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Interaction of Phenylglyoxal with the Human Erythrocyte (Ca^{2+}+Mg^{2+})-ATPase

Evidence for the Presence of an Essential Arginyl Residue

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SUMMARY

Incubation of human erythrocyte membranes with phenylglyoxal irreversibly inhibited (Ca^{2+}+Mg^{2+})-ATPase activity in a pseudo-first order manner, but followed overall second order kinetics. The enzyme exhibited a low affinity ATP-binding site with a K_m of approximately 125 μM. The effects of the inhibitor could be markedly diminished if ATP was also present during phenylglyoxalation. This indicated that phenylglyoxal and ATP were binding at the same site on the enzyme. The concentration-dependent inactivation reduced both the V_{max} and the K_m of the enzyme, but did not change the apparent affinity for Ca^{2+}. Because ATP could protect against inactivation in the absence of Ca^{2+} and Mg^{2+}, we suggest that free ATP can bind at the low affinity site. The modified enzyme was still capable of being activated by calmodulin. Our data indicate that only the ATP site was affected by the inhibitor, whereas the Ca^{2+} sites were not. Since it is established that phenylglyoxal can react with arginyl residues, we conclude that the binding of the ATP to the low affinity site on the human erythrocyte (Ca^{2+}+Mg^{2+})-ATPase involves such a residue.

INTRODUCTION

Human erythrocyte plasma membranes have a Ca^{2+}-activated, Mg^{2+}-dependent, calmodulin-regulated adenosine triphosphohydrolase [(Ca^{2+}+Mg^{2+})-ATPase] activity which is the biochemical expression of the Ca^{2+} pump mechanism (1). It is now recognized that this Ca^{2+} extrusion mechanism is of utmost importance in the regulation of free intracellular Ca^{2+} not only in erythrocytes, but most likely in all other eucaryotic cells. Although major advances recently have been forthcoming owing to the discovery of calmodulin as a regulator of the (Ca^{2+}+Mg^{2+})-ATPase (2) and the Ca^{2+} pump (3), relatively little is known about either the substrate-binding sites or the regulatory sites of this transport enzyme.

Rates of ATP hydrolysis and Ca^{2+} transport are dependent not only on Mg^{2+} and regulated by calmodulin, but are also modulated by Na^+ and K^+. Furthermore, pH, choice of incubation buffers, the lipid environment of the enzyme, and the various methodologies that are used in the membrane and/or vesicle preparations also influence enzymatic activity and Ca^{2+} transport (4). Properties of the human erythrocyte (Ca^{2+}+Mg^{2+})-ATPase include an apparent dissociation constant for Ca^{2+} in the low micromolar range (1 to 10 μM) and two ATP-accepting sites with considerably different affinities (k_1 of 1 to 3.5 μM and k_2 of 120 to 330 μM). The high affinity site is thought of as being the substrate-hydrolyzing site, and the low affinity site has been postulated to regulate turnover (5). Under appropriate conditions, calmodulin can be shown to both lower k_{dCa} and to increase maximal Ca^{2+} transport and substrate hydrolysis severalfold (6).

Several studies on the nature of the active site and the exact substrate specificity of the (Ca^{2+}+Mg^{2+})-ATPase exist. Early experiments by Wolf (7) suggested that the imidazole nitrogen moiety of a histidine residue was involved in both ATP binding and hydrolysis and that an additional ionizable group with a pK_a of 8.2 was involved in substrate hydrolysis. In the same work, it was concluded that Mg-ATP was the substrate and that the enzyme had a K_m of 50 μM at pH 7.0 and in the presence of 2 mM Mg^{2+}. Subsequent work by Rega and co-workers (5, 8) as well as others (9, 10) suggested, on the basis of phosphorylation studies, that free ATP is the substrate and that Mg^{2+} accelerates dephosphorylation of the phosphoenzyme complex (5). On the other hand, more recent reports with the isolated enzyme (11) provide evidence for Mg-ATP as the true ligand at the regulatory, high K_m ATP site of the (Ca^{2+}+Mg^{2+})-ATPase (11, 12). It was therefore concluded that Mg-ATP accelerates dephosphorylation (11). So far, no clear con-

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sensus exists as to the exact substrate requirements of the various enzymatic steps that ultimately lead to Ca^{2+} extrusion across the human erythrocyte membrane.

Arginine residues have been implicated as a crucial part of the active center of a number of enzymes that utilize ATP as a substrate, such as mitochondrial ATPases (13), cardiac myosin S-1 (14), kidney outer medulla (Na^++K^+)-ATPase (15), and brain adenylate cyclase (16). In every case, the substrate was presumed to be negatively charged and to undergo an ionic interaction with a positively charged guanidino group of an essential arginine. The involvement of these arginine residues in substrate binding was demonstrated by incubating the enzyme with arginine-specific reagents such as phenylglyoxal or 2,3-butanedione (13) and other dicarbonyl compounds (17). In this report, we describe the inactivation of the plasma membrane (Ca^{2+}+Mg^{2+})-ATPase of human erythrocytes by phenylglyoxal under several experimental conditions.

EXPERIMENTAL PROCEDURES

Materials

Outdated (32 to 60 days old), packed human erythrocytes from the local blood bank were used for both intact erythrocyte and plasma membrane preparations. Highly purified human erythrocyte calmodulin was prepared as described previously (18).

Phenylglyoxal monohydrate was purchased from Aldrich Chemical Company, Inc. Adenosine-5′-triphosphate, crystallized disodium salt, was purchased from Boehringer Mannheim Biochemicals. Other chemicals used were of the highest purity available and purchased from standard laboratory supply sources.

Methods

Plasma membrane preparation. Erythrocytes were washed three times by centrifugation (5 min; 3000 rpm) in isotonic NaCl (154 mM), and the "buffy" top layer was removed by aspiration. Washed and packed cells were hemolyzed in 14 volumes of 20 mM imidazole buffer, pH 7.4 at 4° and centrifuged at 39,200 × *g* for 20 min. The resultant supernatant was discarded, and the pellet was resuspended and washed two times in 20 mM imidazole, 0.1 mM EGTA,[1] pH 7.4 at 4°. The membrane pellet was washed two more times with the initial hemolysis buffer (20 mM imidazole) followed by a final wash with 40 mM imidazole, 40 mM histidine buffer, pH 7.1 at 4°, respectively. The pellet was resuspended in the latter buffer to give about half of the initial packed cell volume resulting in a membrane protein concentration of 7 to 9 mg/ml.

Membrane protein concentrations were determined according to the method of Lowry *et al.* (19).

Phenylglyoxal pretreatments of plasma membrane preparations. Erythrocyte plasma membranes at a concentration of 5 mg/ml protein were incubated in a total volume of 1.5 ml containing imidazole buffer, pH 7.4. Phenylglyoxal (0.3 to 3 mM), $MgCl_2$ (3 mM), $CaCl_2$ (100 μM), ATP (3 to 6 mM), and EGTA (0.1 mM) were added as indicated. Time of incubation varied from 0 to 60 min depending on the type of experiment. The complete incubation mixture at 37° had a pH of 7.17. Incubation was terminated by addition of 7.5 ml of ice-cold imidazole buffer, pH 7.4, and centrifugation at 39,200 × *g* for 4 min repeated twice. Membrane pellets were resuspended in imidazole buffer to give a suspension of approximately 2 mg/ml of protein. These protein concentrations were reassayed later for subsequent calculations of specific ATPase activities. In phenylglyoxal time course experiments, incubations were stopped by placing the reaction vials immediately in ice water followed by a centrifugation and washing step with ice-cold imidazole buffer.

Phenylglyoxalation of intact cells. For experiments involving whole cell preparations, erythrocytes were washed as in the initial steps of the plasma membrane preparation. Washed and packed cells were resuspended to give a hematocrit of 25% in a medium containing 144 mM NaCl, 10 mM Tris, pH 7.4 at 4°, and then incubated for 30 min at 37°. After the incubation, phenylglyoxal was added to the cell suspension to give a final concentration of 1 mM and was further incubated for an additional 60 min. Hemolysis at the end of the incubation period was negligible. ATP levels were determined after 30-min incubation (before the addition of phenylglyoxal) essentially by the standard luciferin-luciferase method (20). After incubation, phenylglyoxal was removed by washing the cells twice in 154 mM NaCl. Subsequently, plasma membranes were prepared and assayed for protein content as described above.

(Ca^{2+}+Mg^{2+})-ATPase assays and inorganic phosphate determination. ATPase assays and inorganic phosphate determinations were similar to previously described methods (21) with the following modifications. The incubation medium was reduced to 1.0 ml containing a fixed membrane protein concentration of 200 μg/ml. The incubation medium also contained 18 mM histidine, 18 mM imidazole, 15 mM KCl, 80 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.1 mM ouabain, pH 7.1. ATPase assays were started with the addition of ATP to the incubation medium. Membranes were incubated for 60 min at 37° and reactions were terminated by the addition of 1.0 ml of 2% sodium dodecyl sulfate. The phosphomolybdate complex was measured spectrophotometrically at 750 nm.

Free Ca^{2+} concentrations in the complete incubation medium (including membrane protein) were determined at 37° with an ion-selective electrode (Orion No. 93200).

All ATPase assay incubations and inorganic phosphate measurements were carried out in duplicate or triplicate unless otherwise stated. Data of individual experiments from one or several membrane preparations assayed on different days are reported as means and standard error of the mean. Membrane preparations were used within 5 days of preparation.

RESULTS

Time dependence of phenylglyoxal inactivation of the (Ca^{2+}+Mg^{2+})-ATPase. Incubation of human erythrocyte plasma membranes with phenylglyoxal at 37°, pH 7.17, resulted in a time- and concentration-dependent inactivation of the (Ca^{2+}+Mg^{2+})-ATPase. Fig. 1A shows the kinetics of inactivation obtained with different phenylglyoxal concentrations. Rates of inactivation appeared to obey first order kinetics until 97% of the initial enzyme activity was lost. The remaining (Ca^{2+}+Mg^{2+})-ATPase activities after 60-min exposure to 0.3, 1.0, and 3.0 mM phenylglyoxal were 67, 18, and 1%, respectively. The half-times ($t_{1/2}$) of inactivation were 102.0 min for the lowest concentration of phenylglyoxal and 22.5 and 6.5 min for the intermediate and the highest concentration of phenylglyoxal. From the control experiment, it can be seen that the membrane preparation was remarkably stable under these conditions.

Fig. 1B summarizes data from experiments similar to those shown in A. In these experiments, 6 mM ATP was added 10 min before the addition of phenylglyoxal. ATP considerably reduced inactivation by phenylglyoxal. Remaining enzyme activities after 60 min in this situation were 90 (0.3 mM phenylglyoxal), 69 (1.0 mM phenylglyoxal), and 13% (3.0 mM phenylglyoxal). The half-times of inactivation were 300, 97, and 23.5 min, respectively.

[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

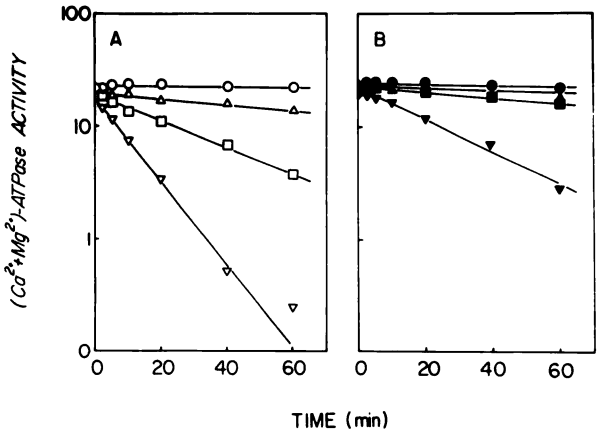

FIG. 1. *Time course of inactivation of human erythrocyte $(Ca^{2+}+Mg^{2+})$-ATPase by phenylglyoxal*

A, enzyme activities from membranes pretreated for 0 to 60 min with 0 (control incubation) (○), 0.3 (△), 1 (□), and 3 mM (▽) phenylglyoxal in 20 mM imidazole buffer at pH 7.17 and 37°. The preincubation medium contained 200 μM Ca^{2+}. Phenylglyoxal and Ca^{2+} were removed as described (see Methods), and membrane activities were measured as described. B, ATP protection of time-dependent inactivation by phenylglyoxal. $(Ca^{2+}+Mg^{2+})$-ATPase activities from membranes pretreated with 0 (●), 0.3 (▲), 1 (■), and 3.0 mM (▼) phenylglyoxal in the presence of 6 mM ATP. Other experimental conditions were the same as those described for A. Results are expressed as nmol of P_i mg^{-1} min^{-1} and represent the means of duplicates from three (A) or four (B) independent experiments.

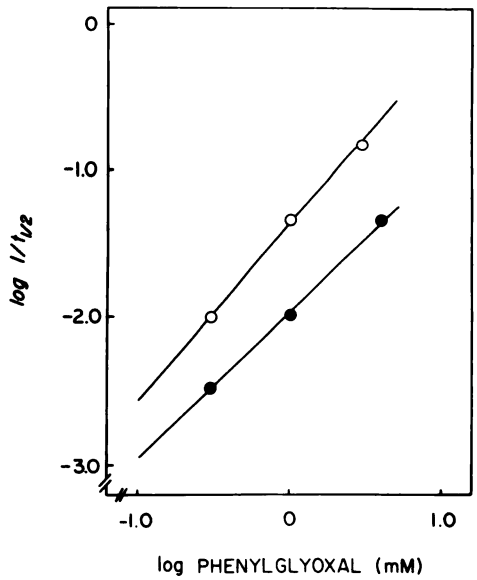

FIG. 2. *Phenylglyoxal incorporation in the presence and the absence of ATP*

Reciprocal log plot of calculated $t_{1/2}$ versus the log of inhibitor concentration. ○, in the absence of ATP during preincubation (slope = 1.2; r = 0.995); ●, experiments in the presence of 6 mM ATP (slope = 1.0; r = 0.996).

Data from Fig. 1 were replotted to generate the lines shown in Fig. 2. This secondary plot of log $(1/t_{1/2})$ versus the log of phenylglyoxal concentrations yielded a straight line with a slope of 1.2 (r = 0.9995) in the absence of ATP during pretreatment of membranes (○). When ATP (6 mM) was added to protect against phenylglyoxalation, another straight line with a slope of 1.00 (r = 0.9957) was generated (●). A slope with a value near 1 from such a plot indicates that inactivation by phenylglyoxal resulted from the irreversible modification of 1 arginyl residue/substrate-binding site of erythrocyte $(Ca^{2+}+Mg^{2+})$-ATPase. On the average, the rate of inactivation of the enzyme by 1 mM phenylglyoxal in the absence of ATP was approximately three times greater than in the presence of 6 mM ATP.

Although Ca^{2+} (200 μM) was present during the inactivation reaction by phenylglyoxal, it should be emphasized that in other experiments 6 mM ATP was able to protect against inactivation in the complete absence of Ca^{2+} and/or Mg^{2+} ions. In fact, when Ca^{2+} and Mg^{2+} were omitted and EGTA (100 μM) added during ATP protection experiments, a 61.7% inhibition was reduced to 23.1% in the presence of 6 mM ATP (Table 1). The largest inactivation by phenylglyoxal was observed in membranes that were pretreated in the simultaneous presence of 0.2 mM Ca^{2+} and 3 mM Mg^{2+} (80.9%). This inactivation was reduced to 56.2% by the addition of 6 mM ATP during the 30-min preincubation.

The double reciprocal plot in Fig. 3 shows the dependence of inactivation rates on phenylglyoxal concentrations in the presence of 6 mM ATP and in the absence of ATP during incubation. The lines for both conditions could be extrapolated to go through the origin, which is characteristic of nonsaturation kinetics. Other workers have observed similar phenomena (22). From Fig. 3, the second order rate constants in the absence and the presence of ATP were calculated to be 0.620 and 0.173 M^{-1} sec^{-1}, respectively.

Kinetic properties of residual plasma membrane $(Ca^{2+}+Mg^{2+})$-ATPase activity: the substrate concentration activity relationship. Although, mainly on the basis of phosphorylation studies, two ATP sites with large differences in affinity have been reported, this study deals with only the low affinity, regulatory ATP site. According to the proposal that Ca^{2+} is responsible for phosphorylation while Mg^{2+} regulates dephosphorylation, our experimental conditions [measuring hydrolysis product in the presence of high $MgCl_2$ (3 mM)] should favor the expression of only the low affinity site.

TABLE 1

Effects of ATP on phenylglyoxalation under various preincubation conditions

Preincubation was for 30 min at 37° in 20 mM imidazole buffer, pH 7.4 at 4°. Membranes were then washed and assayed under standard conditions as described under Methods. For each preincubation medium condition, the activity in the absence of phenylglyoxal (PG) and/or ATP was defined as 100%. Data are derived from two independent experiments in duplicate and are expressed as percentage of means ± standard error.

Preincubation medium	Control	PG (3 mM)	PG (3 mM) and ATP (6 mM)
	% $(Ca^{2+}+Mg^{2+})$-ATPase activity remaining		
0.2 mM Ca^{2+}	100	36.8 ± 2.3	51.0 ± 16.8
3.0 mM Mg^{2+}	100	25.4 ± 11.6	53.4 ± 12.0
0.2 mM Ca^{2+}, 3.0 mM Mg^{2+}	100	19.1 ± 6.4	43.8 ± 2.7
0.1 mM EGTA	100	39.3 ± 16.6	76.9 ± 18.0

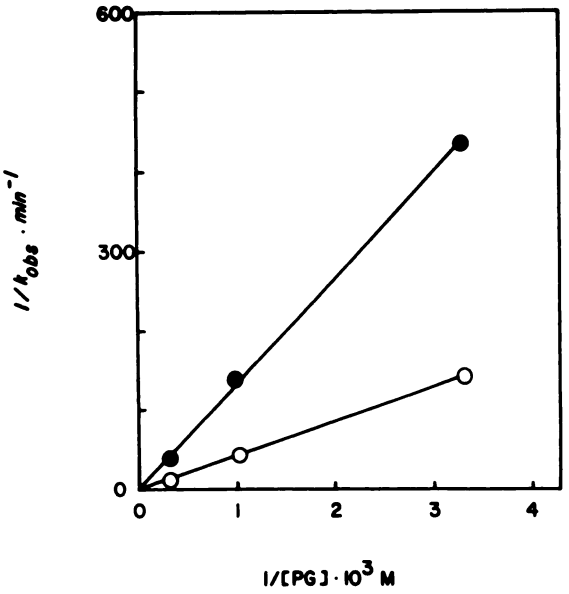

FIG. 3. *Inactivation kinetics of (Ca^{2+}+Mg^{2+})-ATPase by phenylglyoxal with and without ATP protection*

Double reciprocal plot of the apparent inactivation rate constant (k_{obs}) versus the phenylglyoxal (*PG*) concentration. Data are replotted from Figs. 1 and 2. ○, without ATP; ●, with 6 mM ATP.

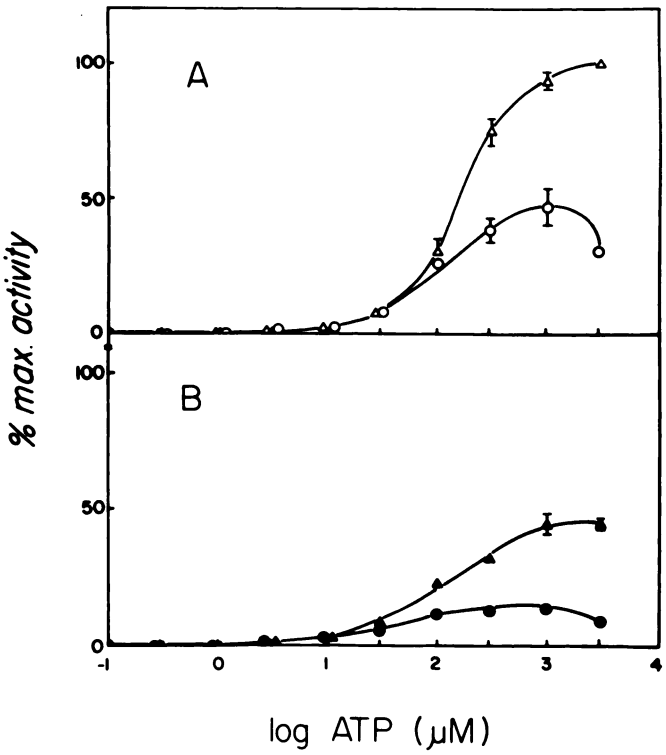

FIG. 4. *Substrate concentration activity relationship of basal and calmodulin-activated (Ca^{2+}+Mg^{2+})-ATPase*

Effects of ATP concentrations in the absence (○) and presence (△) of 0.26 μg/ml calmodulin (A). Effects of ATP concentrations on membranes which were pretreated for 30 min at 37° with 2 mM phenylglyoxal (B). All other conditions were the same as in A. In all experiments, 3 mM Mg^{2+} and 19 μM Ca^{2+} were present. Results are expressed as the mean percentage of three experiments, where the maximal activity from control experiments in the presence of calmodulin (△) was defined as 100%. This mean specific activity was 22.2 ± 3.77 nmol P_i mg^{-1} min^{-1} ($n = 3 \pm$ SE).

The effect of calmodulin on the substrate-velocity plot is shown in Fig. 4A. Replotting the data on a double reciprocal plot revealed only one apparent K_m of about 100 μM for the basal and one apparent K_m of about 160 μM for the calmodulin-stimulated activity.

Membranes pretreated with 2 mM phenylglyoxal for 30 min exhibted reduced maximal activities when tested as a function of substrate concentration (Fig. 4B). The K_m for ATP of the calmodulin-stimulated enzyme activity was 125 μM, whereas the K_m for basal (Ca^{2+}+Mg^{2+})-ATPase activity was reduced to 38 μM. In both types of membranes, activation by calmodulin was approximately 2- to 3-fold at 3 mM ATP.

Double reciprocal plots of substrate concentrations in the range of 0.1 to 1.0 mM versus ATP hydrolysis activities for the control and the phenylglyoxal-modified (1, 2, and 3 mM phenylglyoxal) enzymes are shown in Fig. 5. Fig. 5A represents the mean (Ca^{2+}+Mg^{2+})-ATPase activities of two or three independent experiments (n = 2 for 2 mM phenylglyoxal) from membranes which were phenylglyoxalated in the absence of ATP. The mean V_{max} of the control enzyme was calculated at 14.9 nmol of P_i mg^{-1} min^{-1} with a K_m of 125 μM. The V_{max} for ATP hydrolysis decreased in a phenylglyoxal concentration-dependent manner, whereas, similar to data shown in Fig. 4B, the apparent K_m for the substrate decreased to 67 and 50 μM in membranes after pretreatment with 1 and 2 mM of the inactivator, respectively. Pretreatment of the enzyme with 3.0 mM phenylglyoxal resulted in a further decrease of V_{max} to 2.5 nmol of P_i mg^{-1} min^{-1}. Interestingly, at this concentration, no further increase in the substrate affinity (50 μM) was observed. Fig. 5B shows the effects of 3 mM ATP when added to the preincubation mixtures while the membranes were exposed for 30 min to the various indicated concentrations of phenylglyoxal. The line from the control experiment in the presence of 3 mM ATP was essentially identical to the control data in the absence of ATP seen in Fig. 5A. As expected, ATP markedly reduced the inactivation by phenylglyoxal as demonstrated by the smaller changes in the V_{max} by hydrolysis. Surprisingly, the presence of 3 mM ATP apparently caused all phenylglyoxal concentrations tested to decrease the K_m for the substrate to approximately 50 μM.

No evidence for direct chemical interaction between phenylglyoxal and ATP was found by ultraviolet spectral analysis (results not shown).

The transport substrate concentration activity relationship. In addition to being the transport substrate of the Ca^{2+} pump ATPase, free Ca^{2+} ions are positive effectors of the ATPase and transport activities (Fig. 6). The curve for the control experiment had an apparent $k_{dCa^{2+}}$ of 5 μM, a V_{max} of 12 nmol mg^{-1} min^{-1}, and a Hill coefficient of 0.993 (r = 0.984). The V_{max} of the enzyme from membranes that were pretreated with 1 and 2 mM phenylglyoxal was decreased to 5.6 and 3.1 nmol mg^{-1} min^{-1}, respectively. The extrapolated values for the apparent dissociation constants of phenylglyoxalated membranes were also 5 μM and did not change from the value for the control enzyme (inset).

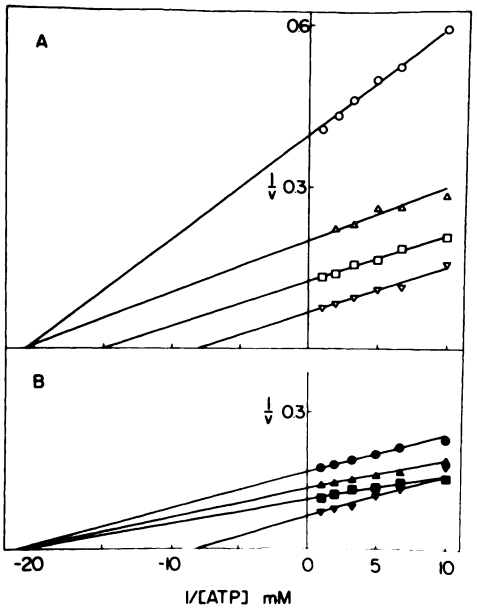

FIG. 5. *Double reciprocal plots of substrate concentration activity relationships of partially inactivated basal (Ca^{2+}+Mg^{2+})-ATPase activities in the presence and absence of ATP*

A, effects of ATP concentrations (0.1 to 1.0 mM) on (Ca^{2+}+Mg^{2+})-ATPase from control membranes (▽) and membranes that were pretreated with 1 (□), 2 (△), and 3 mM (○) phenylglyoxal for 30 min. B, same as A except 3 mM ATP was present during preincubation of control and phenylglyoxal-treated membranes. Results are expressed as specific activities and are the means of two to three independent experiments run in duplicate. Units of *V* are in nmol of P_i mg^{-1} min^{-1}.

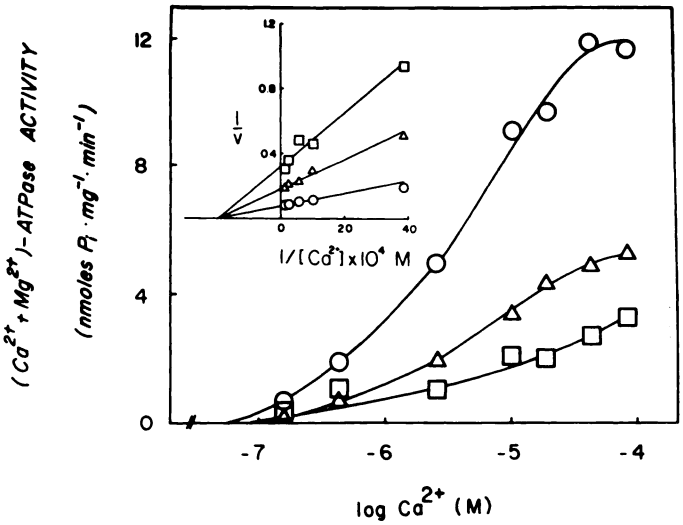

FIG. 6. *Ca^{2+} concentration effect relationship of (Ca^{2+}+Mg^{2+})-ATPase of control and phenylglyoxalated membranes*

Membranes were incubated for 60 min with various concentrations of free Ca^{2+}, as determined by ion-selective electrode and other conditions as specified in Methods. Membranes were preincubated for 30 min in the absence (○) and presence of 1 (△) and 2 mM (□) phenylglyoxal. Inset, double reciprocal plot of data from concentrations ranging from 2.6 to 82 μM free Ca^{2+}.

Fig. 7 again demonstrates that 5 mM ATP can reduce inactivation by phenylglyoxal. As in the previous experiment, only enzyme velocity was affected, although this was to a lesser degree than that observed in the absence

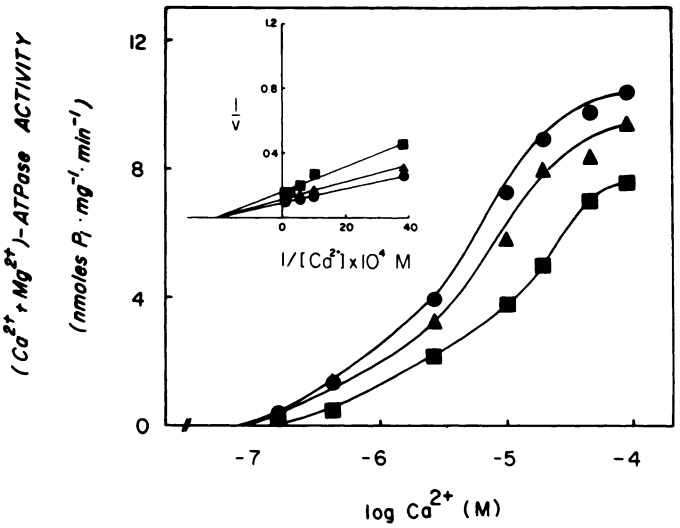

FIG. 7. *Ca^{2+} concentration activity relationship of (Ca^{2+}+Mg^{2+})-ATPase from control and phenylglyoxalated membranes: protection by ATP*

Membranes were preincubated for 30 min in the absence (●) and presence of 1 (▲) and 2 mM (■) phenylglyoxal. Other experimental conditions were as in Fig. 6 except for the presence of 5 mM ATP during the preincubation with phenylglyoxal. Inset, double reciprocal plot of data from Ca^{2+} concentration range of 2.6 to 82 μM.

of ATP. In contrast, from the insets of Figs. 6 and 7, it is clear that phenylglyoxal pretreatment did not affect the apparent affinity of the enzyme for Ca^{2+}.

Phenylglyoxalation of intact erythrocytes. The following experiments were carried out to investigate the sidedness of site(s) affected by phenylglyoxal. Outdated and washed erythrocytes were incubated for 30 min at 37° in a Tris-NaCl buffer, pH 7.4. The hematocrit of the solution was adjusted to 25%. At this point, an aliquot of cell suspension was removed for ATP determination. In three independent batches of erythrocytes, ATP levels on the average were 0.471 ± 0.03 mmol $liter^{-1}$ cells.

Phenylglyoxal (1 mM) was added after 30 min and the cells were incubated for an additional 60 min. Phenylglyoxal was then removed by washing the erythrocytes twice with isotonic NaCl and then membranes were prepared as described above. Fig. 8 shows the observed Ca^{2+} concentration effect relationship of such membranes in the presence and the absence of added calmodulin. The curves obtained were essentially identical to control data from erythrocytes that were not exposed to phenylglyoxal. In membranes from phenylglyoxal-pretreated cells and in membranes from unmodified cells, calmodulin (0.26 μg/ml) caused a modest shift of the apparent $k_{dCa^{2+}}$ (from 6 to 2.5 μM) and a more prominent increase (5.5- and 6.3-fold) in maximal enzyme velocity at 10 μM free Ca^{2+}.

DISCUSSION

After 20 years since the discovery of the erythrocyte plasma membrane Ca^{2+} pump ATPase, it is now recognized by most that the enzyme is quintessential in normal intracellular Ca^{2+} homeostasis and the obligatory Ca^{2+} extrusion process in erythrocytes. Recently, evidence has accumulated that plasma membrane (Ca^{2+}+Mg^{2+})-ATP-

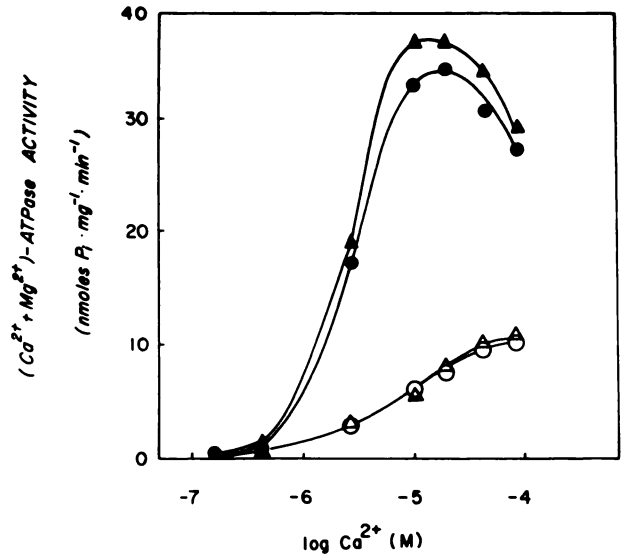

FIG. 8. *Lack of effects of phenylglyoxal on intact erythrocytes*
Basal (open symbols) and calmodulin-activated (closed symbols) (Ca^{2+}+Mg^{2+})-ATPase activities of a representative experiment from membranes which were derived from control (▲, ●) and phenylglyoxal (1 mM, 60 min at 37°)-pretreated intact erythrocytes (△, ○).

ases also play important roles in other, more complex animal cells (23). The calmodulin-regulated Ca^{2+} pump ATPase is modulated by a number of hormones (24, 25) and is subject to pharmacological modification (18, 26).

The use of phenylglyoxal as a tool for establishing functionally essential arginine residues in a number of enzyme systems has been invaluable (17). Under proper conditions, phenylglyoxalation can be highly selective and has been used by Bjerrum *et al.* (27) to distinguish two peptide regions of the erythrocyte anion transporter. Marcus *et al.* (13) reported that arginyl residues are important for hydrolytic function of mitochondrial ATPase in heart and liver.

From work presented here, it is clear that human erythrocyte (Ca^{2+}+Mg^{2+})-ATPase is also inactivated by relatively low concentrations of phenylglyoxal. The inactivation process follows pseudo-first order kinetics and takes place most likely at a recognition site for ATP, since the nucleotide can be shown to protect significantly against inhibition. Interestingly, the protection afforded by ATP can take place in the absence of either Ca^{2+} or Mg^{2+}. From Table 1, it appears that the protective effects of ATP are most prominent when EGTA is present during preincubation with phenylglyoxal. This may indicate that free ATP can bind to the site where phenylglyoxalation takes place.

Based on data seen in Fig. 2, we suggest that one arginine residue is modified in the absence or the presence of ATP (13, 28). A plot of the rate of inhibition (k_{obs}) versus inhibitor concentration with lines extrapolating through the origin indicates that the enzyme and phenylglyoxal do not form a measurable reversible complex before covalent inactivation. Thus, the overall reaction between enzyme and inactivator follows overall second order kinetics. Similar phenomena have been found by others using a variety of irreversible enzyme-inhibitor complexes (22, 29).

Garrahan and Rega (8) and Muallem and Karlish (12) reported that human erythrocyte (Ca^{2+}+Mg^{2+})-ATPase has two ATP-binding sites that have different apparent K_m values: 2.5 and 145 μM. Both sites are thought to be occupied during ATP catalysis under optimal conditions (100 μM Ca^{2+}, 3 mM Mg^{2+}, and ≤1 mM ATP). Richards *et al.* (5) suggest that the high affinity site is responsible for catalysis and that the low affinity site regulates turnover. Unmasking the high affinity substrate site would require reducing the Mg^{2+} to micromolar concentrations. Thus, our membrane preparations, in the presence of 3 mM Mg^{2+} and 19 μM Ca^{2+} tested over an extended range of ATP concentrations, exhibit only the low affinity site in the presence and the absence of a maximally stimulating concentration of calmodulin (Fig. 4A). In phenylglyoxalated membranes and in the presence of maximally stimulating amounts of calmodulin, the low affinity for the substrate was maintained while the affinity for the substrate in basal membranes was increased significantly (Fig. 4B). From these data and the complex inhibition kinetics seen in Fig. 5, it may be postulated that phenylglyoxal preferentially interacts with the high K_m (turnover regulation) site for ATP. Although alternative interpretations, such as ATP producing a conformational change and thus preventing phenylglyoxalation elsewhere (i.e., the catalytic site), should not be neglected, we consider it a less likely possibility. Based on data presented in Fig. 5B, we favor the former interpretation of a direct interaction at the substrate-binding site. In these experiments, where inactivation by phenylglyoxal was decreased by ATP, presumably by competing for the same arginyl site, only a latent relatively high affinity site (approximately 50 μM) was expressed in the modified enzyme. While this affinity is increased as compared to the one before modification, it does not reach the value for the previously reported high affinity (catalytic) site of 1 to 3.5 μM. The observation that in phenylglyoxal-pretreated membranes assayed in the presence of calmodulin (Fig. 4B) the high K_m site was maintained is difficult to interpret. One possible explanation may be that calmodulin, which is known to regulate enzyme turnover in several enzyme systems (30), can override or substitute for the turnover regulation afforded by the high K_m ATP site and thus reestablish ATP sensitivity for the ATPase and Ca^{2+} pump activity. Whatever the case may be, the data presented are consistent with the existence of two ATP sites on the enzyme of which phenylglyoxal modifies the low affinity site, which has an essential arginine in the ATP-binding domain.

The effects of phenylglyoxal on the transport substrate (Ca^{2+}) concentration activity relationship are clear-cut. The reduction in enzyme velocity takes place, in this case, without changing the affinity for the positive effector, Ca^{2+}. This inactivation was also reduced by ATP. These observations were predictable, since inactivation by phenylglyoxal is tantamount to removing the enzyme from the influence of substrate and effectors. This, by itself, should not affect the affinity of the enzyme for Ca^{2+}, irrespective of the site of action of the inhibitor.

Results presented in Fig. 8 corroborate the general

consensus (1), that the ATP-binding sites are situated on the inner surface of the membrane. In intact cells, phenylglyoxalation of the outer plasma membrane surface appeared to have no effect on the enzymatic expression of the ATPase or its regulation by calmodulin. The data, furthermore, suggest that phenylglyoxal either does not cross the cell membrane barrier, or if it does, that endogenous ATP levels are sufficient to protect against inactivation.

This report describes supportive evidence for the presence of a functionally essential arginine amino acid residue in the high K_m substrate-binding site of the $(Ca^{2+}+Mg^{2+})$-ATPase of human erythrocytes. From Ca^{2+} concentration effect relationship analysis in nonmodified and phenylglyoxalated membranes, it appears that the inactivation process did not change the affinity of Ca^{2+} at its binding site (Figs. 6 and 7) or calmodulin interaction with the enzyme (Fig. 4). The irreversible inactivation of $(Ca^{2+}+Mg^{2+})$-ATPase by phenylglyoxal could be minimized by ATP under a variety of preincubation conditions. Notably, ATP was able to protect against inactivation in the total absence of either Ca^{2+} or Mg^{2+} ions, which supports the contention that free ATP can be a ligand, at least at the low affinity substrate site. The data presented suggest that covalent modification of a functionally relevant arginine residue, which leads to irreversible inactivation of the enzyme, is limited to the high K_m ATP-binding site on the inner surface of the Ca^{2+} transport enzyme of the plasma membrane. We propose the use of phenylglyoxal and other dicarbonyl compounds as potentially powerful tools for the elucidation of details regarding the various effector sites on the $(Ca^{2+}+Mg^{2+})$-ATPase in particular and in the Ca^{2+} transport phenomenon in general.

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