Transmembrane Gradient and Ligand-Induced Mechanisms of Adenosine 5′-Triphosphate Synthesis by Sarcoplasmic Reticulum Adenosinetriphosphatase†

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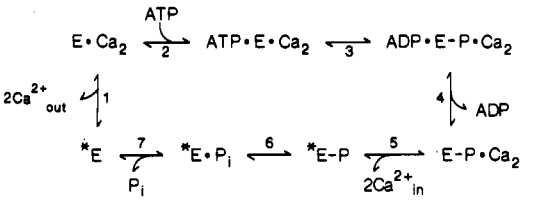
ABSTRACT: A series of experiments was performed in order to characterize ATP formation by sarcoplasmic reticulum adenosinetriphosphatase (ATPase). Comparative measurements were obtained by using native and leaky vesicles, in the presence and in the absence of a transmembrane Ca^{2+} gradient. ATP formation was started by addition of ADP to phosphoenzyme obtained by preincubation with acetyl phosphate and Ca^{2+} or by addition of ADP and Ca^{2+} to phosphoenzyme obtained by preincubation with inorganic phosphate (P_i) in the absence of Ca^{2+}. Transient-state measurements were carried out to obtain a kinetic characterization of phosphoenzyme formation following addition of ATP to enzyme preincubated with Ca^{2+} (10^2 s^{-1}) in the forward direction of the cycle and for ATP formation following addition of ADP to the phosphoenzyme–calcium complex (3×10^2 s^{-1}) in the reverse direction of the cycle. The rate constants of ATP association (4.5×10^6 M^{-1}) with and dissociation (50 s^{-1}) from the catalytic site were also obtained. A slow (k_{app} = 20 s^{-1}) step for ATP formation was observed when millimolar Ca^{2+} and ADP were added to phosphoenzyme obtained with P_i. This demonstrates a transition of this phosphoenzyme to a rapidly reactive state, before the occurrence of phosphoryl transfer to ADP. A match of the ATP hydrolysis and Ca^{2+} gradient potentials is consistent with ATP formation in the presence of a Ca^{2+} gradient but does not explain ATP formation in the absence of a gradient. A formulation is then introduced considering all the equilibrium constants for the partial reactions of the ATPase cycle. This analysis yields overall equilibria that explain transient formation of ATP even in the absence of a Ca^{2+} gradient as a consequence of reequilibration of intermediate enzyme states following ligand interactions with the phosphoenzyme. An additional finding obtained during this experimentation is that a rise of intrinsic fluorescence occurs when calcium binds to the nonphosphorylated enzyme but not when calcium binds to the enzyme in the phosphorylated intermediate state. This suggests that high-affinity binding and low-affinity binding occur in different protein locations and calcium is displaced from one to another as a consequence of enzyme phosphorylation.

It was originally discovered by Makinose and Hasselbach [1971; see also Yamada et al. (1972)] that if sarcoplasmic reticulum (SR) vesicles are filled with Ca^{2+} by active transport, using acetyl phosphate as a substrate in the presence of [^{32}P]P_i, addition of ADP [together with ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to reduce the Ca^{2+} concentration outside the vesicles] is followed by efflux of Ca^{2+} from the vesicles and formation of [γ-^{32}P]ATP. These and other experiments on enzyme phosphorylation with inorganic phosphate (P_i) (Makinose, 1972) in the presence of a transmembrane Ca^{2+} gradient suggested that osmotic mechanisms may be involved in the reversal of the SR Ca^{2+} pump to yield ATP. It was subsequently found that formation of ATP (Knowles & Racker, 1975; de Meis & Tume, 1977) can be obtained in single-cycle experiments even in the absence of a Ca^{2+} gradient across the SR membrane. This finding raised questions regarding the source of free energy for net synthesis of ATP in the absence of an electrochemical transmembrane gradient. Analysis of these phenomena, however, has been limited by the lack of kinetic and equilibrium data obtained in strictly comparable conditions (i.e., pH, electrolyte concentrations, ATP trapping systems, etc.). Therefore, considerable uncertainty has remained on just how ATP synthesis in the absence of a transmembrane electrochemical gradient can be reconciled with chemiosmotic mechanisms of energy transduction.

We describe in this paper a series of experiments carried out in order to characterize stoichiometries and kinetics of phosphorylation reactions and to establish the occurrence of isomeric enzyme transitions leading to ATP synthesis in comparative experiments with and without a transmembrane Ca^{2+} gradient. ATP was measured specifically as the product, in the absence of ATP-trapping systems. For the purpose of preliminary orientation, we reproduce (Scheme I) a version of the reaction scheme given by de Meis and Vianna (1979) for the ATPase cycle.

Scheme I



Even though detailed analysis and simulation of certain kinetic measurements require additional steps (Froehlich & Taylor, 1975; Fernandez-Belda et al., 1984), Scheme I includes the basic mechanistic features of the enzyme and transport cycle and provides the simplest way to explain our experimental plan. With our experiments we completed the characterization of partial reactions and isomeric transitions postulated by Scheme I and obtained estimates of related constants. We also found that the ATP synthesis observed in single-cycle experiments cannot be explained by simply relating the free energy of ATP hydrolysis to the transmembrane electrochemical gradient, as is usually done for multiple turnover experiments. Single-cycle experiments, dealing with product stoichiometry corresponding to that of the enzyme, require that

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intermediate enzyme states and related constants be considered. We were then able to account satisfactorily for the free energy required for transient ATP synthesis in the absence of a Ca^{2+} transmembrane gradient, on the basis of ligand-induced reequilibration of intermediate enzyme states.

Materials and Methods

Native SR vesicles were isolated from rabbit hind leg white muscle as previously described (Eletr & Inesi, 1972). Leaky vesicles were obtained by detergent extraction of native vesicles (Meissner et al., 1973) or by the addition of the ionophore A23187. Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Free Ca^{2+} concentrations were estimated from total calcium and EGTA by computations (Fabiato & Fabiato, 1979) taking into account the binding constant for the EGTA·Ca complex (Schwartzenbach et al., 1957), the EGTA·H^+ dissociation (Blinks et al., 1982), and the H^+, Mg^{2+}, and nucleotide concentrations when present.

Reagents. Carrier-free [^{32}P]P_i was obtained from New England Nuclear and purified (de Meis & Tume, 1977) before use. Acetyl [^{32}P]phosphate was prepared according to Kornberg et al. (1956).

Fluorescence intensity was measured in an Aminco-Bowman spectrofluorometer equipped with a thermostated cell holder (25 °C) and a cuvette stirrer. Excitation wavelength was 290 nm, and the emission intensity was measured at 330 nm. Rapid kinetic measurements were carried out with a Dionex D-137 stopped-flow spectrofluorometer equipped with a controlled-temperature bath (25 °C) and a 75-W mercury-doped xenon lamp. The excitation wavelength (290 nm) was selected with a monochromator, and the emitted light was passed at 90° through a 0-54 Corning cutoff filter.

Rapid-mixing experiments for measurements of phosphoenzyme decay and ATP synthesis were carried out with the aid of a Dionex D-133 multimixing apparatus or a Froehlich-Berger chemical quench-flow apparatus. Both instruments have a temperature-controlled (25 °C) block. In these experiments, the [^{32}P]phosphoenzyme formed during a 1-min preincubation of SR ATPase with acetyl [^{32}P]phosphate (25 °C) was mixed with an equal volume of medium containing ADP; then, at serial time intervals (3 ms–1 s), the reaction was quenched with 6.5% Cl_3CCOOH and nonradioactive ATP. The quenched samples were cooled in ice and centrifuged at 5000 rpm for 10 min (10 °C). The pellets were utilized for determination of [^{32}P]phosphoenzyme by measurements of radioactivity and protein following repeated washings. An aliquot (1 mL) of the supernatant was brought to neutral pH, filtered (Millipore, 0.45; 8 μM), and passed through a PARTISIL-10 SAX (250 × 46 mm) anion-exchange column (Regis Chemical Co.) in a Waters high-pressure liquid chromatographic system. The loaded column was washed for 14 min with 200 mM potassium phosphate (pH 6.0), and then the ATP was eluted with 500 mM potassium phosphate (pH 6.0). The elution flow was monitored continuously at 254 nm with a Waters 441 UV detector, and 1-mL sequential samples were collected for determination of radioactivity.

In other experiments phosphorylation of SR ATPase was obtained with [γ-^{32}P]ATP or [^{32}P]P_i as substrate, and the phosphoenzyme was measured as described above. Reaction mixtures and other details of various experiments are given in the legends of the figures.

Results

ATP Formation following Addition of ADP to Phosphoenzyme Formed with Acetyl Phosphate in the Presence of Ca^{2+}, Using Native SR Vesicles. The kinetics of ATP synthesis, through reversal of the catalytic and transport cycle of SR ATPase, are most simply demonstrated by first phosphorylating the enzyme and loading SR vesicles with calcium by utilization of acetyl [^{32}P]phosphate as the substrate and then adding ADP and EGTA to obtain [γ-^{32}P]ATP. Thereby, only one cycle of ATP synthesis occurs since further phosphoenzyme formation by acetyl phosphate is prevented by the low Ca^{2+} in the outside medium, and phosphorylation by P_i does not occur due to low P_i concentration. Under these conditions Pickart and Jencks (1982) obtained biphasic kinetics of phosphoenzyme decay and ATP formation. The biphasic kinetics were attributed to rapid formation of enzyme-bound ATP, followed by a slower phase that is rate-limited by slow dissociation of the nucleotide from the enzyme, as in

$$\text{E–P·Ca}_2 + \text{ADP} \xrightleftharpoons{\text{very fast}} \text{E–P·Ca}_2\text{·ADP} \xrightleftharpoons{\text{fast}} \text{E·Ca}_2\text{·ATP} \xrightleftharpoons{\text{slow}} \text{E·Ca}_2 + \text{ATP} \quad (1)$$

With our experiments we improved the time resolution and confirmed unambigously the biphasic kinetics of ATP synthesis (Figure 1). The fast component of ATP synthesis occurs with a rate constant $\cong$300 s^{-1}. This is in agreement with the rapid decay of phosphoenzyme observed by Froehlich and Heller (1985) upon addition of ADP. The apparent time constant for the slower kinetic phase is approximately 50 s^{-1}. In the presence of saturating ADP, the fraction of phosphoenzyme undergoing rapid phosphoryl transfer to ATP is approximately 70%. The stoichiometric ratio of ATP formation from phosphoenzyme is nearly 1 during the rapid kinetic component but becomes less than 1 during the slower phase.

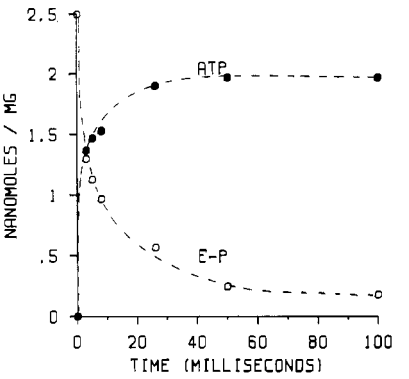

FIGURE 1: Phosphoryl transfer from [^{32}P]phosphoenzyme to ADP in the presence of a Ca^{2+} concentration gradient. Native SR vesicles (4.5 mg/mL) were preincubated with 2 mM acetyl [^{32}P]phosphate in the presence of 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and 1.05 mM $CaCl_2$. Following a 60-s preincubation, ADP and EGTA were added to yield 1.5 and 10 mM concentrations, respectively. The reaction was stopped at fractional time intervals with trichloroacetic acid and nonradioactive ATP to yield 6.6% and 0.33 mM, respectively. The quenched samples were processed for determination of [^{32}P]-phosphoenzyme and [^{32}P]ATP. Reaction temperature was 25 °C.

Phosphoenzyme Formation following Addition of ATP to Enzyme Preincubated with Ca^{2+}. Further kinetic characterization of the reaction of ATP with E·Ca_2 was obtained by adding various concentrations of [γ-^{32}P]ATP to enzyme preincubated with Ca^{2+} and resolving the time course of phosphoenzyme formation by rapid quench methods (Figure 2A). These measurements can be considered on the basis of the reactions

$$\text{ATP} + \text{E·Ca}_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{ATP·E·Ca}_2 \underset{k_{rev}}{\overset{k_{phos}}{\rightleftharpoons}} \text{ADP·E–P·Ca}_2 \quad (2)$$

It should be pointed out that the experimental measurements

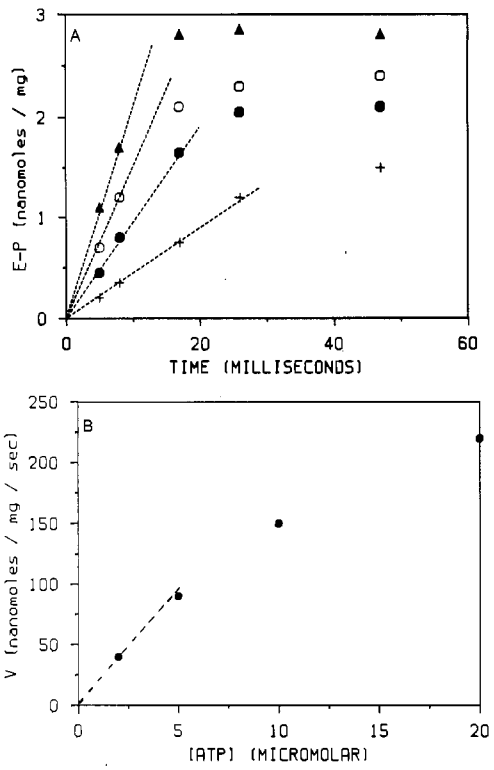

FIGURE 2: Enzyme phosphorylation with ATP. Native SR vesicles (0.1 mg/mL) were preincubated with 20 mM MOPS (pH 6.8), 80 mM KCl, 2 mM $MgCl_2$, and 50 μM $CaCl_2$. The reaction was started by the addition of [γ-^{32}P]ATP to yield 2, 5, 10, or 20 μM concentrations. Quenching was obtained at serial times by the addition of perchloric acid and nonradioactive P_i to yield 0.125 and 2 mM concentrations, respectively. The quenched samples were processed for determination of [^{32}P]phosphoenzyme. Reaction temperature was 25 °C. In (A) the phosphoenzyme levels are plotted vs. time for the different ATP concentrations: (+) 2, (●) 5, (○) 10, and (▲) 20 μM ATP. In (B), the initial velocities of phosphorylation are plotted vs. the ATP concentration.

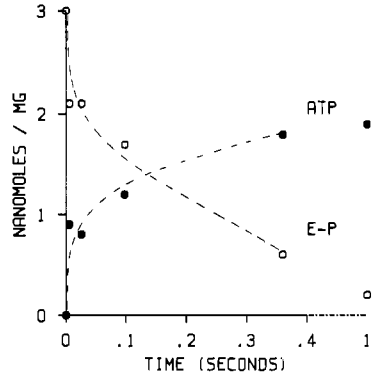

FIGURE 3: Phosphoryl transfer from [^{32}P]phosphoenzyme to ADP in the absence of a Ca^{2+} concentration gradient. SR ATPase assembled in leaky vesicles (4.5 mg/mL) was preincubated with 2 mM acetyl [^{32}P]phosphate in the presence of 50 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, and 20 mM $CaCl_2$. Following a 60-s preincubation, ADP and EGTA were added to yield 1.5 and 10 mM concentrations, respectively. Quenching and sample processing were as described for Figure 1. Reaction temperature was 25° C.

determine E–P independent of ADP or Ca^{2+} complexation and of various phosphoenzyme states that may be formed in the enzyme cycle (cf. Scheme I). The observed constants may include conformational transitions that occur as a consequence of ATP binding and/or enzyme phosphorylation.

For the reactions above, k_{off} and k_{rev} were derived from the measurements shown in Figure 1 and are 50 s^{-1} and 300 s^{-1}, respectively.

Estimate of k_{phos}. We now assume that ADP dissociates rapidly (Pickard & Jenks, 1982, 1984; Froehlich & Heller, 1985) from the phosphorylated enzyme and that negligible reversal occurs, as demonstrated by the very low ADP–ATP exchange at low ADP concentrations (not shown). We consider then that even though phosphoenzyme hydrolysis is limited by a slow step (10 s^{-1}), significant phosphoenzyme hydrolysis occurs in the *steady state*, as demonstrated by the dependence of the final levels of phosphoenzyme on the ATP concentration (Figure 2A). We have shown, however, that phosphoenzyme hydrolysis begins after a lag period following addition of ATP and formation of phosphoenzyme (Fernandez-Belda et al., 1984). For these reasons, we assume that the *initial phase* of the phosphorylation reaction includes negligible reversal and/or hydrolysis. The initial velocities of phosphoenzyme formation are then obtained from Figure 2A and expressed as functions of [ATP] in a double-reciprocal plot. Extrapolation of this plot to infinite [ATP] (not shown) yields V_{max} = 400 nmol mg^{-1} s^{-1}. If we consider that $v = k_{phos}[E{\cdot}Ca_2{\cdot}ATP]$ and that, at infinite [ATP], $[E{\cdot}Ca_2{\cdot}ATP] = [E_{tot}]$, we conclude that $k_{phos} = V_{max}/[E_{tot}]$.

$[E_{tot}]$ was measured separately by phosphorylating the ATPase catalytic sites with P_i in highly favorable conditions (Barrabin et al., 1984). The resulting estimate for k_{phos} is approximately 100 s^{-1}.

Estimate of k_{on}. To determine the value of k_{on}, we assume that at very low concentrations the rate-limiting step is k_{on}-[ATP]. Therefore, $v = k_{on}[ATP][E_{tot}]$; then, $k_{on} = v/[ATP][E_{tot}]$. This can be derived from the slope of the plot (Figure 2B) of initial velocity vs. [ATP]. The initial portion of this plot is linear and extrapolates to 0. Our estimate for k_{on} is 4.5 × 10^6 M^{-1} s^{-1}.

ATP Formation following Addition of ADP to Phosphoenzyme Formed with Acetyl Phosphate in the Presence of Ca^{2+}, Using Leaky Vesicles. The kinetics of ATP synthesis shown in Figure 1 were obtained with native vesicles in which the internal Ca^{2+} concentration had been raised by preincubation with acetyl phosphate. In this system, addition of ADP and EGTA started one cycle of ATP synthesis in the presence of a transmembrane Ca^{2+} gradient. We then carried out a set of experiments under comparable conditions, using leaky vesicles in order to avoid formation of a transmembrane Ca^{2+} gradient. High (10 mM) Ca^{2+} was present inside and outside the vesicles during the preincubation with acetyl phosphate. In these experiments we again found biphasic kinetics of ATP formation within the first 100 ms of reaction (Figure 3), with a nearly stoichiometric ratio of phosphoryl transfer from the phosphoenzyme to ADP. A significant fraction (approximately half) of the phosphoenzyme, however, underwent slower (up to 500 ms) decay without forming ATP. It is likely that the latter decay was due to hydrolytic cleavage of phosphoenzyme that was not saturated with calcium at the times of ADP addition and proceeded in the forward, rather than in the reverse, direction of the catalytic cycle.

We conclude that the phosphoenzyme obtained by preincubation with a phosphorylating substrate in the forward direction of the cycle can transfer its phosphate to ADP very rapidly within a single reverse cycle, independent of the presence of a Ca^{2+} gradient across the SR membrane, as long as calcium is bound to the phosphoenzyme. In the experiment reported in Figure 3, a sequence of events analogous to that reported in Figure 1 took place within a single cycle. In fact, calcium was first bound to the phosphoenzyme during the preincubation in the presence of 10 mM Ca^{2+}. Then it was released in parallel with ATP formation since EGTA was added with ADP to initiate the phosphoryl-transfer reaction.

ATP Formation following Addition of Ca^{2+} and ADP to Phosphoenzyme Formed with P_i in the Absence of Ca^{2+}. The experiments described above were performed with enzyme phosphorylated with acetyl phosphate in the presence of Ca^{2+} to obtain phosphoenzyme–calcium complex which is reactive to ADP. On the other hand, the behavior of the phosphoenzyme with no calcium bound can be best studied by phosphorylating the enzyme with P_i (Masuda & de Meis, 1973; Kanazawa & Boyer, 1973) in the absence of Ca^{2+} (reverse direction of partial reactions 7 and 6 in Scheme I), yielding the equilibrium

$$E + P_i \rightleftharpoons E{\cdot}P_i \rightleftharpoons {}^*E\text{–}P \qquad (3)$$

Contrary to the phosphoenzyme formed with acetyl phosphate or ATP, this phosphoenzyme does not form ATP upon addition of ADP, unless millimolar Ca^{2+} is also added with ADP (Knowles & Racker, 1975; de Meis & Tume, 1977). This suggests that calcium binding to the low-affinity sites produces transition of the phosphoenzyme to a state reactive to ADP. Consistent with this assumption, the kinetics of ATP formation following addition of millimolar calcium and ADP to phosphoenzyme formed with P_i were found by de Meis and Tume (1977) to be rather slow. Their measurements, however, were obtained at pH 6 and in the absence of K^+ (in order to obtain high levels of phosphoenzyme with P_i) and therefore under conditions that are not directly comparable to those of the experiments with acetyl phosphate. In order to observe the kinetics of ATP formation under conditions identical with those of the experiments with enzyme preincubated with acetyl phosphate, we then phosphorylated the enzyme (leaky vesicles) with P_i at pH 6 and in the absence of K^+ and then jumped the pH to 6.8 and raised the K^+ concentration to 80 mM upon addition of calcium and ADP. We found that net ATP synthesis can still be obtained under these conditions. However, the phosphoenzyme decay and ATP formation are much slower (compare Figure 4 to Figure 1). The stoichiometric ratio of phoshoenzyme transformation to ATP is also much lower, although more favorable ratios (de Meis & Tume, 1977) are obtained under special conditions (i.e., in the absence of K^+ and presence of ATP-trapping systems).

The apparent rate constant of ATP formation following addition of Ca^{2+} and ADP to E–P formed with P_i is approximately 20 s^{-1} (Figure 4B). This value is much lower than that (300 s^{-1}) observed (Figure 1) for phosphoryl transfer to ADP from phosphoenzyme formed with ATP or acetyl phosphate and is evidently related to a slow transition preceding the transfer.

Fluorescence Changes following Addition of Ca^{2+} to Phosphoenzyme Formed with P_i in the Absence of Ca^{2+}. The (millimolar) calcium requirment for ATP formation upon addition of ADP to phosphoenzyme formed with P_i in the absence of Ca^{2+} indicates that calcium binding to the phosphoenzyme produces a slow transition rendering the phosphoryl-transfer reaction possible. We then investigated whether this transition is accompanied by a change of intrinsic fluorescence, in analogy to the effect produced by micromolar calcium on the high-affinity sites of the nonphosphorylated enzyme. To this effect, we first obtained comparative measurements of intrinsic fluorescence following addition of calcium (in the absence of ADP) to SR ATPase in the nonphosphorylated state or following phosphorylation with P_i. Examples of intrinsic fluorescence rise following addition of low or high Ca^{2+} to enzyme preincubated with EGTA in the absence (therefore E) or in the presence of P_i (under conditions yielding mostly *E–P) are shown in Figure 5. It is clear that in all cases the intrinsic fluorescence rise is much slower when

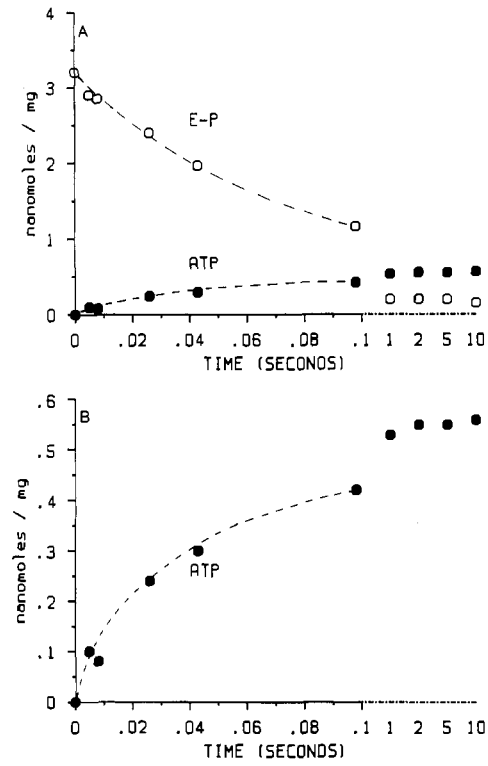

FIGURE 4: Phosphoenzyme decay and ATP formation following addition of ADP and Ca^{2+} to SR ATPase phosphorylated with P_i. SR ATPase assembled in leaky vesicles was preincubated (1 mg/mL) for several minutes with 5 mM [^{32}P]P_i in the presence of 5 mM 2-(*N*-morpholino)ethanesulfonic acid–tris(hydroxymethyl)aminomethane (MES–Tris) (pH 6.0), 10 mM EGTA, and 10 mM $MgCl_2$. This mixture was then mixed in a chemical quench-flow system with an equal volume of a medium containing 200 mM MOPS (pH 6.8), 160 mM KCl, 10 mM $MgCl_2$, 40 mM $CaCl_2$, and 4 mM ADP. Serial quenching and determination of phosphoenzyme and ATP were carried out as described for Figure 1. Reaction temperature was 25 °C. ATP formation is shown in expanded scale in (B). Following the burst of phosphoryl transfer and ATP formation shown above, further formation of [γ-^{32}P]ATP is obtained by prolonged incubation (not shown). Contrary to the early burst, the slow formation of [γ-^{32}P]ATP occurs in the absence of significant EP levels and is reduced by the myokinase inhibitor bis(adenosyl) pentaphosphate. Therefore, it is likely that this very slow phenomenon is actually due to (nonradioactive) ATP formation from ADP (catalyzed by myokinase contaminations) and then to [^{32}P]$P_i \rightleftharpoons$ ATP exchange operated by the SR ATPase.

Ca^{2+} is added to the phosphorylated rather than to the nonphosphorylated enzyme. This behavior suggests that the phosphoenzyme undergoes hydrolytic cleavage before the intrinsic fluorescence rise. We then carried out parallel measurements of phosphoenzyme and fluorescence levels following addition of calcium.

When Ca^{2+} is added to yield concentrations in the *micromolar* range to the enzyme phosphorylated with P_i, the phosphoenzyme decays (Figure 6A) due to a calcium-induced transformation of the enzyme to a form that cannot be phosphorylated by P_i, as in

$$^*E\text{-}P \xrightleftharpoons{P_i} {}^*E \xrightleftharpoons{2Ca^{2+}} E{\cdot}Ca_2 \qquad (4)$$

where *E–P is a low-fluorescence state with low calcium affinity ($K_d \cong 10^{-3}$ M) and E is a state with high affinity ($K_d \cong 10^{-6}$ M) for Ca^{2+}. This transformation is accompanied by an increase in intrinsic (tryptophan) enzyme fluorescence, which is known to accompany high-affinity calcium binding to SR ATPase (Dupont, 1976). It is shown in Figure 6A that the change of intrinsic fluorescence proceeds in parallel with

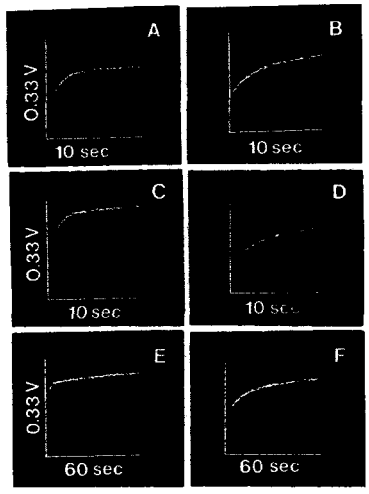

FIGURE 5: Changes of intrinsic fluorescence following addition of Ca^{2+} to nonphosphorylated or phosphorylated enzyme. SR ATPase assembled in leaky vesicles was preincubated (0.3 mg/mL) for several minutes in the absence (A, C, and E) or in the presence (B, D, and F) of 10 mM P_i in the presence of 100 mM MES–Tris (pH 6.1), 10 mM $MgCl_2$, and 4 mM EGTA. This mixture was mixed in a stopped-flow apparatus with an equal volume of a medium containing 100 mM MES–Tris (pH 6.1), 10 mM $MgCl_2$, and $CaCl_2$ to yield 100 μM (A and B) or 10 mM (C, D, E, and F) concentrations.

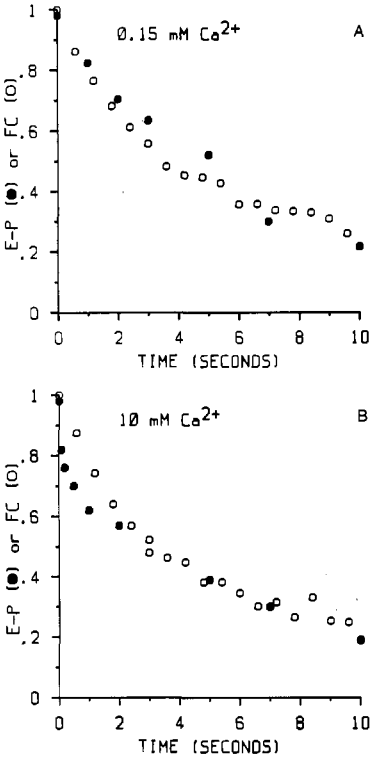

FIGURE 6: Fractional change in phosphoenzyme (decay) and fluorescence (rise) levels following addition of Ca^{2+} to enzyme phosphorylated with P_i. SR ATPase assembled in leaky vesicles was preincubated (0.3 mg/mL) for several minutes with 10 mM P_i or $[^{32}P]P_i$ in the presence of 100 mM MES–Tris (pH 6.1), 10 mM $MgCl_2$, and 4 mM EGTA. This mixture was then mixed with an equal volume of a medium containing 100 mM MES–Tris (pH 6.1), 10 mM $MgCl_2$, and $CaCl_2$ to yield 0.15 (A) or 10 mM (B) Ca^{2+} concentrations, with a stopped-flow apparatus to monitor fluorescence changes as described for Figure 5 or with a chemical quench-flow system for determination of $[^{32}P]$phosphoenzyme at serial times as described for Figure 4. Reaction temperature was 25 °C.

the decrease of phosphoenzyme level. Therefore, we conclude that addition of *micromolar* Ca^{2+} displaces the equilibrium in favor of $E{\cdot}Ca_2$ (see Scheme I) due to high-affinity calcium binding to E and that the phosphoenzyme level decays as a consequence of this displacement.

Table I: Approximate Kinetic and Equilibrium Constants for the Phosphorylation Reaction of SR ATPase ($E{\cdot}Ca_2$) with ATP[a]

reactions	constants
$ATP + E{\cdot}Ca_2 \rightleftharpoons ATP{\cdot}E{\cdot}Ca_2$	$k_{on} = 4.5 \times 10^6\ M^{-1}\ s^{-1}$ $k_{off} = 50\ s^{-1}$ $K_a = 1.0 \times 10^5\ M^{-1}$
$ATP{\cdot}E{\cdot}Ca_2 \rightleftharpoons ADP{\cdot}E{\sim}P{\cdot}Ca_2$	$k_{phos} = 100\ s^{-1}$ $k_{rev} = 300\ s^{-1}$ $K = 0.33$

[a] These values were obtained from experiments conducted at 25 °C, at pH 6.8, and in the presence of 80 mM KCl and 2–5 mM Mg^{2+} (Figures 1 and 2). The reactions listed refer to the actual experimental measurements and do not state explicitly enzyme transitions that are known to accompany these reactions and are relevant to the coupling mechanism of catalysis and transport.

We then investigated the effect of (millimolar) calcium binding (in the absence of ADP) to the low-affinity sites of the phosphoenzyme obtained with P_i. It is shown in Figure 6B that, following addition of millimolar Ca^{2+} to the phosphoenzyme formed with P_i, a change of intrinsic fluorescence does not precede but rather follows phosphoenzyme decay. This indicates that the higher fluorescence is a specific consequence of calcium binding to the high-affinity sites of the nonphosphorylated enzyme. Therefore, the phosphoenzyme must lose its phosphate before developing the calcium-induced fluorescence rise. This observation is of considerable interest, inasmuch as it suggests that high-affinity calcium binding to the nonphosphorylated enzyme and low-affinity calcium binding to the phosphorylated enzyme occur in different protein sites. In this view, calcium would be displaced from one location to the other, as a consequence of enzyme phosphorylation.

Discussion

The experiments shown in Figures 1 and 2 yield values for the kinetic and equilibrium constants (Table I) for ATP association with and dissociation from the enzyme–calcium complex of the SR ATPase. They also provide values for the rate constants of phosphoenzyme formation following addition of ATP to enzyme preincubated with Ca^{2+} in the forward direction of the cycle and for ATP formation following addition of ADP to $E{\sim}P{\cdot}Ca_2$ in the reverse direction of the cycle. The rate constant for phosphoenzyme formation (100 s^{-1}) is only slightly lower than that published originally by Froelich and Taylor (1975). The rate constants for ATP binding and dissociation and for ATP formation, which are here demonstrated experimentally, are similar to those chosen by Pickart and Jencks (1982) and Froehlich and Heller (1985) in their analysis.

It is noteworthy that the phosphoryl-transfer reactions may be preceded or accompanied by functionally relevant enzyme transitions. For instance, the biphasic kinetics of ATP synthesis in single-cycle experiments, originally observed by Pickart and Jencks (1982), include a fast phase attributed to phosphoryl transfer from the phosphoenzyme to ADP (300 s^{-1}) and a slow phase attributed to dissociation of newly synthesized ATP from the enzyme (50 s^{-1}). On the other hand, when the phosphoenzyme is obtained under different conditions, a significant fraction does not reach with ADP [Figure 3; see also Froehlich and Heller (1985)]. To avoid ambiguities in the attribution of various kinetic components to ADP-sensitive and ADP-insensitive fractions of the phosphoenzyme pool, we carried out a separate set of experiments with enzyme phosphorylated with P_i in the absence of Ca^{2+} (reactions 7 and 6 in Scheme I) and therefore with a phosphoenzyme pool

totally nonreactive to ADP. We then measured the kinetics of its transition to the ADP-sensitive state upon addition of high (10 mM) Ca^{2+} and ADP. In this case we did not observe the rapid phase of ATP synthesis, but rather we observed slow kinetics (k_{app} = 20 s^{-1}) that include the phosphoenzyme transition as well as the phosphoryl transfer and other possible steps. The low rate of the calcium-induced phosphoenzyme transition is also revealed by the low stoichiometric ratio of ATP formation (Figure 4), indicating that hydrolytic cleavage and calcium binding to the dephosphorylated enzyme (reactions 6, 7, and 1 in Scheme I) proceed at a rate that competes significantly with the calcium-induced phosphoenzyme transition to the ADP-sensitive form (reversal of steps 5 and 4 in Scheme I). All these observations indicate strongly that the mechanism of calcium-induced destabilization of the phosphoenzyme (Jencks, 1980; Tanford, 1981a; Inesi, 1985), which is instrumental in promoting phosphoryl transfer from the phosphoenzyme to ADP, entails a slow conformational change in parallel with reequilibration of intermediate enzyme states.

Our comparative experiments in the presence and in the absence of a gradient demonstrate clearly that, in addition to thermodynamic limitations, kinetic constraints can also limit ATP synthesis within the time frame of experimental measurements [see also Guimaraes-Motta and de Meis (1980)]. For instance, even though in optimal kinetic conditions relatively large amounts of ATP can be formed by dispersion of a Ca^{2+} gradient and repeated reversal of ATPase cycles (Makinose & Hasselbach, 1971; Chaloub et al., 1979), in the presence of the same gradient ATP synthesis can be limited to only a single enzyme cycle (Figure 1) if the P_i concentration is lower than that required to again phosphorylate the enzyme following the first cycle. In this case, similar amounts of ATP are formed in the presence (Figure 1) and in the absence (Figure 3) of a Ca^{2+} gradient. On the other hand, in the absence of a gradient, a lower stoichiometry of phosphoryl transfer to ADP is obtained when the phosphoenzyme is formed with P_i in the absence of Ca^{2+} (Figure 4), as compared to acetyl phosphate in the presence of Ca^{2+} (Figure 3). Such a lower stoichiometry is due to the prevalence of kinetic pathways leading to hydrolytic cleavage (steps 6 and 7 in Scheme I) over those leading to phosphoryl-transfer (steps 5, 4, and 3) reactions.

As for thermodynamic limitations, we consider first that, in an energy-transducing system such as the SR pump, the free energy realized through dissipation of an electrochemical gradient is commonly estimated according to

$$\Delta G = -RT \ln ([Ca^{2+}_{in}]/[Ca^{2+}_{out}]) + nF\Delta V \quad (5)$$

Since charge imbalance is assumed to be compensated in SR by rapid fluxes of electrolytes (other than Ca^{2+}), the electrical gradient is usually neglected and only the Ca^{2+} gradient is considered (Tanford, 1981b). Accordingly, a 3 order of magnitude Ca^{2+} gradient (e.g., 10^{-3} M Ca^{2+}_{in} and 10^{-6} M Ca^{2+}_{out}, at 25 °C) yields 4 kcal mol^{-1} Ca^{2+} or 8 kcal per reverse enzyme cycle (2 mol of calcium are translocated per cycle). The free energy requirement for formation of relatively large amounts of ATP under conditions permitting repeated reverse cycles in the presence of catalytic enzyme concentrations is then accounted for by eq 5, which, however, is totally independent (and noninformative) of the mechanism of transduction. On the other hand, for the analysis of single-cycle experimentation conducted in the presence of high enzyme concentration, we introduce various enzyme states in the equilibrium equation, stating explicitly a reaction mechanism. In fact, a minimal number of sequential reactions derived from Scheme I and their approximate equilibrium constants can be written for the SR ATPase as shown in Table II. In standard conditions (i.e., in the presence of 1 M concentrations of Ca^{2+}_{in}, Ca^{2+}_{out}, ATP, ADP, and P_i) the overall equilibrium [$(3 \times 10^{12}$ $M^{-2})(1 \times 10^5$ $M^{-1})(0.3)(7 \times 10^{-4}$ M)$(3 \times 10^{-6}$ $M^2)(1)(1 \times 10^{-2}$ M)] can be calculated to be 2×10^6, yielding −8.3 kcal mol^{-1} of "standard" free energy per cycle in the forward direction, which corresponds to the free energy of hydrolysis of 1 mol of ATP to ADP, and can be utilized for transport of Ca^{2+} against a concentration gradient (Inesi et al., 1980; Pickart & Jencks, 1984; Inesi, 1985). This shows that the overall analysis is correct, even though it is certain that some of the reactions listed include more than a single step.

Table II

step	reaction	constants	ref
1	$*E + 2Ca^{2+}_{out} \rightleftharpoons E{\cdot}Ca_2$	3×10^{12} M^{-2}	Inesi et al., 1980
2	$E{\cdot}Ca_2 + ATP \rightleftharpoons ATP{\cdot}E{\cdot}Ca_2$	1×10^5 M^{-1}	Figure 2
3	$ATP{\cdot}E{\cdot}Ca_2 \rightleftharpoons ADP{\cdot}E{-}P{\cdot}Ca_2$	0.3	Figures 1 and 2
4	$ADP{\cdot}E{-}P{\cdot}Ca_2 \rightleftharpoons E{-}P{\cdot}Ca_2 + ADP$	7×10^{-4} M	Pickart & Jencks, 1984
5	$E{\sim}P{\cdot}Ca_2 \rightleftharpoons *E{-}P + 2Ca^{2+}_{in}$	3×10^{-6} M^2	Coan et al., 1979
6	$*E{-}P \rightleftharpoons *E{\cdot}P_i$	1	Inesi et al., 1982
7	$*E{\cdot}P_i \rightleftharpoons *E + P_i$	1×10^{-2} M	Inesi et al., 1982

If the pertinent equilibrium constants are corrected by the concentrations of ligands (Hill, 1960) present in typical experiments on ATP synthesis through dissipation of a Ca^{2+} gradient (i.e., 1×10^{-2} M Ca^{2+}_{in}, 1×10^{-6} M Ca^{2+}_{out}, 2×10^{-3} M ADP, 1×10^{-2} M P_i, and 1×10^{-7} M ATP), the overall equilibrium constant is 9.3×10^{-5}, yielding a free energy change of +5.6 kcal mol^{-1}, which, in the reverse direction of the cycle, can be utilized for ATP synthesis through dissipation of the gradient. This is also predicted by eq 5. Analysis of the partial reactions, however, takes into account the role of binding and dissociation events in the mechanism of energy transduction.

We now consider the single cycle of ATP synthesis observed in the absence of a Ca^{2+} gradient. Assuming that the preincubation mixture (formation of *E–P from P_i) contains 1×10^{-2} M P_i, 1×10^{-8} M Ca^{2+}_{in}, 1×10^{-8} M Ca^{2+}_{out}, 1×10^{-7} M ATP, and 1×10^{-7} M ADP, the overall equilibrium constant is 1.9×10^8, with a ΔG of −11.5 kcal mol^{-1}. When the concentrations of Ca^{2+}_{in}, Ca^{2+}_{out}, and ADP are changed to 1×10^{-2}, 1×10^{-2}, and 2×10^{-3} M, respectively, the overall equilibrium is shifted to 9.5×10^3, corresponding to a ΔG of −5.5 kcal mol^{-1}. Therefore, approximately 6 kcal mol^{-1} are available for displacement of the equilibrium upon ligand binding, making it possible to obtain a change in the distribution of intermediate states and a transient formation of ATP. This can be detected if the reaction is quenched at appropriate times.

It is of interest that if 10^{-6} M (rather than 10^{-2} M) Ca^{2+}_{in} and Ca^{2+}_{out} are added with ADP, no significant ATP formation is observed, even though the calculated free energy change is the same. This is due to the different pathways prevailing in the reequilibration process, when only the high-affinity (reactions 6, 7, and 1 in Scheme I), as opposed to the low-affinity (reactions 5, 4, 3, 2, and 1 in Scheme I), sites are occupied by calcium.

Our observations demonstrate that a reaction-cycle analysis which is based on the partial reactions has the advantage of being explicit with respect to the mechanism of energy transduction and clarifies the role of ligand binding and dissociation in coupling of catalysis and cation transport.

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A New Acylphosphatase Isoenzyme from Human Erythrocytes: Purification, Characterization, and Primary Structure†

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ABSTRACT: A new acylphosphatase from human erythrocytes was isolated by an original purification procedure. It is an isoenzyme of the well-characterized human skeletal muscle acylphosphatase. The erythrocyte enzyme shows hydrolytic activity on acyl phosphates with higher affinity than the muscle enzyme for some substrates and phosphorylated inhibitors. The sequence was determined by characterizing the peptides purified from tryptic, peptic, and *Staphylococcus aureus* V8 protease digests of the protein, and it was found to differ in 44% of the total positions as compared to the human muscle enzyme. About one-third of these differences are in the form of strictly conservative replacements. The protein consists of 98 amino acid residues; it has an acetylated NH_2-terminus and does not contain cysteine: Ac-Ala-Glu-Gly-Asn-Thr-Leu-Ile-Ser-Val-Asp-Tyr-Glu-Ile-Phe-Gly-Lys-Val-Gln-Gly-Val-Phe-Phe-Arg-Lys-His-Thr-Gln-Ala-Glu-Gly-Lys-Lys-Leu-Gly-Leu-Val-Gly-Trp-Val-Gln-Asn-Thr-Asp-Arg-Gly-Thr-Val-Gln-Gly-Gln-Leu-Gln-Gly-Pro-Ile-Ser-Lys-Val-Arg-His-Met-Gln-Glu-Trp-Leu-Glu-Thr-Arg-Gly-Ser-Pro-Lys-Ser-His-Ile-Asp-Lys-Ala-Asn-Phe-Asn-Asn-Glu-Lys-Val-Ile-Leu-Lys-Leu-Asp-Tyr-Ser-Asp-Phe-Gln-Ile-Val-Lys-OH.

Acylphosphatase was first described as acetylphosphatase by Lipmann (1946). The enzyme is a small protein (M_r 11 000–12 000) characterized by a specific phosphomonohydrolase activity. It catalyzes the hydrolysis of compounds with the general structure R–COO–PO_3^{2-}, such as 1,3-diphosphoglycerate and carbamyl phosphate (Ramponi, 1975), succinyl phosphate (Berti et al., 1977), and the β-aspartyl phosphates that are present in phosphorylated intermediates

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