Reduction in Water Activity Greatly Retards the Phosphoryl Transfer from ATP to Enzyme Protein in the Catalytic Cycle of Sarcoplasmic Reticulum $Ca^{2+}$-ATPase*

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Hiroshi Suzuki and Tohru Kanazawa‡
From the Department of Biochemistry, Asahikawa Medical College, Asahikawa 078, Japan

Cys-674 of the sarcoplasmic reticulum $Ca^{2+}$-ATPase was labeled with N-acetyl-$N$'-(5-sulfo-1-naphthyl)ethylendiamine without a loss of the catalytic activity. The ATP-induced drop in the fluorescence of the label, which was shown in our previous studies to reflect the conformational change upon formation of the calcium-enzyme $ATP$ complex, was followed by the stopped-flow method. The subsequent phosphoenzyme formation was followed by the rapid quenching method. Effects of a partial substitution of organic solvents for water in the medium on the conformational change and phosphoenzyme formation were investigated in the presence of $100 \mu M$ $CaCl_2$ at pH 7.5, 0°C. The rate of the conformational change increased with increasing ATP concentration (0.1-100 $\mu M$) and was unaffected by 30% (v/v) dimethyl sulfoxide. In contrast, the rate of phosphoenzyme formation decreased sharply with increasing concentration of dimethyl sulfoxide (20-40% (v/v)), even when phosphoenzyme formation was saturated with ATP. N,N-Dimethylformamide and glycerol had essentially the same effects as dimethyl sulfoxide. These results show that the reduction in water activity does not affect the rate of the conformational change upon formation of the calcium-enzyme $ATP$ complex, but greatly retards the subsequent phosphoryl transfer from ATP to the enzyme protein. This strongly suggests that in this early stage of the catalytic cycle water plays a critical role in ensuring the rapid turnover of the enzyme.

Finally, ADP-insensitive $EP$ is hydrolyzed to liberate $P_i$. This catalytic cycle is fully reversible.

The and Hasselbach (10) showed previously that the rate of $EP$ hydrolysis is markedly reduced by Me$_2$SO. Later, de Meis et al. (11-13) found that $EP$ formation from $P_i$ in the reverse reaction is greatly favored by substituting organic solvents such as Me$_2$SO, DMF, and glycerol for water in the medium. They further suggested that, in contrast to the catalytic site of ADP-insensitive $EP$, which is thought to be hydrophobic (11), the catalytic site of ADP-sensitive $EP$ is hydrophilic. According to this proposal, a change in water activity within the catalytic site may be essential for the conformational transition from ADP-sensitive $EP$ to ADP-insensitive $EP$ (11, 14, 15). However, the role of water in the formation of ADP-sensitive $EP$ from ATP in the early stage of the catalytic cycle has not yet been explored.

Previously, we labeled Cys-674 of the enzyme by EDANS selectively without a loss of the catalytic activity and found that the fluorescence of bound EDANS decreases greatly upon formation of the calcium-enzyme-substrate complex in the initial step of the catalytic cycle (16-18). This fluorescence drop reflects a conformational change in the vicinity of the $ATP$-binding site, because Cys-674 is surrounded by amino acid residues which contribute the conformation of the ATP-binding site (19-25). This conformational change is immediately followed by the phosphoryl transfer from ATP to the enzyme protein (16-18).

In this study, we have investigated the effects of organic solvents (Me$_2$SO, DMF, and glycerol) on the conformational change in the calcium-enzyme-substrate complex and $EP$ formation from ATP by using EDANS-labeled SR vesicles. The conformational change has been followed by the stopped-flow measurements of the fluorescence of bound EDANS and $EP$ formation followed by the continuous-flow rapid quenching method. The results demonstrate that the reduction in water activity by addition of the organic solvents does not appreciably affect the rate of the conformational change, but greatly retards the subsequent phosphoryl transfer. These findings suggest that in this early stage of the catalytic cycle water plays a critical role in ensuring the rapid turnover of the enzyme.

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles—SR vesicles were prepared from rabbit skeletal muscle and stored at ~80°C as described previously (26). The $Ca^{2+}$-dependent ATPase activity determined at 25°C in a mixture containing 0.01 mg of SR vesicles/ml, 1.33 $\mu M$ $A_23187$, 0.2 $mM$ $[\gamma-32P]ATP$, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.4 mM EGTA, 0.1 mM $KCl$, and 20 mM MOPS-Tris (pH 7.0) was 2.51 ± 0.05 nmol of $P_i$/mg/min (n = 3). The content of phosphorylation site in this preparation was 4.46 ± 0.08 $nmol/mg$ (n = 6) when determined with $[\gamma-32P]ATP$ according to Bar-rabin et al. (27).

Labeling of SR Vesicles with I-EDANS—SR vesicles were labeled with I-EDANS as described previously (16). The content of bound EDANS was about 1 mol per mole of phosphorylation site. The $Ca^{2+}$-
dependent ATPase activity was not impaired by this labeling, being 
\[2.53 \pm 0.04 \mu\text{mol of Pi/mg/min (n = 3)}\]. The content of the phosphorylation site determined as described above was 
\[4.31 \pm 0.06 \text{nmoI/mg (n = 6)}\].

Loading of EDANS-labeled SR Vesicles with Ca\(^{2+}\)—EDANS-labeled SR vesicles were passively loaded with Ca\(^{2+}\) by incubation for 12–16 h at 4 °C in a mixture containing 10 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), 0.1 mM KCl, and 20 mM Tris/HC1 (pH 7.5) in the absence of organic solvents in the presence of 30% (v/v) Me\(_2\)SO (B), 30% (v/v) DMF (C), or 30% (v/v) glycerol (D) were mixed with ATP in the same medium at 0 °C by the stopped-flow method. ATP concentrations after the mixing are indicated in the figure.

Steady-state Measurements of Fluorescence—The steady-state fluorescence intensity of bound EDANS was measured on a computer-interfaced spectrofluorometer as described previously (17). The excitation and emission wavelengths were set to 380 and 475 nm, respectively.

Stopped-flow Measurements of Fluorescence—Rapid kinetic measurements of the fluorescence of bound EDANS were made by using a stopped-flow spectrofluorometer interfaced with a personal computer which was programmed to accumulate the digitized data, as described.
Concentrations used were 0% (●), 20% ( ), 30% (▲), and 40% (△, ×) (w/v). [γ-32P]ATP concentrations after the mixing were 100 (○, ▲, △) and 300 (×) μM. For comparison, the ATP-induced fluorescence change (●) was determined in the presence of 30% (w/v) DMF, otherwise same as described in the legend to Fig. 2.

Effect of Reduction in Water Activity on SR Ca2+-ATPase

Effect of glycerol on the kinetics of EP formation. EP formation was performed as in Fig. 2 except that Me2SO was replaced by glycerol. Glycerol concentrations used were 0% (○), 20% (●), 30% (▲), and 40% (△, ×) (v/v). [γ-32P]ATP concentrations after the mixing were 100 (○, ▲, △) and 300 (×) μM. For comparison, the ATP-induced fluorescence change (●) was determined in the presence of 30% (v/v) glycerol, otherwise same as described in the legend to Fig. 2.
Effect of Reduction in Water Activity on SR Ca\(^{2+}\)-ATPase

Fig. 5. Dependence of the extent of fluorescence drop on concentrations of AMP-PCP and ADP. A, the extent of the AMP-PCP- or ADP-induced fluorescence drop attained in the steady state was determined at 0 °C by adding small volumes of AMP-PCP (○, ●) or ADP (▲, ○) to 2.4 ml of a suspension of EDANS-labeled SR vesicles (0.025 mg/ml) in a medium containing 10 mM MgCl\(_2\), 0.1 mM KCl, 20 mM Tris/HCl (pH 7.5), and 0.1 mM CaCl\(_2\) in the absence (○, ▲) or presence (●, ○) of 40% (v/v) Me\(_2\)SO. In all the measurements, the total volumes of added AMP-PCP or ADP were less than 0.05 ml. The fluorescence drop was corrected for dilution upon each addition. The data in the low AMP-PCP or ADP concentration range in upper panel were replotted in lower panel. B, the measurements were performed in the presence of 5 mM EGTA in place of CaCl\(_2\), otherwise same as described in A. The symbols correspond to those in A. The solid lines in A and B show least squares fit to a Michaelis equation.

agreement with the previously reported results (10, 11). When 40% (v/v) Me\(_2\)SO was present, an increase in the ATP concentration from 100 to 300 μM gave no increase in the rate of EP formation and in the steady-state level of EP. It is, therefore, clear that EP formation at saturating concentrations of ATP was greatly retarded by Me\(_2\)SO. For comparison, the EDANS fluorescence drop induced by 100 μM ATP was followed by the stopped-flow method in the presence of 100 μM Ca\(^{2+}\) and 30% (v/v) (Fig. 2) or 40% (v/v) (not shown) Me\(_2\)SO. The fluorescence drop was much faster than EP formation under the same conditions. All these findings demonstrate that Me\(_2\)SO does not affect the rate of the ATP-induced fluorescence drop but greatly retards the subsequent phosphoryl transfer from ATP to the enzyme protein.

In order to see whether the observed effect of Me\(_2\)SO on EP formation is due to reduction in the water activity or is specific to this organic solvent, effects of two other organic solvents, DMF and glycerol, were investigated in the same way as described in Fig. 2 except that DMF or glycerol was used in place of Me\(_2\)SO. A similar retardation of EP formation was found with either DMF (Fig. 3) or glycerol (Fig. 4), although glycerol was somewhat less effective than Me\(_2\)SO and DMF. In fact, the rate of EP formation decreased 20-fold by 40% (v/v) DMF and 13-fold by 40% (v/v) glycerol. An increase in the ATP concentration from 100 μM to 300 μM again gave no increase in the rate of EP formation which was retarded by 40% (v/v) DMF or glycerol. The steady-state level of EP was unaffected by these organic solvents, being 3.65 nmol/mg at 40 s in the presence of 40% (v/v) DMF and 4.05 nmol/mg at 30 s in the presence of 40% (v/v) glycerol (not shown). The EDANS fluorescence drop in the presence of 30% (v/v) DMF or glycerol was much faster than EP formation under the same conditions. These results are essentially the same as those obtained with Me\(_2\)SO, strongly suggesting that the observed retardation of EP formation is due to the reduction in water activity. It is evident that the observed effects of the organic solvents are not due to changes in the dielectric constant of the medium, because the dielectric constant decreases considerably upon addition of DMF or glycerol but only slightly upon addition of Me\(_2\)SO. Indeed, the dielectric constants of water, 40% (v/v) Me\(_2\)SO, 40% (v/v) DMF, and 40% (v/v) glycerol at 0 °C are 88.1, 85.1, 77.1, and 75.7, respectively (29).

Effects of Me\(_2\)SO, DMF, and Glycerol on the Affinity of Ca\(^{2+}\)-ATPase for AMP-PCP and ADP—Concentration dependencies of the EDANS fluorescence drop on AMP-PCP (a nonhydrolyzable ATP analog which is incapable of phosphorylating the enzyme) and ADP were determined by the steady-state measurements in the absence and presence of 40% (v/v) Me\(_2\)SO, 40% (v/v) DMF, and 40% (v/v) glycerol (Fig. 5 and Table I). When Me\(_2\)SO was added in the presence of Ca\(^{2+}\) and absence of organic solvents, the fluorescence decreased with increasing concentration of AMP-PCP (Fig. 5A). The K\(_m\) value obtained is consistent with the previously reported affinity of the catalytic site of the enzyme for AMP-PCP (30, 31). This is in accord with our previous conclusion (17) that the fluorescence drop reflects a conformational change occurring upon formation of the calcium-enzyme-substrate complex. When Me\(_2\)SO was added to give 40% (v/v), the affinity of the enzyme for AMP-PCP increased greatly, and the K\(_m\) became 50-fold lower than that in the absence of organic solvents. The K\(_m\) value for ADP obtained in the presence of Ca\(^{2+}\) and absence of organic solvents is also consistent with the previously reported affinity of the catalytic site for ADP (32). When Me\(_2\)SO was added to give 40% (v/v), the K\(_m\) for ADP again decreased greatly. Similar results were obtained with DMF, although DMF was less effective than Me\(_2\)SO. On the other hand, 40% (v/v) glycerol caused no appreciable decrease in the K\(_m\) for AMP-PCP and ADP.

The K\(_m\) for AMP-PCP and ADP in the absence of Ca\(^{2+}\) decreased only slightly when Me\(_2\)SO was added to give 40%
(v/v) and rather increased to some extent when DMF or glycerol was added to give 40% (v/v) (Fig. 5B and Table I). These results are in contrast to those obtained in the presence of Ca\(^{2+}\). Accordingly, the conformational change responsible for the ATP (or its analog)-induced fluorescence drop in the presence of Ca\(^{2+}\) is distinct from that in the absence of Ca\(^{2+}\). It is, therefore, very likely that the observed effects of Me\(_2\)SO and DMF on the affinity for AMP-PCP or ADP in the presence of Ca\(^{2+}\) are specific to the Ca\(^{2+}\)-activated enzyme.

**DISCUSSION**

The mechanism of the phosphoryl transfer retardation induced by the reduction in water activity and a possible role of water in the early stage of the catalytic cycle may be conveniently discussed in terms of the following reaction scheme proposed previously (16-18),

\[
Ca^z \cdot E + S \rightleftharpoons Ca^z \cdot E' \cdot S \rightleftharpoons Ca^z \cdot E'P
\]

**REACTION 1.**

where E, E’, and E” denote different conformational states of the Ca\(^{2+}\)-activated enzyme, and S denotes the substrate (ATP or its analog). E’P represents ADP-sensitive EP. Our previous findings (16-18) revealed that most of the ATP-induced fluorescence drop (Fig. 1), as well as the whole of the fluorescence drop induced by nonhydrolyzable ATP analogs (Fig. 5) in the presence of Ca\(^{2+}\), occurs upon the conformational change in Step 1. The present results (Figs. 2-4) show that the reduction in water activity markedly increases the activation energy for the phosphoryl transfer in Step 2. This strongly suggests that in this early stage of the catalytic cycle water plays a critical role in ensuring the rapid turnover of the enzyme, although the stereochemical analysis of the phosphoryl transfer in the SR Ca\(^{2+}\)-ATPase (33) previously presented convincing evidence for the in-line displacement mechanism of the phosphoryl transfer in which water molecules are not directly involved.

The increase in the activation energy for the phosphoryl transfer by the reduction in water activity may be possibly due to unstabilization of the transition state in Step 2. This suggests that the transition state is stabilized by hydration when the water activity has not been reduced. The data (Table I) showing the lack of the glycerol-induced shift of the equilibrium between Ca\(_2^z\)E + S and Ca\(_2^z\)E’S imply that the energy levels of both Ca\(_2^z\)E + S and Ca\(_2^z\)E’S are equally raised, rather than equally lowered, by the addition of glycerol, because glycerol is less hydrophilic than water and thus unstabilizes hydrated substrates such as ATP and its analogs (cf. Ref. 11). This gives a support to the above possibility that the observed increase in the activation energy for the phosphoryl transfer is due to unstabilization of the transition state in Step 2 rather than stabilization of Ca\(_2^z\)E’S. However, it should be noted that the suggested hydration of the transition state is seemingly out of harmony with the well known tight binding of transition state analogs to amino acid residues within the catalytic site (34-36).

The results (Fig. 1) show that the addition of any of the three organic solvents causes no change or only a slight decrease in the rate of the conformational change in Step 1. This finding indicates that the energy levels of CaE + S and the transition state in Step 1 are almost equally raised by the reduction in water activity. In contrast, the Me\(_2\)SO- or DMF-induced large reductions in the \(K_m\) for formation of Ca\(_2^z\)E’S (Table I) indicate that the rise in the energy level of Ca\(_2^z\)E’S comparable with that in the energy level of CaE + S is not induced by the addition of Me\(_2\)SO and DMF. It is, therefore, likely that the energy level of Ca\(_2^z\)E’S is less sensitive to these two considerably hydrophobic solvents than that of CaE + S. This is consistent with the possible hydrophobic tertiary structure of the ATP-binding site, which was proposed previously by Taylor and Green (37) on the basis of the predicted secondary structure of the enzyme. Although the reason why the effect of glycerol on the relative energy levels of CaE + S and Ca\(_2^z\)E’S is different from those of Me\(_2\)SO and DMF remains obscure, this difference might be possibly due to the fact that glycerol is less hydrophilic than Me\(_2\)SO and DMF (11).

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Effect of Reduction in Water Activity on SR Ca\textsuperscript{2+}-ATPase

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