Kinetic and Thermodynamic Control of ATP Synthesis by Sarcoplasmic Reticulum Adenosinetriphosphatase*

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Energy interconversion by the sarcoplasmic reticulum (SR) membrane vesicles was demonstrated experimentally in both forward (Hasselbach and Makinose, 1961) and reverse (Makinose and Hasselbach, 1971) directions of the calcium pump. The interconversion was accounted for by considering the ATP synthesis observed in the absence of a Ca$^{2+}$ gradient, where the phosphoenzyme obtained with acetylphosphate provides the simplest conditions for kinetic studies on Ca$^{2+}$-ATP synthesis.

Other previously characterized parameters, satisfactory simulations of single and multiple cycle ATP synthesis, in the presence and in the absence of a Ca$^{2+}$ gradient, were obtained.

Several experimental parameters, critical to the analysis of ATP synthesis by sarcoplasmic reticulum ATPase, were determined experimentally.

1) The phosphorylated enzyme intermediate obtained with acetylphosphate in the presence of a Ca$^{2+}$ gradient was shown to be entirely ADP sensitive but quite stable in the absence of added ADP. On the contrary, the phosphoenzyme obtained with ATP is unstable due to the ADP formed during the phosphoryl transfer reaction. For this reason, addition of ADP to [32P]phosphoenzyme obtained with [32P]acetylphosphate provides the simplest conditions for kinetic studies on [γ-32P]ATP synthesis.

2) The dissociation rate constant of newly synthesized ATP (in the reverse direction of the ATPase cycle) was measured experimentally and found to be 16 s$^{-1}$. This value agrees well with the dissociation rate constant determined for adenyl-5'-yl imidophosphate bound to this enzyme.

3) ATP synthesis observed in the absence of a Ca$^{2+}$ gradient was shown to be a kinetic overshoot due to ligand-induced perturbation of a limited number of partial reactions and occurring before equilibration of the entire system. Most of the ATP formed under these conditions was subsequently hydrolyzed as the overall equilibrium was reached.

4) Based on these and other (previously characterized) parameters, satisfactory simulations of single and multiple cycle ATP synthesis, in the presence and in the absence of a Ca$^{2+}$ gradient, were obtained.

Energy interconversion by the sarcoplasmic reticulum (SR) membrane vesicles was demonstrated experimentally in both forward (Hasselbach and Makinose, 1961) and reverse (Makinose and Hasselbach, 1971) directions of the calcium pump. The interconversion was accounted for by considering simply the ATP and Ca$^{2+}$ gradient potentials in solution which, at equilibrium, are related as in:

$$K_{\text{ATP}} \cdot \frac{[\text{ATP}]}{[\text{ADP}][P_i]} = \frac{[\text{Ca}^{2+}]_{\text{in}}^2}{[\text{Ca}^{2+}]_{\text{out}}^2}$$

where $K_{\text{ATP}}$ is the equilibrium constant for ATP hydrolysis in the absence of linked reactions. Electrical charge imbalance is generally neglected, assuming compensation by other electrolytes.

Equation 1 accounts for most experimental findings but does not account for the ATP synthesis obtained by addition of ADP and Ca$^{2+}$ in the absence of a transmembrane Ca$^{2+}$ gradient (Knowles and Racker, 1975; de Meis and Tume, 1977).

Subsequently (Inesi et al., 1980; Pickart and Jencks, 1984), analysis of this system was carried out considering a minimal number of sequential reactions and their equilibrium constants obtained at neutral pH, in the presence of 80 mM KCl and 5 mM MgCl$_2$, and at 25°C temperature (Fernandez-Belda and Inesi, 1986):

$$E + 2Ca^{2+}_{\text{out}} \rightleftharpoons E \cdot Ca_2 \quad 3 \times 10^{12} \text{M}^{-2}$$

$$E \cdot Ca_2 + ATP \rightleftharpoons ATP \cdot E \cdot Ca_2 \quad 1 \times 10^{9} \text{M}^{-1}$$

$$ATP \cdot E \cdot Ca_2 \rightleftharpoons ADP \cdot E \cdot P \cdot Ca_2 \quad 0.3$$

$$ADP \cdot E \rightleftharpoons E \cdot P \cdot Ca_2 + ADP \quad 7 \times 10^{-4} \text{M}$$

$$E \rightleftharpoons P \cdot Ca_2 \quad 3 \times 10^{-6} \text{M}^2$$

$$E \rightleftharpoons P \rightleftharpoons E \cdot P_i \quad 1$$

$$E \cdot P_i \rightleftharpoons E + P_i \quad 1 \times 10^{-2} \text{M}$$

The advantage of this analysis is to be explicit with respect to the coupling mechanism of enzyme catalysis and active transport and to allow understanding of the Ca$^{2+}$ gradient potential in terms of calcium binding to and dissociation from the ATPase in various interactive states with ATP, ADP, and P$_i$. However, while the equilibrium constant for the sequential reactions have been determined experimentally to an acceptable approximation, some of the related kinetic constants have not. Furthermore, analysis based on the equilibrium constants for the partial reactions and the concentrations of ligands yields overall free energies relations identical to those obtained by the use of Equation 1 but still does not explain the ATP synthesis experimentally observed in the absence of a Ca$^{2+}$ gradient.

With the experiments described in this manuscript, we have optimized conditions for measurements of ATP synthesis in the presence and in the absence of a transmembrane Ca$^{2+}$ gradient and determined directly the rate constant for dissociation of newly synthesized ATP from the enzyme, which was suggested to be a rate-limiting step (Pickart and Jencks, 1982). We have also determined the kinetic regulation of phosphoenzyme turnover and are able to explain unambiguously the ATP synthesis observed in the absence of a transmembrane Ca$^{2+}$ gradient (Knowles and Racker, 1975; de Meis and Tume, 1977), in terms of a kinetic overshoot due to ligand-induced perturbation of a limited number of partial reactions, before equilibration of the entire system is reached.
MATERIALS AND METHODS

SR vesicles were isolated from rabbit skeletal muscle as previously described by Eletr and Inesi (1972). Leaky vesicles were obtained by the addition of the ionophore A23187 to the native vesicles (10 μm final concentration). Protein concentration was determined after the procedure of Lowry et al. (1951), using bovine serum albumin as a standard.

Free calcium concentrations were estimated from total calcium and EGTA by computations (Fabiato and Fabiato, 1979) based on the binding constant for Ca-EGTA complex given by Schwartzschild et al. (1987) and taking into account pH, Mg2+, K+, Pi, and nucleotides concentrations as well as the EGTA-H dissociation constants given by Blanks et al. (1982).

[^32]P[ATP, [γ-32]P]ATP, and [α-32]P[AMP-PNP were purchased from Du Pont-New England Nuclear. [α-32]P[AMP-PNP was obtained from ICN Radiochemicals, the ionophore A23187 from Behring Diagnostics, and all other reagents from Sigma.

Carrier-free [32]P[ATP was purified according to de Meis and Tume (1957) and taking into account pH, M2+, K+, Pi, and nucleotides concentrations. Khornberg et al. (1956). [γ-32]P[ATP synthesis was determined by measuring the radioactivity remaining in quenched reaction media after phosphate extraction as phosphomolybdate with 2-butanol/benzene (1:1 v/v) as described by de Meis and Carvalho (1974). Acetyl [32]P[ATP was completely hydrolyzed in the assay media under our conditions, yielding acids of about [32]P[AMP-PNP.

Rapid mixing experiments were carried out in a Froehlich-Berger chemical quench-flow apparatus, with temperature control set at 25°C. The reaction was quenched with 0.25 M PCA and 13 mM Pi at serial time intervals. The quenched samples (~2 ml) were cooled in ice and centrifuged at 5000 rpm for 10 min at 0°C. The pellets were washed four times with 5 ml of 0.125 M PCA and 20 mM Pi, and protein concentration and radioactivity were measured to determine the partial differential equations. All other reagents from Sigma.

[^32]P[ATP was synthesized as described by Blinks et al. (1986).[^\text{32}P]ATP synthesis was then started by adding ADP and EGTA to produce reversal of the calcium pump. Under these conditions a simple cycle of ATP formation is observed within the first 100 ms of reaction, accompanied by phosphoenzyme decay (Fig. 1A; see also Pickart and Jencks, 1982; Froehlich and Heller, 1985; Fernandez-Belda and Inesi, 1986). Synthesis of ATP presents a burst within the first 10 ms following addition of ADP, followed by a slower phase that reaches a plateau within approximately 30 ms. This curve of ATP synthesis can be fitted with two exponentials of 300 and 60 s -1 rate constants, accounting for 1.7 and 1.0 nmol of ATP/mg of protein, respectively. This biphasic presteady state kinetic behavior has been attributed to rapid formation of ATP- E' -Ca from ADP -E' -P- Ca, followed by a slow phase related to ADP dissociation and/or transition of ADP-insensitive to ADP-sensitive phosphoenzyme (Picart and Jencks, 1982; Froehlich and Heller, 1985; Fernandez-Belda and Inesi, 1986), as in:

\[
\text{ADP} \cdot \text{E}' - \text{P} \cdot \text{Ca} \xrightarrow{1} \text{ADP} \cdot \text{E}' - \text{P} \cdot \text{Ca} + 2\text{Ca}^{2+} \xrightarrow{2} E + \text{ATP} + 2\text{Ca}^{2+}
\]

where 2 is fast and 3 is slow, thereby limiting the rate of internal re-equilibration of reaction 2.

It is apparent in Fig. 1A that the fast phase of ATP synthesis is matched by a reduction of phosphoenzyme level, proceeding with an ATP/phosphoenzyme ratio of approximately 1. The slower phase of phosphoenzyme decay, however, proceeds with lower efficiency, indicating that a fraction of phosphoenzyme yields P instead of ATP. It is possible that calcium loading was obtained by incubating SR vesicles with acetyl [32]P[AMP-PNP (10 μM) binding was determined in the presence and in the absence of acetylphosphate. The filters were washed with 1 ml of 1.5 mM ADP in 50 mM Mops, pH 6.8, 80 mM KCl, and 5 mM MgCl₂ before use, to minimize nonspecific binding to the filters.

Calcium loading was obtained by incubating SR vesicles with 2.27 mg/ml with 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1.05 mM [α-32]P[AMP-PNP, and 1 mM acetyl phosphate. The reaction was quenched at different time intervals by addition of 10 mM LaCl₃, 20 mM Mops, pH 6.8, and 80 mM KCl. An aliquot containing 50 mM Mops, pH 6.8, and 80 mM KCl. The filter was solubilized with 5 ml of dimethyloformamide for determination of [α-32]P[AMP-PNP. In the experiments on ATP synthesis, 2 ml of supernatant were drawn out soon after the first centrifugation for [γ-32]P[ATP determination.

Rapid filtration experiments were performed with the aid of a BioLogic Rapid-filtration System. For enzyme-bound ATP determination, SR vesicles were incubated in a medium containing 2 mM acetyl [32]P[AMP-PNP, 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, and 1.05 mM CaCl₂ (the free calcium concentration ≈ 60 μM). After 1.5 min of incubation, 70 μg of protein were placed on a Millipore filter (DAPW, 0.65-μm pore size) and a solution of 1.5 mM ADP, 10 mM EGTA, in 50 mM Mops, pH 6.8, 80 mM KCl, and 5 mM MgCl₂ was passed through the filter for different time intervals. Ten filters per each time point were collected in 5 ml of 10% trichloroacetic acid at room temperature (10 min). The filters were then washed four times with 1 ml of 1.5 mM ADP, 10 mM EGTA, 50 mM Mops, pH 6.8, 80 mM KCl, and 5 mM MgCl₂, then, they were washed with the same solution at the same periods of time used for enzyme-bound ATP determination, and the [32]P[AMP-PNP retained on the filters was measured.

In the [α-32]P[AMP-PNP (10 μM) binding was determined in the presence and in the absence of calcium in the same conditions as above, in the absence of acetylphosphate. The filters were washed with 1 ml of 1.5 mM ADP in 50 mM Mops, pH 6.8, 80 mM KCl, and 5 mM MgCl₂ before use, to minimize nonspecific binding to the filters.

Calcium loading was obtained by incubating SR vesicles with 2.27 mg/ml with 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1.05 mM [α-32]P[AMP-PNP, and 1 mM acetyl phosphate. The reaction was quenched at different time intervals by addition of 10 mM LaCl₃, 20 mM Mops, pH 6.8, and 80 mM KCl. An aliquot containing 60 μg of protein was placed on a filter (Millipore, HAWP, 0.45-μm pore size) and washed with 4 ml of quenching solution (10 mM LaCl₃, 20 mM Mops, pH 6.8, and 80 mM KCl). The filter was solubilized with 5 ml of dimethylformamide for determination of [α-32]P[AMP-PNP.

Simulations of transient kinetic reequilibrations were calculated on a Zenith 158 computer using an iterative Runge-Kutta solution of the partial differential equations.

2 G. Inesi, unpublished results.
this phenomenon is related to the interference of competing pathways in a complex mechanism of catalysis, whereby some phosphoenzyme proceeds to hydrolysis cleavage instead of being utilized for phosphoryl transfer to ADP (Froehlich and Heller, 1985; Fernandez-Belda and Inesi, 1986). An alternative explanation is that a small fraction of the SR vesicles are leaky and do not build up a Ca²⁺ concentration gradient during the preincubation with acetyl[³²P]phosphate. The [³²P]phosphoenzyme of leaky vesicles then would not be able to form ATP upon addition of ADP (since the Ca²⁺ concentration inside the vesicles is too low) and would proceed directly to hydrolytic cleavage.

It is noteworthy that, following the first cycle, ATP formation continues at a low rate in the time scale of seconds, even though the level of phosphoenzyme is very low (Fig. 1B). This is probably not due to further phosphoryl transfer from acetylphosphate to the enzyme and then to ADP because the enzyme cannot utilize acetylphosphate after EGTA is added to the medium. An indirect effect of myokinase (i.e. production of nonradioactive ATP from ADP and exchange with [³²P]P, to form radioactive ATP) was ruled out using an inhibitor of the myokinase activity. The most likely explanation for this slow ATP formation is incorporation of [³²P]P, (produced by acetylphosphate hydrolysis) into the enzyme in the absence of external Ca²⁺ (EGTA is present), and subsequent transfer of this phosphoryl moiety to ADP in the presence of high intravesicular Ca²⁺ (see simulation in Fig. 6B in which 0.1 mM P, was assumed to be present). Steady state ATP formation from P, and ADP in the presence of a transmembrane gradient has been previously demonstrated (Makinose, 1972; Yamada et al., 1972).

**ATP Synthesis and Phosphoenzyme Decay in the Absence of a Ca²⁺ Gradient—Complete reversal of the ATPase cycle in the absence of a Ca²⁺ gradient has been demonstrated with leaky vesicles by first phosphorylating the enzyme with P, in the absence of Ca²⁺ (ADP-insensitive phosphoenzyme), and then adding high (mM) Ca²⁺ and ADP to produce ATP (Masuda and de Meis, 1973; Knowles and Racker, 1975; de Meis and Tume, 1977; de Meis and Inesi, 1982). When this type of experiment is carried out under conditions commonly used for studies of ATPase catalysis, the rapid burst of ATP is not observed. On the contrary, ATP synthesis occurs at a lower rate and with lower efficiency (Fernandez-Belda and Inesi, 1986) owing to significant competition of phosphoenzyme cleavage with reverse cycling.

In the experiment shown in Fig. 2, [³²P]phosphoenzyme was first made with acetylphosphate in the presence of Ca²⁺, under conditions identical to the experiment of Fig. 1. The resulting Ca²⁺ gradient was then collapsed suddenly by adding a divalent cation ionophore together with ADP and EGTA when the reverse cycle was started. It is apparent in Fig. 2A that the single cycle of ATP synthesis occurs with kinetics and efficiency identical to those of the experiment in Fig. 1A, despite the Ca²⁺ gradient collapse that was produced by the ionophore (checked independently by stopped flow spectrometry in the presence of metallochromic indicators). This indicates that nearly all the phosphoenzyme obtained with acetylphosphate is ADP-sensitive and remains so for a considerable time.

It is shown in Fig. 2B that the ATP formed during the first reverse cycle undergoes slow breakdown, which is accelerated by the presence of Ca²⁺ in the medium. Therefore, in the absence of Ca²⁺ gradient, the ATP formed by stoichiometric phosphoryl transfer to ADP is then utilized in the forward direction of the catalytic cycle. This is quite the opposite to that observed in the presence of a Ca²⁺ gradient (Fig. 1B).
substrate. Under these conditions, decay of the phosphoenzyme obtained by utilization of acetylphosphate proceeds very slowly within the time scale of seconds (Fig. 3). On the contrary, the phosphoenzyme obtained by utilization of ATP undergoes rapid decay with a half-time of approximately 100 ms (Fig. 3). If, however, an ATP-regenerating system is present, the phosphoenzyme decay occurs within the same slow kinetics, whether formed with ATP or acetylphosphate (Fig. 3). This indicated that the instability of the phosphoenzyme obtained with ATP is due to the effect of the ADP formed during ATP utilization.

It is of interest that phosphoenzyme decay is much faster following collapse of the Ca\(^{2+}\) gradient (Fig. 4) than in loaded vesicles (Fig. 3). In the experiment shown in Fig. 4, \([^{32}P]\)phosphoenzyme was formed by utilization of \([^{32}P]\)acetylphosphate, and its decay observed following a chase with nonradioactive substrate. It is clear that the decay occurs with nearly identical kinetics whether the reaction mixture does or does not include 50 \(\mu\)M ATP. On the other hand, a significant acceleration of the decay is observed when 1 mM ATP is added, consistent with the previously observed turnover activation by high ATP concentrations. This indicates that the intermediate obtained with acetylphosphate is intrinsically identical to that formed with ATP.

**Experimental Measurements of the Dissociation of Newly Formed ATP from the Enzyme**—The experiments described above demonstrate that most of the phosphoenzyme formed with acetylphosphate in vesicles filled with calcium is ADP-sensitive. Therefore, it can be excluded that a slow transition of ADP-insensitive to ADP-sensitive phosphoenzyme accounts for the slow phase of ATP synthesis in our single cycle experiments (Figs. 1A and 2A). The slow phase has been attributed to rate-limiting dissociation of newly formed ATP from the enzyme (Pickart and Jencks, 1982; Fernandez-Belda and Inesi, 1986). However, the rate of ATP dissociation was never measured directly. In fact, in all experiments published so far the ATP measured includes the contribution of enzyme-bound ATP (ATP-'E'-Ca\(^{2+}\)) and ATP dissociated to the medium because the reaction has been always quenched with acid and the protein denatured before determination of ATP. An alternative possibility is to separate the vesicles from the medium at serial times with the aid of a rapid filtration device.

In this case, the kinetics of ATP dissociation from the enzyme can be determined by measuring the radioactivity remaining with the enzyme on the filters. It is shown in Fig. 5A that within the first 20 ms after the addition of ADP most of the newly synthesized ATP is still bound to the enzyme, and it is then released very slowly. The experimental curve of ATP dissociation was fitted to the following equation:

\[
B = \frac{A \cdot k_1}{k_2 - k_1} e^{-k_2 t} - e^{-k_1 t}
\]

This equation derives from a reaction scheme including two sequential steps, as in

\[
a \xrightarrow{k_1} b \xrightarrow{k_2} c
\]

where \(k_1\) is the apparent rate constant for formation of enzyme-bound ATP, and reaction 2 is the dissociation of newly formed ATP from the enzyme. The latter can be assumed to be irreversible since the ATP is removed from the filter as it dissociates from the enzyme. \(B\) is a transient intermediate (the experimentally measured, enzyme-bound ATP) that appears and then disappears. The best fit yields values of 128 and 16 s\(^{-1}\) for \(k_1\) and \(k_2\), respectively. In this case \(k_2\) is the rate constant for dissociation of newly formed ATP from the

**FIG. 4. Phosphoenzyme decay in the absence of a calcium gradient.** Native SR vesicles (0.5 mg/ml) were phosphorylated at 20°C with 1 mM [\(^{32}P\)]acetylphosphate for 90 s, in a medium containing 50 mM MOPS, pH 6.8, 80 mM KCl, 5 mM MgCl\(_2\), 1 mM EGTA, and 1.05 mM CaCl\(_2\). [\(^{32}P\)]Phosphoenzyme decay was initiated by a 10-fold volume dilution with either 50 mM nonradioactive acetylphosphate (\(\Delta\)), or 50 \(\mu\)M ATP (O), or 1 mM ATP (\(\bigcirc\)), in a medium of the same electrolyte composition as the preincubation medium. Quenching at serial time intervals was obtained by addition of 0.25 M PCA and 20 mM P. The figure shows only the time course of phosphoenzyme decay, starting 20–100 ms following dilution to ensure dissociation of radioactive substrate from the phosphorylation site.

**FIG. 5. Determination of the dissociation rate constant of nucleotide from SR-ATPase.** A, native SR vesicles (0.08 mg/ml) were phosphorylated as described in Fig. 1, then 70 \(\mu\)g of protein aliquots were placed on filters, and a solution containing 1.5 mM ADP, 10 mM EGTA in 50 mM Mops, pH 6.8, 80 mM KCl, and 5 mM MgCl\(_2\) was passed through the filters for different time intervals using a rapid filtration device. The filter was processed for determination of enzyme-bound \([\gamma-^{32}P]\)ATP as described under "Materials and Methods." B, Native SR vesicles (0.08 mg/ml) were preincubated with 10 \(\mu\)M AMP-PNP containing [\(\alpha-^{32}P\)]AMP-PNP in 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl\(_2\), 1 mM EGTA, in the absence (O) and in the presence (\(\bigcirc\)) of 1.05 mM CaCl\(_2\). After 2 min of incubation, 70 \(\mu\)g of protein aliquots were placed on filters and a solution of 1.5 mM ADP and 10 mM EGTA was passed through the filter for different time intervals. The filters were then solubilized in 1 ml of dimethylformamide and the remaining radioactivity determined by scintillation counting. A blank was obtained for each time interval under the same conditions without protein. The continuous lines represent the fitting to the equations shown in the text obtained with a dissociation rate constant of 16 s\(^{-1}\) (Equation 4) for ATP and 17 s\(^{-1}\) (a nonexponential equation) for AMP-PNP dissociation.
enzyme, and its low value demonstrates its role in limiting the slow phase in the single cycle of ATP formation (Figs. 1 and 2, and simulations in Figs. 6 and 7).

The nucleotide dissociation constant was also determined under the same conditions using the nonhydrolyzable ATP analogue, AMP-PNP. This nucleotide was incubated for 2 min in the presence and in the absence of 10 μM calcium. Aliquots were then placed on filters and washed for increasing time intervals with ADP and EGTA (Fig. 5B). In this case the nucleotide dissociation is expected to be first order since no synthesis or binding of the nucleotide occur during the wash out. The observed dissociation rate constant of approximately 17 s⁻¹ is in good agreement with the constant obtained from the experiment shown in Fig. 5A.

It is of interest that the rate constant of AMP-PNP dissociation is the same in the presence and in the absence of calcium (Fig. 5B).

**DISCUSSION**

The Reaction Scheme—Reaction scheme 2 includes a sufficient number of steps to explain the coupling mechanism of catalysis and transport, as well as to allow satisfactory analysis of the energy interconversions. However, analysis of kinetic experiments requires more complex schemes. We have previously proposed a branched pathway following phosphoryl transfer from ATP to SR ATPase, in the forward direction of the cycle. The branched pathway includes rapid transition of ADP⁻⁻⁻⁻⁺⁺⁺⁺⁻⁺⁺⁺⁺ to ADP⁻⁻⁻⁻⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺ (lower phosphorylation potential) and then release of ADP, or release of ADP first and then slow transition of E⁻⁻⁻⁻⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺ to E⁻⁻⁻⁻⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺ (Scheme 6). This branching was first suggested by the very slow turnover of the phosphoenzyme obtained in the presence of high intravesicular Ca²⁺ and a strong ATP-regenerating system to prevent accumulation of ADP (Nakamura et al., 1986).

The rationale for the other partial reactions included in Scheme 6 has been discussed previously (de Meis and Vianna, 1979; Ikemoto, 1982, Inesi, 1985). As Scheme 6 was used for analysis and simulations discussed below, a list of rate constants for the partial reactions and their origin is provided in Table II.

**Kinetic Regulation**—In our present work we have measured experimentally (Fig. 5) the rate constant for dissociation of newly formed ATP from SR ATPase and found it to be 16 s⁻¹. It is then demonstrated that when ATP synthesis is started by addition of ADP to ADP-sensitive phosphoenzyme (Fig. 1), ATP dissociation is a rate-limiting factor for completion of a single reverse cycle (Scheme 6; see also Pickart and Jencks, 1982). Simulation of a single reverse cycle following addition of ADP to E⁻⁻⁻⁻⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺ (based on reaction Scheme 6 which includes the 16 s⁻¹ rate constant in k₄⁻, for dissociation of newly formed ATP) is shown in Fig. 6. The simulation is in good agreement with the experimentally observed transients of EP decay and ATP formation (Fig. 1), as well as of ATP dissociation from the enzyme (Fig. 5). It is important to realize that if values of 8 or 32 s⁻¹ are used for k₄⁻ in the simulations, significant disagreement with the experimental results is obtained.

Considering the high potential derived from the Ca²⁺ concentration gradient still present across the SR membrane following the first reverse cycle, one would expect ATP synthesis to proceed toward equilibration. In fact this is observed (Fig. 1B). The rate of ATP formation following the first cycle, however, is very slow, owing to the kinetic limitation imposed in this case by enzyme phosphorylation. A simulation of this experiment is shown in Fig. 6, demonstrating that the behavior observed experimentally is in fact predicted by Scheme 6. This indicates that the dissociation rate constant can kinetically limit ATP synthesis. This is an example demonstrating how, in the presence of a nearly identical thermodynamic drive, the rate of ATP synthesis can vary greatly depending on the partial reaction that becomes kinetically limiting under a particular set of conditions.

A more interesting case is presented by the experiment shown in Fig. 2, in which a single cycle of ATP synthesis was obtained, even though the transmembrane Ca²⁺ gradient was collapsed (with an ionophore) upon addition of ADP. In fact, the single cycle kinetics (Fig. 2A) are identical to those observed in the presence of the gradient (Fig. 1A). This can be explained by a ligand-induced shift of equilibrium within a limited number of partial reactions (reactions 8-4 in Scheme...
calcium binding and steady state ATP velocity. The direction of the cycle, which is Ca\textsuperscript{2+} dependent. Again, the ATP level decays at a rate which is dependent on the Ca\textsuperscript{2+}

including its distribution between enzyme-bound and dissociated forms. The equilibrium of ligand-induced ATP synthesis in the absence of a transmembrane Ca\textsuperscript{2+} gradient is a kinetic overshoot which will ultimately approach the predicted equilibrium at rates dictated by the particular set of experimental conditions.

Our experimental observations and analysis suggest an important generalization regarding the flow of biological systems regulated by enzyme catalysis. It is apparent that kinetic constraints may render portions of linked reaction chains thermodynamically "closed," thereby allowing (within a limited time frame) substrate flow against the overall thermodynamic drive.

### TABLE I

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\textsuperscript{*} Derived from analysis of the Ca\textsuperscript{2+} concentration dependence of calcium binding and steady state ATP velocity. 

\textsuperscript{\#} Derived from dissociation constants of Ca\textsuperscript{2+} from phosphorylated enzyme (Coan et al., 1979). 

\textsuperscript{\#} Rate constants measured experimentally. 

\textsuperscript{\#} From Fernandez-Belda and Inesi (1986). 

\textsuperscript{\#} Rate constants experimentally determined by Inesi et al. (1982). 

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