Cooperative Calcium Binding and ATPase Activation in Sarcoplasmic Reticulum Vesicles*

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(Received for publication, November 13, 1979)

High affinity calcium binding which is involved in ATPase activation was measured following equilibration of sarcoplasmic reticulum vesicles with Ca^{2+} buffers in chromatography columns. Binding occurred with $K_{\text{app}} = 2.3 \times 10^6$ M$^{-1}$ and with a maximal level of 8 to 10 nmol per mg of protein. This value corresponds to 2 calcium ions per ATPase phosphorylation site, since the maximal level of phosphorylated enzyme intermediate obtained in the presence of ATP was 4 to 5 nmol per mg of protein.

Analysis of calcium binding as a function of Ca^{2+} concentration demonstrates a cooperative mechanism including binding to a first site, followed by development of higher affinity and binding to a second site. Accordingly, the best fit of the equilibrium binding curve requires two constants, with a 2 orders of magnitude increase in the second as compared to the first constant. Scatchard and Hill plots also demonstrate a highly cooperative ($n_H = 1.82$) binding mechanism.

High affinity calcium binding is accompanied by a protein conformational change which is revealed by EPR spectroscopy of spin-labeled preparations and is consistent with the analysis developed for the equilibrium binding data. It is apparent that such a conformational change is an integral part of the cooperative binding mechanism and of the enzyme activation following calcium binding.

Kinetic experiments on enzyme phosphorylation upon addition of ATP to enzyme preincubated with Ca^{2+}, as compared to addition of ATP and Ca^{2+} to enzyme deprived of Ca^{2+}, revealed that the Ca^{2+}-induced enzyme activation is a slow step which is influenced by substrate (e.g. ATP) binding.

In conclusion, equilibrium and kinetic analysis indicate the following mechanism for high affinity calcium binding and ATPase activation:

$$E + Ca^{2+} \rightarrow E\cdot Ca \rightarrow E\cdot Ca + Ca^{2+} \rightarrow E\cdot Ca_2.$$ 

Appropriate rate constants for these and subsequent partial reactions generate satisfactory simulations of initial ATPase cycles.

Calcium binding to specific sites of the sarcoplasmic reticulum ATPase is an absolute requirement for enzyme activation and active transport of the divalent cation. Of the various classes of binding sites found in sarcoplasmic reticulum vesicles (1-4), only high affinity sites which are attributed to the ATPase protein (5-7) appear to be specifically involved in enzyme activation. However, owing to considerable experimental difficulty in measurement of high affinity binding, there are still some uncertainties in the value of the binding constant (0.8 to $5.0 \times 10^7$ M$^{-1}$) and in the stoichiometry of binding (0.5 to 2.1 sites/ATPase unit). Most importantly, none of the binding studies completed so far has demonstrated cooperativity among high affinity sites, even though transport of 2 calcium ions/ATPase cycle (8, 9), and the Ca^{2+} concentration dependence of enzyme velocity (10-13) suggest cooperative interactions.

We have found that equilibration of sarcoplasmic reticulum with EGTA-Ca buffers in chromatography columns presents unique advantages for determination of high affinity binding. Therefore, we have carried out a detailed study clarifying the stoichiometry of the high affinity calcium sites with respect to the enzyme phosphorylation sites, and demonstrating their highly cooperative behavior. We have also found that a conformational change, detected by an ATPase spin label, is associated with calcium binding and enzyme activation. Estimates of the rate constants for the corresponding protein transition, were obtained by analysis of rapid kinetic experiments on enzyme phosphorylation.

EXPERIMENTAL PROCEDURES

Fragmented sarcoplasmic reticulum was prepared from rabbit hind leg muscle as previously described (14). The resulting membrane vesicles were characterized by ultrastructural and electrophoretic analysis (15), indicating that a band which migrates in the $M_r = 100,000$ range, and includes a polypeptide chain identified with the ATPase enzyme (16), accounts for the largest fraction of the total membrane protein. These vesicles exhibited satisfactory transport activity and were used in most experiments reported here.

Alternatively, the sarcoplasmic reticulum vesicles were fractionated further on sucrose gradients (5), and the fraction containing vesicles with a higher ATPase content was used in some experiments.

Total protein was determined by the Folin method, standardized with bovine serum albumin. Closely matching values were obtained with the biuret method, while nitrogen determination by the Nessler method gave approximately 20% higher values than expected. This difference was attributed, at least in part, to phospholipid nitrogen.

Calcium Binding—Calcium binding was measured in equilibrium conditions by the column chromatography method originally described by Hummel and Dreyer (17). Accordingly, columns (1.5 x 30.0 cm) filled with Bio-Gel P-30 (50 to 100 mesh) were equilibrated with an elution medium containing 5 mM MOPS, pH 6.8, 80 mM KCl, 10 mM MgCl$_2$, 40 to 100 μM $[^{45}]$CaCl$_2$, and various concentrations of EGTA to yield the desired $p_C$.

Total calcium was measured by atomic absorption spectrometry or by double wavelength photometry and titration with EGTA in the presence of murexide. Estimates of free Ca$^{2+}$ were obtained with the aid of a computer program taking into account the binding constant.

* This work was supported by Grants HL-16607 from the National Institutes of Health and a grant from the Muscular Dystrophy Association of America. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: MOPS, morpholinopropanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether) N,N',N'-'tetraacetic acid.
for EGTA-Ca (18), pH, and competitive effects of Mg²⁺, K⁺, and nucleotides present, as described by Fabiato and Fabiato (19).

The sarcoplasmic reticulum sample was prepared by diluting 5 to 15 mg of protein into 10 ml of elution medium, centrifuging down the vesicles, and resuspending the sediment in 1 to 2 ml of elution medium. This concentrated suspension was placed on top of the column and chromatography was allowed to proceed at a rate of 0.4 ml/min. Radioactive calcium and protein concentration were then measured in the serial samples collected at the end of the column.

ATPase Phosphorylation and P Production—ATPase phosphorylation and P production were obtained in the presence of 20 mM Mops-KOH pH 6, 80 mM KCl, 5 mM MgCl₂, 4 to 50 µM [γ³²P]ATP, 0.1 mM CaCl₂, and 0.05 to 0.3 mg of sarcoplasmic reticulum protein/ml. Rapid mixing and quenching were carried out with a Durrum Dionex D-133 multimeter which permits reaction times varying between 17 ms and 10 s through electronically controlled flow and aging procedures. Standardisation of the instrument with regards to mixing and reaction times was previously described (20).

Quenching was obtained in 3.5% Cl₃COCOOH and 0.1 mM P₄, and the denatured protein was washed four times by centrifugation and resuspension in 0.125 mM perchloric acid and 2 mM P₄. The final pellet was dissolved in 0.25 ml of 0.1 N NaOH, 2% Na₂CO₃, and 2% sodium dodecyl sulfate, and then 2.0 ml of water was added. The dissolved sample was used for determination of [³²P]P₄ and protein.

The supernatant obtained following the first centrifugation mixture was used for [³²P]P₄ determination. To this effect, 2 ml of the supernatant were extracted with 0.5 ml of 1% chloroform, then resuspended and filtered through 0.45-µm Millipore filters. The filters were washed twice with 1.0 ml of 5% Cl₃COCOOH, and the filtrate collected. Ten microliters of 100 mM cold Pi, 0.3 ml of acetone, and 1.5 ml of 5% NH₄-molybdate in 2.5 N H₂SO₄ were then added in succession to the filtrate and the sample was vortexed. Following extraction with 2.0 ml of 1:1 isobutyl alcohol/benzene and centrifugation, an aliquot of the organic phase was collected for determination of radioactivity. The entire procedure was carried out at 2-4°C.

Spin Labeling—Spin labeling of sarcoplasmic reticulum vesicles with 2,2,6,6-tetramethyl-4-aminonitrosomethane (N-iodoacetomethane) and 1-oxyl (Syvvar) was obtained as previously described (21). The labeled sarcoplasmic reticulum retained full ATPase activity.

EPR spectra were obtained with a JOEL-ME-1X electron spin resonance spectrometer. Sarcoplasmic reticulum vesicles were spun down from the labeling mixture, resuspended in cold medium (20 mM MOPS, pH 6.8, 80 mM KCl) to a concentration of 15 to 18 mg/ml and microvolumes of concentrated EGTA, MgCl₂, CaCl₂, and AMP-P(NH)₂ solutions were added as required. Aliquots of the reaction mixture were then scanned over a 106 G range, with a modulation width of 2.0 G, a response time of 3 ms, and a scan rate of 100 G/10 min. In some experiments, selected portions of the spectrum were scanned at a 1-min interval.

Computations—Computations were carried out with the assistance of a North Star Horizon Microcomputer, equipped with a 32 K memory and dual floppy disc drives.

RESULTS

Ca²⁺ Binding to Sarcoplasmic Reticulum Vesicles—Measurement of Ca²⁺ binding to sarcoplasmic reticulum at low Ca²⁺ concentrations presents considerable difficulty owing to interference of endogenous calcium in the experimental manipulation of total calcium concentrations. Treatment with chelating agents gives no assurance of complete removal of the contaminant, while the naticrate of the membrane may be altered by such a treatment (22).

We found that equilibration in chromatography columns (17) presents unique advantages for calcium-binding studies, since loss or acquisition of calcium, and exchange of isotopes, occur as the sarcoplasmic reticulum vesicles move down the column into fresh medium, while the exposed medium is delayed by the porous gel. For this reason, the peak of radioactivity associated with the sarcoplasmic reticulum vesicles at the void volume, yields an accurate value for calcium bound at the Ca²⁺ concentration determined by the experimental design. On the other hand, a deep trough and other small perturbations observed on the radioactivity base-line at the end of the elution volume, represent the initial equilibration and isotope exchange. A good separation of the radioactivity peak associated with the protein, from the trough in the baseline, gives assurance of complete equilibration (Fig. 1).

Another very useful expedient is the use of the EGTA-Ca buffers permitting the occurrence of significant calcium binding to sarcoplasmic reticulum, while the free Ca²⁺ concentration in the medium undergoes only negligible variations. In addition to EGTA-Ca buffers, the equilibration media used in our experiments contained KCl, MgCl₂, and a H⁺ buffer, in concentrations comparable to those used for ATP-dependent Ca²⁺ transport.

The amount of calcium bound to sarcoplasmic reticulum as a function of Ca²⁺ concentration is shown in Fig. 2A. The resulting plot indicates the presence of three classes of binding sites, which are titrated within the 10⁻⁷ to 10⁻⁵ M, 10⁻⁵ to 10⁻⁴ M, and 10⁻⁴ M Ca²⁺ concentration ranges. The presence of different classes of binding sites was previously reported (1-3, 6). This study is focused on the high affinity binding sites which are primarily involved in transport activation.

Trituration of High Affinity Binding Sites—It is shown in Fig. 2B that within the 0.1 to 1.0 µM Ca²⁺ concentration range, approximately 8 nmol of calcium can bind to 1 mg of sarcoplasmic reticulum protein. Somewhat higher values (9 to 11 nmol of calcium/mg of protein) were obtained with ATPase-rich vesicles which were purified in sucrose gradients (5).

It is apparent in Fig. 2B that the shape of the high affinity sites titration curve does not correspond to the simple behavior of a single binding unit with one binding constant. This can be best appreciated by using the apparent Kₗ obtained from the half-saturation point to generate a binding curve for a simple binding mechanism:

\[ E + Ca^{2+} \rightarrow E \cdot Ca \]

where each E is an independent binding site. For this mechanism, formation of the complex was computed according to:

\[ E \cdot Ca = \frac{K_{1}E_{max}[Ca^{2+}]}{1 + [Ca^{2+}]} \]

The resulting binding curve is shown with a dotted line in Fig. 2B, and clearly does not fit the experimental curve. Rather, the experimental data suggest a cooperative, two-step sequential binding mechanism: binding to an initial site, followed by development of higher affinity and binding to a second site, as in:

\[ E + Ca^{2+} \rightarrow \frac{K_{1}}{E \cdot Ca + Ca^{2+}} \rightarrow \frac{K_{2}}{E \cdot Ca_{2}} \]

\[ CPM \times 10^{3} \]

\[ 0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \]

\[ 0 \quad 0.1 \quad 0.2 \quad 0.3 \quad 0.4 \quad 0.5 \quad 0.6 \quad 0.7 \quad 0.8 \quad 0.9 \quad 1.0 \]

\[ OD \]

FIG. 1. Elution of radioactive calcium (upper) and sarcoplasmic reticulum protein (lower) from a chromatography column, for determination of calcium binding by the method of Hummel and Dreyer (17). See "Calcium Binding" for experimental details.
where $E$ corresponds to a two-site binding unit. This mechanism becomes apparent through the following considerations:

A general binding equation for any two-site binding unit, may be expressed (23) as:

$$\text{Calcium bound} = \frac{K_1[Ca^{2+}] + 2K_2K_3[Ca^{2+}]^2}{1 + K_1[Ca^{2+}] + K_2[Ca^{2+}]^2}$$

A titration curve can then be fit to the experimental data using a nonlinear least squares regression analysis, as shown in Fig. 2B. An accurate fit requires a 2 order of magnitude increase in the second, as compared to the initial binding constant. Best apparent values for $K_1$ and $K_3$ are $1.2 \pm 0.3 \times 10^6$ M$^{-1}$ and $5 \pm 0.6 \times 10^6$ M$^{-1}$, respectively. The large difference between these two equilibrium constants indicates a cooperative mechanism.

A further demonstration of the cooperative mechanism is provided by a Scatchard plot of the experimental data, which yields a nearly perfect parabolic curve (Fig. 3A). In addition, when the experimental and computed values are plotted as a function of $\text{Ca}^{2+}$, a straight line with a saturation intercept of 8 nmol of calcium/mg of protein is obtained. Both the parabolic shape of the usual Scatchard plot and the linearity of the $\text{Ca}^{2+}$ form, indicate that the cooperative mechanism involves binding of 2 calcium ions in a sequential manner (24) and, as shown in Fig. 3A, values of $K_1$ and $K_3$ used in conjunction with the two-site binding equation, accurately predict the shape of the Scatchard plot. Consistent with the analysis, a Hill plot (Fig. 3B) yields a slope of 1.82, also indicating that the two sites are involved in a highly cooperative binding mechanism.

**High Affinity Binding and Protein Conformation**—Selective labeling of sarcoplasmic reticulum ATPase with the spin probe 2,2,6,6-tetramethyl 4-amino (N-iodoacetamide) piperidine-1-oxyl provides a convenient method for detection of conformational changes by EPR spectroscopy (26, 27). Spectral changes related to conformational effects are observed following binding of substrates (e.g. ATP, AMP-P(NH)P, ADP, ITP, acety~phosphate) to the catalytic site of spin-labeled sarcoplasmic reticulum ATPase, and further change (Fig. 4) is then produced by the addition of calcium (21).

It is of interest to compare the $\text{Ca}^{2+}$ concentration dependence of an empirical spectral parameter to that of $\text{Ca}^{2+}$ binding to the high affinity sites. As is apparent in the $\text{Ca}^{2+}$ titration curves, changes in the EPR spectra ($K_{\text{app}} = 2.4 \times 10^6$ M$^{-1}$, Fig. 5A) and calcium binding ($K_{\text{app}} = 2.3 \times 10^6$ M$^{-1}$, Fig. 2B) occur within the same $\text{Ca}^{2+}$ concentration range. A Scatchard plot of the spectral data (Fig. 5B) demonstrates the same parabolic behavior as the $\text{Ca}^{2+}$ binding data, and accordingly a Hill plot yields a slope of $1.9 \pm 0.1$. Empirically, this suggests a direct relationship between $\text{Ca}^{2+}$ binding and the conformational change observed in the spectrum. More specific evidence is obtained, however, in using the $\text{Ca}^{2+}$ binding constants to fit the spectral data. When a general binding equation, similar to that shown above is expressed in terms of the spectral parameters, values of $K_1$ and $K_3$, although obtained separately in the binding experiments, give a precise fit to both the titration curve and the Scatchard plot, as shown in Fig. 5.

It is then apparent that a conformational change is associ-
activation of sarcoplasmic reticulum ATPase requires 0.1 to 1.0 μM Ca$^{2+}$ (9), indicating an involvement of the high affinity calcium binding sites in the activation mechanism. In fact, Ca$^{2+}$ specifically required for formation of a phosphorylated enzyme intermediate by transfer of the ATP terminal phosphate onto the enzyme protein (28-31). Maximal levels of acid-stable phosphorylated intermediate obtained in these conditions are 4.0 nmol/mg of protein (Fig. 6) in the usual preparations of sarcoplasmic reticulum vesicles, and approximately 5 nmol/mg in the ATPase-rich vesicles obtained by sucrose gradient centrifugation (5). Considering a corresponding calcium binding of 8 to 10 nmol/mg, it is apparent that the stoichiometry of enzyme activation includes 2 calcium ions per phosphorylation site.

An important kinetic feature of the Ca$^{2+}$ involvement in enzyme regulation is that the phosphorylation occurs with considerable delay when ATP and Ca$^{2+}$ are added simultaneously to sarcoplasmic reticulum preincubated with EGTA, in contrast to ATP added to sarcoplasmic reticulum preincubated with Ca$^{2+}$ (Fig. 6). This observation is in agreement with those made by Sumida et al. (32), and indicates that enzyme activation occurs through a rather slow transition associated with calcium binding.

An even slower (5 s$^{-1}$) conformational effect was detected by Dupont and Leigh (33), monitoring changes in protein fluorescence upon addition of calcium to sarcoplasmic reticulum vesicles in the absence of ATP. This transition appears related to that observed by us in spin-labeled sarcoplasmic reticulum, as well as to the Ca$^{2+}$-induced enzyme activation. In fact, all of these phenomena display a Ca$^{2+}$ concentration dependence which is within the range required for occupancy of the high affinity binding sites. However, as shown in Fig. 6, it is apparent that in the presence of ATP the Ca$^{2+}$-induced transition occurs more rapidly than the fluorescence change measured in the absence of ATP.

It is noteworthy that even the reversal of the Ca$^{2+}$-depend-
ent transition is rather slow. Such a reversal can be estimated by adding EGTA to sarcoplasmic reticulum preincubated with Ca\(^{2+}\), and monitoring the loss of ATPase reactivity to ATP (33, 34) which is dependent on calcium occupancy of the high affinity sites. In addition, the appearance of ATPase reactivity to Pi, (35, 36), which is dependent on Ca\(^{2+}\) dissociation from the high affinity sites, can also be monitored following addition of EGTA (20). From these measurements, a 15 to 30 s\(^{-1}\) time constant is obtained for the transition associated with dissociation of calcium-enzyme complex. These values are comparable to those obtained by measuring fluorescence changes following addition of EGTA to sarcoplasmic reticulum preincubated with calcium (33).

In conclusion, kinetic studies on enzyme phosphorylation and protein conformation, indicate that binding and, conversely, dissociation of calcium from high affinity sites, are associated with the occurrence or reversal of a slow protein transition. The conformation obtained in the presence of calcium corresponds to the active state, with respect to ATP utilization.

**DISCUSSION**

It is clear that the high affinity calcium binding is an intrinsic property of the ATPase protein, since this binding remains associated with the enzyme following extraction of extrinsic proteins from the sarcoplasmic reticulum vesicles (5). Furthermore, the relevance of high affinity calcium binding to enzyme activation is demonstrated by the good agreement between the Ca\(^{2+}\) concentration dependence of calcium binding (Fig. 2) and that of ATP utilization for calcium transport (10-13).

Involvement of 2 calcium ions per ATPase cycle was originally suggested by a 2:1 stoichiometric ratio between the rates of calcium transport and ATP utilization by sarcoplasmic reticulum vesicles (8, 9). Our measurements reported here show that in sarcoplasmic reticulum vesicles, 1 mg of protein contains 8 to 10 nmol of high affinity (\(K_{app} = 2.3 \times 10^6 \text{ M}^{-1}\)) calcium binding sites. Considering that a maximal level of 4 to 5 nmol of phosphorylated intermediate/mg of protein is formed in the presence of ATP, we conclude that in the native membrane each enzyme unit consists of two high affinity calcium binding sites and one phosphorylation site.

Analysis of the equilibrium binding data demonstrates a high degree of cooperativity between two interacting calcium sites, resulting in a marked increase in affinity following occupation of the first site. It is apparent that such a cooperative binding mechanism includes a protein conformational change. In fact, analysis of our spin label observations shows a Ca\(^{2+}\) concentration dependence and a cooperative behavior which are identical to those obtained with the binding data. This demonstrates a tight relation between Ca\(^{2+}\) binding and protein conformational effects.

Calcium binding results in activation of a specific step of the ATPase cycle, consisting of enzyme phosphorylation through transfer of ATP terminal phosphate to the ATPase protein. The phosphorylation reaction proceeds with a 85 to 150 s\(^{-1}\) rate constant at 25°C (20, 37) when ATP is added to the enzyme preincubated with calcium. On the other hand, phosphorylation is definitely slower when ATP and Ca\(^{2+}\) are added to the enzyme preincubated with EGTA (Fig. 6), even though the kinetics of calcium binding to high affinity (\(K_{app} \approx 10^6 \text{ M}^{-1}\)) sites are expected to be very rapid. Therefore, an additional step associated with calcium binding is suggested by the slow kinetics of enzyme phosphorylation.

An event which is likely to confer slow kinetics to a cooperative binding mechanism producing enzyme activation is the occurrence of protein conformational changes which, for the sarcoplasmic reticulum ATPase, have been demonstrated by spin label experiments (21, 38), studies of —SH reactivity (39, 40), and measurements of intrinsic protein fluorescence (33). The latter experiments are of particular interest inasmuch as they include kinetic measurements showing a slow fluorescence increase upon addition of calcium to sarcoplasmic reticulum preincubated with EGTA and a fluorescence decrease upon addition of EGTA to sarcoplasmic reticulum preincubated with calcium.

Taken together, kinetic measurements of ATPase phosphorylation and protein conformational changes indicate that calcium binding to, and dissociation from high affinity binding sites of sarcoplasmic reticulum ATPase are associated with occurrence of a slow protein transition. Experimentally, the transitions induced by Ca\(^{2+}\), or conversely by EGTA were observed by Dupont and Leigh (33) to occur with time constants of 5 s\(^{-1}\) and 30 s\(^{-1}\), in the absence of ATP. These authors attributed their finding to a slow step added to a simple binding mechanism (\(E + Ca^{2+} \rightarrow E.Ca \rightarrow E.Ca^2\)).

Our demonstration of a two-step cooperative mechanism for calcium binding permits an alternate reaction sequence, by inserting the slow protein transition between the two binding steps:

\[
E + Ca^{2+} \rightarrow E.Ca \rightarrow E.Ca^2 \rightarrow E.Ca^3
\]

where \(1\) and \(3\) are the two binding steps, \(2\) is the slow transition, and \(E^1\) and \(E^2\) are enzyme states corresponding to occupancy of one or both calcium binding sites. Introduction of Step 2 (slow transition) requires adjustment of the apparent binding constants (\(K_1\) and \(K_2\)), based on the magnitude of \(K_2\).

It must be understood that a unique advantage of this mechanism is that, in the presence of Ca\(^{2+}\), it allows the

**Fig. 7.** An illustration of simulated kinetics for intermediates and products formation upon addition of Ca\(^{2+}\) to ATPase (A), or EGTA to ATPase calcium complex (B). The simulation assumes the following mechanism for high affinity binding:
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reaction to reach nearly total saturation of E with calcium, even though the reverse rate constant for the slow transition is faster than the forward constant ($k_{-2} = 60 \text{ s}^{-1}$). This is due to trapping of E in the $E'\cdot Ca$ form, owing to the very large $K_c$. An illustration of the reaction sequence upon addition of $Ca^{2+}$ to E or, conversely addition of EGTA to $E'\cdot Ca_{2\theta}$, is given in Fig. 7, in which simulated kinetics of intermediates and product formation is shown to be nearly identical to that obtained experimentally by Dupont and Leigh (33) monitoring protein fluorescence.

Time constants and large free energy changes suggest that this reaction may contribute significantly to the mechanism of $Ca^{2+}$ translocation. In this regard, it is noteworthy that rapid kinetic experiments on enzyme phosphorylation (Fig. 6) indicate that, in the presence of substrate (e.g. ATP), the calcium-induced enzyme transition (e.g. activation) is completed within a time much faster than 1 s, which is the time indicated by fluorescence changes following addition of calcium in the absence of ATP (35). It is likely that substrate binding provides a lower energy path for the transition.

In fact, an estimate of the time constant for the calcium-induced enzyme transition in the presence of ATP, may be obtained by considering a minimal number of reactions (42, 43) occurring upon addition of $Ca^{2+}$ and ATP to the enzyme preincubated with EGTA (E):

$$E + Ca_{2\theta} \rightarrow E\cdot Ca$$  \hspace{1cm} (1)

$$ATP + E\cdot Ca \rightarrow ATP\cdot E\cdot Ca$$  \hspace{1cm} (2)

$$ATP\cdot E\cdot Ca \rightarrow ATP\cdot E'\cdot Ca$$  \hspace{1cm} (3)

$$ATP\cdot E'\cdot Ca + Ca_{2\theta} \rightarrow ATP\cdot E''\cdot Ca_{2\theta} \rightarrow (E''\cdot Ca_{2\theta} + ATP)$$  \hspace{1cm} (4)

$$ATP\cdot E''\cdot Ca_{2\theta} \rightarrow ADP\cdot E'' \rightarrow P\cdot Ca_{2\theta}$$  \hspace{1cm} (5)

In this mechanism, binding of the first $Ca^{2+}$ and ATP is very rapid. On the other hand, enzyme phosphorylation (Reaction 5) is dependent on occupancy of both $Ca^{2+}$ binding sites and related protein transformation (Reactions 3 and 4). It was previously shown (41-43) that $Ca^{2+}$ translocation is completed upon enzyme phosphorylation. At this time, a reduction in binding affinity (Reaction 6) is followed by $Ca^{2+}$ dissociation (Reactions 7 and 8) on the inner side of the membrane. Thereafter, the enzyme returns to its original state and undergoes rate-limiting cleavage.

Based on this scheme, it is possible to obtain good fits to experimental determinations of phosphoenzyme ($E'' \rightarrow P + E''\cdot P + E\cdot P$) and P; production (Fig. 8A) by assuming $k_3$ and $k_{-10} = 110 \text{ s}^{-1}$, and $k_3$ and $k_{-10} = 240 \text{ s}^{-1}$ for the reversible transition of E to $E'$ in the presence of ATP, as opposed to the 5 and 60 s$^{-1}$ in the absence of ATP. It should be strongly emphasized that satisfactory fitting of the data shown in Fig. 8 does not imply a unique solution for the ATPase reaction mechanism which may well include additional steps (37).

![Figure 8](http://www.jbc.org/)
Therefore, some of the rate constants used may not be directly expressing single reactions, but only rate-limiting steps in subgroups of partial reactions. Nevertheless, the partial reactions considered here are sufficient to test the calcium binding mechanism derived from our equilibrium studies, in terms of kinetic regulation of the transient state following addition of Ca\(^{2+}\) and ATP to the enzyme preincubated with EGTA. Furthermore, it should be noted that the forward and reverse rate constants used in the simulations yield an overall equilibrium constant which is close to that given for hydrolysis of ATP terminal phosphate (Fig. 8B).

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