J. Membrane Biol. 102, 217–224 (1988)

The Journal of
Membrane Biology

Studies on Inactivation of Anion Transport in Human Red Blood Cell Membrane by Reversibly and Irreversibly Acting Arginine-Specific Reagents

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Summary. A chromophoric derivative of phenylglyoxal, 4-hydroxy-3-nitrophenylglyoxal (HNPG), known to be highly selective for modification of arginine residues in aqueous solution is found to be a potent inhibitor of anion transport across the red cell membrane. In contrast to the action of all other arginine-specific reagents used under the experimental conditions in this laboratory, the action of HNPG on sulfate transport is completely reversible. Hence, a kinetic analysis of its inhibitory effect on SO_4^{2-} self-exchange could be performed. The effect of increasing chloride concentration on the inhibitory potency of HNPG is consistent with the concept that Cl^- and HNPG compete for the same site on the anion transporter. The IC_{50} value for the inhibition of SO_4^{2-} exchange with HNPG is about 0.13 mM at pH 8.0 and 0.36 mM at pH 7.4, and the Hill coefficient for the interaction between the transporter and the inhibitor is near one at both pH's. HNPG is able to protect the transport system against inhibition with the (under our experimental conditions) irreversibly acting arginine specific reagent, phenylglyoxal. Partial inactivation of the transport system with phenylglyoxal lowers the maximal rates of SO_4^{2-} and chloride exchange but does not modify the apparent K_s for the substrate anions. Reversibly acting anion transport inhibitors known to interact with the DIDS binding site like salicylate, tetrathionate, APMB, DNDS, and flufenamate are able to protect the transport system against phenylglyoxalation. Other inhibitors like phloretin and phlorizin have no effect.

Key Words erythrocyte anion transporter · arginine · substrate binding site

Introduction

Band 3 is the major transmembrane protein of the human red blood cell membrane. This protein is multifunctional; besides its main function in facilitating anion exchange across the plasma membrane (Cabantchik & Rothstein, 1974; Zaki et al., 1975), it plays a role in anchoring the cytoskeleton to the lipid bilayer (Bennet & Steinbuck, 1980). This protein also contains binding sites for many cytoplasmic proteins (Strapazon & Steck, 1976; Shaklai, Yguerabide & Ranney, 1977). The gene sequence of mouse band 3 suggests as many as 13 transmembrane segments (Kopito & Lodish, 1985). In spite of intensive studies on this protein, the molecular mechanisms by which this protein transports substrates across the cellular membrane is still largely unknown. Stilbene disulfonate derivatives such as SITS, DIDS and H_2DIDS are potent inhibitors of band 3-mediated anion transport. These agents bind to a site which is only accessible from the extracellular side of the membrane. The covalent binding sites for these compounds are either one or two lysine residues located on the 17k and 35k fragments comprising the transmembrane domain (Jennings & Passow, 1979). Further studies have shown that neither of these lysine residues appears to participate directly in anion translocation (Passow et al., 1980). Studies on reversibly acting stilbendisulfonate derivatives have shown that these compounds compete with the substrate anions for the transport site (Shami, Rothstein & Knauf, 1978).

The site of action of these reversibly acting inhibitors is still not yet clearly defined. It is also not yet clear if their effect is a steric one. On the other hand, Zaki (1981–1984), Zaki and Julien (1983, 1985*a*,*b*) and Julien and Zaki (1987) have found that arginine-specific reagents are potent inhibitors of sulfate exchange across the red blood cell membrane. Wieth, Bjerrum and Borders (1982) also found that chloride exchange in red cells can be inactivated by phenylglyoxal. The site of action of these compounds has been found not to be identical to the site of action of either the covalently or non-covalently binding site of the H_2DIDS (Julien & Zaki, 1987). We have also found that the loaded transporter is unable to react with the covalently binding, arginine-specific reagent, phenylglyoxal (Zaki & Julien, 1985*a*,*b*). The action of this compound under the experimental conditions used in our laboratory is irreversible and the mechanism of inhibition could not be studied with the classic kinetic analysis. In this paper, a chromophoric derivative of phenylglyoxal, 4-hydroxy-3-nitrophenylgly-

oxal, is found to be a completely reversible anion transport inhibitor. This compound enabled us to study the mechanism of inhibition by classical kinetic analysis and to determine the functional role of the inhibitory site. Because of the structural similarities between HNPG and PG, we believe that resolution of the mechanism of inhibition and identification of the site of action of this reversibly acting agent will be a great help for defining the functional role and the site of action of the irreversibly acting arginine-specific reagents. The mechanism of action of both the reversibly and the irreversibly acting arginine-specific reagents and their interaction with other noncovalently acting anion transport inhibitors has been studied. A preliminary report of this work has been previously published (Zaki & Julien, 1985*b*, 1986).

Abbreviations

HNPG, 4-hydroxy-3-nitrophenylglyoxal; PG, phenylglyoxal; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; APMB, 2-(4′-aminophenyl)-6-memylbenzenethiazol-3′, 7-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate.

Materials and Methods

All experiments were performed with human erythrocytes from healthy donors. Blood was obtained from the Red Cross in Frankfurt/Main and stored at 4°C in acid/citrate/dextrose buffer. The cells were used after no more than 4 days of storage. Resealed ghosts were prepared essentially as described by Zaki et al. (1975).

Cells were hemolyzed at 0°C at a cell/medium ratio of 1 : 20 in medium containing 4 mM $MgSO_4$ and 1.45 mM acetic acid. Five minutes after hemolysis, sucrose, gluconate, citrate and HEPES were added from a concentrated stock solution to obtain a final concentration of 200 mM sucrose, 27 mM gluconate, 25 mM citrate, and 5 mM HEPES in the hemolysate.

After centrifugation the ghosts were resuspended and resealed in standard medium containing (mM): 200 sucrose, 27 gluconate, 25 citrate, 5 HEPES and 1 Na_2SO_4. The pH was either 7.4 or 8.0 as indicated in the Figure legends. Modification of resealed ghosts was done with 4-hydroxy-3-nitrophenylglyoxal. The reaction of the resealed ghosts with HNPG was carried out at a hematocrit of 10% in standard medium at 37°C. The concentrations of HNPG are indicated in the Figures.

Flux measurements and calculation of the rate constants were done as described previously (Zaki et al., 1975). Calculation of SO_4^{2-} flux was done according to Schnell (1972). Transport is expressed as percent of the residual activity relative to a control value in the same media used for the reaction but without the inhibitor.

The kinetic data were fitted with a least-squares method by a nonlinear regression.

Materials

4-hydroxy-3-nitrophenylglyoxal (HNPG) was synthesized by the procedure of Fodor and Kovács (1949) with some modifications according to Borders et al. (1979). The reagent recrystallizes from hot water as the monohydrate m. p. 101 to 102°C. Analysis: found Department of Chemistry (Wolfgang Goethe University, Frankfurt/Main). C, 45.14%; H, 3.31%; N, 6.64%. Calculated for $C_8H_5NO_6$. C, 45.08%; H, 3.31%; N, 6.57%.

Results

Inhibition of Sulfate Exchange Across the Red Blood Cell Membrane with 4-Hydroxy-3-Nitrophenylglyoxal (HNPG)

As illustrated in Fig. 1, HNPG is a potent inhibitor of anion transport in resealed ghosts at both pH 7.4 and 8.0. The inhibitory effect increases with increasing pH. This is in agreement with previous studies by Yamasaki, Vega and Feeney (1980) who have found that the reaction between HNPG and arginine residues increases with pH. It is also shown that the inhibitory effect of HNPG is completely reversible. Removal of the agent by washing after an incubation time of more than 90 min at both the pH 7.4 and 8.0 at the concentrations indicated in Fig. 1 causes the transport system to regain its complete activity. The presence of 50 mM borate does not affect the rate of the reaction or its reversibility. From the data in Fig. 1, the Hill coefficient for the interaction between the transporter and the inhibitor and the apparent IC_{50} at both pH's were calculated. The value for Hill coefficient was found to be 1.03 at pH 7.4, and 0.9 at pH 8.0. IC_{50} was found to be 0.36 mM at pH 7.4 and 0.13 at pH 8.0.

Interaction Between the Binding Site of HNPG and the Binding Site of Phenylglyoxal

In order to find out whether the binding site of this compound is identical with the binding site of the (under our experimental conditions) irreversibly acting arginine-specific reagent phenylglyoxal (Zaki, 1982, 1983), the following experiments have been performed.

Resealed ghosts were modified with the phenylglyoxal at either 2.5 or 5 mM in absence or presence of 5 mM of HNPG. After an incubation time of 60 min at either pH 7.4 or 8.0 at 37°C, the unreacted modifiers were removed by washing and $^{35}SO_4^{2-}$ efflux was measured. Figure 2 represents the results of this type of experiment.

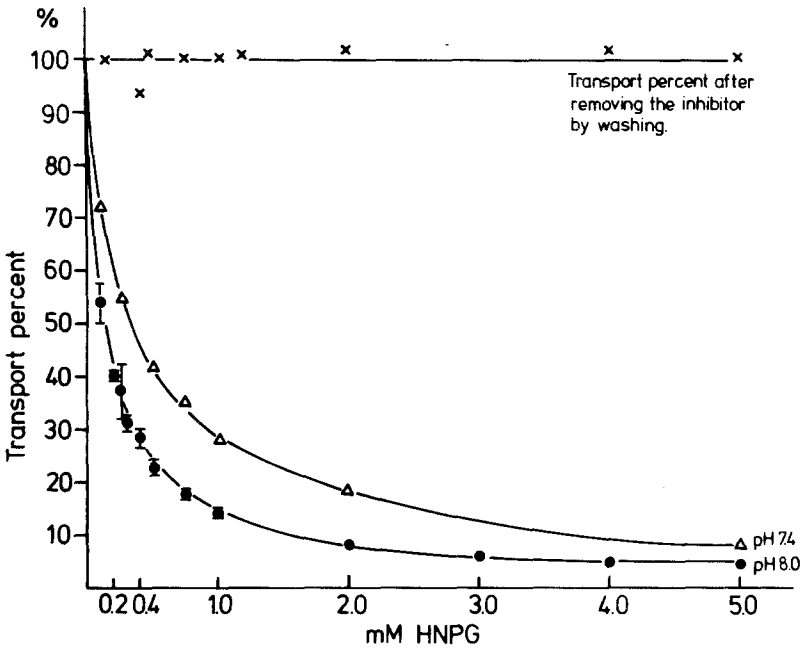

Fig. 1. Inactivation of sulfate equilibrium exchange in resealed ghosts with HNPG at pH 7.4 and 8.0. *Ordinate:* The rate of $^{35}SO_4$ efflux in percent of control value without HNPG. *Abscissa:* HNPG concentration (mM), temp. 37°C, pH 7.4 (△) and pH 8 (●). (×) represents the transport activity after removing the inhibitor by washing and the fluxes were measured in standard medium containing 1 mM SO_4 (*see* Materials and Methods)

Table. Kinetic parameter of the partially modified anion transport system

Conditions	Kinetic parameters for:			
	Sulfate medium, pH 7.4		Chloride medium, pH 8.0	
	V_{max} (min^{-1})	K_s (mM)	V_{max} (min^{-1})	K_s (mM)
control	3.73	28.7	0.97	13.7
partially modified ghosts	2.4	28.2	0.398	13.4

One notices that 5 mM HNPG protects the transport system almost completely against inhibition with either 2.5 or 5.0 mM of PG at pH 7.4. The same result is also found at pH 8.0 (last column). This suggests that both PG and HNPG act on the same site.

Effect of Modification by Phenylglyoxal on the Kinetic Parameter for Transport Activity

Resealed ghosts were first modified with 1 mM phenylglyoxal at pH 7.4 for 30 min at 37°C (under these conditions, the transport is inhibited to about 50%). The excess of the inhibitor was removed by washing. The modified ghosts were then resuspended at various concentrations of either chloride (pH 8.0) or SO_4^{2-} (pH 7.4) ions and sulfate efflux was measured. A control was run with unmodified ghosts.

The apparent K_m value for the modified and unmodified ghosts were not significantly altered while the value for V_{max} decreased by about one-half as indicated in the Table. This is also shown in Figs. 3 and 4.

Similar results have been found with chloride ions when the efflux was measured at pH 7.4. [The effect of sulfate ions at pH 8.0 has not been measured since it was previously shown that their protective effect is very low at higher pH's (Zaki, 1983)].

This suggests that modification with phenylglyoxal completely abolishes the transport activity and that the residual activity in the treated sample is the activity derived from unmodified molecules with normal activity. This means that the transporter is either completely inhibited by PG or not at all ("all-or-none" behavior). These results are in close agreement with those of previous investigators (Wieth et al., 1982).

Effect of Reversible Acting Anion Transport Inhibitors on Phenylglyoxalation of Resealed Ghosts

In order to get some information about the molecular structure of the site of activation of the arginine-specific reagents, the interaction of some reversibly acting anion transport inhibitors on the rate of inac-

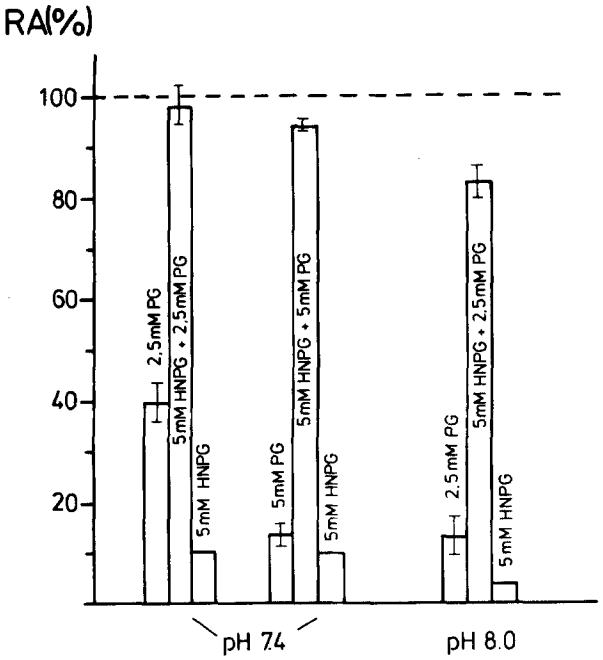

Fig. 2. Interaction between HNPG binding site and the binding site of phenylglyoxal. The results in Fig. 2 summarize the effect of HNPG on phenylglyoxalation of the resealed ghosts. The *Ordinate* = residual activity in percent of control value without the inhibitors. In each category, the first column represents the effect of either 2.5 or 5 mM PG. The second column shows the protection caused by HNPG when present during the incubation of the resealed ghosts with PG (for more details *see* text). The third column represents the effect of HNPG when present in the flux medium.

$$\text{Residual activity (Ra)} \frac{\text{rate constant of } {}^{35}SO_4 \text{ efflux in presence of HNPG}}{\text{rate constant of } {}^{35}SO_4 \text{ efflux in absence of HNPG}}$$

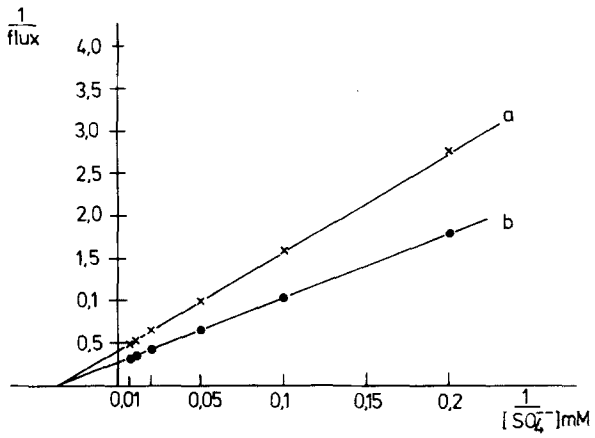

Fig. 3. Effects of modification by phenylglyoxal on the kinetic parameter for anion transport activity. The resealed ghosts were first modified (*a*) or not (*b*) by incubation with 1 mM phenylglyoxal for 30 min under standard conditions at pH 7.4. The rate constants of $^{35}SO_4$ exchange in the modified and unmodified transport systems were measured in a flux medium containing increasing concentration of sulfate ions at pH 7.4

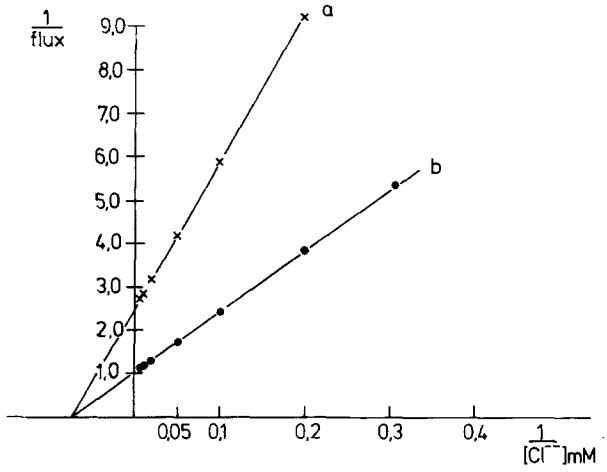

Fig. 4. Same as Fig. 3 except that flux medium contained increasing concentration of chloride ions at pH 8.0

tivation of resealed ghosts with phenylglyoxal has been studied. In this type of experiment, the resealed ghosts were first incubated with the reversibly acting inhibitors as indicated in Figs. 5 and 6. After 5 min of incubation, phenylglyoxal was added and the ghosts were further incubated for 45 min at 37°C, either at pH 7.4 or 8.0. After removal of the reversibly acting inhibitors and the excess of phenylglyoxal by washing, flux measurements were performed. Figures 5 and 6 show that neither phloretin nor phlorizin protect the transport system against inactivation by phenylglyoxal while salicylate, tetrathionate, APMB and flufenamate are able to do so. Similar results were found for these inhibitors at both pH 7.4 and 8.0 (all data are shown in the Figs.). Figure 7 indicates that DNDS is able to protect the transport system against inhibition with phenylglyoxal at pH 7.4. This also applies pH 8.0.

Competition Between Chloride and HNPG for the Sulfate Binding Site

Since the interaction between HNPG and the transport system are completely reversible, competition studies could be performed. For this purpose, ghosts with various chloride concentration have been prepared. In each group, SO_4^{2-} self-exchange has been measured in the absence and presence of the various concentrations of HNPG and chloride. The data for the effect of HNPG on SO_4^{2-} exchange are shown by a Hunter-Downs plot in Fig. 8. This plot has the advantage that a straight line is obtained over the entire Cl^- concentration range de-

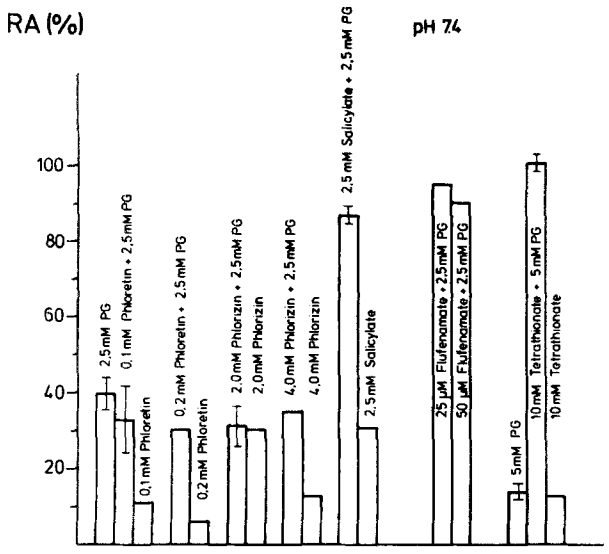

Fig. 5. Effect of reversible acting inhibitors on phenylglyoxal binding site. Resealed ghosts were exposed to 2.5 mM phenylglyoxal in the presence of the inhibitors shown in the Fig. at pH 7.4. *Ordinate:* The rate constant of $^{35}SO_4^{2-}$ exchange in percent of control value in the same medium as in the columns without inhibitors. The first two inhibitors, phloretin and phlorizin, offered no protection against inactivation with phenylglyoxal. The other three inhibitors, salicylate, flufenamate and tetrathionate are able to protect the transport system against inhibition with phenylglyoxal

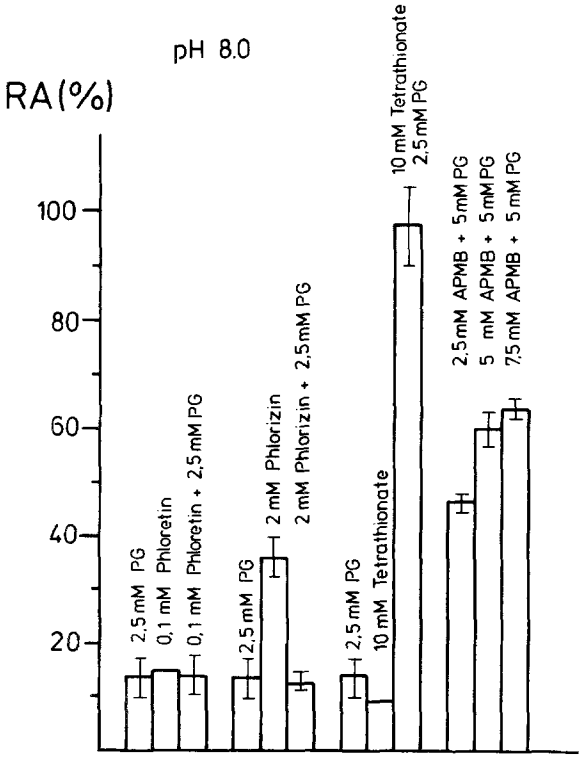

Fig. 6. Represents the effect of the reversible acting inhibitor when present during phenylglyoxalation of resealed ghosts at pH 8.0

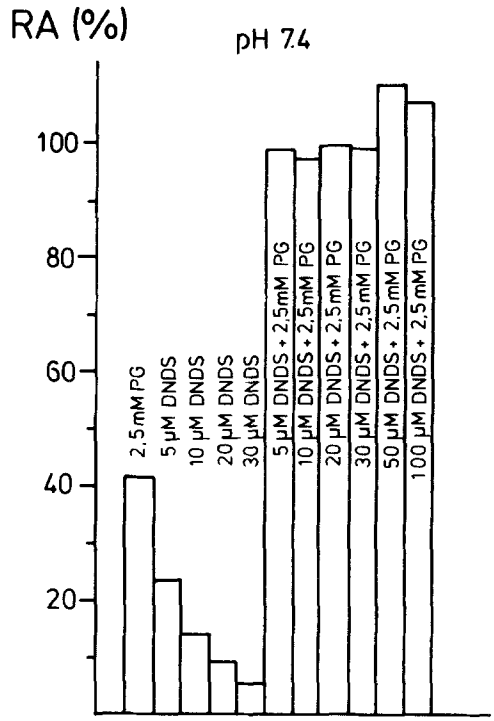

Fig. 7. Interaction between DNDS and phenylglyoxal binding site. Resealed ghosts were exposed to 2.5 mM phenylglyoxal in presence of the various concentrations of the reversible acting anion transport inhibitor DNDS indicated in the Figure. The first five columns indicate the effect of DNDS when added to the flux medium. The last columns represent the protective effect of DNDS against phenylglyoxalation of the resealed ghosts at pH 7.4. *Ordinate:* The rate constant of $^{35}SO_4^{2-}$ exchange in percent of control value without inhibitors

spite the presence of two anion binding sites (Knauf et al., 1978). The apparent K_i at zero chloride concentration is about 100 μM at pH 8.0. This value is similar to the value calculated from the Hill equation. By increasing the Cl^- concentration in the medium, more HNPG is required to inhibit SO_4^{2-} exchange by 50%. This shows that the substrate anion and HNPG compete with each other.

The x-intercept of the plot, which corresponds to the chloride concentration required to half saturate the HNPG binding site, is only 19 mM; this is not significantly different from the value reported for half saturation of the substrate site (Passow, 1986). This behavior is indicative of competitive inhibition.

Figure 8 shows also that the apparent K_i depends linearly on the free HNPG concentration as expected from a competitive relationship between HNPG and the substrate anion. This indicates that HNPG inhibits SO_4^{2-} self-exchange by acting at the substrate site.

When the data of the above-mentioned experiments are plotted on a Hanes plot (Fig. 9), the data

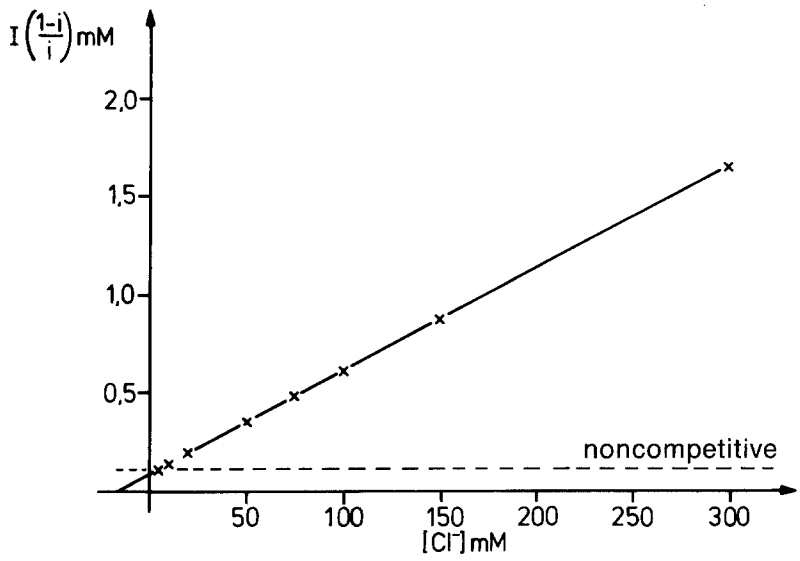

Fig. 8. Hunter-Downs plot of apparent K_i for inhibition by HNPG calculated as $I\ (1 - i)/i$ *vs.* chloride concentration. The data were obtained form experiments in which either chloride or HNPG concentration was varied. (×) Fitted points achieved by use of nonlinear regressions analysis of at least six experimental data. The broken line represents the expected result for noncompetitive inhibition

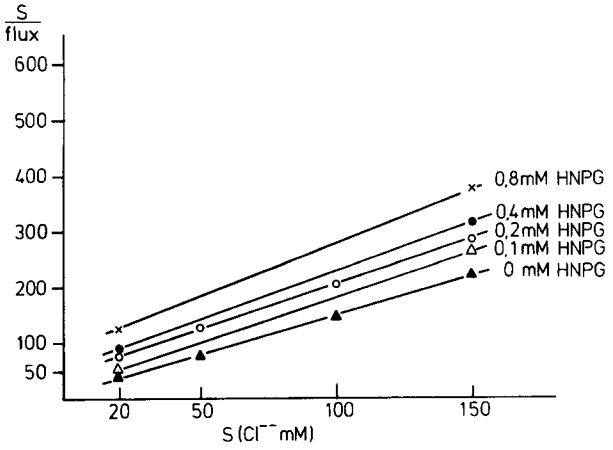

Fig. 9. Hanes plot of chloride concentration in millimoles divided by sulfate equilibrium exchange flux is calculated according to Schnell (1972). The data are taken from the same experimental data used for Fig. 8. The straight lines with the same slopes represent the data points either under the HNPG concentration indicated in the Figure or the data points of a control value without inhibitor.

points under the HNPG concentration of 0.1, 0.2, 0.4 and 0.8 mM lie on straight lines parallel to the data points of the control without the inhibitor. The behavior that all the lines of the various concentration of the inhibitor have the same slope as the control is also indicative of a competitive inhibition.

A Dixon plot (Fig. 10) (1/flux versus inhibitor concentration) yields straight lines, providing that only one inhibitor molecule combines with each functional site over the concentration range examined. The slope and x-intercept vary with a substrate concentration. This behavior is also indicative of linear competitive inhibition. The lines intersect at a point and its x-intercept gives the apparent K_i at pH 8.0 which is found to be 125 μM. This value is not significantly different from the value calculated from Hunter-Downs and Hill equations.

Discussion

With the present data we have been able to show that the arginine-specific reagent HNPG is a competitive inhibitor of anion transport in the red cell membrane. This may be interpreted that this inhibitor is acting on the anion binding site. On the other hand, it is still unknown if this inhibitor is able to recruit the transport site from one side of the membrane to the other. Recruitment experiments are not feasible since the inhibitor is able to penetrate the membrane.

Although the product of the reaction between HNPG and soluble protein molecules has been found to be quite stable (Borders et al., 1979), we see only a reversible interaction between HNPG and the anion transporter. Also under the same experimental conditions, the binding of two other phenylglyoxal derivatives, the para-nitrophenylglyoxal and the para-hydroxyphenylglyoxal is found to be quite stable throughout the experimental time period (Zaki & Julien, 1986). The stoichiometry of the reaction between phenylglyoxal (Takahashi, 1968), HNPG (Borders et al., 1979) and the guanido groups have been found to be 2 : 1. The first molecule of the reagent condenses reversibly with the guanido group to form a glyoxaline ring which then reacts rapidly with a second molecule of phenylglyoxal to form the final product. So the reversibility of

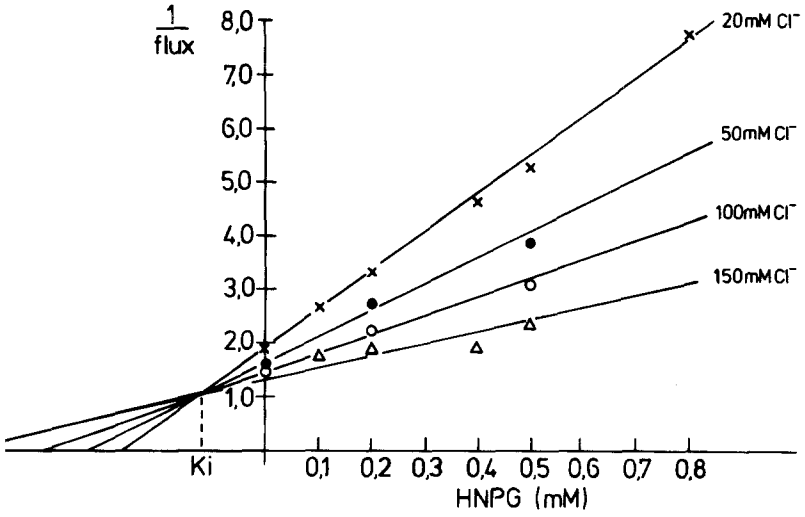

Fig. 10. Dixon plot (1/flux versus inhibitor concentration). When the data used for Fig. 9 are plotted according to a Dixon plot, the data points for each chloride concentration yields a straight line with a slope and x-intercept vary with the substrate concentration. The lines intersect at a point and its x-intercept gives the apparent K_i for HNPG at pH 8.0

the reaction of HNPG with the anion binding site may be explained as follows: HNPG contains an -OH group in the para position and a NO_2 group in the meta position of the phenylglyoxal molecule. This structure may produce a steric hindrance for another HNPG molecule to react and stabilize the complex between the first HNPG molecule and the guanidyl residue in the transport site. A second explanation may be that the existence of a hydrogen bridge between the OH and the NO_2 groups in the HNPG molecule will reduce the electronic character of the molecule and hence reduce its interacting with a certain group (possibly a negatively charged group) in the vicinity of the HNPG binding site, or because of the relatively hydrophilic character of the molecule it is unable to interact with a hydrophobic environment of the protein which is found to increase the potency of a series of reversibly acting inhibitors (Barzilay, Ship & Cabantchik, 1979).

Kinetic analysis of the partially phenylglyoxal modified transporter showed that apparent K_s value for the substrate anion chloride and sulfate was not altered. However, the V_{max} of transporter decreased in proportion to the inactivation of the transport system. These results suggest that modification of the transporter completely abolished its activity and that the residual activity derived from unmodified transporters with normal active sites.

Although previous results have shown that the binding site of the arginine-specific reagents is not identical to the binding site of H_2DIDS (Zaki, 1981; Julien & Zaki, 1987) and DIDS (Falke & Chan, 1986), the results on the effect of DNDS, APMB, salicylate, flufenemate and tetrathionate on the PG binding site have shown that a certain interaction must exist. All these compounds have been able to protect the transport system against inactivation with phenylglyoxal. Our results concerning the protective effect of flufenamate contrast the finding of Wieth et al. (1982) who found that flufenamate under their experimental condition (where phenylglyoxal was only allowed to react with the extracellular side of the membrane) is unable to protect the transport system against inactivation. Protection of the transport system with these bulky reagents could be due to modification of a nonessential residue near the transport binding site and cause a steric blocking of the latter and hence lead to protection of the transport site against phenylglyoxylation. The inactivation of the transport system with these compounds may be also due to charge modification (since all these compounds are negatively charged) or conformational changes leading to the burial of the transport site.

Falke and Chan (1986) have classified anion transport inhibitors in three classes: the transport site inhibitors, channel blockers and translocation inhibitors. Our kinetic studies (Julien & Zaki, 1986) and the studies in this work are in close agreement with their results that phenylglyoxal directly interacts with the substrate binding site. On the other hand, we have found that both the competitive inhibitor DNDS and the noncompetitive inhibitor flufenamate are very effective in protecting the transport system against inhibition with phenylglyoxal. The results do not allow us to determine the basis of the protection, so we are not able to exclude the idea that the effect of DNDS may have allosteric character, since the nature of the interaction between the transport site and phenylglyoxal differ from that with DNDS. Alternatively, one must assume that the deprotonated guanidine residue known to react with PG (Takahashi, 1968) is adjacent to positively charged groups which interact

with DNDS. The further characterization of the HNPG binding site is currently under investigation.

We thank Professor H. Passow, Dr. Phil Wood for comments on the manuscript, Mr. E. Betakis for preparing a part of the HNPG and his cooperation during some of the experimental work and Ms. Marion Dirscherl for typing the manuscript. This study is part of the dissertation of T. Julien, Max-Planck-Institute für Biophysik, 6000 Frankfurt, West Germany.

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Received 25 September 1987; revised 10 December 1987