Partial Reactions in the Catalytic and Transport Cycle of Sarcoplasmic Reticulum ATPase†

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ABSTRACT: Stopped flow and rapid quench techniques were combined to measure enzyme phosphorylation, P_i production, H^+ liberation, and Ca^{2+} transport in the millisecond time scale, following addition of ATP, ITP, or P_i to sarcoplasmic reticulum vesicles. A number of constants for partial reactions of the catalytic and transport cycle were obtained experimentally. Furthermore a comparison of rapid kinetic measurements with previous observations (mostly made in steady-state conditions) leads to the following conclusions. (a) Phosphorylation of one enzyme molecule is rapidly followed by transfer of two calcium molecules across the membrane and release of Ca^{2+} inside the vesicles. The rapid Ca^{2+} translocation step is responsible for immediate utilization of the free energy transferred from ATP to the enzyme, into vectorial transport of Ca^{2+}. On the other hand, accumulation of ADP leads to utilization of this free energy for synthesis of ATP at a fast rate in the reverse reaction. (b) Following the initial burst of protein phosphorylation and Ca^{2+} translocation, Ca^{2+} transport proceeds at linear rates and is related with a constant stoichiometric ratio to H^+ production. Maximal Ca^{2+} transport velocity in the first 400 ms is 70–100 nmol mg^{-1} s^{-1}. (c) The structure of the substrate not only determines its affinity for the enzyme (de Meis, L., & de Mello, M. C. F. (1973) *J. Biol. Chem. 248,* 3691), but also affects the rate of the calcium translocation step subsequent to enzyme phosphorylation. This is attributed to conformational effects induced by the substrate on the enzyme. (d) The dependence of the initial levels of phosphorylated enzyme on substrate concentration follows a simple, as opposed to the diphasic pattern exhibited by transport and hydrolytic activities. This indicates that, while saturation of the catalytic site is obtained in the presence of 0.1 mM ATP, turnover activation is produced by 1.0 mM ATP. (e) The enzyme reaction with orthophosphate (Masuda, H., & de Meis, L. (1973) *Biochemistry 12,* 4581; Kanazawa, T., & Boyer, P. D. (1973) *J. Biol. Chem. 248,* 3163) occurs at different rates in the presence, as opposed to the absence, of a transmembrane Ca^{2+} gradient. This suggests that the enzyme resides in equilibrium between two different states, characterized by exposure of the Ca^{2+} binding sites on the inner or outer membrane surface, respectively.

Characterization of the ATPase and Ca^{2+} transport activities of sarcoplasmic reticulum (SR) was initially obtained by steady-state experimentation. It was shown that vesicular fragments of SR membrane accumulate Ca^{2+} in the presence of ATP (Ebashi & Lipman, 1962; Hasselbach & Makinose, 1961, 1963), and that Ca^{2+} transport is coupled to ATP hydrolysis with a Ca^{2+}/ATP ratio of 2:1 (Martonosi & Feretos, 1964; Weber et al., 1966). The coupling mechanism is carried out by the ATPase protein which is the main component of the SR membrane (Mac Lennan, 1970; Meissner et al., 1973; Racker, 1972). The catalytic cycle includes transfer of the ATP terminal phosphate to the enzyme (Yamamoto & Tonomura, 1967; Makinose, 1969), resulting in acid stable phosphorylation of an aspartyl residue (Degani & Boyer, 1973; Bastide et al., 1973).

Adding to the steady-state investigations, rapid mixing techniques were more recently used for the time resolution of transient state phenomena (Inesi & Scarpa, 1972; Froehlich & Taylor, 1975, 1976; Boyer et al., 1977). In our laboratory we have combined stopped-flow and rapid-quench techniques for determination of protein phosphorylation, P_i production, H^+ liberation, and Ca^{2+} transport in the millisecond time scale. In this manner we were able to study a number of partial reactions in the ATPase-transport cycle.

Materials and Methods

Sarcoplasmic reticulum vesicles were isolated from rabbit white skeletal muscle as previously described (Eletr & Inesi, 1972). Protein concentration was estimated by the Folin method.

Optical measurements of H^+ production were obtained by monitoring light absorption changes undergone by phenol red. For this purpose an Aminco DW2 dual-wavelength spectrophotometer, modified to reduce chopper noise in the signal, was used. The reaction was initiated by addition of ATP to the reaction mixture using a regenerative stopped-flow apparatus (Chance et al., 1967). Alternatively, a Durrum dual-detector stopped flow spectrophotometer was used. Absorption changes were continuously recorded in storage oscilloscope.

Measurements of H^+ production were done in a medium containing 80 mM KCl, 100 μM $CaCl_2$, 8 mM $MgCl_2$, 100 μM phenol red, 2 mM Mops[1] (pH 7.1), 0.6–1.0 mg of SR protein/mL, and 0.1–1.0 mM ATP. Calibration was obtained by stepwise addition of equimolar amounts of HCl and inorganic phosphate. A linear response to additions up to 50 nmol/mL of HCl + P_i was obtained. A reference wavelength of 480 nm and a measuring wavelength of 560 nm were used.

Rapid mixing and quenching experiments were carried out with the aid of a Durrum D-133 multimixing apparatus whose performance in the millisecond time scale was checked both

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[1] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; PEP, phosphoenolpyruvate.

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in terms of mixing rates and time calibration.

The efficiency of the mixing chambers was tested separately with a stopped-flow optical system equipped with an identical mixing chamber. The dead time was measured by following the decrease in transmitted light at 590 nm upon the reaction of 0.01 M $Fe(NO_3)_3$ in 0.1 N H_2SO_4 with 0.01 M KCNS. Extrapolation from the initial slope of the transmittance-dropoff produced by the reaction back to the 100% transmission line indicates that complete mixing was obtained in less than 1 ms.

The accuracy of time calibration was tested by following the alkaline hydrolysis of 2,4-dinitrophenyl acetate (Gutfreund, 1969), for which a second-order rate constant (inset to Figure 1) of 52 M^{-1} s^{-1} was obtained, in close agreement with that obtained by optical stopped-flow measurements (Barman & Gutfreund, 1964) and by quenching techniques (Froehlich et al., 1976). The curves in Figure 1 intercept the origin indicating that the times shown reflect the actual reaction times down to 20 ms.

Radioactive Ca^{2+}-Uptake Measurements by Rapid Quenching Techniques. In addition to the murexide method, we are able to monitor the transient Ca^{2+} uptake by the radioactive Ca^{2+} tracer method, using the Durrum multimixer (Kurzmack et al., 1977). Uptake was started with ATP and stopped by rapidly quenching with high concentrations of EGTA. The Ca^{2+} accumulated inside the vesicles was thus retained for more than 30 s thereafter, allowing sufficient time for collection of the sample and separation of the vesicles from the medium by filtration (Martonosi & Feretos, 1964).

The reaction was started by mixing two equal volumes, one containing SR and the other ATP in addition to all other reagents. The final concentrations in the reaction mixture were 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 30–35 μM $^{45}CaCl_2$, 0.15–0.40 mg of SR protein/mL and 0.02–3.0 mM ATP or ITP. Rapid quenching was obtained with an equal volume of 20 mM Mops, 80 mM KCl, 10 mM EGTA. The quenched solution (1.0–1.5 mL) was collected and filtered within 15–20 s through a Swinnex containing a Millipore filter (13 mm, Type HA.45 μm) and 0.5 mL of the filtrate was used for determination of radioactivity.

Blanks were prepared by inverting the sequence of addition of ATP and quenching solution. SR suspension and quenching solution were placed in the first two syringes and allowed to react for 50–100 ms to ensure removal of radioactive calcium from the vesicles before addition of ATP with the third syringe. No detectable radioactivity was found missing from the blank filtrates when compared with the original solutions. Parallel experiments showed that no phosphoenzyme was formed in the blank samples.

Phosphoenzyme Formation and P_i Production. Final concentrations were 40 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3 mM PEP, 20 units of pyruvate kinase/mL, 1 μM–1 mM [γ-^{32}P]ATP or [γ-^{32}P]ITP and 0.02–0.04 mg of SR protein/mL for the low ATP (1–10 μM) or 0.2–0.4 mg/mL for the high ATP and ITP experiments. The quenching solution was 7% Cl_3CCOOH plus 0.2 mM P_i carrier. For low protein assays, three to four 2-mL samples of the quenched reaction were collected in sequence for each reaction time and combined. Acid-stable phosphoprotein was pelleted, resuspended in 1.0 mL of ice-cold 5% Cl_3CCOOH, and deposited on a glass fiber filter (Gelman no. 61630) prewashed with 1 mL of 2 mM ATP or ITP. The protein on the filter was washed with 30–45 mL of ice-cold 5% Cl_3CCOOH plus 1 mM pyrophosphate. The filter was counted in a liquid scintillation mixture. The supernatant containing $^{32}P_i$ produced and the remaining nucleotide was mixed with an equal volume of a

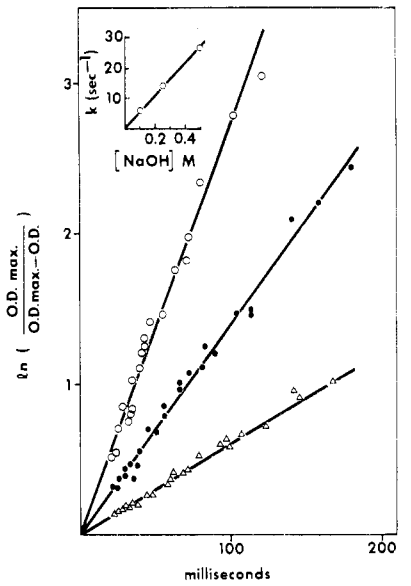

FIGURE 1: Hydrolysis of 2–4-dinitrophenyl acetate in the presence of (△) 0.1, (●) 0.25, and (○) 0.5 M NaOH. OD_{max} refers to the maximal absorbance change, and OD to the absorbances at the times indicated. The lines are fitted by the least-square method, and the rates obtained are plotted.

suspension of 1 g% acid-washed charcoal, followed by filtration through Millipore (HA 0.45 μm). The $^{32}P_i$ in the filtrate was then extracted as a phosphomolybdate complex in isobutyl alcohol–benzene as previously described (Verjovski-Almeida & de Meis, 1977). Aliquots from the organic phase were counted to determine the amount of $^{32}P_i$ produced.

Enzyme Phosphorylation with $^{32}P_i$. Reaction mixtures contained 100 mM Mops (pH 6.8), 80 mM KCl, 30 mM $MgCl_2$, 50 μM $CaCl_2$, 10–20 mM $^{32}P_i$, 5–10 mM EGTA, 0.4–0.6 mg of SR protein/mL. The reaction was started by rapidly mixing equal volumes of two solutions, one containing SR and the other $^{32}P_i$ + EGTA in addition to all other reagents. The reaction was quenched with 7% Cl_3CCOOH plus 200 mM carrier P_i and 2-mL samples were collected and kept ice cold. Acid-stable phosphoprotein was pelleted by centrifugation and washed three to four times with 3 mL of ice-cold 5% Cl_3CCOOH. Subsequently, it was dissolved in 0.5 mL of methylbenzethonium hydroxide and heated for 15 min at 60 °C. After addition of 1.5 mL of 0.5 N HCl, it was counted in a liquid scintillation mixture. When Ca-loaded vesicles were used, SR was preincubated for 2–3 h at room temperature in a solution containing 100 mM Mops, 80 mM KCl, 30 mM $MgCl_2$, 40 mM $CaCl_2$. This suspension was placed in one of the two mixing syringes. The other syringe contained extra EGTA and an appropriate amount of Tris-Cl (pH 9.0) to maintain the pH at 6.8 after EGTA chelates Ca^{2+}. After rapid mixing, the final concentrations in the reaction mixture were 100 mM Mops (pH 6.8), 80 mM KCl, 30 mM $MgCl_2$, 20 mM $CaCl_2$, 10–20 mM $^{32}P_i$, 40 mM EGTA, 0.4–0.6 mg of SR protein/mL. Quenching and subsequent steps were the same as described above. In all experiments with $^{32}P_i$, blanks were obtained by acid denaturation of the protein before addition of $^{32}P_i$ + EGTA.

Decay of the phosphoenzyme formed with $^{32}P_i$ was observed following addition of calcium to prevent further phosphorylation. For each sample, the phosphoenzyme was freshly formed from $^{32}P_i$ and Ca-loaded vesicles by the procedure described above except that the reaction (3 mL final volume) was started by hand mixing, and acid quenching was omitted. Rather the phosphoenzyme suspension was quickly placed into one of the

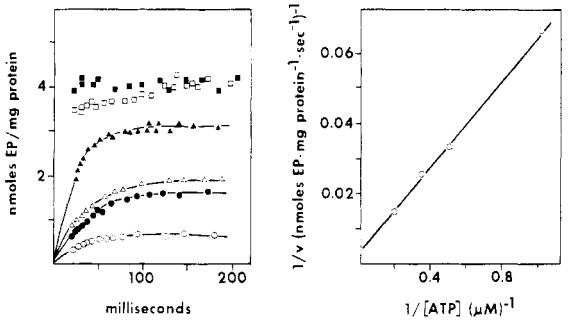

FIGURE 2: Enzyme phosphorylation by ATP. The time curves on the left are obtained in the presence of (○) 1, (●) 2, (△) 2.9, (▲) 5, (□) 100 μM and (■) 1 mM ATP (See Materials and Methods). At 100 μM and 1 mM ATP near-maximal levels of phosphoenzyme were reached within 20 ms, and it was impossible to measure the initial velocity. Those obtained at low ATP concentrations are plotted on the right.

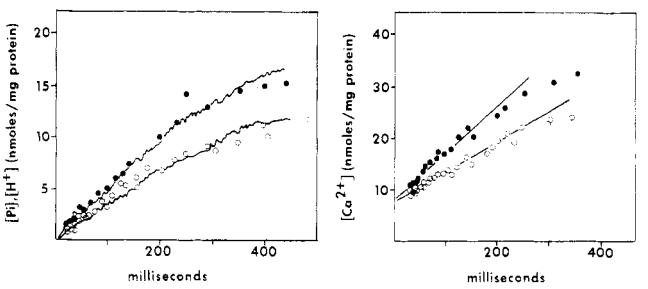

FIGURE 3: Production of phosphate, liberation of H^+ and Ca^{2+} transport following addition of ATP. (Left) P_i production determined in acid quenched samples (○, ●) and H^+ liberation monitored optically in stopped-flow experiments (continuous traces). The proton traces were derived by subtracting light absorption changes obtained in the absence of ATP, from those obtained in the presence of ATP. (Right) Ca^{2+} transport determined in EGTA-quenched samples (○, ●). The ATP concentrations were 100 μM (○) and 1 mM (●).

syringes of the multimixer (10 to 15 s). The second syringe contained 100 mM Mops (pH 6.8), 80 mM KCl, 30 mM $MgCl_2$, 40 mM $CaCl_2$, and Tris-Cl to maintain the pH at 6.8. The rapid mixer was actuated at 18–22 s after the hand mixing step. The concentrations after rapid mixing were 100 mM Mops (pH 6.8), 80 mM KCl, 30 mM $MgCl_2$, 5–10 mM $^{32}P_i$, 30 mM $CaCl_2$, 20 mM EGTA, 0.2–0.3 mg of SR protein/mL. Thus, the phosphoenzyme formed in the presence of excess EGTA over $CaCl_2$ (hand mixed reaction) was exposed to an excess $CaCl_2$. The mixture was then quenched by 7% Cl_3CCOOH plus 200 mM carrier P_i at successive time intervals in the milliseconds range. Washing of the acid denatured protein and subsequent steps were performed as described above.

[^{14}Ca]ATP Formation. Reaction mixtures contained 5 mM ATP, 2 mM [^{14}C]ADP (8×10^5 cpm/μmol), 8 mM $MgCl_2$, 50 mM Mops (pH 6.8), 80 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM EGTA, and 0.7 mg of SR protein/mL. The reaction was started by rapid mixing SR with ATP and [^{14}C]ADP and quenched with 7% Cl_3CCOOH. The acid-quenched suspensions (0.2 mL) were kept ice cold and the pH was adjusted to 7.0 with 5 N NaOH. The denatured protein was pelleted by centrifugation and discarded. The nucleotides in supernatant were then separated by thin-layer continuous flow ascending chromatography in PEI-cellulose plates with 0.7 M LiCl (Randerath & Randerath, 1976). For each sample a 20-μL aliquot was spotted on the plate and chromatographed for 1.5 h at room temperature or 3 h in the cold room. The spots were observed under UV light and compared with standards of ATP, ADP, and AMP. No AMP was present in the samples. ATP and ADP spots were separately cut out of the plates and dropped in vials. One milliliter of 0.7 M $MgCl_2$–20 mM Tris-Cl (pH 7.4) was added and left for 1 h at room temperature to elute the nucleotides from the plates. Scintillation mixture was added and the vials were counted. Blanks were obtained by acid denaturation of the protein before the addition of ATP + [^{14}C]ADP.

Reagents [γ-^{32}P]ATP, [^{14}C]ADP, and $^{32}P_i$ were obtained from ICN Radioisotope Div., Calif.; $^{32}P_i$ was further purified as described by Cross & Boyer (1973). [γ-^{32}P]ITP was prepared by the method of Glynn & Chappell (1964). ATP concentrations were measured in stock solutions by absorbance at 260 nm using an extinction coefficient of 15 400 M^{-1} cm^{-1}. All experiments were done at 25 °C.

Results

Enzyme Phosphorylation with ATP. The presence of a phosphorylated intermediate (EP) in the reaction mechanism of SR ATPase is well established (Yamamoto & Tonomura, 1967; Makinose, 1969; Martonosi, 1969; Inesi et al., 1970), and the time dependence of its formation was previously measured (Kanazawa et al., 1971; Froehlich & Taylor, 1975). However, these measurements were obtained either at low temperature, or in the presence of relatively low ATP:enzyme concentration ratios, and in the absence of an ATP regenerating system.

In our experiments we lowered the enzymic site concentrations to levels at least five times lower than the concentrations of ATP, in order to minimize ADP build-up and reverse phenomena. In fact, we obtained identical results in the presence and in the absence of an ATP regenerating system.

At high ATP concentrations (100 μM) maximal levels of EP were reached too rapidly to permit time resolution of the initial rising phase. On the other hand, low ATP concentrations yielded time curves which could be extrapolated to zero (Figure 2). We then chose the initial velocities for estimates of the rate constant, since the initial part of the time curve is expected to be the least sensitive to reverse reactions and, as opposed to the half-time, is not dependent on the steady-state concentration of product.

A double-reciprocal plot of the initial velocities obtained in the range 1–5 μM vs. ATP concentration gives a maximal velocity of 340 nmol mg^{-1} s^{-1} and an apparent K_m of 2×10^{-5} M (Figure 2). Based on the maximal levels of phosphoenzyme (4 nmol/mg of protein) obtained in the presence of large ATP excess and in conditions favoring phosphoenzyme build-up (high Ca^{2+}), the maximal velocity of phosphorylation can be transformed into a first-order rate constant (ATP·E + k → ADP·EP) which is 85 s^{-1} at 25 °C. This value is similar to that obtained by Froehlich & Taylor (1975).

Hydrolytic Reaction and Ca^{2+} Uptake in the Presence of ATP. The simplest reaction mechanism predicts that the phosphoenzyme undergoes hydrolytic cleavage according to:

$$EP + H_2O \rightleftharpoons E + P_i + H^+$$

Therefore, the reaction is expected to yield H^+ and P_i in parallel. In fact, it is shown in Figure 3 that the time courses of P_i and H^+ production are nearly identical. Furthermore, H^+ and P_i are related to Ca^{2+} transport with a 2:1 stoichiometric ratio, following an initial burst of Ca^{2+} translocation which is completed within the first 20 ms (Figure 3, right).

It should be pointed out that an initial burst of P_i production was previously noticed within the first 50 ms of reaction and attributed to formation of an acid-labile phosphate·membrane

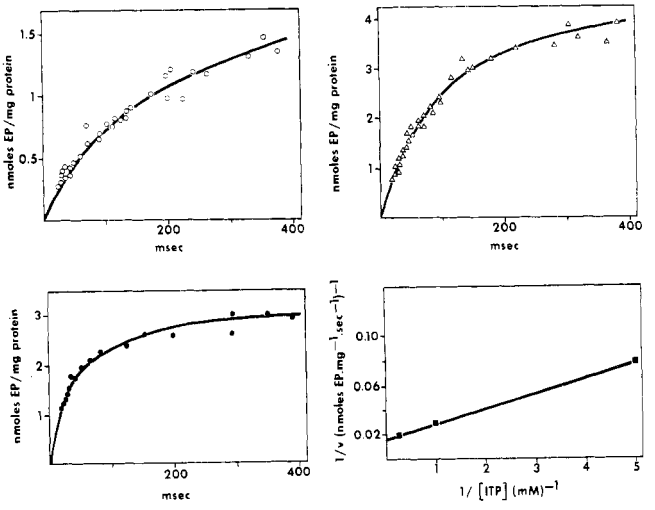

FIGURE 4: Enzyme phosphorylation by ITP. Phosphorylation of the enzyme was followed at (○) 0.2, (△) 1, and (●) 5 mM ITP. Note the different verticle axis scales used. The initial velocities are plotted on the bottom right, and the double-reciprocal plot yields a maximal velocity of 64 nmol of EP mg^{-1} s^{-1}.

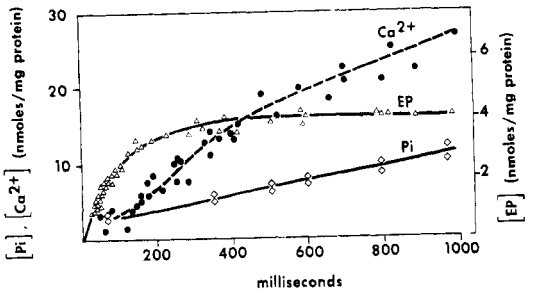

FIGURE 5: Enzyme phosphorylation by ITP Ca^{2+} uptake and P_i production during the transient state. The ITP concentration was 1 mM. Phosphoenzyme (△) and inorganic phosphate (◇) were assayed by the acid-quench method, and Ca^{2+} uptake (●) by the EGTA quenching method.

complex (Froehlich & Taylor, 1975). Another apparent P_i burst was reported to extend for a longer time and was attributed to a decline of the enzyme turnover occurring within the first second following addition of ATP (Kanazawa et al., 1971).

In previous experiments we also noticed in the early part of the reaction an excess P_i production over that expected on the basis of H^+ and Ca^{2+} transport measurements. These inconsistencies which were noted more frequently at high ATP concentrations, have disappeared when we changed the $^{32}P_i$ determination procedure, to combine charcoal absorption of nucleotide and P_i extraction in organic solvents, rather than precipitation of phosphomolybdate with triethylamine.

With regard to Ca^{2+} uptake, a linear initial behavior was previously monitored with metallochromic indicators in stopped-flow experiments (Inesi & Scarpa, 1972) and it is confirmed here. It is important to note that in EGTA rapid quench experiments the added EGTA (10 mM) removes Ca^{2+} bound to transport sites previous, but not following enzyme phosphorylation (Kurzmack et al., 1977). The EGTA quench method demonstrates that the Ca^{2+} initially bound to the high affinity sites is rapidly translocated across the membrane as a consequence of enzyme phosphorylation. Following this initial phenomenon, Ca^{2+} transport occurs at a constant rate.

The absence of an initial burst of Ca^{2+} uptake in the time curves obtained with metallochromic indicators, as opposed to those determined by EGTA quench, is due to the different baselines (absence of ATP) in the two methods. In the presence of metallochromic indicators Ca^{2+} is bound to the high affinity sites even in the absence of ATP. On the contrary, the EGTA quench removes Ca^{2+} from high affinity sites, unless these sites become unavailable on the outer surface of the vesicles as a consequence of enzyme phosphorylation.

Another interesting observation is that, while the maximal levels of phosphoenzyme are not increased by raising the ATP concentration from 100 μM to 1.0 mM (Figure 2), the early phase of hydrolytic and Ca^{2+} transport activities is further increased (Figure 3), consistent with previous steady-state observations (Inesi et al., 1967; Yamamoto & Tonomura, 1967). Therefore, we were able to measure near-maximal velocities of H^+ production and Ca^{2+} transport in the early phases of reaction, and (based on a concentration of 4 nmol of activated sites/mg of protein) we obtained a turnover number of 5–7 and 10–12 s^{-1} for the hydrolytic activity coupled to Ca^{2+} transport, in the presence of 0.1 and 2.0 mM ATP, respectively. These constants are within the same order of magnitude of those observed by Inesi & Scarpa (1972) and Froehlich & Taylor (1975).

The rate constant of Ca^{2+} uptake is approximately twice that of the hydrolytic reaction, consistent with the 2:1 stoichiometric ratio previously established for Ca^{2+} transport and ATP hydrolysis (Weber et al., 1966).

Enzyme Phosphorylation with ITP. ATP can be replaced by other substrates such as ITP, that have a lower affinity for the enzyme (Makinose & The, 1965; de Meis & de Mello, 1973). ITP was used here in an attempt to determine the dependence of various partial reactions on the nucleoside moiety of the substrate, and to resolve the fast Ca^{2+} translocation burst that follows phosphoenzyme formation.

As shown in Figure 4, the rate of phosphorylation of the enzyme with ITP was slower than with ATP and increased in the range of 0.2–5.0 mM ITP. A double-reciprocal plot of the initial velocities of phosphorylation (tangents to the time curves) vs. ITP concentrations gives a maximal velocity of phosphorylation of 63 nmol mg^{-1} s^{-1} and an apparent K_m of 7×10^{-4} M. The first-order rate constant for the transfer of the terminal phosphate from ITP to the enzyme site (ITP·E + k — IDP + EP) is 16 s^{-1} (25 °C), based on a maximal amount of 4 nmol/mg of SR protein for the ITP·E complex. This constant is five times lower than that obtained for enzyme phosphorylation with ATP.

It should be pointed out that, in our experiments with intact vesicles, maximal levels of EP were formed in the presence of low outside Ca^{2+}, while "leaky" vesicles require high Ca^{2+} (Souza & de Meis, 1976; Verjovski-Almeida & de Meis, 1977). This difference is likely due to the ability of our vesicles to concentrate Ca^{2+} inside their lumen.

Ca^{2+} Uptake and P_i Production in the Presence of ITP. Phosphoenzyme formation, Ca^{2+} uptake, and P_i production, measured in the presence of 1 mM ITP, are displayed in Figure 5. The concentration of phosphorylated intermediate rises during an initial transient phase lasting approximately 300 ms. During this transient phase and with considerable delay with respect to enzyme phosphorylation, a burst of Ca^{2+} uptake is observed. After steady-state levels of phosphoenzyme are reached and the initial burst of Ca^{2+} uptake is over, further uptake proceeds at a constant rate of 22 nmol mg^{-1} s^{-1}. This linear part of the uptake curve intercepts the vertical axis at 8 nmol of Ca^{2+}/mg of SR protein, indicating that during the transient phase of phosphorylation approximately 2 mol of

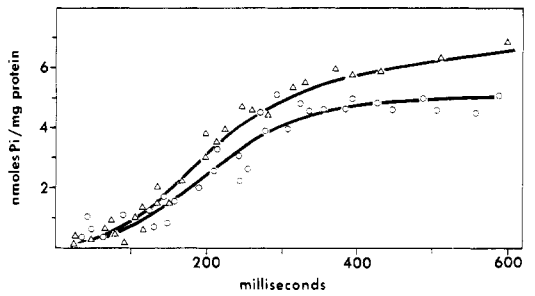

FIGURE 6: Early P_i burst in the presence of ITP with and without Ca^{2+}. The reaction was started by addition of 0.2 mM ITP in the presence of 0.1 mM $CaCl_2$ (△) or 1 mM EGTA (○). Other required cofactors are described under Materials and Methods). The reaction was stopped by acid quenching.

Ca^{2+} was rapidly taken up per mol of phosphoenzyme formed.

In the time curve of P_i production in the presence of ITP, a linear portion can be identified better than in the curves obtained with ATP. Extrapolation to the vertical axis indicates an initial burst of P_i production corresponding to 2.5–5.0 nmol/mg of protein, which is completed within the first 100 ms of reaction in the presence of 1 mM ITP. It is apparent that this burst corresponds to the one that Froehlich & Taylor (1975) were able to observe in the presence of ATP. It is shown in Figure 6 that the P_i burst is independent of Ca^{2+} and not necessarily related to Ca^{2+} transport.

Enzyme Phosphorylation with P_i. The ATPase enzyme can be phosphorylated with P_i either in the absence of Ca^{2+} (Kanazawa & Boyer, 1973; Masuda & de Meis, 1973), or in the presence of high Ca^{2+} inside the vesicles and very low Ca^{2+} outside (Makinose, 1972; Yamada & Tonomura, 1972). The phosphorylated protein formed in the two cases is different with respect to its ability to form ATP upon addition of ADP (Beil et al., 1977). In the former case the phosphoenzyme does not form ATP unless Ca^{2+} is added with ADP (Knowles & Racker, 1975; de Meis & Tume, 1977). In the latter case, cyclic phosphorylation and ATP formation are coupled to Ca^{2+} efflux, and a reversal of the Ca^{2+} pump is observed (Barlogie et al., 1971; Makinose & Hasselbach, 1971).

In our experiments we were able to resolve the early phase of enzyme phosphorylation when P_i and EGTA were added to vesicles previously loaded with Ca^{2+} by passive equilibration (Figure 7A). In control experiments with radioactive calcium tracer we checked that the vesicles were loaded with 350 nmol of calcium/mg of protein and that, following EGTA addition, the vesicles retained calcium for at least 2 min. Therefore, since EGTA binds very rapidly calcium outside but does not penetrate the vesicles at significant rates (Weber et al., 1966), a Ca^{2+} gradient was maintained across the SR membrane throughout the time course of enzyme phosphorylation.

It is shown in Figure 7A that the time course of phosphorylation in the presence of Ca^{2+} gradient exhibits a lag period followed by a linear rise which then bends to asymptotic levels. It should be pointed out that the lag period is not due to a delay in the EGTA effect, since the outside Ca^{2+} is removed by EGTA within 20 ms (Figure 7B) and inside Ca^{2+} is not completely removed for the entire duration of the phosphorylation reaction. Rather it is likely that the lag period is related to conversion of the enzyme to a different form, previous to phosphorylation:

$$E \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} E^* + P_i \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} E^* \cdot P_i \underset{k_{-3}}{\overset{k_3}{\longleftrightarrow}} E^*\text{-}P$$

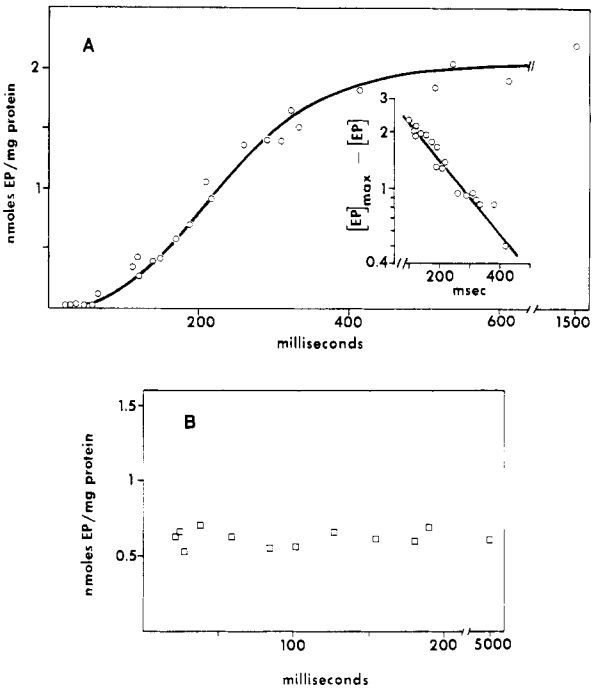

FIGURE 7: Enzyme phosphorylation by P_i in the absence and presence of a Ca^{2+} gradient. The reaction was started by the addition of 10 mM P_i plus 40 mM EGTA in previously loaded (A) as opposed to nonloaded vesicles (B). Following acid quenching the phosphoenzyme formed was measured. The inset in A shows the same data points in a logarithmic scale, with the exclusion of a lag period of 90 ms (see text).

More specifically, one may consider the equilibria:

$$[E \cdot Ca]_{out} \overset{K_1}{\longleftrightarrow} [Ca_{out}] + [E_{out}] \overset{K_2}{\longleftrightarrow} [E_{in}] + [Ca^{2+}{}_{in}] \overset{K_3}{\longleftrightarrow} [E \cdot Ca]_{in}$$

where E_{out} and E_{in} are forms of the enzyme with binding sites facing the outside of the vesicles in a high affinity state, and the inside in a low affinity state, respectively. It is clear, then, that in the presence of high Ca^{2+} both outside and inside the vesicles, the enzyme is mostly in the E_{out} state, since K_1 is approximately three orders of magnitude larger than K_3.

It is expected that in this system when $Ca^{2+}{}_{out}$ is chelated by the addition of high EGTA (as in the experiment illustrated in Figure 7A), $[E\text{-}Ca]_{out}$ becomes negligible and E_{out} shifts predominantly to E_{in} due to the presence of high $Ca^{2+}{}_{in}$. In the assumption that the enzyme conformation reacting with P_i is E_{in} (de Meis & Boyer, 1978), the lag period observed in Figure 7A may be explained with the transformation of E_{out} to E_{in}, if k_1 is comparable to k_3.

An approximate estimate for the rate constant of phosphorylation may be obtained, excluding a lag period (90 ms) by extrapolation of the linear portion of the phosphorylation curve to the baseline. The remaining data can be satisfactorily fitted with one exponential and a correlation coefficient = 0.95. In this manner a rate constant of 4.5 s^{-1} is obtained for enzyme phosphorylation with P_i in the presence of Ca^{2+} inside the vesicles at 25 °C.

As opposed to the experiments on phosphorylation of vesicles previously loaded with Ca^{2+}, we were unable to obtain time resolution of enzyme phosphorylation when P_i and EGTA were added to vesicles which were not previously loaded with Ca^{2+} (Figure 7B). In these conditions, steady-state levels of phosphoenzyme of 0.7 nmol/mg of protein were reached within 20 ms, and no further increase was obtained even in the presence of P_i concentrations as high as 10 mM. These experiments

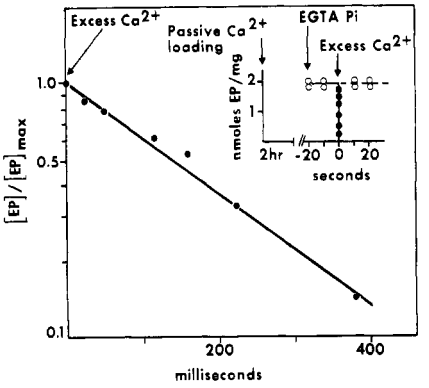

FIGURE 8: Decay of phosphoenzyme formed from P_i. Ca^{2+}-loaded vesicles were phosphorylated by addition of 10 mM P_i plus 40 mM EGTA (○). The inset shows that the phosphoenzyme level was maintained for at least 40 s. The rapid mixing experiment was initiated by adding excess Ca^{2+} to the phosphoenzyme suspension to prevent further phosphorylation. The decay was then monitored by quenching with acid and measuring the acid stable phosphorylation (●).

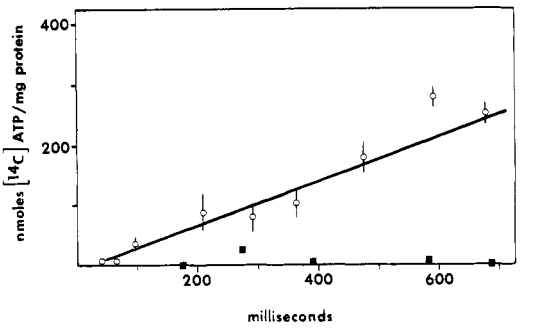

FIGURE 9: Adenosine moiety exchange between ADP and ATP. The reaction was started by addition of 5 mM ATP and 2 mM [^{14}C]ADP in the presence of 3 mM EGTA (■) or 0.5 mM $CaCl_2$ and 0.5 mM EGTA (○) and other cofactors (Materials and Methods). Following acid quenching, the nucleotides were separated and the radioactivity incorporated into ATP was measured.

suggest that the enzyme deprived of Ca^{2+}, both inside and outside the vesicles, resides in a state which may be phosphorylated at very rapid rates, but requires high P_i concentrations (de Meis, 1976; Beil et al., 1977). Specific conformational features of such an enzyme state have been shown repeatedly (Champeil et al., 1976; Coan & Inesi, 1977; Murphy, 1978).

Decay of the Phosphoenzyme Formed with P_i. The phosphoenzyme formed with P_i in the presence of a transmembrane Ca^{2+} gradient (high Ca^{2+} inside the vesicles) can be maintained at steady-state levels for several seconds. However, if Ca^{2+} is added to the outside medium to reach concentrations exceeding EGTA, protein phosphorylation is quenched and the phosphoenzyme undergoes an exponential decay (Figure 8). The rate constant of dephosphorylation is 5 s^{-1}, at 25 °C. This value is nearly identical with that obtained in the hydrolytic reaction coupled to Ca^{2+} transport in the presence of low ATP. This indicates that the transition of the dephosphorylated enzyme (E_{in}) to the form that binds the calcium in the outside medium (E_{out}) is not rate limiting.

It should be noted that when phosphoenzyme formed from P_i in the *absence* of a Ca^{2+} gradient is exposed to high Ca^{2+}, the half-time of dephosphorylation is 2-4 s (de Meis & Tume, 1977; Rauch et al., 1977) which is a reaction too slow to be a step in the cycle.

Phosphoryl Transfer from Phosphoenzyme to ADP. Complete reversal of the Ca^{2+} pump, including phosphorylation of ADP in the presence of P_i and a transmembrane Ca^{2+} gradient, was previously demonstrated (Barlogie et al., 1971). The entire cycle of reversal includes several partial reactions, including the relatively slow enzyme phosphorylation with P_i, which is likely to be rate limiting.

In order to estimate specifically the rate constant for ADP phosphorylation, we applied the rapid quench method to detection of adenosine moiety exchange between ADP and ATP, as in

$$\begin{array}{ccc} & & E + [^{14}C]ATP + Ca^{2+}{}_{out} \\ & & \uparrow k_2 \\ E + ATP + [^{14}C]ADP + Ca^{2+}{}_{out} & \xrightarrow{k_1} & Ca\cdot EP + [^{14}C]ADP \\ & & \downarrow k_3 \\ & & E + P_i + Ca^{2+}{}_{in} \end{array}$$

This exchange reaction was previously described (Ebashi & Lipman, 1962; Hasselbach & Makinose, 1962) and found to be totally Ca^{2+} dependent (Inesi & Almendares, 1968). With our rapid mixing experiments we have confirmed that no exchange takes place if the Ca^{2+} concentration in the medium is reduced below 10^{-8} M by the addition of EGTA.

In reactions started by rapid mixing of 5 mM ATP and 2 mM [^{14}C]ADP with SR and required cofactors, we found that the amount of [^{14}C]ATP formed within 700 ms was less than 4% of the total pool of ATP and less than 10% of the initial pool of [^{14}C]ADP. Therefore, under these conditions, reversal of steps 1 and 2, i.e., utilization of both nonlabeled ADP and [^{14}C]ATP formed from the forward reactions, was negligible.

In our experiments the velocity of formation of [^{14}C]ATP (Figure 9) was 350 nmol mg^{-1} s^{-1}, which is clearly identical with V_{max} for enzyme phosphorylation with ATP (Figure 2). This demonstrates that in the exchange reaction k_1 is rate limiting, and k_2 must be higher than k_1. In fact, in parallel experiments with 5 mM [γ-^{32}P]ATP we found that addition of 2 mM ADP reduces the steady-state level of EP from 4 to less than 1 nmol/mg of protein.

These experiments indicate that the rate of reversal of the phosphorylation step is faster than its forward rate. The steady-state rates of ADP-ATP exchange were already reported to be high when compared with the steady-state rates of P_i production (Hasselbach & Makinose, 1962; Inesi & Almendares, 1968).

Discussion

The objective of this paper is to provide experimental information on the kinetic behavior of partial reactions of the Ca^{2+}-dependent ATPase. The reactions covered by our experimentation can be outlined in a sequence similar to those proposed by Souza & de Meis (1976) and de Meis & Boyer (1978):

$$ATP + E_{out} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ATP\cdot E_{out} \quad (1)$$

$$ATP\cdot E_{out} + Ca^{2+}{}_{out} \overset{K_{Caout}}{\rightleftharpoons} ATP\cdot E\cdot Ca_{out} \quad (2)$$

$$ATP\cdot E\cdot Ca_{out} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\sim P\cdot Ca_{out} + ADP \quad (3)$$

$$E\sim P\cdot Ca_{out} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E\text{-}P\cdot Ca_{in} \quad (4)$$

$$E\text{-}P\cdot Ca_{in} \overset{K_{Cain}}{\rightleftharpoons} E_{in}\ P + Ca^{2+}{}_{in} \quad (5)$$

$$E_{in}\text{-}P + H_2O \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} E_{in} \cdot P_i \qquad (6)$$

$$E_{in} \cdot P_i \overset{k_7}{\rightleftharpoons} E_{in} + P_i \qquad (7)$$

$$E_{in} + P_i \rightarrow + H^+$$

$$E_{in} \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} E_{out} \qquad (8)$$

where E_{out} and E_{in} refer to the enzyme with Ca^{2+} binding sites exposed to the outer or inner membrane surface, and displaying high and low affinity for Ca^{2+}, respectively. Proton release may occur in steps 6 or 7, depending on the extent to which P_i bound to the enzyme can affect the pK_a of the carboxyl group on the phosphorylation site.

The constants evaluated in our experiments are $K_{ATP} = 2.0 \times 10^{-5}$ M; $K_{ITP} = 7 \times 10^{-4}$ M; $k_3 = 85\ s^{-1}$ with ATP and 16 s^{-1} with ITP; $k_{-3} \gg 85\ s^{-1}$; $k_6 = 7$ and $12\ s^{-1}$, in the presence of 0.1 mM and 2.0 mM ATP, respectively; $k_{-6} = 4.6\ s^{-1}$. Furthermore, based on this reaction scheme the following inferences can be made: $k_8 > k_{-8}$ and $k_8 > k_6$, $k_{-8} \cong k_{-6}$.

Substrate Dependence. As previously reported (Makinose & The, 1965; de Meis & de Mello, 1973), ATP and ITP can be both utilized as substrate for the enzyme reaction coupled to Ca^{2+} transport. Our K_m values for the two nucleotides are 2×10^{-5} and 7×10^{-4} M. These values were obtained by measuring velocities of enzyme phosphorylation, rather than final product. Our 2.0×10^{-5} M K_m for ATP is nearly identical with the 3.5×10^{-5} M K_d determined by Pang & Briggs (1977) for dissociation of the ATP·enzyme binding complex.

An intriguing feature of the ATP dependence of steady-state ATPase activity is an apparent saturation of 0.1 mM ATP, followed by a further activation at 1.0 mM ATP (Inesi et al., 1967; Yamamoto & Tonomura, 1967; Neet & Green, 1977). Our experiments show that such a diphasic dependence on substrate concentration does not involve the maximal levels (Figure 2) of acid stable phosphoenzyme, in the early part of the reaction. Therefore, the activation of transport and hydrolytic activities observed at high ATP concentrations (Figure 3) must be related to an effect on the phosphoenzyme turnover.

In comparative experiments on the utilization of ATP and ITP as substrates, we observed not only different K_m values, but also different rate constants for enzyme phosphorylation. In fact, we found that k_3 is $85\ s^{-1}$ in the presence of ATP, as compared with $16\ s^{-1}$ in the presence of ITP. Even more interesting is the fact that the initial Ca^{2+}-uptake burst (Figure 5) displays a lag period relative to the phosphorylation curve. This indicates a slower rate constant for Ca^{2+} translocation (k_4) following phosphorylation of the enzyme with ITP, as compared with ATP. This behavior suggests that either the nucleoside remains bound to the phosphorylated enzyme or another nucleotide molecule binds to an additional regulatory site (de Meis & de Mello, 1973; Froehlich & Taylor, 1975; Eckart et al., 1977). It is apparent that the structure of the substrate not only determines its binding affinity, but also affects catalytic and transport mechanisms. Conformational effects of nucleotide and nucleoside binding were previously demonstrated by spin-labeling methods (Landgraf & Inesi, 1969).

Enzyme Phosphorylation with Orthophosphate. One of the most interesting features of the SR Ca^{2+} pump is its reversal including phosphorylation of the ATPase enzyme with orthophosphate. In fact, this partial reaction is extremely sensitive to the Ca^{2+}, as indicated by the following findings. (a) Enzyme phosphorylation does not occur in the presence of Ca^{2+} on the outer surface of the vesicles (Kanazawa & Boyer, 1973; Masuda & de Meis, 1973). (b) The rate constants of enzyme phosphorylation are different, depending on whether high Ca^{2+} concentrations are present inside the vesicles or not (compare Figures 7A and 7B). (c) Phosphoryl transfer from the enzyme to ADP occurs only in the presence of a transmembrane Ca^{2+} gradient (Makinose & Hasselbach, 1971) or upon addition of high Ca^{2+} concentrations (Knowles & Racker, 1975; de Meis & Tume, 1977).

It is clear that the absence, as opposed to the presence of Ca^{2+} on high affinity binding sites, confers a specific conformation to the enzyme, as demonstrated by spectroscopic observations (Champeil et al., 1976; Coan & Inesi, 1977) and by the kinetics of -SH groups reactivity (Murphy, 1978). The conformation in the presence of Ca^{2+} is an absolute requirement for phosphorylation of the enzyme with ATP, while it prevents enzyme phosphorylation with orthophosphate. The opposite is observed in the absence of Ca^{2+}.

Furthermore, when a Ca^{2+} concentration gradient across the membrane is established by addition of EGTA to vesicles preequilibrated with high Ca^{2+}, the enzyme undergoes a transition to a different state (E_{in}), which is phosphorylated with Pi at slower rate (Figures 7A and 7B). This transition is also consistent with the lag period in Figure 7A and corresponds to recycling of the enzyme from a form with Ca^{2+} binding sites exposed to the outer, as opposed to the inner surface of the membrane (reversal of step 8, in the reaction scheme given above).

Coupling of Enzyme and Transport Activities. The study of partial reactions leading to ATP hydrolysis is informative with respect to mechanisms of catalysis and demonstration of rate-limiting steps. Furthermore, measurements of forward and reverse rate constants determine the net free energy change per each step and evaluate the stability of various states in the reaction sequence. On the other hand, the most intriguing feature of the SR ATPase reaction is its coupling to Ca^{2+} transport. To this effect it is desirable to single out partial reactions permitting free energy transfer within the reacting system, and leading to calcium translocation across the membrane.

Based on a $K_m = 2 \times 10^{-5}$ M ($\cong K_d$ for ATP·E) and a $K_{Ca_{out}} = 1 \times 10^6\ M^{-1}$, it is apparent that a sizable free energy loss accompanies the first two steps in our reaction sequence, indicating that ATP·E·Ca is much more stable than E. Independent of the free energy loss, this complex retains its calcium binding site exposed on the outer surface of the membrane. In fact, substituting ATP with the analogue AMP-PNP (Yount et al., 1971) which is bound but not utilized for enzyme phosphorylation, it was shown that Ca^{2+} can be rapidly removed from the nucleotide·enzyme complex by adding EGTA to the medium outside the vesicles (Kurzmack et al., 1977). On the contrary, if enzyme phosphorylation is permitted to proceed in the presence of ATP (step 3), the bound calcium becomes rapidly unavailable for chelation by EGTA on the outer surface of the vesicles. These experiments (cf. Figures 2, 3, and 5) indicate that phosphorylation of 1 mol of enzyme is necessary, for 2 mol of calcium to be removed from the outer surface of the membrane and to be translocated toward the interior of the vesicles.

In addition to this calcium movement, it can be shown that phosphorylation of the enzyme results in an approximately three order of magnitude reduction in its affinity for calcium (Ikemoto, 1976) to approximately $1 \times 10^3\ M^{-1}$ (Verjovski-Almeida & de Meis, 1977). Such a shift in equilibrium for the

Ca^{2+} binding reaction between steps 2 and 4 requires a free energy input ($\Delta G^\circ = RT \ln (K_{Ca_{in}}/K_{Ca_{out}})$ of 4.1 kcal mol^{-1}. Considering that 2 mol of calcium is involved in each transport cycle, the total free energy input is 8.2 kcal per cycle in standard conditions. This value is compatible with the ΔG° of ATP.

These considerations indicate that enzyme phosphorylation (step 3) is in fact the partial reaction permitting free-energy transfer from ATP to the enzyme protein. Relaxation of this unstable intermediate (step 4) is then accompanied by translocation of Ca^{2+} and reduction in affinity of the calcium binding site. This step accounts for vectorial requirements and energy transduction in coupling of enzyme catalysis to transport activity.

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