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Differential reactivity of lysine residues of the red blood cell Ca^{2+} pump involved in the E_1–E_2 conformational equilibrium

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1. Modification of Lys residues of the Ca^{2+}-ATPase from human red blood cells with methyl acetimidate (MA) inhibited up to 70% of the Ca^{2+}-ATPase activity. Furthermore, calmodulin-activated *p*-nitrophenyl phosphatase activity was fully inhibited at non-limiting concentrations of MA. 2. Treatment with MA inhibited phosphorylation of the Ca^{2+}-ATPase. 3. When the enzyme was treated with 7.2 mM-MA in the presence of 100 μM-Ca^{2+}, Ca^{2+}-ATPase activity was decreased by 33%, whereas when the membranes were treated with MA in the presence of 50 μM-VO_4^{3-}, this activity was decreased by only 8%. 4. When membranes were either proteolysed or preincubated with 1 mM-Ca^{2+}, MA quickly inactivated the Ca^{2+}-ATPase ($k = 1.2$ min^{-1}). On the other hand, inactivation of membranes preincubated in the absence of Ca^{2+} and Mg^{2+} was slow ($k = 0.08$ min^{-1}). 5. When the activity was measured in the absence of calmodulin, MA decreased to the same extent the values of K_{Ca} (the apparent dissociation constant for Ca^{2+}) and $V_{max.}$, but in the presence of calmodulin the treatment decreased $V_{max.}$ only. 6. The results are consistent with the idea that MA reacts readily with the Ca^{2+}-ATPase when the enzyme is in an E_1 conformation, but not an E_2 conformation, and that, reciprocally, treatment of the enzyme with MA shifts the enzyme to E_1. 7. Provided that Ca^{2+} is present, ATP, with low apparent affinity ($K_{0.5} = 195$ μM), protected against inactivation by MA. However, MA treatment did not change the K_m values of either the high-affinity or the low-affinity site for ATP, suggesting that protection results from a shift to a conformation in which the Lys residues are inaccessible to MA.

INTRODUCTION

The existence of two major conformations, E_1 and E_2, has been shown for the Ca^{2+}-ATPase of red blood cell membranes by various criteria: the rapid formation of the phosphorylated intermediate from ATP in the presence of Ca^{2+} for E_1 and the ability to react with water for E_2P [1,2], the pattern of proteolysis [3,4] and spectroscopic studies of the purified enzyme [5]. However, little is known about the structural changes involved in this transition or the relationship between these changes and the functional properties of the enzyme.

Chemical modification of amino acid residues has been of great help in studying the relationship between structure and function of other cation-transporting ATPases. For instance, modification of a Lys residue by fluorescein isothiocyanate (FITC) has been used to identify and study the ATP-binding site of the Na,K-ATPase [6], the sarcoplasmic reticulum Ca^{2+}-ATPase [7] and the H,K-ATPase of the gastric mucosa [8]. Pyridoxal phosphate has been utilized to study the effect of modification of Lys residues of the Na,K-ATPase [9], and carbodi-imides have been helpful in studying the function of carboxyl groups on the Na,K-ATPase [10] and the Ca^{2+}-ATPase of sarcoplasmic reticulum [11]. Furthermore, FITC has been used to study the properties [12] and the primary structure [13] of the active site of the Ca^{2+}-ATPase from red blood cells, whereas carbodi-imides were employed to examine the role of carboxyl groups in enzyme activity in the absence and the presence of calmodulin [14,15].

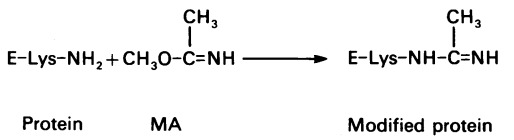
We have studied the effects of chemical modification of the Ca^{2+}-ATPase with methyl acetimidate (MA), a probe for Lys residues that to our knowledge has not been used previously to study cation-transporting ATPases. The modification produced by MA on Lys residues [16] is shown in Scheme 1.

Unlike the probes mentioned above, modification of amino groups by MA does not alter their net charge, since the resulting amidine has a similar pK value; furthermore, MA has little effect on the size of the lysine residue. Thus the effects produced by MA treatment should be ascribed to the modification of the residue itself rather than to a general effect produced by a charge change or by the introduction of a bulky group.

Our results indicate that MA reacts selectively with the E_1 conformation of the enzyme, and that as consequence of this reaction the Ca^{2+}-ATPase is partially inactivated. Thus MA can be used as a new tool to study the $E_1 \rightleftharpoons E_2$ equilibrium in Ca^{2+}-ATPase.

MATERIALS AND METHODS

Fresh blood from haematologically normal adults collected in acid/citrate/dextrose solutions was always used. Red blood cell membranes were prepared using the procedure of Gietzen *et al.* [17], as follows. Red blood cells (washed 3 times with 150 mM-NaCl) were lysed in 8 vol. of lysing solution (1 mM-EGTA/15 mM-Tris/HCl, pH 7.4) at 4 °C. Membranes were centrifuged at 17000 *g* for 20 min and then washed twice with lysing solution. Then the membranes were suspended in 8 vol. of lysing solution, incubated for 15 min at 37 °C in this solution



Scheme 1. Modification of lysine residues by MA

Abbreviations used: FITC, fluorescein 5′-isothiocyanate; MA, methyl acetimidate; CDTA; *trans*-1,2-diaminocyclohexane-*NNN′N′*-tetra-acetic acid.

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and centrifuged at 17000 *g* for 20 min. This step was repeated once. Then the membranes were washed with 8 vol. of 15 mM-Tris/HCl (pH 7.4), resuspended in 1 vol. of the same solution and stored at −20 °C. This procedure yields membranes devoid of endogenous calmodulin. Calmodulin was purified from bovine brain as described by Kakiuchi *et al.* [18].

For treatment with trypsin, the membranes were washed and suspended in medium containing 120 mM-KCl, 30 mM-Tris/HCl (pH 7.4 at 37 °C), 0.5 mM-EGTA and 10 μg of trypsin/ml (60 units/mg). The mixture was incubated at 4 °C, and after 4 min trypsin action was terminated by the addition of soybean trypsin inhibitor (final concentration 200 μg/ml). Then membranes were sedimented (10000*g*; 5 min) and resuspended in 150 mM-Bicine-K (pH 8.3 at 25 °C). Control experiments (not shown) indicated that, after this treatment, the Ca^{2+}-ATPase activity was about 80% of the initial and was insensitive to calmodulin. *p*-Nitrophenyl phosphatase activity was measured by estimating the release of *p*-nitrophenol from *p*-nitrophenyl phosphate [19]. The incubation medium contained 120 mM-KCl, 6.25 mM-$MgCl_2$, 30 mM-Tris/HCl (pH 7.40 at 37 °C), 10 mM-*p*-nitrophenyl phosphate, 1.0 mM-EGTA, 120 nM-calmodulin, 1 mM-ouabain, 60–80 μg of membrane protein/ml and the concentration of $CaCl_2$ necessary to obtain 1.0 μM free Ca^{2+}. Ca^{2+}-ATPase activity was measured at 37 °C in a similar medium to that used for the estimation of the *p*-nitrophenyl phosphatase activity, but without the addition of *p*-nitrophenyl phosphate and with a final concentration of 2 mM-ATP and the free Ca^{2+} concentrations indicated in Tables and Figures. The concentration of $MgCl_2$ in such media was 3.75 mM. The release of P_i from ATP was estimated by the procedure of Fiske & SubbaRow [20]. To estimate the Ca^{2+}-dependent activity, Ca^{2+}-ATPase activities measured in similar media to those described above but without the addition of $CaCl_2$ were subtracted from overall values. The reaction was started by transferring the tubes to a 37 °C water bath. The free Ca^{2+} concentration in the incubation medium was measured with an IS-561 Ca^{2+} electrode [21]. [γ-^{32}P]ATP was prepared according to the procedure of Glynn & Chappell [22], and protein was estimated by the method of Lündahl [23].

Incubation of the calmodulin-free membranes with MA was performed at 25 °C in 50 mM-Bicine-K (pH 8.3 at 25 °C). Except where otherwise indicated, the incubation time was 30 min. After this treatment, membranes were washed twice with 15 mM-Tris/HCl (pH 7.4 at 37 °C)/40 mM-KCl/1.8 mM-$MgCl_2$ and resuspended in the same buffer at a total protein concentration of 0.5–0.8 mg/ml.

SDS/PAGE, staining of the gels and autoradiographic procedures were carried out as described in [24].

MA was obtained from Pierce. EGTA, *trans*-1,2-diaminocyclohexane-*NNN'N'*-tetra-acetic acid (CDTA), ATP (grade I), Tris, SDS, trypsin, soybean trypsin inhibitor and *p*-nitrophenyl phosphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Salts and reagents were of analytical reagent grade.

The experiments presented in the Results section were chosen as representative of 2–4 experiments. Each of the measurements was performed in triplicate and the individual values did not differ from the mean by more than 5%. Curves were fitted to the experimental results by least-squares non-linear regression by using the algorithm of Gauss–Newton with optional damping [25]. The concentration variables were assumed to have negligible error and the velocity variation to be homoscedastic. The equations used to fit the experimental points were chosen on the basis of their best fitting, as judged by the minimum S.D. of the regression. The S.D. of the regression is the sum of the square errors divided by the number of parameters. Calculations were performed with an Epson Equity III+ microcomputer. The program used allows us to fit any function with up to two independent variables and up to 15 adjustable parameters and their S.D. values [26].

RESULTS

Inhibition of Ca^{2+}-ATPase activity by preincubation of the membranes with MA

Fig. 1 shows the results of an experiment in which red blood cell membranes were preincubated for 30 min in the presence of MA. The membranes were then extensively washed and Ca^{2+}-ATPase activity was measured. Such treatment inhibited the Ca^{2+}-ATPase activity in a MA-concentration-dependent fashion. The remaining Ca^{2+}-ATPase activity was 63% of the control activity in the absence of calmodulin, and 30% of control in the presence of 60 nM-calmodulin. The MA concentration required for half-maximal inhibition ($K_{0.5}$) was 4.0 ± 0.8 mM when the Ca^{2+}-ATPase activity was measured in the absence of calmodulin, and 3.3 ± 0.9 mM in its presence. The fact that the inhibition of the Ca^{2+}-ATPase by MA was only partial raises the question of whether MA is able to modify Lys residues in only a fraction of the total population of Ca^{2+}-ATPase molecules, or whether all Ca^{2+}-ATPase molecules are modified and this produces a slower turnover of the enzyme. To provide an answer to this question, the effect of MA on calmodulin-dependent *p*-nitrophenyl phosphatase activity was tested. Fig. 1 also shows that non-limiting concentrations of MA fully inhibited *p*-nitrophenyl phosphatase activity. Under the conditions used in the experiment in Fig. 1 the $K_{0.5}$ for inhibition by MA was 3.01 ± 0.07 mM. This result suggest that all Ca^{2+}-ATPase molecules were modified by MA rather than only a fraction of them.

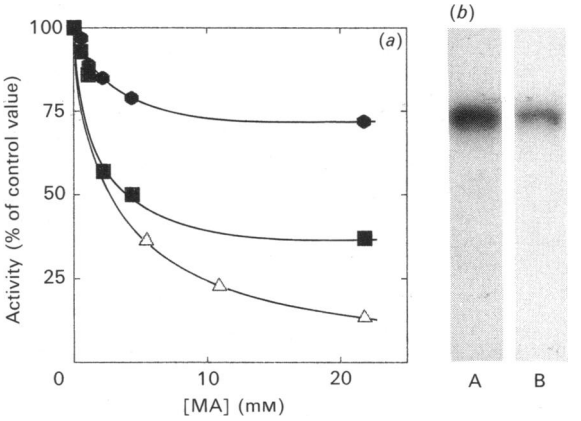

Fig. 1. Effect of MA treatment on the Ca^{2+}-ATPase and Ca^{2+}-phosphatase activities in red blood cell membranes

(*a*) Red blood cell membranes were incubated with MA as described in the Materials and methods section. After such treatment, Ca^{2+}-ATPase in the absence (●) and in the presence (■) of calmodulin, and calmodulin-activated *p*-nitrophenyl phosphatase (△) activities were measured. (*b*) Effect of MA treatment on the phosphorylation of the Ca^{2+} pump by ATP. Red blood cell membranes pretreated with 0 (lane A) or 10 (lane B) mM-MA were phosphorylated with [γ-^{32}P]ATP. Membranes (2.5 mg of protein/ml) were incubated at 4 °C in medium containing 25 mM-Tris/HCl (pH 7.0 at 4 °C), 100 μM-Ca^{2+}, 20 μM-La(III) and 50 μM-[γ-^{32}P]ATP (specific radioactivity 2×10^9 c.p.m./μmol). After 40 s the reaction was stopped by addition of a cold solution containing 7% trichloroacetic acid, 2 mM-ATP and 50 mM-P_i. The mixture was centrifuged for 10 min at 3000 *g* and the pellet was washed three times with the same solution, except that ATP was omitted. SDS/PAGE was then performed and the radioactive bands were revealed by autoradiography as described in the Materials and methods section.

Table 1. Incubation of Ca^{2+}-ATPase with MA in the presence of Ca^{2+} or VO_4^{3-}

Red blood cell membranes were incubated with 0, 1.5 or 3.0 mM-MA as described in the Materials and methods section, in the presence of 100 μM-Ca^{2+} or 50 μM-VO_4^{3-} in the inactivation medium. After treatment, membranes were carefully washed with 3 x 10 vol. of 15 mM-Tris/HCl (pH 7.4 at 37 °C) and Ca^{2+}-ATPase activity was measured in the absence of calmodulin.

MA concentration (mM)	Ca^{2+} Activity (μmol/h per mg of protein)	Ca^{2+} Inhibition (%)	VO_4^{3-} Activity (μmol/h per mg of protein)	VO_4^{3-} Inhibition (%)
0	2.01±0.02	0	1.95±0.05	0
1.8	1.51±0.03	25	1.85±0.02	5
7.2	1.35±0.04	33	1.79±0.02	8

Fig. 1(*b*) shows an autoradiogram of isolated membranes incubated at 4 °C for 40 s with 50 μM labelled ATP and subsequently separated by electrophoresis in gels containing 0.2% SDS. Phosphorylation was carried out in the presence of 50 μM-Ca^{2+} and 20 μM-La(III) for control and MA-treated membranes (lanes A and B respectively). Although a single band corresponding to the phosphorylated intermediate of red blood cell Ca^{2+}-ATPase [27] was observed for both treated and control untreated membranes, the radioactivity in this band was clearly greater for control membranes. Therefore it can be concluded that treatment with MA inhibits Ca^{2+}-ATPase phosphorylation.

The calmodulin-binding site of the Ca^{2+}-ATPase contains one Lys residue that is a potential target for the action of MA [28]. To test whether the effect of MA was due to a modification of this Lys residue by the reagent, Ca^{2+}-ATPase activity was measured as function of calmodulin concentration in control and MA-treated membranes. Membrane Ca^{2+}-ATPase was stimulated about 1.8-fold by calmodulin (at 38 μM free Ca^{2+}) in both cases. The $K_{0.5}$ value for calmodulin stimulation was 7.6±1.0 nM in control and 7.1±1.1 nM in MA-treated membranes (results not shown). These results indicate that the observed inhibition of the Ca^{2+}-ATPase was not due to a failure of MA-modified Ca^{2+}-ATPase to bind and/or be stimulated by calmodulin.

Differential sensitivity of the E_1 conformation of the Ca^{2+}-ATPase to be inactivated by MA

It is known that different ligands stabilize Ca^{2+}-ATPase in different conformations [1,29], i.e., Ca^{2+} stabilizes the ATPase in the E_1 conformation and VO_4^{3-} stabilizes the enzyme in the E_2 conformation. To test whether the Lys residues modified by MA were differentially exposed in the two conformations of the pump, red blood cell membranes were treated with different concentrations of MA in the presence of either 50 μM-VO_4^{3-} or 100 μM-Ca^{2+}. The treated membranes were then extensively washed and Ca^{2+}-ATPase activity was measured in the standard media (Table 1). It is evident that the Ca^{2+}-ATPase activity of control membranes preincubated without MA was the same whether the preincubation medium contained Ca^{2+} or VO_4^{3-}. It can also be seen that when the membranes were treated in the presence of 100 μM-Ca^{2+}, activity decreased with MA concentration down to 67% of the control. When the preincubation medium contained 50 μM-VO_4^{3-}, activity was barely decreased by

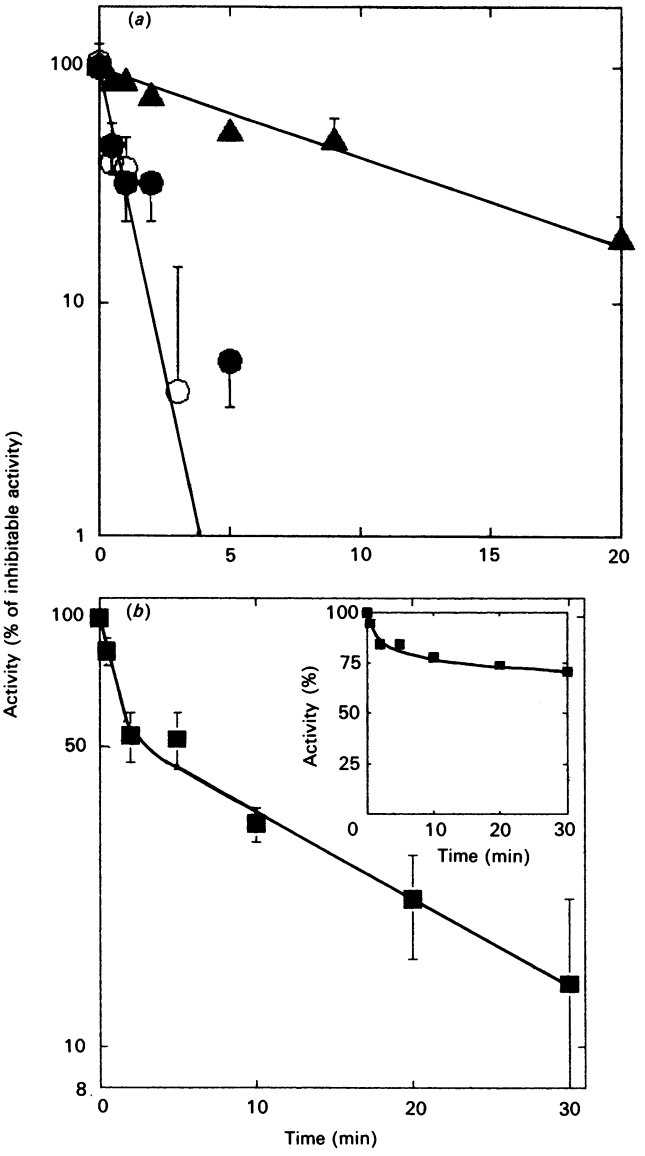

Fig. 2. Time course of inactivation of Ca^{2+}-ATPase by MA

(*a*) Red blood cell membranes were either proteolysed as described in the Materials and methods section (○) or suspended in Bicine buffer and preincubated at 37 °C for 15 min with 100 μM-Ca^{2+} (●) or 1 mM-CDTA (▲). The suspension was then equilibrated at 25 °C and MA was added to give a final concentration of 3 mM. The reaction was stopped at various time points by dilution in 1 vol. of glycine-K (100 mM; pH 7.0 at 25 °C). The membranes were washed with Tris/HCl (pH 7.4 at 37 °C) and the Ca^{2+}-ATPase activity in the absence of calmodulin was measured. The activity measured at zero time minus the activity estimated at infinite time by non-linear regression is set at 100%. (*b*) A suspension of red blood cell membranes in Bicine buffer was preincubated at 37 °C for 15 min with 2 mM-EGTA plus 3 mM-Mg^{2+}, and then the MA treatment was carried out as described for (*a*). Ca^{2+}-ATPase activity was measured in the absence of calmodulin. The inset shows residual activity as a function of time of incubation with MA.

MA treatment, to 92% of the control value. These results suggest that the modifications induced by MA to the Ca^{2+}-ATPase are dependent on the conformation of the enzyme, E_1 being more sensitive than E_2.

The time course of inactivation of the Ca^{2+}-ATPase by 3.0 mM-MA was monitored in conditions that shift the conformational equilibrium towards either E_1 or E_2 (Fig. 2*a*). E_1 was produced

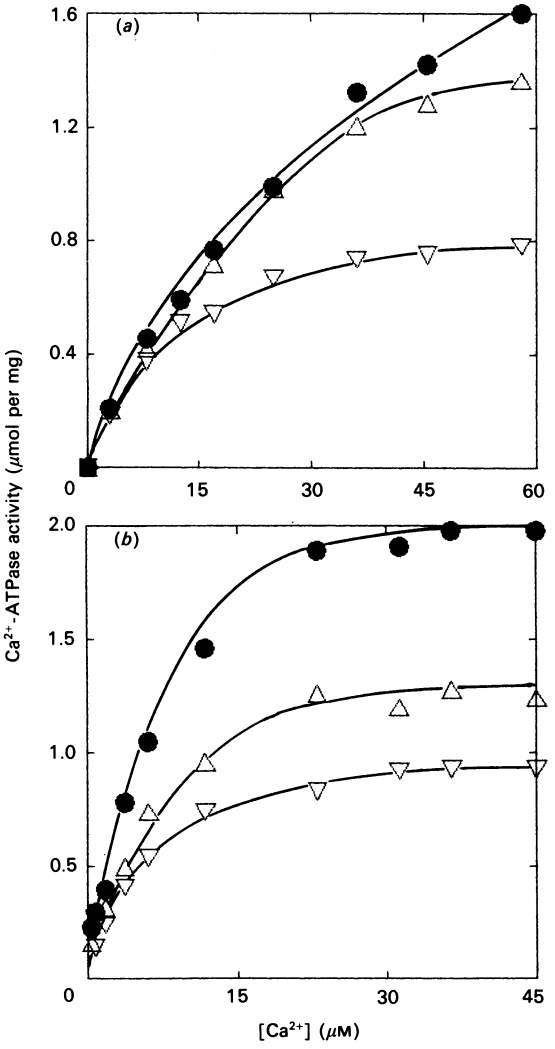

Fig. 3. Ca^{2+}-ATPase activity as a function of Ca^{2+} concentration in MA-treated membranes

Red blood cell membranes devoid of calmodulin were treated with 0 mM-MA (●), 1.8 mM-MA (△) or 7.2 mM-MA (▽) as described in the Materials and methods section. Ca^{2+}-ATPase activity was then measured in the absence (*a*) or in the presence (*b*) of calmodulin, in media containing different Ca^{2+} concentrations.

in two ways; either by preincubating the membranes in the presence of 1 mM-Ca^{2+} or by mild proteolysis with trypsin [1]. The E_2 conformation was favoured by preincubating the membranes with 1 mM-CDTA. In membranes preincubated with Ca^{2+}, activity decayed monoexponentially, with a pseudo-first-order constant of 1.2 ± 0.2 min^{-1}. The remaining activity at infinite time was 71 ± 1% of the activity at $t = 0$. For the proteolysed membranes similar values were obtained: 1.5 ± 0.6 min^{-1} for the pseudo-first-order constant and residual activity of 78 ± 2%. On the other hand, when the preincubation medium contained 1 mM-CDTA and no added divalent cations, activity decayed with a pseudo-first-order constant of 0.08 ± 0.02 min^{-1}. The residual activity was in this case 78 ± 2%. When preincubation media contained 2 mM-EGTA plus 3 mM-$MgCl_2$ (Fig. 2*b*), activity decayed with preincubation time along a biphasic curve. The pseudo-first-order constant for the fast component was 1.4 ± 0.6 min^{-1} and for the slow component it was 0.06 ± 0.03 min^{-1}.

Table 2. Kinetic parameters of the Ca^{2+}-dependence of Ca^{2+}-ATPase activity in MA-treated membranes

The best-fit values and their S.E.M. values were obtained by adjusting eqns. (1) and (2) by non-linear regression to the data in Fig. 3(*a*) and Fig. 3(*b*) respectively:

$$v = \frac{V_{max.}}{(1 + K_{Ca}/[Ca^{2+}])} \quad (1)$$

$$v = \frac{V_{max.}}{(1 + K_{Ca}/[Ca^{2+}])^2} \quad (2)$$

K_{Ca} is the apparent dissociation constant for Ca^{2+}. For the significance of the equations, see ref. [38].

	−Calmodulin		+Calmodulin	
MA concentration (mM)	K_{Ca} (μM)	$V_{max.}$ (μmol/h per mg of protein)	K_{Ca} (μM)	$V_{max.}$ (μmol/h per mg of protein)
0	38±4	2.6±0.1	2.7±0.3	2.3±0.1
3	21±3	1.9±0.1	2.4±0.3	1.42±0.05
12	9±1	0.87±0.04	2.0±0.2	1.00±0.03

The results of the experiment shown in Fig. 2 suggest that, in the presence of Ca^{2+} (E_1 conformation), a population of Lys residues of Ca^{2+}-ATPase is readily attacked by MA. These Lys residues are not accessible in the absence of Ca^{2+} (E_2), since in this case the enzyme is only slowly inactivated by the reagent. In the presence of Mg^{2+}, but the absence of Ca^{2+}, the inactivation curve is biphasic, with both a rapid and a slow phase, suggesting that in this condition the enzyme would be distributed between the E_1 and E_2 conformations. It is worth noting that in the presence of Mg^{2+} alone, Adamo *et al.* [1] found a value of $k = 0.08$ min^{-1} for the rate of conversion of E_2 to E_1, which is comparable to the value for the rate constant for the slow phase of the curve shown in Fig. 2(*b*). At present it is not known whether the slow phase of the inactivation curve reflects the slow transition from E_2 to E_1 in the absence of Ca^{2+} or the reaction of MA with a Lys residue in the E_2 conformation.

Effect of preincubation with MA on the Ca^{2+}-dependence of the Ca^{2+}-ATPase

In Fig. 3 the results of an experiment in which the Ca^{2+}-ATPase activity was measured as a function of Ca^{2+} concentration are shown, in membranes preincubated for 30 min with different concentrations of MA from 0 to 7.2 mM. Activities were estimated in the absence (Fig. 3*a*) and in the presence (Fig. 3*b*) of 300 nM-calmodulin. The kinetic parameters for the curves in Fig. 3 are shown in Table 2. It is evident that when Ca^{2+}-ATPase activity was measured in the absence of calmodulin, both $V_{max.}$ and K_{Ca} were decreased by pretreatment with MA. On the other hand, when the activity was measured in the presence of calmodulin, MA-treated membranes showed lower $V_{max.}$ values but the same K_{Ca} values as control curves. These results may indicate that the E_1 conformation (with high affinity for Ca^{2+}) is stabilized by MA. The change in the apparent affinity for Ca^{2+} would not be apparent in the presence of calmodulin because in this condition the enzyme would be mostly shifted to the E_1 conformation.

Protection by ATP against inactivation of Ca^{2+}-ATPase by MA

Fig. 4 shows the result of an experiment in which red blood cell membranes were preincubated for 30 min with different concentrations of MA (0–21.8 mM) in the absence and in the

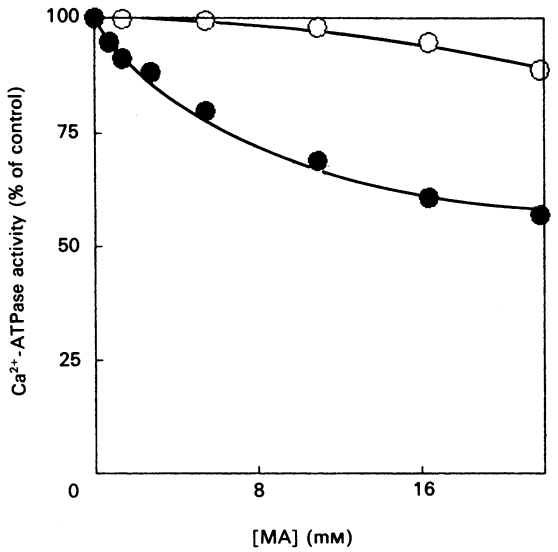

Fig. 4. Protection by ATP against inactivation of the Ca^{2+}-ATPase by MA

Red blood cell membranes were treated with different MA concentrations in the absence (●) or in the presence (○) of 5 mM-ATP. Ca^{2+}-ATPase activity was measured in the absence of calmodulin.

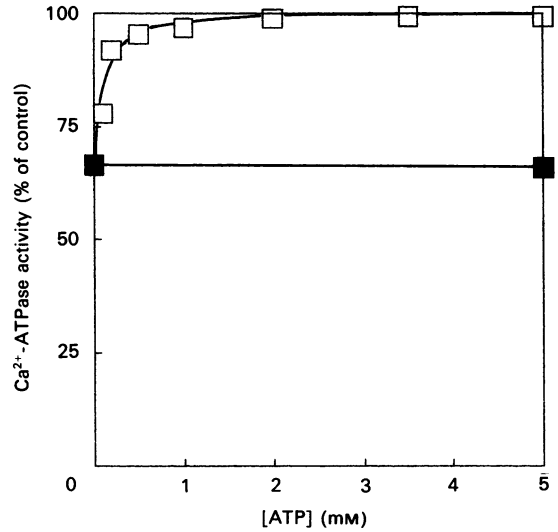

Fig. 5. Inactivation of Ca^{2+}-ATPase by MA as a function of ATP concentration

Red blood cell membranes were incubated as described in the Materials and methods section with 4 mM-MA and different ATP concentrations. This procedure was carried out in the presence of either 100 μM-Ca^{2+} (□) or 1 mM-CDTA (■). Ca^{2+}-ATPase activity was measured in the absence of calmodulin.

presence of 5 mM-ATP. When the preincubation medium did not contain ATP, activity decayed with increasing MA concentration to about 55% of the activity of the control membranes. However, when preincubation medium contained 5 mM-ATP, activity remained unchanged in membranes treated with MA concentrations up to 10.9 mM, and 21.8 mM-MA inhibited activity by less than 10%. Thus ATP protected the Ca^{2+}-ATPase against inactivation produced by MA.

In the experiment shown in Fig. 5, inactivation of the enzyme by treatment of the membranes with 6.85 mM-MA was measured as a function of ATP concentration in the preincubation medium. When the preincubation medium contained Ca^{2+}, ATP protected the Ca^{2+}-ATPase against inactivation by MA, with a $K_{0.5}$ of 195 ± 50 μM. This value is similar to the K_m value for the low-affinity site for ATP of the Ca^{2+}-ATPase. This suggests that ATP protected against inactivation by MA by binding to its low-affinity site in the Ca^{2+}-ATPase. In the absence of divalent cations (1 mM-CDTA), protection by ATP was not observed. When preincubation with MA was carried out in medium containing Mg^{2+} but no Ca^{2+}, ATP was also ineffective in protecting the Ca^{2+}-ATPase against inactivation by MA (results not shown). These results indicate that Ca^{2+}, as well as ATP at the low-affinity site, are required to protect the enzyme against inactivation by MA.

DISCUSSION

We have tested the effect of modification of Lys residues by MA on the activity of the Ca^{2+}-ATPase from red blood cell membranes. This reagent specifically causes addition of an acetimidine group on primary amino groups, without changing the charge of the residue. Since in the Ca^{2+} pump the *N*-terminal end is blocked [30], the primary amino groups that are susceptible to modification by MA are on Lys residues. Thiol groups could probably react with the imido ester as suggested by the structure of iminothiolane, a cyclic thioimido ester formerly thought to be 4-mercaptobutyrimidate [32]. However, since the thioimido ester readily reacts with amino groups (releasing the thiol) [32], treatment of the enzyme with glycine, utilized to quench the action of MA, would reverse the hypothetical modification of cysteine residues. Slight modification of Lys residues resulted in up to 70% inhibition of Ca^{2+}-ATPase activity. This indicated the importance of these residue(s) for the activity of the enzyme.

The most important conclusion that can be drawn from the results in the present paper is that there is a Lys residue(s) that is more reactive (i.e. probably more exposed to the aqueous milieu) in the E_1 than in E_2 conformation. The experimental evidence that led us to this conclusion is as follows. (1) When MA treatment was carried out in the presence of Ca^{2+}, which promotes formation of the E_1 conformation [1], Ca^{2+}-ATPase activity was inhibited to a greater extent by MA than when the treatment was performed in the presence of VO_4^{3-}, which promotes E_2 formation [4,33]. (2) When the membranes were preincubated with Ca^{2+} or were subjected to mild proteolysis with trypsin, the Ca^{2+}-ATPase was quickly inactivated by MA. From measurements of the rate of phosphorylation by ATP, it was recently concluded that both Ca^{2+} and mild proteolysis shift the ATPase to the E_1 conformation [1]. On the other hand, when membranes were preincubated in the absence of divalent cations (a condition where the Ca^{2+}-ATPase is in the E_2 conformation [1]), inactivation by MA was much slower. It is worth mentioning that when membranes were preincubated with Mg^{2+} but no Ca^{2+}, inactivation of the Ca^{2+}-ATPase by MA followed biphasic kinetics, with a rapid phase and a slower phase, suggesting that in such conditions a fraction (but not all) of the Ca^{2+}-ATPase molecules are in the E_1 conformation. This is consistent with the results reported by Adamo *et al.* [1], who concluded that Mg^{2+} alone shifts the Ca^{2+}-ATPase only partially to the E_1 conformation.

If MA reacts preferentially with the E_1 conformation, it is to be expected that this reagent shifts the $E_1 \rightleftharpoons E_2$ equilibrium towards E_1. As E_1 has a higher apparent affinity for Ca^{2+} than E_2, it is, in turn, likely that the MA-treated ATPase would have a higher apparent affinity for Ca^{2+} than the untreated enzyme. This

was indeed the case when, after treatment of the membranes with MA, Ca^{2+}-ATPase activity was measured in the absence of calmodulin (see Fig. 3*a*). As calmodulin alone displaces the conformational equilibrium to E_1 [29], it is not surprising that in the presence of this protein both the MA-treated and the untreated Ca^{2+}-ATPase have the same apparent affinity for Ca^{2+}.

The fact that inactivation of Ca^{2+}-ATPase by MA was partial raises the question of whether only a fraction of the Ca^{2+}-ATPase molecules was modified by the reagent or, on the contrary, all of the enzyme molecules acquired a lower turnover number upon reaction with MA. Regarding this matter, it is noteworthy that treatment of the membranes with MA led to almost complete inactivation of Ca^{2+}/calmodulin-dependent *p*-nitrophenyl phosphatase, an enzymic activity that undoubtedly belongs to the Ca^{2+} pump [34]. This result suggests that Lys residues were modified in almost all enzyme molecules.

Several Lys residues of functional importance have been identified in the Ca^{2+}-ATPase from red blood cell membranes. A Lys residue is part of the calmodulin-binding site [27]. However, treatment of red blood cell membranes with MA did not change either the maximum effect or the $K_{0.5}$ of the enzyme for calmodulin when measured at an optimal Ca^{2+} concentration. Furthermore, the trypsin-treated enzyme, which lacks the calmodulin-binding domain [35], was also inactivated by the reagent. From these results it can be concluded either that the residue(s) modified by MA is not within the calmodulin-binding site, or that such modification, if it does occur, has no detectable effect on activity. Therefore a modification elsewhere in the ATPase molecule must be responsible for the observed inactivation.

An interesting feature of the inactivation of Ca^{2+}-ATPase by MA is that, provided Ca^{2+} is present, ATP protects against this inactivation. The results in Fig. 4 might be interpreted on the basis that other reagents, such as FITC, react with a Lys residue present in the ATP-binding site [13]. However, there are two main differences in the mode of action of MA compared with that of FITC. (1) Ca^{2+} is essential for protection by ATP against inactivation by MA; Mg^{2+} is not a substitute for Ca^{2+}. On the other hand, ATP was protective against inactivation by FITC even in the presence of EGTA [12]. (2) Although in the presence of Ca^{2+}, ATP was protective against inactivation by MA, the enzyme treated with this reagent had the same K_m value for ATP as the intact ATPase. Provided that all of the ATPase molecules were modified by the reagent (see above), this is not the expected result if MA modified a Lys residue within the ATP-binding site. A change in the K_m value for ATP was not seen on the FITC-modified Ca^{2+}-ATPase either, but this result was expected, since the molecules modified by FITC did not contribute to the Ca^{2+}-ATPase activity observed in such membranes [12]. MA-modified ATPase molecules, however, still retain some ATPase activity. Thus modification of Lys residues within the ATP-binding site should be excluded as an explanation for the inactivation of the enzyme by MA. Alternatively, the data support the idea that protection results from a shift to a conformation in which the Lys residues are inaccessible to MA rather than competition between MA and ATP. From the concentration of ATP required for the protection to be manifest, the hypothetical conformational changes would be promoted by binding of ATP to its low-affinity site in the Ca^{2+}-ATPase (see Fig. 5).

In this study, red blood cell membranes, but not purified Ca^{2+}-ATPase, were treated with MA. As the Ca^{2+}-ATPase is less than 0.1 % of the total membrane protein [36], it might be argued that the observed effects on Ca^{2+}-ATPase activity are an indirect consequence of the modification of Lys residues on other membrane proteins. Although at present we cannot provide definitive evidence against such interpretation, it should be pointed out that MA specifically lowered the amount of Ca^{2+}-ATPase phosphoenzyme (see Fig. 1*b*).

MA reacts selectively with Lys residues involved with the $E_1 \rightleftharpoons E_2$ equilibrium. This fact makes this reagent a useful tool to study structural and functional properties of these conformations and the effects of different ligands on the $E_1 \rightleftharpoons E_2$ transition. Finally, it would be interesting to compare the effects other reagents that modify Lys residues, such as *N*-acetylimidazol (reported to inactivate the Na,K-ATPase [37]) with the action of MA.

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