Formation of the ADP-Insensitive Phosphoenzyme Intermediate in the Sarcoplasmic Reticulum Ca^{2+}-ATPase of Which both Cys344 and Cys364 Are Modified by *N*-Ethylmaleimide†

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ABSTRACT: Sarcoplasmic reticulum vesicles were pretreated with *N*-ethylmaleimide under the conditions in which both Cys344 and Cys364 (SH_D) of the Ca^{2+}-ATPase are selectively modified. Effects of the modification on the transition of the phosphoenzyme intermediate (EP) from ADP-sensitive form to ADP-insensitive form and on the formation of ADP-insensitive EP from P_i were examined without added K^+. At pH 7.0−8.0 in totally aqueous media, the EP transition and the EP formation from P_i were almost completely inhibited by the SH_D modification. The formation of ADP-insensitive EP from ATP and from P_i in the SH_D-modified enzyme occurred to some extent at pH 6.0−6.5 and were greatly increased by addition of dimethyl sulfoxide at pH 6.0−8.0. The inhibition by the SH_D modification of the EP formation from P_i in the absence of dimethyl sulfoxide was attributed to a decrease in the equilibrium constant for the EP formation from the enzyme-P_i-Mg complex. When 40% (v/v) dimethyl sulfoxide was present, almost all the phosphorylation sites in the SH_D-modified enzyme were phosphorylated with ATP at pH 6.0 or with P_i at pH 6.0−7.0, and all the EP formed was ADP-insensitive. These results lead to the possibility that the previously reported exclusion of water from the catalytic site upon the EP transition and upon the EP formation from the enzyme-P_i-Mg complex is inhibited by the SH_D modification. The present study has revealed the conditions in which the enzyme is released from the inhibition by this modification. The modification of SH_D, which brackets the phosphorylation site (Asp351), may provide a useful tool for the analysis of conformational changes at the phosphorylation site occurring in the catalytic cycle.

The SR[1] Ca^{2+}-ATPase catalyzes Ca^{2+} transport coupled to ATP hydrolysis (*1*, *2*), and its primary structure has been revealed (*3*). This enzyme has the catalytic site in the cytoplasmic domain and the transport sites in the transmembrane domain (*4*). In the catalytic cycle, the enzyme is activated by Ca^{2+} binding to the transport sites on the cytoplasmic side of the SR membrane, and then the γ-phosphoryl group of Mg·ATP bound to the catalytic site is transferred to Asp351 (*3*, *5*−*7*) to form ADP-sensitive EP, which can react with added ADP to form ATP (*8*−*10*). In the subsequent conformational transition, this EP is converted to ADP-insensitive form. Concurrently, the affinity of the transport sites for Ca^{2+} is greatly reduced, and the Ca^{2+} is released into the lumen. Finally ADP-insensitive EP is hydrolyzed to liberate P_i. This EP can also be formed from the enzyme and P_i in the presence of Mg^{2+} and absence of Ca^{2+} by reversal of the late step of the catalytic cycle (*11*, *12*). This phosphorylation occurs through the enzyme-P_i-Mg complex which is formed by random binding of P_i and Mg^{2+} to the enzyme (*13*−*15*).

Cys344 and Cys364 are suitable for the analysis of conformational changes at the phosphorylation site, because these residues bracket the phosphorylation site and because both the residues are selectively modified by maleimide derivatives under specified conditions (*16*, *17*). Previously, both Cys344 and Cys364 were modified by fluorescently labeled maleimides (*18*), and the distance between these bound maleimides was determined. The results showed a large spatial separation between the bound maleimides of 36 Å (*18*). Determinations of the changes in this distance occurring in the catalytic cycle, if possible, will provide important information on the conformational changes at the phosphorylation site. However, the modification of both Cys344 and Cys364 (SH_D) results in almost complete inhibition of the EP transition (*19*−*22*) and the EP formation from P_i (*20*), causing serious problems that have been interfering with such determinations. On the other hand, each of Cys344 and Cys364 is functionally nonessential because the modification of either of these residues by maleimide derivatives causes no loss of the enzyme activity (*16*, *17*, *19*, *23*).

In the present study, we examined effects of the modification of SH_D by NEM on the EP transition and the EP formation from P_i at different pH values in the presence of different concentrations of Me_2SO. The results showed that the EP transition and the EP formation from P_i can take place in the SH_D-modified enzyme provided these reactions are performed in the presence of high concentrations of Me_2SO

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[1] Abbreviations: SR, sarcoplasmic reticulum; EP, phosphoenzyme; SH_D, Cys344 and Cys364 of the Ca^{2+}-ATPase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; NEM, *N*-ethylmaleimide.

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and/or at acidic pH. The results suggest that the previously reported exclusion of water from the catalytic site on the EP transition and on the EP formation from P_i (*24*−*26*) is inhibited by the SH_D modification.

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles. SR vesicles were prepared from rabbit skeletal muscle and stored at −80 °C as described previously (*21*). The Ca^{2+}-dependent ATPase activity determined at 25 °C in a mixture containing 0.01 mg of SR vesicles/mL, 1.33 μM A23187, 0.2 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.4 mM EGTA, 0.1 M KCl, and 20 mM MOPS-Tris (pH 7.0) was 2.51 ± 0.05 μmol of P_i mg^{-1} min^{-1} (n = 3). The content of phosphorylation site determined under the conditions of Barrabin et al. (*27*) was 4.46 ± 0.08 nmol/mg (n = 6) and 4.94 ± 0.02 nmol/mg (n = 3) when determined with [γ-^{32}P]ATP and with $^{32}P_i$, respectively.

Modification of SH_D of the SR Ca^{2+}-ATPase by NEM. SR vesicles were treated with NEM under the conditions described by Kawakita et al. (*16*) as follows. The vesicles (3 mg/mL) were incubated with 1.5 mM NEM at 30 °C for 20 min in a mixture containing 1 mM adenyl-5′-yl imidodiphosphate, 50 μM $CaCl_2$, 5 mM $MgCl_2$, 0.1 M KCl, and 40 mM TES−NaOH (pH 7.0). The excess reagent was removed by gel filtration through a Sephadex G-50 column equilibrated with 0.1 mM $CaCl_2$, 0.1 M KCl, and 5 mM MOPS-Tris (pH 7.0). The resulting NEM-treated vesicles were collected by centrifugation and stored as above. It was previously shown that approximately 2 mol of NEM/mol of Ca^{2+}-ATPase binds rapidly to the Ca^{2+}-ATPase during the initial 20-min period of the incubation under these conditions (see Figure 5 of ref *16*), that the modification of 2 mol of cysteine residues/mol of Ca^{2+}-ATPase results in an almost complete loss of the Ca^{2+} transport activity without loss of the capacity to form EP from ATP (see Figures 5 and 6 of ref *16*), and that these modified residues consist of 1 mol of Cys344 and 1 mol of Cys364 per mole of Ca^{2+}-ATPase (*17*, *23*). In agreement with these previous results, in the present study the Ca^{2+}-dependent ATPase activity was almost completely suppressed by the NEM treatment, whereas the content of the phosphorylation site, 4.35 ± 0.04 nmol/mg (n = 6), determined with [γ-^{32}P]ATP as described above was unaffected. The content of phosphorylation site, 4.87 ± 0.05 nmol/mg (n = 3), determined with $^{32}P_i$ in the presence of 40% (v/v) Me_2SO as described above also was unaffected by the NEM treatment.

Determination of EP. Continuous-flow−rapid-quenching measurements of EP formation from [γ-^{32}P]ATP were made at 10 °C as described previously (*21*). For the determination of the total amount of EP, the reaction was started by mixing equal volumes of solutions from two syringes, one containing SR vesicles in a medium and the other containing [γ-^{32}P]-ATP in the same medium. The reaction was quenched with trichloroacetic acid containing carrier ATP and P_i, and then bovine serum albumin was added unless otherwise stated. The denatured vesicles were washed repeatedly with perchloric acid containing P_i and PP_i, and the radioactivity was determined as described previously (*21*). For the determination of the amount of ADP-insensitive EP, the reaction was started as described above, and at different times thereafter an equal volume of an ADP/EGTA mixture in the same medium as above was added from the third syringe to give 10 mM ADP and 5 mM EGTA. At 100−200 ms after this addition, the reaction was quenched by spouting the reaction mixture into a cuvette containing trichloroacetic acid, and the amount of EP remaining was determined as described above. ADP-sensitive EP entirely disappeared before the acid quenching, whereas ADP-insensitive EP was not significantly decomposed before the quenching. When the reaction was long enough, the procedures above were carried out by manual pipetting and the time course of the decomposition of ADP-insensitive EP was measured between 1 and 6 s after the addition of the ADP/EGTA mixture. The amount of ADP-insensitive EP was obtained by extrapolation of the remaining EP to the time of addition of the ADP/EGTA mixture in a semilogarithmic plot of the time course. EP formation from $^{32}P_i$ was performed at 20 °C. The reaction was quenched with trichloroacetic acid containing carrier P_i and PP_i, and the denatured vesicles were washed repeatedly with perchloric acid and subjected to the determination of the protein concentration, radioactivity, and amount of EP as described previously (*28*).

Other Methods. NEM was purchased from Nacalai Tesque (Kyoto, Japan). ATP and ADP were obtained from Yamasa Biochemicals (Chiba, Japan). Adenyl-5′-yl imidodiphosphate was from Sigma. [γ-^{32}P]ATP was obtained from NEN Life Science Products. $^{32}P_i$ was purified according to Kanazawa and Boyer (*11*). Protein concentrations were determined by the method of Lowry et al. (*29*) with bovine serum albumin as a standard. Concentrations of free P_i and free Mg^{2+} were calculated as described previously (*28*). Data were analyzed by the nonlinear least-squares method as described previously (*28*).

RESULTS

Time Course of Accumulation of ADP-Insensitive EP from ATP. EP formation from ATP was followed with NEM-untreated and -treated vesicles without added K^+ (Figure 1), which accelerates hydrolysis of ADP-insensitive EP and suppresses its accumulation (*30*). The total amounts of EP in both the NEM-untreated and -treated vesicles increased rapidly after addition of ATP and reached almost the same steady-state level in about 1−2 s at pH 7.0 and 6.0 in the absence or presence of 20% (v/v) Me_2SO.

When EP formation was performed with the untreated vesicles at pH 7.0 in the absence of Me_2SO, the transition of EP from ADP-sensitive form to ADP-insensitive form was much slower than the formation of total EP and reached a steady-state level in about 10 s (Figure 1A). The accumulated ADP-insensitive EP amounted to 70% of the total EP at the steady state.

When EP formation was performed with the NEM-treated vesicles at pH 7.0 in the absence of Me_2SO, the accumulation of ADP-insensitive EP was entirely suppressed (Figure 1B). These results show that the EP transition was blocked by the NEM treatment under these conditions, consistent with previous findings (*19*−*22*). Thus the characteristics of the NEM-treated enzyme in the present study agree with those of the SH_D-modified Ca^{2+}-ATPase reported previously (*16*−*22*).

When the pH of the medium was lowered to 6.0 in the absence of Me_2SO, the ADP-insensitive EP in the untreated

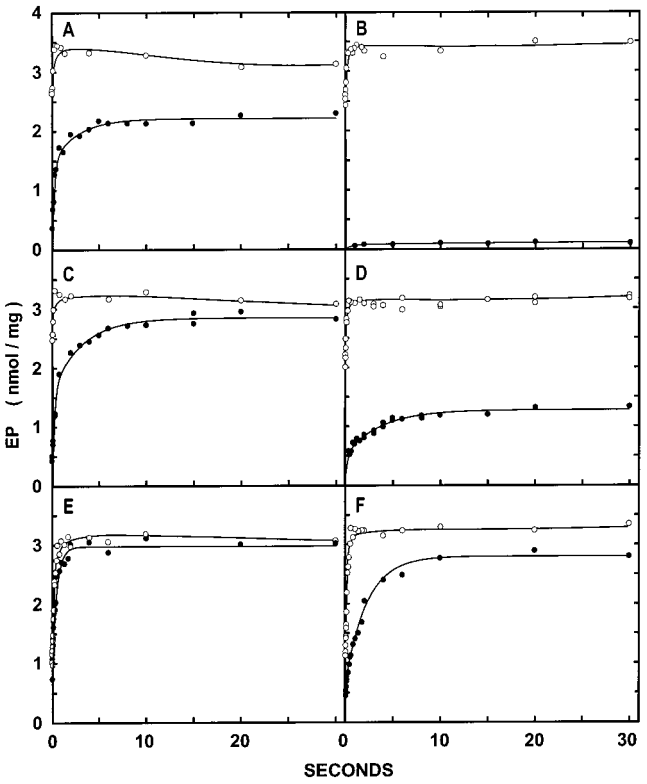


FIGURE 1: Time course of accumulation of ADP-insensitive EP from ATP. NEM-untreated (A, C, E) or NEM-treated (B, D, F) SR vesicles (0.05 mg/mL) were preincubated with 2 μM A23187 at 10 °C for 30 min in the following medium, and then mixed with an equal volume of 100 μM [γ-^{32}P]ATP in the same medium by the continuous-flow−rapid-quenching method or by manual pipetting. Compositions of the media: (A, B) 50 mM MOPS−LiOH (pH 7.0), 0.5 mM $CaCl_2$, 0.4 mM EGTA, 10 mM $MgCl_2$, and 0.1 M LiCl; (C, D) 50 mM MES−LiOH (pH 6.0) in place of MOPS−LiOH, otherwise as in A and B; (E, F) 20% (v/v) Me_2SO included in the medium, otherwise as in C and D. At different times after the start of the reaction, the total amount of EP (○) and the amount of ADP-insensitive EP (●) were determined. Lines drawn under solid circles show the least-squares fit of the data to two exponentials. The first-order rate constant and the maximum amount of EP in the first exponential are (A) 4.66 s^{-1} and 1.47 nmol/mg, (B) 1.55 s^{-1} and 0.07 nmol/mg, (C) 4.84 s^{-1} and 1.61 nmol/mg, (D) 3.86 s^{-1} and 0.51 nmol/mg, (E) 35.1 s^{-1} and 1.14 nmol/mg, and (F) 41.8 s^{-1} and 0.60 nmol/mg. Those in the second exponential are (A) 0.38 s^{-1} and 0.75 nmol/mg, (B) 0.07 s^{-1} and 0.04 nmol/mg, (C) 0.32 s^{-1} and 1.24 nmol/mg, (D) 0.27 s^{-1} and 0.77 nmol/mg, (E) 1.95 s^{-1} and 1.84 nmol/mg, and (F) 0.44 s^{-1} and 2.19 nmol/mg.

vesicles increased, and almost all the total EP was ADP-insensitive at the steady state (Figure 1C). At this pH, the ADP-insensitive EP in the NEM-treated vesicles amounted to 40% of the total EP at the steady state (Figure 1D). The result shows that the transition of EP in the NEM-treated vesicles occurs at this pH, although the steady-state level of ADP-insensitive EP was much lower than that of ADP-insensitive EP in the untreated vesicles.

When EP formation was performed at pH 6.0 in the presence of 20% (v/v) Me_2SO, the EP transition in the untreated vesicles was very rapid and almost all the total EP was ADP-insensitive even at 25 ms after the start of the reaction (Figure 1E). The ADP-insensitive EP in the NEM-treated vesicles was greatly increased by the addition of Me_2SO at pH 6.0 and amounted to 88% of the total EP at the steady state (Figure 1F). However, the EP transition in the NEM-treated vesicles was much slower than that in the untreated vesicles.

The time courses of the EP transition in the NEM-untreated and -treated vesicles under all the conditions tested were well fit by two exponentials, but not by single exponentials. The rates of the EP transition in the fast and slow phases were greatly increased by addition of Me_2SO, although the increase of the rate in the slow phase with the NEM-treated vesicles was much less pronounced. The ratio between the maximum amount of ADP-insensitive EP in the fast phase and that in the slow phase varied depending on the conditions used.

Effects of pH and Me_2SO on Accumulation of ADP-Insensitive EP from ATP at the Steady State. The steady-state levels of total EP and ADP-insensitive EP were determined 20 s after addition of ATP at pH 6.0−8.0 in the presence of different concentrations of Me_2SO (Figure 2). The amounts of total EP formed with the NEM-untreated and -treated vesicles were very close to the contents of the phosphorylation site under all the conditions examined. When Me_2SO was absent, ADP-insensitive EP in the untreated vesicles comprised a large part of the total EP at pH 6.0−8.0 (Figure 2A). It increased when 10% (v/v) Me_2SO was added (Figure 2B) and comprised all the total EP in the presence of 20% (Figure 2C) or 40% (Figure 2D) (v/v) Me_2SO at pH 6.0−8.0.

When EP formation was performed with the NEM-treated vesicles at pH 7.0−8.0 in the absence of Me_2SO, ADP-insensitive EP did not accumulate to any detectable extent (Figure 2A). ADP-insensitive EP increased with decreasing pH and/or with increasing concentrations of Me_2SO, and almost all the total EP was ADP-insensitive at pH 6.0 in the presence of 40% (v/v) Me_2SO (Figure 2D).

EP Formation from P_i. The equilibrium levels of EP formed from P_i in the NEM-untreated and -treated vesicles were determined at pH 6.0−8.0 in the absence or presence of 40% (v/v) Me_2SO (Figure 3). When EP formation was performed with the untreated vesicles in the absence of Me_2SO, an appreciable level of EP was found at pH 6.0 and this level decreased with increasing pH. When 40% (v/v) Me_2SO was present, EP formation increased greatly and almost all the phosphorylation sites were phosphorylated regardless of pH variations from 6.0 to 8.0. These results are in good agreement with the findings reported previously (*24*).

When EP formation was performed with the NEM-treated vesicles in the absence of Me_2SO, no appreciable level of EP was found at pH 7.0−8.0. This is again consistent with the finding reported previously (*20*). In contrast, at lower pH values (6.0 and 6.5) a significant level of EP was found, although this level was lower than that obtained with the untreated vesicles. When 40% (v/v) Me_2SO was present, EP formation increased greatly at all the pH values tested, and almost all the phosphorylation sites were phosphorylated at pH 6.0−7.0.

Equilibrium Analysis of EP Formation from P_i. The effect of the SH_D modification on EP formation from P_i in the absence of Me_2SO at pH 6.0 (Figure 3) was analyzed according to Scheme 1 (*13*−*15*), in which E, E·P_i·Mg, and EP denote the dephosphoenzyme; the ternary complex of the enzyme, P_i, and Mg^{2+}; and the phosphoenzyme, respectively. K_1, K_2, K_3, and K_4 are the dissociation constants for Mg^{2+} or P_i at each step, and K_5 is the equilibrium constant for the formation of EP·Mg. This reaction scheme leads to

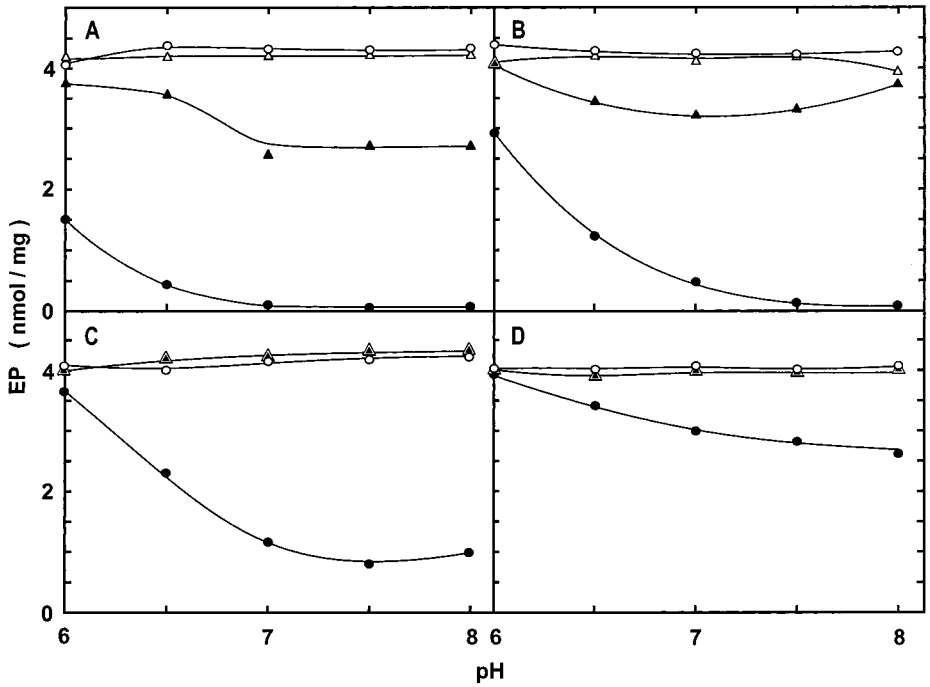


FIGURE 2: Effects of pH and Me_2SO on accumulation of ADP-insensitive EP from ATP at the steady state. NEM-untreated (△, ▲) or NEM-treated (○, ●) SR vesicles (0.5 mg/mL) were preincubated with 6 μM A23187 in a medium containing 0.5 mM $CaCl_2$, 0.4 mM EGTA, 10 mM $MgCl_2$, 0.1 M LiCl, and either 50 mM MES−LiOH (pH 6.0−6.5) or 50 mM MOPS−LiOH (pH 7.0−8.0) in the presence of different concentrations of Me_2SO, and then mixed with [γ-^{32}P]ATP, otherwise as in Figure 1. Me_2SO concentrations used were 0% (A), 10% (B), 20% (C), and 40% (D) (v/v). At 20 s after the start of the reaction, the total amount of EP (○, △) and the amount of ADP-insensitive EP (●, ▲) were determined. In these measurements, the acid-quenched vesicles were washed with perchloric acid without addition of bovine serum albumin, and then subjected to the determination of the protein concentration, radioactivity, and amount of EP as described previously (*28*).

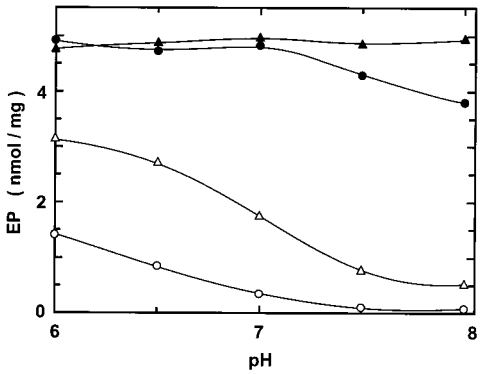
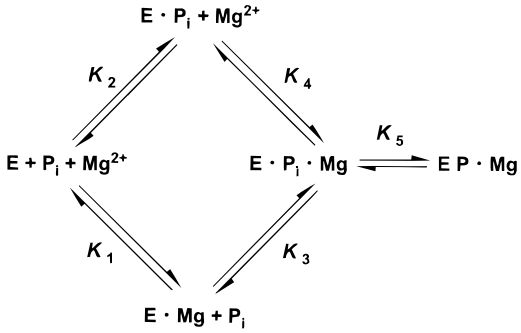


FIGURE 3: Effect of the NEM treatment on EP formation from P_i. NEM-untreated (△, ▲) or NEM-treated (○, ●) SR vesicles (0.5 mg/mL) were phosphorylated with 10 mM $^{32}P_i$ for 1 min (○, △), or with 1 mM $^{32}P_i$ in the presence of 40% (v/v) Me_2SO for 10 min (●, ▲), in a medium containing 2 mM EGTA, 10 mM $MgCl_2$, 0.1 M LiCl, and either 50 mM MES−LiOH (pH 6.0−6.5) or 50 mM MOPS−LiOH (pH 7.0−8.0).

$$[EP\cdot Mg]/(\epsilon K_5) = 1/(1 + K_5 + K_4/[Mg^{2+}] + K_3/[P_i] + K_1K_3/[Mg^{2+}][P_i])$$

for the equilibrium level of EP ([EP·Mg]) as a function of free Mg^{2+} concentration and free P_i concentration, where ϵ is the content of the phosphorylation site determined with P_i (see Experimental Procedures). These constants were determined graphically from data obtained by varying one substrate and keeping the other constant (Figure 4 and Table 1), as described previously (*13*). Double-reciprocal plots of the EP level versus the free Mg^{2+} concentrations in the presence of different fixed free P_i concentrations (Figure 4A, C) or versus the free P_i concentrations in the presence of different fixed free Mg^{2+} concentrations (Figure 4B, D) yielded a linear relationship. The intersection in Figures 4A and 4C gave the value for K_1 and that in Figures 4B and 4D gave the value for K_2. K_3, K_4, K_5, and $[EP\cdot Mg]_{max}$ ([EP] at $[P_i] \rightarrow \infty$ and $[Mg^{2+}] \rightarrow \infty$) were obtained from secondary plots (Figure 4E, F). The values thus determined are summarized in Table 1.

Scheme 1



The values for K_1, K_2, K_3, K_4, and K_5 obtained with the untreated vesicles are in good agreement with those obtained previously under similar conditions by equilibrium and kinetic analyses (*31*). The values for K_1 and K_2 obtained with the NEM-treated vesicles were essentially the same as those obtained with the untreated vesicles. K_3 and K_4 were reduced to some extent by the NEM treatment. K_5 was substantially reduced by this treatment.

DISCUSSION

The present study demonstrates that the transition of EP from ADP-sensitive form to ADP-insensitive form and the

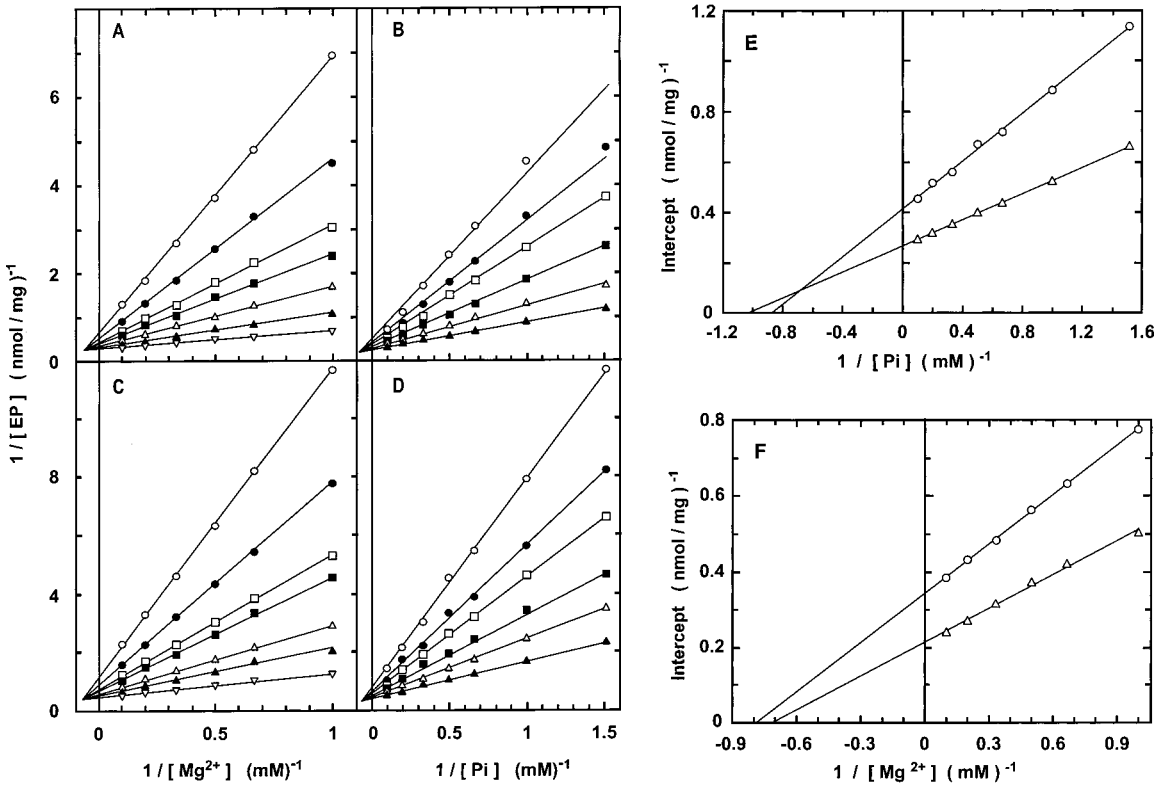


FIGURE 4: Double-reciprocal plots of EP formation at various P_i and Mg^{2+} concentrations. NEM-untreated (A, B) or NEM-treated (C, D) SR vesicles were phosphorylated for 1 min in the presence of different concentrations of free P_i and free Mg^{2+} in a medium containing 2 mM EGTA, 0.1 M LiCl, and 50 mM MES−LiOH (pH 6.0), otherwise as in Figure 3. (A, C) Dependence of EP formation on free Mg^{2+} concentration. Free P_i concentrations (mM): (○), 0.66; (●), 1; (□), 1.5; (■), 2; (△), 3; (▲), 5; (▽), 10. (B, D) Dependence of EP formation on free P_i concentration. Free Mg^{2+} concentrations (mM): (○), 1; (●), 1.5; (□), 2; (■), 3; (△), 5; (▲), 10. (E) The intercept at $1/[Mg^{2+}] = 0$ in A (△) or C (○) was replotted against the reciprocal of free P_i concentration. (F) The intercept at $1/[P_i] = 0$ in B (△) or D (○) was replotted against the reciprocal of free Mg^{2+} concentration.

Table 1: Effect of NEM Treatment on the Equilibrium Constants for EP Formation from P_i[a]

	vesicles	
	untreated	NEM-treated
K_1 (mM)	15.7	14.3
K_2 (mM)	11.9	15.0
K_3 (mM)	6.3	2.5
K_4 (mM)	8.9	2.8
K_5	5.4	1.2
$[EP{\cdot}Mg]_{max}$[b] (nmol/mg)	4.17	2.65
ϵ[c] (nmol/mg)	4.94	4.87

[a] The dissociation constants (K_1, K_2, K_3, and K_4), equilibrium constant (K_5), and maximum level of EP formation ($[EP{\cdot}Mg]_{max}$) were obtained from the plots in Figure 4. [b] The reciprocal of the average of intercepts in Figure 4E,F. [c] The content of the phosphorylation site determined with $^{32}P_i$ (see Experimental Procedures).

formation of EP from P_i can take place in the SH_D-modified Ca^{2+}-ATPase provided these reactions are performed in the presence of high concentrations of Me_2SO and/or at acidic pH (Figures 1−3). This suggests that the modification of SH_D may provide a useful tool for the analysis of conformational changes at the phosphorylation site in the catalytic cycle, because SH_D (Cys344 and Cys364) brackets the phosphorylation site and because one of SH_D (Cys344) is located very close to the phosphorylation site (Asp351) in the primary structure. The results also unequivocally show that SH_D is not essential for the EP transition and the EP formation from P_i (reversal of hydrolysis of ADP-insensitive EP).

The data show that the EP transition is inhibited by the SH_D modification and that this inhibition is greatly reduced by addition of Me_2SO (Figures 1 and 2). Previously, de Meis et al. (*24*, *26*) suggested that the EP transition is correlated with the change in water activity within the catalytic site. The observations reported previously with a fluorescent ATP analogue, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)-ATP, also suggested that the polarity of the catalytic site is greatly reduced upon the EP transition from ADP-sensitive form to ADP-insensitive form (*25*, *33*). Therefore, it is likely that the SH_D modification causes inhibition of the exclusion of water from the catalytic site upon the EP transition and as a result induces the inhibition of the EP transition. It is also likely that addition of Me_2SO causes the reduction in water activity within the catalytic site, as shown previously (*24*−*26*, *34*), and as a result induces the relief of the inhibition of the EP transition in the SH_D-modified enzyme.

The observed good curve fitting of the EP transition to two exponentials (Figure 1) indicates that two kinds of kinetically distinct ADP-sensitive EP (one is rapidly converted to ADP-insensitive EP and the other much more slowly) are formed from ATP. The mechanism of the formation of these two kinds of ADP-sensitive EP remains unclear, but it might be possible that this formation is caused by an oligomeric interaction of the Ca^{2+}-ATPase. The increase in the rate of the EP transition in the fast and slow phases by addition of Me_2SO suggests that destabilization of ADP-sensitive EP and/or stabilization of the transition

state of the EP transition is caused by the reduction in water activity.

The equilibrium analysis made according to Scheme 1 (Figure 4 and Table 1) revealed that SH_D is not essential for binding of P_i and Mg^{2+} and that the observed inhibition of EP formation from P_i by the SH_D modification in the absence of Me_2SO (Figure 3) can be attributed to the decrease in K_5, the equilibrium constant for the EP formation from the enzyme-P_i-Mg complex. Dupont and Pougeois (*25*) previously showed that a large number of water molecules are released from the catalytic site on the formation of the acyl phosphate bond from P_i and that this exclusion of water is essential for the formation of the stable acyl phosphate bond. Furthermore, de Meis et al. (*24*, *32*) showed that K_5 increases greatly with a decrease in water activity. Therefore, the observed substantial decrease in K_5 by the SH_D modification (Table 1) and almost full phosphorylation of the phosphorylation sites in the presence of Me_2SO in the SH_D-modified enzyme (Figure 3) are consistent with the possibility that the SH_D modification results in the inhibition of the exclusion of water from the catalytic site upon the EP formation from the enzyme-P_i-Mg complex.

It was shown previously (*31*) that the affinity of the enzyme for different ionization states of P_i, as well as the protonation of some residues of the enzyme, is involved in the optimal EP formation from P_i at acidic pH in the absence of Me_2SO. The pH dependence of EP formation from P_i in the absence of Me_2SO with the untreated vesicles (Figure 3) is consistent with this view. The partial relief by reducing pH of the SH_D modification-induced inhibition of EP transition (Figures 1 and 2) and EP formation from P_i (Figure 3) raises the possibility that protonation of a residue(s) of the SH_D-modified enzyme may play a significant role in the exclusion of water from the catalytic site. Alternatively, it is possible that protonation of a residue(s) of the SH_D-modified enzyme may induce a conformational change that results in a partial relief of the SH_D modification-induced inhibition of EP transition and EP formation from P_i.

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