Coincidence of H⁺ Binding and Ca²⁺ Dissociation in the Sarcoplasmic Reticulum Ca-ATPase during ATP Hydrolysis*

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H⁺ and Ca²⁺ concentration changes in the reaction medium following MgATP addition at pH 6.0 were determined with the partially purified Ca-ATPase from sarcoplasmic reticulum vesicles in the presence of 25–50 μM CaCl₂ and 5 mM MgCl₂ at 4 °C. Previously, we showed a sequential occurrence of H⁺ binding and Ca²⁺ dissociation during ATP hydrolysis and further suggested that the H⁺ binding takes place inside the vesicles (Yamaguchi, M., and Kanazawa, T. (1984) J. Biol. Chem. 259, 9526–9531). The present results demonstrate that the H⁺ binding occurred coincidently with Ca²⁺ dissociation from the enzyme upon conversion of the phosphoenzyme (EP) intermediate from the ADP-sensitive form to the ADP-insensitive form in the catalytic cycle of ATP hydrolysis. As KCl decreased in the medium, the extent of the H⁺ binding increased almost proportionately with the extent of either the Ca²⁺ dissociation or the accumulation of ADP-insensitive EP. Both the H⁺ binding and the Ca²⁺ dissociation were prevented by a modification of the specific SH group of the enzyme essential for the conversion of ADP-sensitive EP to ADP-insensitive EP. In the late stage of the reaction, H⁺ dissociation from the enzyme occurred coincidently with Ca²⁺ binding to the dephosphoenzyme which was formed by EP decomposition. These results are consistent with the possibility that the H⁺ ejection during the Ca²⁺ uptake will be limited to Ca²⁺ affinity of the transport sites is greatly reduced and as a result the Ca²⁺ dissociates inside vesicles (8–10, 15–17). Next, ADP-insensitive EP is hydrolyzed, and this hydrolysis is stimulated by K⁺ (11). Finally, the transport sites return to the outer surface of vesicles.

Recently, several investigators (18–24) indicated that H⁺ is ejected from vesicles during the Ca²⁺ uptake. Thus, an important problem to be solved is whether this H⁺ ejection occurs through a specific mechanism directly involving the Ca-ATPase. In this regard, our previous experiment showed a sequential occurrence of H⁺ binding and H⁺ dissociation in the Ca-ATPase during ATP hydrolysis (24). This finding is in favor of the possibility that the Ca-ATPase serves as a H⁺ carrier in the H⁺ ejection.

In the present experiment, we provide evidence that the H⁺ binding and the H⁺ dissociation occur coincidently with the Ca²⁺ dissociation from the enzyme and the subsequent Ca²⁺ binding to the enzyme in the catalytic cycle of ATP hydrolysis. The results are consistent with the possibility that the H⁺ ejection during the Ca²⁺ uptake takes place through a Ca²⁺/H⁺ exchange directly mediated by the membrane-bound Ca-ATPase.

EXPERIMENTAL PROCEDURES

Preparation of Sarcoplasmic Reticulum Vesicles—Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described previously (7). They were suspended in 0.3 M sucrose, 0.1 M KCl, and 1.0 mM MES/Tris (pH 6.0) and stored at −80 °C.

Preparation of Partially Purified Ca-ATPase—Vesicles were treated with deoxycholate according to the method of Meissner and Fleischer (25). The treatment resulted in a partial purification of the Ca-ATPase and a complete loss of the capacity for Ca²⁺ accumulation. All of the measurements in the present experiment were performed at 4 °C by using this purified Ca-ATPase. In order to prevent an undesirable aggregation (26), the enzyme was sonicated for 15 s at 0 °C with a sonicator (Branson, Sonifier-200) immediately before the start of the measurement.

Treatment of the Ca-ATPase with NEM—The purified enzyme (3 mg/ml) was incubated with 1.5 mM NEM in the presence of 1 mM App(NH)p for 20 min by the method of Kawakita et al. (27).

Measurement of pH Changes in the Reaction Medium—pH changes in the reaction medium were determined with a pH meter (Radiometer, PHM84) or determined by following the change in the fluorescence intensity of FITC-dextran with a spectrofluorometer (Shimadzu, RF-503A) as described previously (24). The reaction was initiated by adding 2.2 μl of 30 mM MgATP (30 mM Tris-ATP containing 36 mM MgCl₂, adjusted with Tris to pH 6.0) to 2.2 ml of the medium containing the enzyme and other reagents as described under “Results.” When the enzyme was absent, no significant pH change occurred upon addition of MgATP (see the controls in Fig. 1). Under the present experimental conditions, at pH 6.0, complete hydrolysis of MgATP with an extremely low concentration of the enzyme did not significantly cause H⁺ production and H⁺ absorption.

Measurement of Ca²⁺ Concentration Changes in the Reaction Medium—Ca²⁺ concentration changes in the reaction medium were

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The abbreviations used are: EP, phosphoenzyme; MES, 2-(N-morpholino)ethanesulfonic acid; NEM, N-ethylmaleimide; FITC, fluorescein isothiocyanate, EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′,N″,N‴-tetrasuccinic acid; App(NH)p, adenyly-5′-yl imidodiphosphate.
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determined by two different methods. (a) They were followed with a calcium electrode (Radiometer, F2112Ca). The reaction was initiated by adding 2.5 μl of 30 mM MgATP (pH 6.0) to 2.5 ml of the medium. (b) They were followed with a dual-wavelength spectrophotometer (Shimadzu, UV-300) by using the metallochromic indicator murexide. A wavelength pair of 540 and 700 nm was chosen. The reaction was initiated by adding 2.2 μl of 30 mM MgATP (pH 6.0) to 2.2 ml of the medium.

Determination of EP—Phosphorylation of the Ca-ATPase was carried out with [γ-32P]ATP as described under "Results." The amount of EP was determined essentially in the same way as described previously (28). Rapid kinetic measurements of EP formation were performed with a homemade rapid mixing apparatus (29).

Materials—Na2ATP was purchased from Boehringer Mannheim. Na2ADP, Li2App(NH)p, EGTA, and FITC-dextran (average Mr = 66,000; 0.02 mol of FITC/mol of glucose residue) were obtained from Sigma. Murexide was from Nakarai Chemicals. Removal of free FITC contaminating FITC-dextran and conversion of Na2ATP and Na2ADP into Tris form were performed as described previously (24). [γ-32P]ATP was prepared according to Post and Sen (30). Protein concentrations were determined by the method of Lowry et al. (31) with bovine serum albumin as a standard.

RESULTS

MgATP-induced Changes of H* and Ca2+ Concentrations in the Medium—Changes of H* and Ca2+ concentrations in the reaction medium following addition of 30 μM MgATP at a final concentration were determined with hydrogen and calcium electrodes in the presence of 2.5 mg of the enzyme/ml, 25 μM CaCl2 added, 5 mM MgCl2, and 100 mM KCl (Fig. 1A). The pH of the medium before the start of the reaction was 6.0. After the addition of MgATP, an alkalization of the medium occurred and then the pH returned to the initial level (trace a in Fig. 1A). The data agree with our previous observations (24) that the alkalization and its disappearance represent H* binding and H* dissociation in the Ca-ATPase during ATP hydrolysis.

Under similar conditions, the Ca2+ concentration in the medium increased after the MgATP addition and then it returned to the initial level (trace c in Fig. 1A). These results show Ca2+ dissociation from the enzyme in the early stage of the reaction and Ca2+ binding to the enzyme in the late stage of the reaction (see "Discussion"). When KCl was absent (LiCl was added to maintain a constant ionic strength), the H* binding and the Ca2+ dissociation were substantially enhanced (Fig. 1B). In addition, the H* dissociation and the Ca2+ binding in the late stage of the reaction were markedly delayed. It appeared that the H* binding and the H* dissociation almost coincided with the Ca2+ dissociation and the Ca2+ binding, respectively, either in the presence of KCl or in the absence of KCl. However, it was difficult to exactly compare the time courses since the responses of the electrodes used were slow.

H* Binding and Accumulation of ADP-insensitive EP in the Initial Phase—The H* binding in the initial phase of the reaction was determined fluorometrically with FITC-dextran in the presence (Fig. 2A) and absence (Fig. 2B) of KCl. EP formation was also determined with a rapid mixing apparatus under similar conditions. The H* binding was much slower than EP formation in the presence and absence of KCl. This finding agrees with our previous observations (24). The data showing no detectable H* dissociation and H* binding upon EP formation are consistent with the recent findings from the calorimetric study by Kodama et al. (32).

Contaminant Ca2+ in the reaction mixture was about 15 μM, as determined by atomic absorption spectrophotometry. Most of the Ca2+ was derived from the enzyme. Thus, total Ca2+ concentration in the mixture was estimated to be 40 μM.

Both the H* binding and the accumulation of ADP-insensitive EP were markedly enhanced when KCl was absent. The enhancement of the accumulation of ADP-insensitive EP is compatible with the observation (11) that hydrolysis of ADP-insensitive EP is inhibited in the absence of K*. The time course of the H* binding essentially agreed with that of the accumulation of ADP-insensitive EP either in the presence or absence of KCl.

Comparison of the Time Course between the H* Binding and the Ca2+ Dissociation—In order to compare the time course of the H* binding with that of the Ca2+ dissociation with reasonable accuracy, the Ca2+ dissociation was determined spectrophotometrically by using murexide (Fig. 3). The H* binding was determined with FITC-dextran under the same conditions. The H* binding and the Ca2+ dissociation were markedly enhanced when KCl was absent. This is consistent with the results obtained by using hydrogen and calcium electrodes (cf. Fig. 1). The time course of the H* binding was in good agreement with that of the Ca2+ dissociation either in the presence or absence of KCl.

Extents of H* Binding, Ca2+ Dissociation, and Accumulation of ADP-insensitive EP in the Steady State at Various KC Concentrations—The extents of the H* binding and the Ca2+...
dissociation increased in parallel with the amount of ADP-insensitive EP as KCl was replaced by LiCl, while the total amount of EP remained almost constant (Fig. 4). The ratio of the extent of the H⁺ binding to the amount of ADP-insensitive EP was constant, being about 3 mol/mol over the range of 0–100 mM KCl. On the other hand, the ratio of the extent of the Ca²⁺ dissociation to the amount of ADP-insensitive EP decreased with increasing KCl concentration, being 2 mol/mol in the absence of KCl and 1 mol/mol in the presence of 100 mM KCl. Similar results were obtained when choline chloride was used instead of LiCl (not shown).

Effect of NEM Treatment on H⁺ Binding, Ca²⁺ Dissociation, and ADP Sensitivity of EP—The specific SH group essential for the conversion of ADP-sensitive EP to ADP-insensitive EP was modified with NEM as described under “Experimental Procedures” (Table I). In the presence of KCl, with the nontreated enzyme, the ADP-insensitive EP accumulated to a considerable extent, whereas with the NEM-treated enzyme, it accumulated only to a slight extent. In the absence of KCl, with the nontreated enzyme, most of EP was ADP-insensitive, whereas with the NEM-treated enzyme, a small amount of EP was ADP-insensitive. These results show that this NEM treatment caused a definite inhibition of the conversion of ADP-sensitive EP to ADP-insensitive EP. When MgATP was added to the NEM-treated enzyme, only a slight H⁺ binding occurred in the presence of KCl. When KCl was absent, this H⁺ binding was substantially enhanced, but it was much less than the H⁺ binding with the nontreated enzyme. Under similar conditions with the NEM-treated enzyme, a slight Ca²⁺ binding occurred in the presence of KCl, whereas a significant Ca²⁺ dissociation occurred in the absence of KCl. The Ca²⁺ binding and the H⁺ dissociation in the absence of KCl in the late stage of the reaction were markedly delayed when the enzyme was treated with NEM (not shown).

DISCUSSION

The implications of the results may be discussed conveniently in terms of the minimum scheme (Fig. 5) tentatively proposed for the Ca-ATPase. This scheme is based on present and earlier findings (8–10, 24). E and EP denote the state of the enzyme which has transport sites with a high affinity for Ca²⁺ and a low affinity for H⁺. In this state, the transport sites face on the external medium of vesicles. *E and *EP indicate the state of the enzyme which has transport sites with a low affinity for Ca²⁺ and a high affinity for H⁺. According to this scheme, EP is ADP-sensitive and *EP is ADP-insensitive. The stoichiometries of Ca²⁺ and H⁺ are omitted for simplicity.

The observed alkalinization (Figs. 1–3) agrees with our previous findings (24), which showed a sequential occurrence of H⁺ binding and H⁺ dissociation in the Ca-ATPase during ATP hydrolysis and suggested that the H⁺ binding to the
enzyme occurs inside vesicles. The Ca\(^{2+}\) concentration changes (Figs. 1 and 3) are also consistent with previous findings (15–17, 33) that Ca\(^{2+}\) dissociation and Ca\(^{2+}\) binding in the transport sites occur in Steps 4 and 1, respectively, given in Fig. 5.

The coincidence of the H\(^{+}\) binding and the Ca\(^{2+}\) dissociation (Figs. 1 and 3) gives evidence that the H\(^{+}\) binding occurs in Step 4. This is further supported by the findings (Fig. 2) that the H\(^{+}\) binding almost coincided with the accumulation of ADP-insensitive EP, since the Ca\(^{2+}\) dissociation in Step 4 results from the conversion of ADP-sensitive EP to ADP-insensitive EP (15–17, 33). The enhancement of the H\(^{+}\) binding upon a reduction in KCl concentration corresponded well to the increase in the extent of either the Ca\(^{2+}\) dissociation or the accumulation of ADP-insensitive EP (Fig. 4). This correspondence also adds probability to the H\(^{+}\) binding in Step 4.

The coincidence of the H\(^{+}\) dissociation and the Ca\(^{2+}\) binding in the late stage of the reaction (Fig. 1) suggests that the H\(^{+}\) dissociation occurs between Steps 5 and 1. Step 1 is a strong candidate for this H\(^{+}\) dissociation, because it was previously shown by Inesi and co-workers that the titration of the transport sites with Ca\(^{2+}\) in the absence of ATP was accompanied by a stoichiometric H\(^{+}\) dissociation (20), that the Ca\(^{2+}\) binding to the transport sites was competitively inhibited by H\(^{+}\) (34), and that this H\(^{+}\) competition resulted in an inhibition of EP formation (35).

The inhibition of the conversion of ADP-sensitive EP to
ADP-insensitive EP by NEM treatment (Table I) is in accord with the results reported by Yasuoka-Yabe et al. (36). The prevention of the H* binding by NEM treatment in the presence of KC1 is consistent with the accumulation of ADP-insensitive EP and with lack of the Ca2* dissociation. On the other hand, the significant occurrence of the H* binding to the NEM-treated enzyme in the absence of KC1 corresponded to the accumulation of ADP-insensitive EP and the Ca2* dissociation. The markedly delayed H* dissociation without KC1 in the late stage of the reaction is also consistent with the markedly delayed Ca2* binding. These findings with the NEM-treated enzyme give additional support to the proposal scheme.

Thus, all of the results discussed hitherto are compatible with the possibility that at least at acidic pH the H* ejection during the Ca2* uptake takes place through a Ca2*/H* exchange directly mediated by the Ca-ATPase. However, at present, it is not clear whether the observed H* binding plays an essential role in the process of the Ca2* uptake.

The Ca2* binding to the NEM-treated enzyme in the presence of KC1 (Table I) is in agreement with the previous observations (16, 17, 33) that an additional Ca2* binding takes place upon formation of ADP-sensitive EP. The decrease in the stoichiometric ratio of Ca2* dissociation to ADP-insensitive EP with an increase in KC1 concentration (Fig. 4) could be reasonably explained in terms of this additional Ca2* binding to ADP-sensitive EP.

The stoichiometric ratio of the H* binding to the Ca2* dissociation was 1.5 mol/mol under the conditions in which almost all of the EP formed was ADP-insensitive (Fig. 4). On the other hand, Chiesi and Inesi (20) indicated the stoichiometric ratio of the H* ejection to the Ca2* uptake to be 1.0 mol/mol. Previously Inesi et al. (37) found that functional residues of the enzyme with different pK values (pK 7.7 in **EP** and pK 5.8 in **E-P**) are involved in the reaction of Step 5. This finding is supported by the pH effects on EP hydrolysis (Step 5) (35) and P2/H2O exchange (dynamic reversal of Steps 5 and 6) (38). It seems, therefore, likely that some part of the observed H* binding and H* dissociation is ascribed to the protonation and deprotonation of these residues and is possibly unrelated to the H* ejection.

It is difficult to unequivocally define the sidedness of H* binding from our previous results (24) showing that the alkanilization was enhanced by making the vesicles leaky since, as reported by Meissner and Young (39) and Meissner (40), sarcoplasmatic reticulum membranes are highly permeable to H*. Therefore, the possibility cannot be eliminated that the observed H* binding and H* dissociation in the present study represent conformational changes of the enzyme being not directly related to the H* ejection. Thus, it is still possible that the H* ejection during the Ca2* uptake with the intact vesicles is mediated by a H* channel rather than by an obligate exchange process directly coupled to Ca2* translocation.

Our previous observation (cf. Fig. 7 in Ref. 24) showed that the alkanilization of the medium in the absence of KC1 occurred almost coincidently with the accumulation of ADP-insensitive EP. This finding agrees well with the present results. However, in the previous experiment, the alkanilization in the presence of KC1 appeared to be somewhat faster than the accumulation of ADP-insensitive EP. This rapid alkanilization was not found under the conditions used in the present experiment.

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