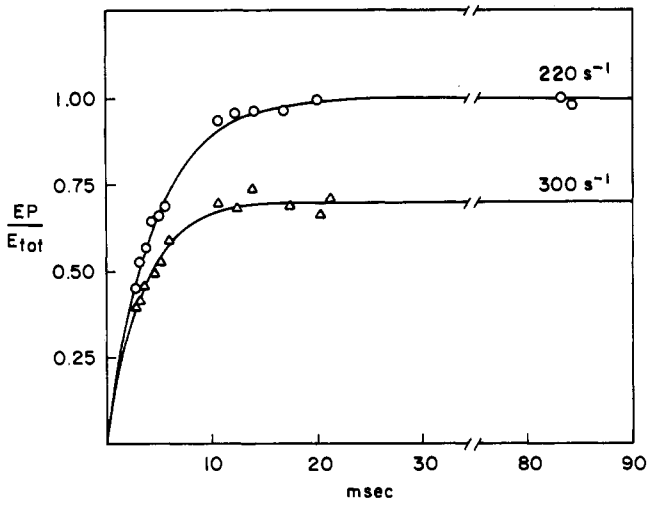

FIGURE 2: Reaction of $^{c}E{\cdot}Ca_2$ with ATP ± EGTA. Final conditions were 40 mM MOPS, pH 7.0, 0.40 mM [^{32}P]ATP, 100 mM KCl, 5.0 mM $MgSO_4$, and 0.40 mM $CaCl_2$ and 0.39 mM EGTA (22 μM free Ca^{2+}) (O) or 0.2 mM $CaCl_2$ and 5.2 mM EGTA (~30 nM free Ca^{2+}) (Δ), 25 °C. Reactions were performed essentially as described in Figure 1, except that syringe B contained 0.40 mM $CaCl_2$ and 0.39 mM EGTA (O) or 10 mM EGTA (Δ), in addition to 0.80 mM [^{32}P]ATP. All other components were present in syringes A and B at the final concentrations. The lines are drawn for first-order rate constants of 220 s^{-1} (O) and 300 s^{-1} (Δ).

s^{-1} predicted for rate-limiting phosphoryl transfer (Figure 1, dashed lines). The fact that k_{obsd} and $[EP]/[E_{tot}]$ are independent of [Mg·ATP] in the range 0.5–1.2 mM, with 1.2–1.8 mM $MgSO_4$ and 0.20–0.24 mM ADP (Table I), rules out the possibility that ADP is competing with ATP for $^{c}E{\cdot}Ca_2$. At lower [ATP], ADP does inhibit the reaction (Table I), consistent with a value of K_i in the range 0.05–0.12 mM reported by others for ADP binding to $^{c}E{\cdot}Ca_2$ (Yates & Duance, 1976; Pickart & Jencks, 1984). It is concluded that a rate-limiting conformational change of the $^{c}E{\cdot}Ca_2{\cdot}ATP$ complex occurs (k_c, eq 2) that results in an intermediate, denoted $^{a}E{\cdot}Ca_2{\cdot}ATP$,

$$^{c}E{\cdot}Ca_2 + ATP \rightleftharpoons {}^{c}E{\cdot}Ca_2{\cdot}ATP \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} {}^{a}E{\cdot}Ca_2{\cdot}ATP \underset{k_{-p}}{\overset{k_p}{\rightleftharpoons}} E{\sim}P{\cdot}Ca_2 + ADP \quad (2)$$

which is active for catalysis of rapid, reversible phosphoryl transfer. The increase in k_{obsd} from 220 to 270 s^{-1} in the presence of ADP, if it is real, might represent a small contribution of the k_{-c} or k_p step to the observed rate constant for approach to equilibrium. The absence of a detectable lag in the phosphorylation kinetics requires that the rate constant for phosphoryl transfer (k_p, eq 2) must be $\geq$1000 s^{-1}, from simulations of the time course (not shown). A limit of $k_p \geq 500$ s^{-1} was obtained by Stahl and Jencks (1984) for phosphorylation of the enzyme through a different route, after a rate-limiting conformational change with k_a = 70 s^{-1} upon the simultaneous addition of ATP and calcium to the enzyme.

Additional evidence for a conformational change of the $^{c}E{\cdot}Ca_2{\cdot}ATP$ intermediate prior to phosphoryl transfer has been obtained by monitoring changes in the ESR spectrum of spin-labeled $^{c}E{\cdot}Ca_2$ upon addition of the nonhydrolyzable analogue AMP-P(NH)P (Coan & Inesi, 1977). A change in ESR spectrum upon addition of ATP was also seen, which probably reflects a conformational change upon phosphorylation of the enzyme (Coan et al., 1979).

Dissociation of Ca^{2+} from $^{c}E{\cdot}Ca_2{\cdot}ATP$. Two independent observations show that Ca^{2+} dissociates from $^{c}E{\cdot}Ca_2{\cdot}ATP$ with a rate constant of k_{-Ca} = 80 s^{-1}. The reaction of $^{c}E{\cdot}Ca_2$ with 0.4 mM [^{32}P]ATP and 5.0 mM EGTA results in the phosphorylation of 70% of the enzyme with k_{obsd} = 300 s^{-1} (Figure 2, triangles). This observed rate constant represents the sum of the first-order rate constants for phosphorylation (k_f, 220 s^{-1}) and irreversible Ca^{2+} dissociation (k_{-Ca}), because Ca^{2+} dissociation prevents phosphorylation but provides a new pathway for the disappearance of $E{\cdot}Ca_2{\cdot}ATP$ (eq 3). The

FIGURE 3: Pulse–chase measurement of ATP dissociation from $^{c}E{\cdot}Ca_2{\cdot}ATP$. Final conditions were 40 mM MOPS, pH 7.0, 100 mM KCl, 5.0 mM $MgSO_4$, 1.96 mM $CaCl_2$, 2.00 mM EGTA (23 μM free Ca^{2+}), and 0.10 mM [^{32}P]ATP (O, Δ) or 0.067 mM [^{32}P]ATP and 3.33 mM nonradioactive ATP (●, ▲), 25 °C. Syringe A contained 0.3 mg/mL passively loaded SRV. Syringe B contained 0.20 mM [^{32}P]ATP, ~30000 cpm/nmol. Syringe C contained ATP chase (10 mM ATP, 10 mM $MgSO_4$, 0.5 mM PEP, 0.01 mg/mL pyruvate kinase). Syringe D contained 2.0 M HCl and 50 mM KH_2PO_4. All other components were present in syringes A, B, and C at the final concentrations. The contents of syringes A and B were mixed for t_1 = 5 (O, ●) or 30 ms (Δ, ▲), followed by ATP chase for varying times t_2 prior to acid quench (●, ▲). For t_2 = 0 (O, Δ) the reactions were quenched with acid after t_1 by reversing the order of addition of the contents of syringes C and D. The dashed lines are drawn for the hydrolysis of EP in loaded vesicles with a k_{obsd} of 2.2 s^{-1}; they extrapolate back to t_2 = 0 at EP/E_{tot} = 1.00 (▲, Δ), 0.83 (●), and 0.50 (O). The solid lines represent phosphorylation of $^{c}E{\cdot}Ca_2$ by 100 μM ATP with k_{obsd} = 150 s^{-1}. The hatched area signifies [^{32}P]$EP{\cdot}Ca_2$ formed as a result of the 5-ms [^{32}P]ATP pulse, which can be ignored.

$$^{c}E{\cdot}Ca_2 + ATP \rightleftharpoons {}^{c}E{\cdot}Ca_2{\cdot}ATP \xrightarrow{k_f = 220\ s^{-1}} E{\sim}P{\cdot}Ca_2 + ADP \quad (3)$$
$$\qquad\qquad\qquad\qquad\qquad\qquad \searrow^{k_{-Ca}} \quad E{\cdot}ATP$$

value of k_{obsd} = 300 s^{-1} gives k_{-Ca} = 300 − 220 = 80 s^{-1}. The observed fraction of total enzyme phosphorylated is equal to $k_f/(k_f + k_{-Ca})$, if all of the Ca^{2+} dissocation occurs from $^{c}E{\cdot}Ca_2{\cdot}ATP$. The observed rate constant of k_{-Ca} = 80 s^{-1} gives a calculated fraction of 220/(220 + 80) = 0.73 phosphorylated enzyme, which agrees well with the observed fraction phosphorylated of 0.70 (Figure 2).

This result shows that, at 400 μM ATP, the pseudo-first-order rate constant for ATP binding is much faster than the first-order rate constant for Ca^{2+} dissociation from $^{c}E{\cdot}Ca_2$ (Sumida et al., 1978) and that Ca^{2+} dissociation occurs from $^{c}E{\cdot}Ca_2{\cdot}ATP$ rather than from a species that is formed after the k_c step (eq 2).

Ikemoto et al. (1981) showed that the reaction of $^{c}E{\cdot}Ca_2$ with 5 μM [^{32}P]ATP and 5 mM EGTA at 4 °C gives 50% phosphorylated enzyme. We observe at 25 °C a hyperbolic relationship between [ATP] and the amount of EP formed that levels off at 70% EP, in the reaction of $^{c}E{\cdot}Ca_2$ with ATP plus 5 mM EGTA; 50% of E_{tot} forms EP at 18 μM ATP under these conditions (J. R. Petithory and W. P. Jencks, unpublished

results). This behavior is consistent with competition between the rate constant for Ca^{2+} dissocation and the pseudo-first-order rate constant for binding of ATP to $^cE{\cdot}Ca_2$.

The rate constant of 80 s^{-1} for Ca^{2+} dissociation from $^cE{\cdot}Ca_2{\cdot}ATP$ is significantly faster than the rate constant of 45–52 s^{-1} measured for Ca^{2+} dissociation from $E{\cdot}Ca_2{\cdot}ATP$ that is formed from the reaction of $E{\sim}P{\cdot}Ca_2$ and ADP in the presence of EGTA (Pickart & Jencks, 1982; N. Stahl and W. P. Jencks, unpublished results). This result is inconsistent with only a single $E{\cdot}Ca_2{\cdot}ATP$ intermediate; it is consistent with two different intermediates that are formed in the forward and reverse directions and are interconverted by a conformational change.

Dissociation of ATP from $^cE{\cdot}Ca_2{\cdot}ATP$. The reaction of 100 μM [^{32}P]ATP and $^cE{\cdot}Ca_2$ for t_1 = 5 ms followed by an acid quench results in 50% phosphorylation (Figure 3, open circles), while quenching after a 30-ms pulse (t_1) gives 100% phosphorylation (open triangles), with $k_{obsd} \simeq 150$ s^{-1}. Addition of a chase of excess nonradioactive ATP after the 5-ms pulse of [^{32}P]ATP, followed by an acid quench after varying times t_2, gives 83% of the total enzyme as [^{32}P]EP (Figure 3, closed circles). A nonradioactive ATP chase following the 30-ms pulse results in a slow disappearance of [^{32}P]EP with k_{obsd} = 2.2 s^{-1} that is due to turnover of EP in calcium-filled vesicles in the presence of 3.3 mM ATP (closed triangles).

The increase in the amount of [^{32}P]EP formed during the nonradioactive ATP chase after the 5-ms pulse of [^{32}P]ATP shows that bound [^{32}P]ATP does not exchange rapidly with the nonradioactive ATP in the medium, in agreement with previous results (Pickart & Jencks, 1982; Shigekawa & Kanazawa, 1982; Stahl & Jencks, 1984; Froehlich & Heller, 1985). The fraction of unphosphorylated enzyme that forms [^{32}P]EP after addition of the unlabeled ATP chase is a measure of the partitioning of $^cE{\cdot}Ca_2{\cdot}$[^{32}P]ATP between phosphorylation (k_f, 220 s^{-1}) and irreversible [^{32}P]ATP dissociation (k_{-ATP}) (eq 4). The fraction of $^cE{\cdot}Ca_2{\cdot}$[^{32}P]ATP that partitions toward phosphorylation is (0.83 − 0.50)/(1.0 − 0.50) = 0.65, which equals $k_f/(k_f + k_{-ATP})$; therefore, k_{-ATP} = 120 ± 20 s^{-1}. This is much faster than the observed rate constant of 35 ± 3 s^{-1} for dissociation of ATP from the $E{\cdot}Ca_2{\cdot}ATP$ complex formed from the reaction of $E{\sim}P{\cdot}Ca_2$ and ADP (Pickart & Jencks, 1982; N. Stahl and W. P. Jencks, unpublished results). This result is inconsistent with a single $E{\cdot}Ca_2{\cdot}ATP$ species; it requires two intermediates that can be formed in the forward and reverse directions and are interconverted by a conformational change.

$$^cE{\cdot}Ca_2 + ATP^* \rightleftharpoons {}^cE{\cdot}Ca_2{\cdot}ATP^* \xrightarrow{k_f = 220\ s^{-1}} E{\sim}P^*{\cdot}Ca_2 + ADP \qquad (4)$$
$$\searrow^{k_{-ATP}} E{\cdot}Ca_2 + ATP^*$$

The phosphorylation reaction acts as a vectorial "switch" (Jencks, 1983) that tells the enzyme whether to bind Ca^{2+} from the exterior or the interior of the vesicle (deMeis & Vianna, 1979). The covalent phosphoenzyme $E{\sim}P{\cdot}Ca_2$ can transfer phosphate to ADP and can dissociate Ca^{2+} into a high concentration of calcium inside the vesicle. This is accomplished via a mutual destabilization of Ca^{2+} and phosphate in $E{\sim}P{\cdot}Ca_2$ (Weber, 1972; Jencks, 1980; Hill & Eisenberg, 1981; Tanford, 1982; Pickart & Jencks, 1984). However, there is no mutual destabilization of phosphate and Ca^{2+} when the phosphate is covalently attached to bound ADP instead of the enzyme (Pickart & Jencks, 1984). It is reasonable to speculate that the site occupied by phosphate is different in $^cE{\cdot}Ca_2{\cdot}ATP$ and $E{\sim}P{\cdot}Ca_2$ (McIntosh & Boyer, 1983; Pickart & Jencks, 1984; Inesi, 1985). MacLennan et al. (1985) proposed that nucleotide binding and phosphorylation occur on different domains of the enzyme. It is possible that the conformational change of the $^cE{\cdot}Ca_2{\cdot}ATP$ complex involves movement of the γ-P of ATP toward the phosphorylation site, aligning it to permit rapid, reversible phosphoryl transfer. It is of interest to note that a monoclonal antibody has been prepared against the Na,K-ATPase that binds with high affinity to the catalytic subunit and inhibits Na^+-dependent EP formation without affecting ATP binding, EP hydrolysis, or K^+-dependent *p*-nitrophenylphosphatase activity (Ball, 1986). It is proposed that the antibody restricts the conformational flexibility of the enzyme that permits transfer of the γ-P of ATP from the nucleotide binding domain to the phosphorylation domain.

We conclude that there are several different conformational changes in the reaction cycle of the calcium ATPase, rather than the single conformational change of the E_1–E_2 model.

Registry No. Ca, 7440-70-2; ATPase, 9000-83-3.

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Primary Structure of Bovine Calpactin I Heavy Chain (p36), a Major Cellular Substrate for Retroviral Protein-Tyrosine Kinases: Homology with the Human Phospholipase A_2 Inhibitor Lipocortin†

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ABSTRACT: An amplified Okayama–Berg plasmid cDNA library was constructed from total poly(A)+ RNA isolated from the Madin–Darby bovine kidney cell line MDBK. This library was screened with a partial murine calpactin I heavy chain (p36) cDNA clone, the identification of which was based on bovine p36 tryptic peptide sequences generated during the course of these studies. The largest p36 cDNA insert (p36/6 of 1.6 kilobase pairs) was fully sequenced by the dideoxy method. The DNA sequence of this insert had an open reading frame of 1014 base pairs and coded for a protein with a molecular weight of 38 481. The deduced protein sequence of 338 residues was concordant with 173 residue positions of p36 determined at the protein level. The 5′- and 3′-ends of p36/6 contained 54 and 307 base pairs of untranslated sequence, respectively. Examination of poly(A)+ RNA prepared from the Madin–Darby cell line indicated a p36 mRNA species of about 1.6 kilobases. Four regions of internal homology, each about 70 amino acid residues in length, were observed in the deduced protein sequence for p36. Thirty-three of the 70 residue positions were conserved in at least three of the four repeating units. A comparison of the derived amino acid sequence for bovine p36 with that previously determined for human lipocortin [Wallner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Chow, E. P., Browning, J. L., Ramachandran, K. L., & Pepinsky, R. B. (1986) *Nature (London) 320*, 77–81] revealed extensive homology (66% overall) and the presence of four repetitive regions in the lipocortin structure. Alignment of the four lipocortin repeats revealed that 32 of the 70 residue positions were conserved. Of these, 26 were observed in corresponding positions within the p36 consensus sequence. A highly conserved region within each of the repeating units of p36 and lipocortin further correlated with a 17 amino acid consensus sequence repeat [Geisow, M. J., Fritsche, U., Hexham, J. M., Dash, B., & Johnson, T. (1986) *Nature (London) 320*, 636–638] recently identified in several calcium-binding proteins, including p36, which interact with biomembranes. These data indicate that p36 and lipocortin are members of the same gene family.

Many of the oncogene products and growth factor receptors are known to be active protein-tyrosine kinases (Bishop & Varmus, 1982; Erikson et al., 1980; Hunter & Cooper, 1985; Hunter & Sefton, 1982; Sefton & Hunter, 1984). Among the major substrates for protein-tyrosine kinases in vivo are polypeptides with apparent molecular weights of 34 000–39 000 as estimated by SDS–PAGE[1] (Radke & Martin, 1979; Erikson & Martin, 1980; Radke et al., 1980; Cooper & Hunter, 1981; Erikson et al., 1981a,b; Fava & Cohen, 1984). These include at least two distinct but related proteins, which have recently been termed calpactins (for calcium-dependent phospholipid- and actin-binding proteins) (Glenney, 1986b). Calpactin I, a complex of p36 and p10, has been shown to be located in the cortical skeleton underlying the plasma membrane in fibroblasts (Greenberg & Edelman, 1983; Nigg et al., 1983; Lehto et al., 1983; Radke et al., 1983) and intestinal epithelial cells (Greenberg et al., 1984; Gould et al., 1986; Gerke & Weber, 1984). Calpactin I isolated from porcine or bovine intestine is a heterotetrameter comprised of two molecules each of p36 and p10 (Gerke & Weber, 1984; Glenney & Tack, 1985). Recently, three independent studies have demonstrated that the 10 000 molecular weight chain (p10) is closely related through its primary structure to the Ca^{2+}-binding S-100 proteins of the brain (Glenney & Tack, 1985; Gerke & Weber, 1985a; Hexham et al., 1986). Cal-

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[1] Abbreviations: poly(A)+, poly(adenylic acid) containing; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; kDa, kilodalton(s); bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; TLCK, N^{α}-*p*-tosyllysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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