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Anion transport in red blood cells and arginine-specific reagents. Interaction between the substrate-binding site and the binding site of arginine-specific reagents

Laila Zaki * and Thomas Julien

Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Strasse 7, 6000 Frankfurt am Main 71 (F.R.G.)

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Phenylglyoxal is found to be a potent inhibitor of sulfate equilibrium exchange across the red blood cell membrane at both pH 7.4 and 8.0. The inactivation exhibits pseudo-first-order kinetics with a reaction order close to one at both pH 7.4 and 8. The rate constant of inactivation at 37°C was found to be 0.12 min^{-1} at pH 7.4 and 0.19 min^{-1} at pH 8.0. Saturation kinetics are observed if the pseudo-first order rate constant of inhibition is measured as a function of phenylglyoxal concentration. Sulfate ions as well as chloride ions markedly decrease the rate of inactivation by phenylglyoxal at pH 7.4, suggesting that the modification occurs at or near to the binding site for chloride and sulfate. The decrease of the rate of inactivation produced at pH 8.0 by chloride ions is much higher than that produced by sulfate ions. Kinetic analysis of the protection experiments showed that the loaded transport site is unable to react with phenylglyoxal. From the data it is concluded that the modified amino acid(s) residues, presumably arginine, is (are) important for the binding of the substrate anion.

Introduction

Anion exchange across the human red cell membrane is mediated by a well characterized integral membrane protein [1,2]. This protein, according to nomenclature of Steck [3], is called band 3. Anion exchange can be inhibited by a large number of compounds. One class of these compounds ~~is~~ the class of stilbenedisulfonate which acts as high-affinity competitive inhibitors of anion exchange when added to the outside of intact cells [4,5]. These compounds do not cross the membrane and bind exclusively to the outward facing unloaded form of the transport system. Other types of inhibitors are found to bind to the loaded transport system and are hence believed to bind to a site which is different from the transport site. A new class of anion transport inhibitors which has been first used in this laboratory are the arginine-specific reagents. It has been found that α-dicarbonyl reagents, which are known for their specificity for arginyl side chains in proteins, are potent inhibitors of anion transport across red cells [6–8]. These results have been confirmed by results from others [9]. It has also been found that the site of action of these compounds is not identical with the site of action of the stilbenedisulfonate inhibitors [6]. On the other hand it has been found that both anion substrates, sulfate and chloride, are able to protect the transport system against inhibition by the arginine-specific reagents [7,8]. It has also been found that complete inactivation at pH 7.4 is accompanied by modification of 2 or 3 arginine residues per band 3 mole-

* To whom correspondence should be addressed.

cule [10]. Under conditions where the cells were inactivated with phenylglyoxal at an extracellular pH of 10.5 and neutral pH inside the cells, phenylglyoxal was found to bind to only one site on the extracellular site of the cells [11]. Since that time more attention has been paid to the essential role of arginine residues in transporting anions across the red cell membrane [12,13]. In the present work the inactivation of anion exchange by phenylglyoxal at pH 7.4 and 8.0 has been studied in more detail. The kinetic analysis of the inactivation process in the presence of the substrate anions, and the nature of the interaction between the binding site of phenylglyoxal and the substrate binding site is reported in this paper.

Materials and Methods

Human erythrocytes from apparently healthy donors were obtained from the Red Cross in Frankfurt and stored at 4°C in acid/citrate/dextrose buffer for 2–4 days: After removal of plasma and buffy coat, the cells were washed three times in isotonic buffer. Resealed ghosts were prepared essentially as in Ref. 1. Cells were hemolyzed at a cell medium ratio of 1 : 20 in medium containing 4 mM $MgSO_4$ and 1.45 mM acetic acid. Five min after hemolysis sufficient amounts of sucrose, gluconate, citrate and Hepes were added to obtain a final concentration of 200 mM sucrose, 27 mM gluconate, 25 mM citrate, 5 mM Hepes in the hemolysate. After centrifugation the ghosts were resuspended and resealed in standard buffer containing, 200 mM sucrose, 27 mM gluconate, 25 mM citrate, 5 mM Hepes and 1 mM Na_2SO_4 (pH 7.4 or 8.0). In the protection experiments, sucrose was partly replaced by either Cl^- or SO_4^{2-} ions at the concentration indicated in Figs. 3 and 4. The reaction of the resealed ghosts with phenylglyoxal was carried out under the conditions given in the figure legends. The kinetic data were fitted with least squares method by nonlinear regression to the equations discussed in the text. $^{35}SO_4^{2-}$ equilibrium exchange was measured after removal of excess phenylglyoxal in a medium with the same composition as that had been used for resealing and reaction with the inhibitors. The flux measurements were done as described previously [1].

Chemicals. Phenylglyoxal (pure) was obtained from Serva, Heidelberg. Hepes was obtained from Calbiochem Boehring. All other substances were from Merck-Darmstadt.

Results

Kinetics of inactivation of sulfate equilibrium exchange by phenylglyoxal

Incubation of resealed ghosts at pH 7.4 and 8.0 with excess phenylglyoxal resulted in a time-dependent inhibition of sulfate exchange. The time-course of inactivation was found to follow pseudo-first-order kinetics until transport is reduced to less than 10% of the initial vlaue. This is indicated by the straight lines obtained in semi-log plots of transport rate versus time (Figs. 1 and 2). The rate of inactivation of the transport system depends on the concentration of phenylglyoxal as shown in Figs. 1 and 2.

Nonlinear fits of single exponentials forced through the points (time, residual activity) = (0, 100) and (∞, 0) agree well with the experimental data at pH 7.4 (Fig. 3). At pH 8.0 and under experimental conditions where the residual activities are less than 10% (at 10 and 5 mM phenylglyoxal, incubation time 30 and 40 min) this procedure does not yield optimal fits. However, when the program is permitted to calculate a value for infinite time, the residual flux after maximal inhibition of the system is 2.5% rather than zero. Applying the same procedure to the data at pH 7.4 yielded residual activities with slightly negative values (about −3.0%). This means that the calculated 2.5% residual flux after maximal inhibition at pH 8.0 is within the range of experimental scatter. Therefore under our experimental conditions phenylglyoxal inhibits the system to at least 97.5.

At low concentrations of phenylglyoxal where there is a linear relationship between the apparent rate constant of inactivation and the concentration of the inactivator (panels (a) in Figs. 1 and 2), the reaction order n with respect to phenylglyoxal is determined according to Eqn. 1 which described the dependence of K_{app} on phenylglyoxal concentrations, [PG].

$$K_{app} = k[PG]^n \quad (1)$$

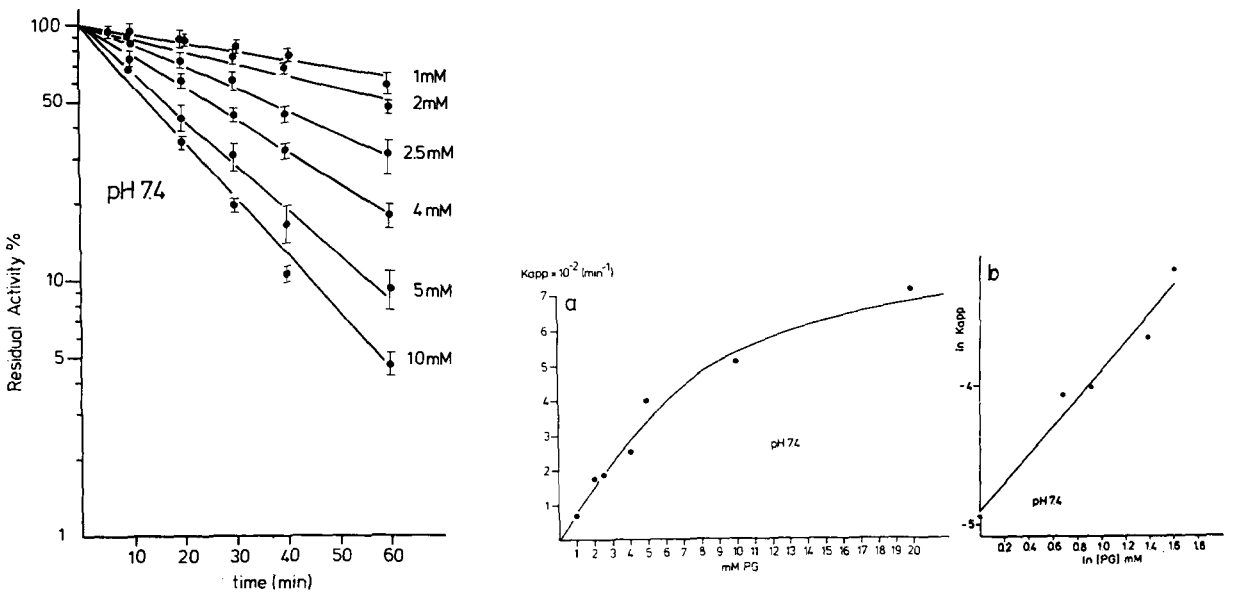


Fig. 1. Semilogarithmic plots of inactivation of sulfate equilibrium exchange by phenylglyoxal. Resealed ghosts were incubated in standard medium at pH 7.4, 37°C at the concentrations of the reagent indicated in the figure. At the times indicated on the abscissa, aliquots were withdrawn, excess phenylglyoxal was removed by washing and the residual activity of $^{35}SO_4^{2-}$ equilibrium exchange was measured. The ordinate presents the residual flux as % of a control value without inhibitor. Flux measurements were done as described in Ref. 1. Panels (a) and (b): linear and double-ln plots of K_{app} vs. phenylglyoxal (PG) concentration. The rate constants (K_{app}) were determined from the individual flux measurements by nonlinear least-squares method.

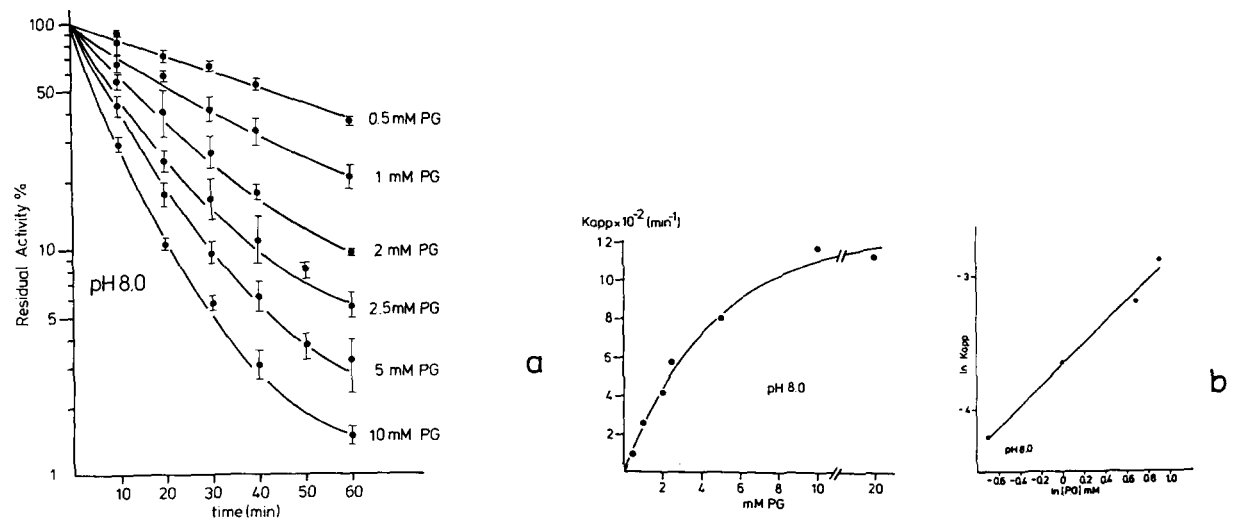


Fig. 2. Semilogarithmic plot of sulfate equilibrium exchange inactivation by phenylglyoxal at pH 8.0. Resealed ghosts were incubated with different concentrations of the reagent (as indicated in the figure), in standard medium at pH 8.0 at 37°C. At the indicated times, aliquots were withdrawn and $^{35}SO_4^{2-}$ equilibrium exchange was measured after removal of excess of phenylglyoxal as described in Ref. 1. Panels (a) and (b): linear primary and double-ln plots of K_{app} vs. phenylglyoxal (PG) concentration.

The k is a proportionality constant and [PG] represents reagent concentration. Thus

$$\ln K_{app} = \ln k + n \ln[PG] \quad (2)$$

From a plot of ln K_{app} vs. ln[PG], n can be obtained from the slope of the line as described by Levy and Ryan [14].

When the linear portion of the curves (panels

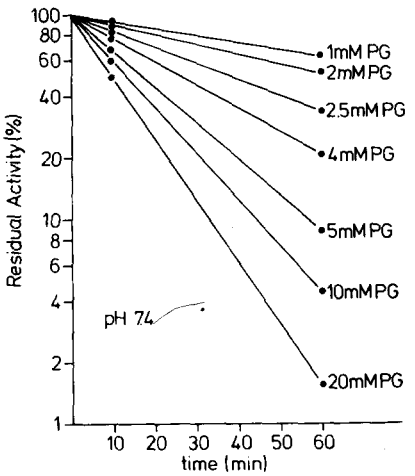


Fig. 3. The straight lines represent nonlinear fits of single exponentials forced through the points (time, residual activity) = (0,100) and (∞,0), for the experimental data in Fig. 1. PG, phenylglyoxal.

(a) in Figs. 1 and 2) were replotted according to Eqn. 2, n was calculated to be 0.96 at pH 7.4 and 0.84 at pH 8.0 (panels (b) in Figs. 1 and 2). Our inhibition kinetics data shows that n is close to one. This would suggest that the reaction of one phenylglyoxal molecule per one band 3 molecule is involved in the rate limiting step in the inactivation process. This is in agreement with earlier results [15–20].

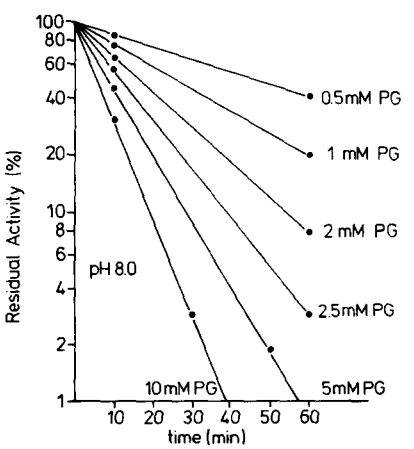


Fig. 4. The straight lines represent nonlinear fits of single exponentials forced through the points (time, residual activity) = (0,100) and (∞,0), for the experimental data in Fig. 2. PG, phenylglyoxal.

The saturation behavior of the rate of inactivation of sulfate flux by phenylglyoxal (panels (a) in Figs. 1 and 2) suggests that the process of inactivation involves association of the reagent with the transporter (T) to form a non-covalent transporter-inhibitor complex (T · I) prior to irreversible modification and inactivation of the transporter (T*I) [21]. The simplest interpretation of the inactivation process is described by the following scheme:

$$T + I \overset{K_I}{\rightleftharpoons} T \cdot I \overset{k}{\rightarrow} T^*I$$

Scheme I.

where K_I is the dissociation constant of the transporter-inhibitor complex T · I, and k the rate constant of inactivation of the transport, T* is the inactivated transporter, I represents the concentration of the inhibitor (phenylglyoxal). The following equation can be derived from this scheme:

$$K_{app} = \frac{k[PG]}{K_I + [PG]}$$
$$\text{or} \quad \frac{1}{K_{app}} = \frac{1}{k} + \frac{K_I}{k[PG]} \qquad (3)$$

Eqn. 3 has the form of the Michaelis-Menten equation.

When the kinetic data were fitted to Eqn. 3 with nonlinear least-squares analysis, k and K_I are calculated to be 0.12 min^{-1} and 12 mM, respectively, at pH 7.4 and 0.19 min^{-1} and 6.46 mM at pH 8.0.

Earlier observations obtained by Takahashi [22] have shown that 2 molecules of phenylglyoxal react with 1 molecule of arginine, with the reaction of the first molecule of phenylglyoxal being rate limiting.

This means that after the binding of one phenylglyoxal molecule to one of the arginine groups of the active site of the anion transport system inactivation occurs and then in a second step another phenylglyoxal molecule is bound, in order to achieve 1 :'2 stoichiometry reported by Takahashi.

Substrate protection experiments

The pseudo-first-order kinetic data were also obtained for the inactivation of sulfate exchange

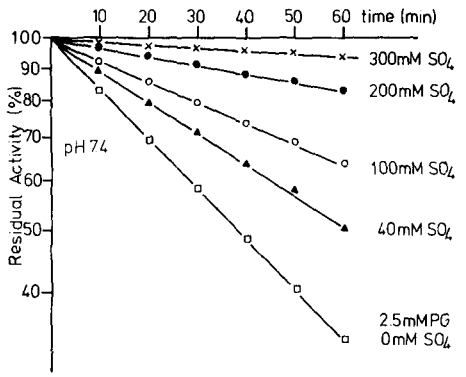
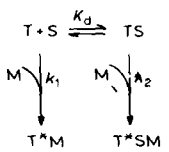


Fig. 5. Protectionof sulfate equilibrium exchange against phenylglyoxal inactivation by SO_4^{2-} ions. Resealed ghosts were equilibrated at the various sulfate concentrations (indicated in the figure) in standard buffer and loaded with [^{35}S]sulfate prior to exposure to 2.5 mM phenylglyoxal. The incubation with phenylglyoxal (PG) was carried out under the same conditions at pH 7.4. Transport is expressed as a percent of the residual activity relative to a control value in the same media without inhibitor. Inset: calculation of K_d value according to the fitted data (see text) for the complex between the transporter and SO_4^{2-} ions. The observed pseudo-first-order rate constants in the absence (K_{app}^{o}) and in the presence of SO_4^{2-} (k_{app}^{s}) were calculated from the data of the main Fig. 5 and plotted according to Scrutton and Utter [23].

by phenylglyoxal in the presence of the substrate anions sulfate and chloride (Figs. 5 and 6) at pH 7.4 and pH 8.0. To ascertain whether sulfate ions and chloride ions completely protected the transport system from inactivation, the effect of different concentrations of these anions on the inactivation rate was determined.

The simplest mechanism for the chemical modification of the transport system is as follows:

Unloaded transporter T, free substrate S and the complex TS are at equilibrium with a dissociation constant K_d. T and TS are inactivated to T* and T*S by the modifier M with the rate constants k_1 and k_2, respectively.



Scheme II.

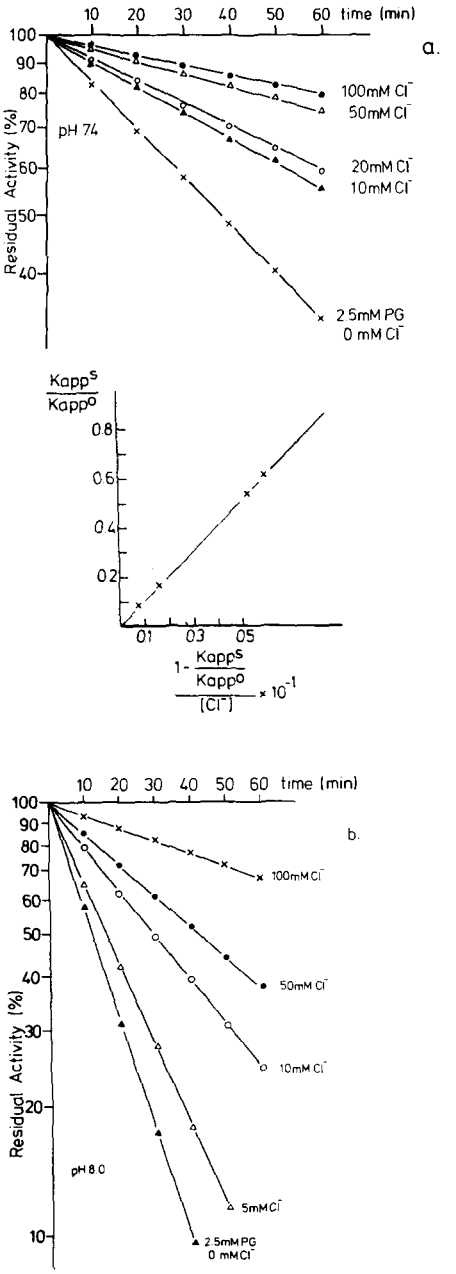


Fig. 6. Protection of sulfate equilibrium exchange against phenylglyoxal inactivation by Cl ions at pH 7.4 (a) and 8.0 (b). Resealed ghosts were incubated with 2.5 mM phenylglyoxal in the presence of different concentrations of chloride ions in standard medium at either pH 7.4 or 8.0, at 37°C. Inset: calculation of K_d values according to the fitted data (see text) for the complex between the transporter and chloride ions. The observed pseudo-first-order rate constants in the absence (K_{app}^{o}) and in the presence of chloride ions (K_{app}^{s}) were calculated from the data of (a) and (b) and plotted according to Scrutton and Utter.

The following equation (according to Scrutton and Utter [23]) holds for the above reaction scheme:

$$\frac{K_{app}^{s}}{K_{app}^{o}} = \frac{k_2}{k_1} + \frac{1 - \frac{K_{app}^{s}}{K_{app}^{o}}}{[S]} K_d \qquad (4)$$

K_{app}^{s} and K_{app}^{o} are the pseudo-first-order rate constants in the presence and absence of the substrate, k_1 and k_2 are the fractional order rate constants for inactivation of T* and T*S, respectively, and K_d is the dissociation constant for the transporter-substrate complex, M is the concentration of the inactivator, S is the corresponding concentrations of either Cl^- or SO_4^{2-}.

When the ratio K_{app}^{s}/K_{app}^{o} of the pseudo-first-order rate constants of inactivation in the presence and absence of S is plotted against $[1 - (K_{app}^{s}/K_{app}^{o})]/[S]$ a straight line passing through the origin would indicate that the substrate anion completely protects the transporter system from inactivation by the modifying reagent ($k_2 = 0$) in Scheme II.

A statistical analysis of the data in Fig. 6(a and b) according to Eqn. 4 for chloride protection gave a value of K_2 equal to 0.0011 min^{-1} and 0.0025 min^{-1} at pH 7.4 and pH 8.0, respectively. In contrast, the value of K_1 (for chloride) was 0.0073 min^{-1} at pH 7.4 and 0.0233 min^{-1} at pH 8.0.

A statistical analysis of the data in Fig. 5 for SO_4^{2-} protection according to Eqn. 4 at pH 7.4 shows that the intercept of the straight line on the ordinate axis is close to 0 and has a negative sign which indicates that within the experimental errors the k_2 value in the presence of SO_4^{2-} ions must be near 0. Thus, we find $k_2 \ll k_1$ for both substrate ions, chloride and sulfate. Assuming $k_2 = 0$, a fit of the data to a straight line passing through the origin yielded a K_d value of 63 mM for sulfate at pH 7.4 and K_d values for Cl^- at pH 7.4 and 8.0 of 14 mM and 10 mM, respectively (insets Fig. 5, 6a and 6b).

Since $K_{app}^{o} = k_1$ M Eqn. 4 can be rearranged to

$$K_{app}^{s} = k_2 M + \left[\frac{K_{app}^{o} - K_{app}^{s}}{S}\right] K_d \qquad (5)$$

Analysis of the data according to Eqn. 5 by the method of least squares gave the same result: $k_2 \ll k_1$. The fitted data gave $K_d = 8.8$ mM for chloride at pH 7.4, and 7.8 mM at pH 8.0. K_d for sulfate = 45.5 mM at pH 7.4.

These results indicate that (within experimental errors) the transporter-substrate complexes may be unable to react with phenylglyoxal.

Discussion

Stilbenedisulfonates have been found to be useful tools in studying anion transport in red blood cells. These compounds act as competitive inhibitors of anion exchange when added to the outside surface of intact cells [4,5]. The possibility that these compounds may produce their effect through allosteric interaction rather than direct steric hindrance of the substrate binding site cannot be excluded. This idea has been first discussed by Passow et al. [24]. Jennings and Adams [25] were able to demonstrate that the binding of one of these derivatives require a part of band 3 that is not directly related to the substrate binding site. Results from other laboratories have shown that the binding of the bulky stilbenedisulfonate compounds to the cell surface cause a large measureable conformational change of band 3 and its environments [26,27]. These compounds also introduce two negative charges to the protein. Therefore, these compounds may give us some information about the extracellular environment of the transporter protein.

In order to obtain direct information about the functional amino acid residue(s) which may participate in binding and translocation of anions across the red cell membrane arginine specific reagents have been used [6–10]. These compounds have the advantage that they cause no change in the protein charge. It has also been reported that phenylglyoxal causes no gross alteration to the protein structure after modification of their functional arginine residues [28].

In the present work the inhibitory effect of one of these reagents on sulfate exchange and its interaction with the substrate anions Cl^- and SO_4^{2-} has been studied in more detail. The results show that phenylglyoxal causes rapid and complete inactivation of sulfate exchange at both pH 7.4 and 8.0.

The reaction rate increased when the pH was

increased. This fact seems to reflect the ionization of arginine residues of the active proteins, because the pH dependence of the inactivation resembles that of arginine-dependent enzymes [29] and arginine itself [30] when modified by phenylglyoxal, although the pK_a of arginine is usually above 12. The reaction order with respect to phenylglyoxal as obtained from kinetics of inactivation Figs. 1 and 2, inset (b) was found to be about 1.0. This may be interpreted as incorporation of a phenylglyoxal in the rate limiting step in the inactivation process [31]. However, modification of 2or 3 orginine residues (or incorporation of a total of 5 to 6 phenylglyoxal residues) per band 3 is required for complete inactivation [10]. From the results in this paper it is still difficult to decide the number of the essential residues precisely because the mechanisms of inactivation of the transport system in our experiment seems to follow Scheme I and at high concentration of phenylglyoxal K_{app} is not equal to $k[PG]^n$ (Figs. 1 and 2, panels (a)).

The results of the protection experiments suggest that the modified amino acids are important for the binding of the substrate anion SO_4^{2-} and Cl^-.

The dissociation constant for the transporter substrate complex at pH 7.4 for SO_4^{2-} and pH 7.4 and 8.0 for chloride has been estimated from the inactivation reaction at different concentrations of the substrate anion and found to be in the range of that previously reported for the transfer site [11,32].

From the data we can conclude that the modified residue(s) may be part of the substrate binding site. This conclusion is in line with the finding of Falke and Chan [33] who found an interaction between chloride binding site(s) in band 3 protein and the binding site of phenylglyoxal.

Thus the essential arginine residue reported in this work is probably at the anion binding site on band 3 protein.

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