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Chemical properties of the anion transport inhibitory binding site of arginine-specific reagents in human red blood cell membranes

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A series of arginine-specific reagents with different size and polarity have been synthesized and their inhibitory potency on sulfate exchange in resealed ghosts has been investigated. The synthesized phenylglyoxal derivatives *p*-nitro-, *p*-methyl-, *p*-hydroxy-, *p*-carboxy-, *p*-sulfo-, and *p*-azido-phenylglyoxal are found to be potent inhibitors of anion transport. The reaction between the cells and azidophenylglyoxal was performed in the dark. Exposure of the modified cells to the light was not followed by an increase in the inhibition. No cross-linking products were visible after gel electrophoresis. The rate of inactivation of sulfate flux with these reagents obeyed pseudo-first-order kinetics and increases with increasing reagents concentration and pH. Prolonged incubation of the cells with these reagents results in almost complete inhibition of the transport system. The positively charged phenylglyoxal derivative 4-(trimethylammonioacetylamido)phenylglyoxal was not able to inhibit the transport system. The hydrophobic character and the electronic properties of these reagents do not correlate with their inhibitory potency. Their electrostatic and steric effects seem to play the major role in their action.

Introduction

A great deal of structure and functional information about anion transport in the red blood cell membrane has been obtained through chemical modification methods

A class of anion transport inhibitors which has been extensively used is the stilbene disulfonates Studies with these compounds have led to the implication of the 96 kDa polypeptide (band 3, [1]) in the mechanism of anion exchange through the red blood cell membrane [2,3] Further studies suggest that the site of action of these compounds is located in a hydrophobic cleft of this protein near the outer surface of the membrane

Another class of anion transport inhibitors first used in this laboratory are the arginine-specific reagents [4–11] The site of action of these compounds does not seem to be identical to the binding site of the stilbene disulfonate derivative, H_2DIDS [10,15]

We also found that the chloride- or sulfate-loaded transporter is unable to react with the covalently binding arginine-specific reagent, phenylglyoxal [9] Our results with [^{14}C]phenylglyoxal (PG) have shown that complete inhibition of the transport system is accompanied by modification of two to three arginine residues [8] It has also been shown that two-thirds of the [^{14}C]phenylglyoxal binding is located on the chymotryptic 60 kDa fragment of band 3 These results are inconsistent with other findings, in which phenylglyoxylation of the red cells was done under extremely unphysiological conditions In these experiments PG was found to bind exclusively to the extracellular site of the 35 kDa fragment [12]

Our recent results with the reversible binding arginine-specific reagent 4-hydroxy-3-nitrophenylglyoxal (HNPG) have shown that it is a competitive inhibitor of anion transport in the red cell membrane [11] This finding suggests that these reagents are interacting with the binding site of the substrate anions Other anion exchange systems like tricarboxylate carrier of the inner mitochondrial membrane [13] and anion transport system at the contraluminal cell side of the renal proximal tubule have also been found to be inhibited by such reagents [14]

Abbreviations PG, phenylglyoxal, NO_2-PG, *p*-nitrophenylglyoxal, CH_3-PG, *p*-methylphenylglyoxal, OH-PG, *p*-hydroxyphenylglyoxal, COOH-PG, *p*-carboxyphenylglyoxal, SO_3-PG, *p*-sulfophenylglyoxal, N_3-PG, *p*-azidophenylglyoxal, TAAA-PG, 4-(trimethylammonioacetylamido)phenylglyoxal, H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate, DNDS, 4,4′-dinitrostilbene-2,2′disulfonate

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In order to obtain information about the local environment and the chemical properties of these essential arginines, we have synthesized a series of phenylglyoxal derivatives of different size and polarity, and their reactivity with these arginines and their inhibitory effect on sulfate exchange in red blood cell membranes have been investigated Preliminary reports of this work have been published previously [15,16]

Materials and Methods

Human Rh^+ blood was obtained from blood bank in Frankfurt and stored at 4° C in acid/citrate/dextrose buffer Cells are used within 3–5 days after withdrawal The experiments were performed with resealed red cell ghosts Resealed ghosts were prepared essentially as in Ref 3

Cells were hemolyzed at 3° C at a cell/medium ratio of 1 20 in medium containing 4 mM $MgSO_4$ and 1 45 mM acetic acid 5 min 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 the resealed ghosts was conducted with the various phenylglyoxal derivatives The modification reactions were carried out at a hematocrit of 10% in standard medium at 37° C The concentration of the reagents and the incubation time are indicated in the figures Flux measurements and calculation of the rate constants were done as described previously [3] Transport is expressed as percent residual activity relative to a control value measured in the same medium as used for the reaction but without the inhibitors

The kinetic data were fitted with a least-squares method by a non-linear regression program

Determination of the lipophilic character of the PG derivatives The lipophilic properties of the different PG derivatives were determined by thin-layer partition chromatography [17] Silica gel (silanized (60) HF 254, Merck) was the stationary phase, a mixture of water and acetic acid was the mobile phase R_M values were calculated according to the following equation [18]

$$R_M = \log\left(\frac{1}{R_F} - 1\right)$$

The R_M values were plotted vs the proportion of water in the mobile phase The intercept of the straight line with the ordinate yielded the R_M value for pure H_2O (R_{M,h_2O}) R_{M,H_2O} is equivalent to the logarithm of a partition coefficient

Chymotrypsin treatment of the cells Treatment of the resealed ghost with extracellular chymotrypsin was performed as described by Zaki [8]

SDS-polyacrylamide gel electrophoresis The procedure was performed as described by Zaki [8]

Phenylglyoxal derivatives Phenylglyoxal derivatives were synthesized from the corresponding acetophenone derivative by the method of Fodor and Kovács [19] The reagents recrystallized from hot water as the monohydrates Analyses were done at the department of chemistry (J W Goethe-University, Frankfurt/Main)

p-Methylphenylglyoxal m p 107–109° C
Analysis found C, 64 9%, H, 5 85%
$C_9H_{10}O_3$ calcd C, 65 05%, H, 6 07%

p-Nitrophenylglyoxal, m p 98–99° C
Analysis found C, 48 06%, H, 3 37%, N, 6 93%
$C_8H_7NO_5$calcd C, 48 72%, H, 3 55%, N, 7 10%

p-Carboxyphenylglyoxal, m p 203–204° C
Analysis found C, 55 05%, H, 3 98%
$C_9H_8O_5$ calcd C, 55 11%, H, 4 11%

p-Sulfophenylglyoxal, m p 260° C
Analysis found C, 37 52%, H, 2 83%
$C_8H_7SO_4Na$ calcd C, 37 8%, H, 2 78%

4-Hydroxyphenylglyoxal, m p 111–112° C
Analysis found C, 57 03%, H, 4 76%
$C_8H_8O_4$ calcd C, 57 14%, H, 4 79%

4-Azidophenylglyoxal, m p 101–103° C
Analysis found C, 51 74%, H, 3 1%, N, 22 02%
$C_8H_5N_3O_2$ calcd C, 52 17%, H, 3 26%,N, 22 82%

4-(Trimethylammonioacetylamido)phenylglyoxal
Analysis found C, 48 36%, H, 6 49% N, 8 38%
$C_{13}H_{21}N_2O_3Cl$ calcd C, 48 67%, H 6 59%, N, 8 73%

Chemicals Phenylglyoxal (pure) was obtained from Serva, Heidelberg, Hepes was obtained from Calbiochem Sulfoacetophenone derivative was obtained from Aldrich, F R G All other substances were obtained from Merck, Darmstadt, F R G

Results

Inactivation of sulfate equilibrium-exchange in resealed ghosts by various phenylglyoxal derivatives

Fig 1 shows the structural formulas of the various derivatives which have been investigated

Kinetics of inactivation of sulfate transport by p-nitrophenylglyoxal and p-methylphenylglyoxal

The sulfate equilibrium exchange was rapidly abolished by treatment of resealed ghosts with *p*-nitrophenylglyoxal

Figs 2 a and b show the irreversible inactivation of sulfate transport with 4-nitrophenylglyoxal (NO_2-PG) The inhibitory effect increases when the pH is increased from 7 4 to 8 0 The inactivation has been found to be both time- and concentration-dependent at both pH

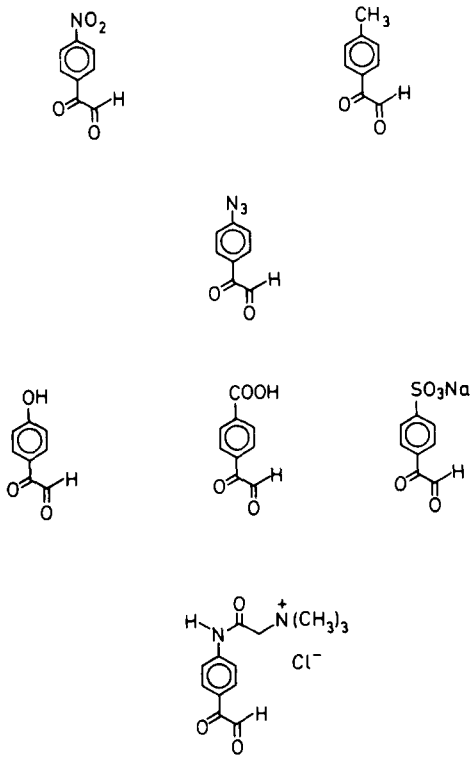

Fig 1 Structure formulas of phenylglyoxal derivatives

values. The time course of the inhibition was found to follow pseudo-first-order kinetics until transport is reduced to less than 10% of the initial value This is indicated by the straight lines obtained in semi-log plots of the transport rate versus time

Essentially similar results have been found with *p*-methylphenylglyoxal (CH_3-PG) (Table I) Table I lists

TABLE I

Rate of inactivation of sulfate efflux in resealed ghosts, by various concentrartions of CH_3-PG at pH 8 0

The apparent rate constants (k_{app}) for the inactivation were calculated from the slopes of the plots of the logarithm of remaining activity vs time

Additions	Apparent rate constant k_{app} (min^{-1})
0 5 mM CH_3-PG	0 018
2 5 mM CH_3-PG	0 036
5 0 mM CH_3-PG	0 048
10 0 mM CH_3-PG	0 068

he effect of different concentrations of CH_3-PG on the rate of inactivation of sulfate transport The apparent rate constant (k_{app}) for the inactivation was calculated from the slopes of the plots of the logarithm of remaining activity vs time The data indicate that the transporter was inactivated by *p*-methylphenylglyoxal with pseudo-first-order kinetics, and that the rate of inactivation depends on the concentration of the reagent

Modification of resealed ghosts with p-hydroxy-, p-carboxy- and p-sulfo-phenylglyoxal

Incubation of the resealed ghosts with the reagents at pH 7 4 and pH 8 0 for 60 min, results in a concentration-dependent loss of sulfate transport

The experiments in Fig 3 show the effect of different concentrations of one of these inhibitors on sulfate exchange In these experiments the cells were exposed to various concentrations of the PG derivatives at pH 8 0 After an incubation time of 60 min, the cells were

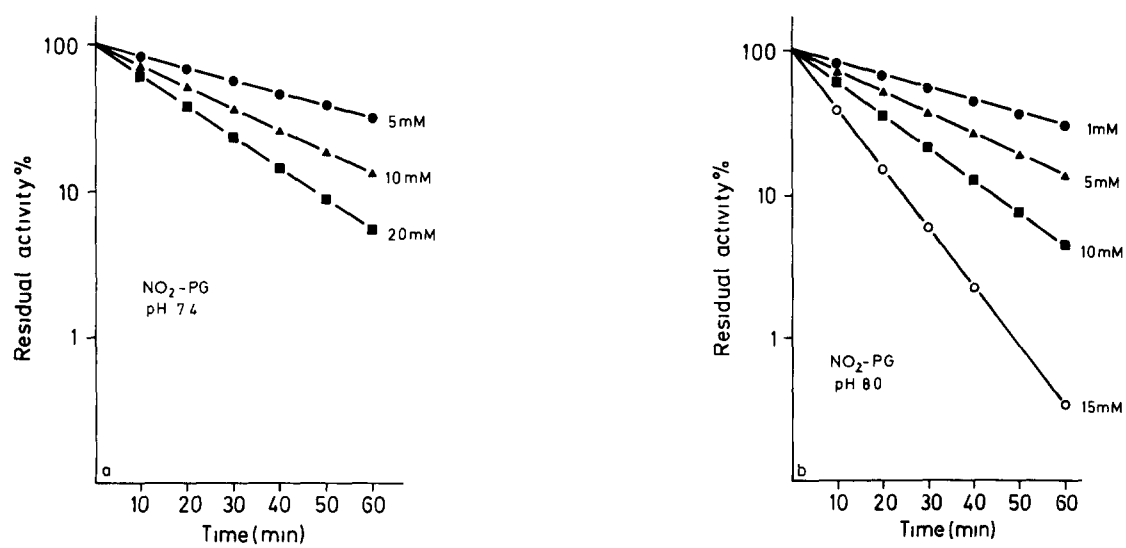

Fig 2 Semilogarithmic plots of the inactivation of sulfate equilibrium exchange by phenylglyoxal derivatives, NO_2-PG Resealed ghosts were incubated in standard medium either at pH 7 4 (a) or pH 8 0 (b) at the concentrations of the reagents indicated in the figures At the time indicated in the abscissa, aliquots were withdrawn, excess of phenylglyoxal derivate was removed by washing and the residual activity of $^{35}SO_3$ equilibrium exchange was measured The ordinate presents the residual flux as percent of a control value without inhibitor Flux measurements were done as described in Ref 3

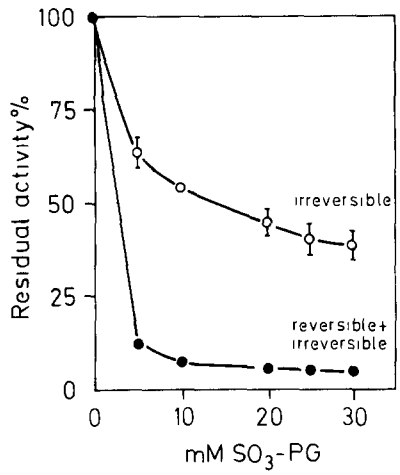

Fig 3 Inactivation of sulfate equilibrium exchange in resealed ghosts with SO_3-PG Ordinate The rate of $^{35}SO_3$ efflux as per cent of control value without inhibitors Abscissa Inhibitor's concentration in mM, temperature 37°C, pH 8 0 The transport activity is presented (•) after removing the excess of the inhibitor by washing and (○) in the presence of the inhibitors

washed by the standard procedure to remove the reversibly bound reagent and sulfate equilibrium exchange was measured (upper curve) In other experiments flux measurements were done in the presence of the inhibitor (lower curve) Obviously, the inhibition observed in presence of PG derivatives in the medium exceeds the inhibition observed after subjecting the cells to the washing procedure

Previous studies by Zaki and Julien [9] suggest that the process of inactivation of sulfate flux by PG involves the association of the reagent with the transporter to form a non-covalent transporter-inhibitor complex prior to irreversible modification by the addition of another PG molecule to the non-covalent transporter-inhibitor complex The occurrence of both forms of binding is directly shown in the experiments presented in Fig 3 In these experiments, the upper curve represents the irreversible inhibition of the transport system after subjecting the cells to the standard washing procedure The lower curves represent the rate of inhibition when the inhibitors are present in the flux medium (reversible and irreversible binding)

In some experiments the resealed ghosts were incubated with the reagents for 60 min After removal of the excess of the reagents by washing, the cells were subdivided into two portions One was used for flux measurements, the other was exposed to an identical PG derivate solution and incubated for another 60, 120 or 180 min, and then subjected to the washing procedure In other experiments the first incubation period was prolonged to 2 or 3 h before washing As shown in Fig 4, the irreversible inactivation by SO_3-PG and OH-PG proceeded until almost complete inactivation was obtained Similar results were obtained with COOH-PG (not shown)

It has been reported that irreversible modification of arginine residues with phenylglyoxal takes place at a stoichiometry of 1 2 The first molecule of the reagent condenses reversibly with the guanidino group to form a glyoxaline ring which then reacts with a second moleculer of phenylglyoxal to form the final product [20] In the case of OH-PG, COOH-PG and SO_3-PG the complex between an arginine residue and the first molecules of these reagents with such bulky head group would sterically hinder the binding of the second molecule and causes the irreversible reaction to proceed more slowly

Time course of inactivation of sulfate equilibrium exchange by p-hydroxy, p-carboxy, and p-sulfophenylglyoxal

Incubation of resealed ghosts with excess of the reagents was done at pH 8 0 Aliquots were withdrawn, at selected intervals, excess of the PG derivative was removed by washing, and the residual activity of sulfate equilibrium exchange was measured The time course of inactivation was found to follow pseudo-first-order kinetics and the rate of inactivation was proportional to reagent concentration (Table II) Upon prolonged incubation complete inactivation was obtained

Modification of resealed ghost with the heterofunctional phenylglyoxal derivative p-azidophenylglyoxal

Bifunctional reagents are good tools for exploring the active center of enzymes These reagents include the hetero-bifunctional cross-linking reagents which have

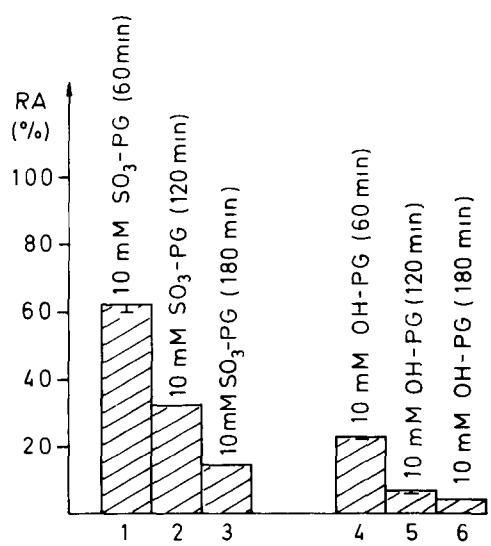

Fig 4 Effect of prolonged incubation of SO_3-PG and OH-PG on sulfate flux Ordinate The rate constant of $^{35}SO_3$ exchange in per cent of control value in the same medium as in the columns without inhibitor The first column represents the effect of SO_3PG after incubation time of 60 min, the second after 120 min, the third column after incubation time of 180 min, columns 4–6 show the same experiments with OH-PG

TABLE II

Rate of inactivation of sulfate efflux by various concentrations of OH-PG, COOH-PG, and SO_3-PG

The values of k_{app} for the inactivation were calculated as in Table I

1 0 mM OH-PG	0 0129
2 5 mM OH-PG	0 0171
5 0 mM OH-PG	0 024
10 0 mM OH-PG	0 0369
20 0 mM OH-PG	0 069
10 0 mM COOH-PG	0 012
20 0 mM COOH-PG	0 018
5 0 mM SO_3-PG	0 0075
30 0 mM SO_3-PG	0 022

two different reactive groups and which can be used for affinity labeling We have synthesized 4-azidophenylglyoxal (N_3-PG) as an arginine-specific affinity label The phenylglyoxal moiety reacts with an arginine residue, whereas when activated with light, the *p*-azidoaryl function generates a nitrene which would react with virtually any group in its vicinity

As shown in Fig 5, the degree of sulfate flux inhibition in the resealed ghosts does not increase after exposure to light (about 20 light flashes) In the dark the PG moiety reacts with the guanidino group responsible for inhibition The results represented in Fig 5 show that in the vicinity of the essential arginine (in a distance of about 9 Å), there is no other essential group

In some experiments the cells were subjected to chymotrypsin digestion either before or after exposure to light The membranes were isolated, dissolved in SDS, and then subjected to SDS-gel electrophoresis Fig 6 shows that the gel pattern does not show any difference between the control and N_3-PG-treated cells either in the dark or after exposure to light

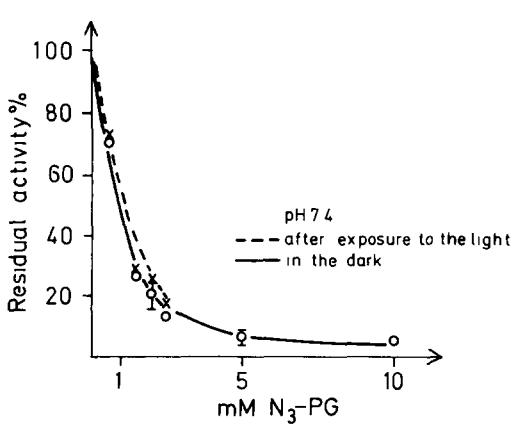

Fig 5 Inactivation of sulfate equilibrium exchange in resealed ghosts with N_3PG The inactivation was done at pH 7 4 either in the dark (○) or after exposure to the flash light (×) Ordinate Penetration rate in percent of control value without N_3-PG Abscissa concentration of N_3-PG in mM, temperature 37 °C, incubation time was 60 min

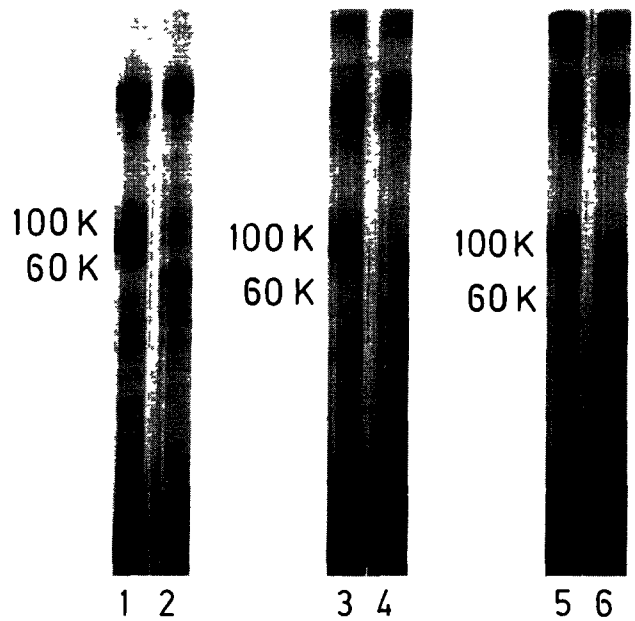

Fig 6 SDS-polyacrylamide gel electrophoretograms of N_3-PG treated and untreated membrane Resealed ghost were treated with 5 mM N_3-PG for 1 h at pH 7 4 in the dark After removal of excess N_3-PG the cells were subdivided into two portions, one was exposed to the light and the other was not For chymotrypsin treatment the ghosts were subjected either before or after exposure to the light Lane 1, control, lane, control + Chymo, lane 3, + 5 mM N3-PG in the dark lane 4, probe No 3 + Chymo, lane 5, + 5 mM N3-PG + light, lane 6, probe No 5 + Chymo

The gel electrophoretograms (Fig 6) also show that adjacent segments of the band 3 polypeptide (the 35 kDa and the 60 kDa fragment obtained after treatment with extracellular chymotrypsin) cannot be cross-linked by N_3-PG

The inactivation process of sulfate transport with N_3-PG was found to be concentration dependent at both pH 7 4 and pH 8 0 and obeyed pseudo-first-order kinetics until more than 97% of the original activity was lost (Table III)

Effect of positively charged 4-(trimethylammonioacetylamido)phenylglyoxal (TAAA-PG) on anion transport

Incubation of resealed ghosts with TAAA-PG up to a concentration of 10 mM causes no inhibition of sulfate flux at both pH 7 4 and pH 8 0 The bulky and

TABLE III

Effect of various concentrations of N_3-PG on sulfate transport in resealed ghosts

The values of k_{app} for the inactivation were calculated as in Table I

Reagent	Apparent rate constant (min^{-1})
1 0 mM N_3-PG	0 029
5 0 mM N_3-PG	0 087
10 0 mM N_3-PG	0 117

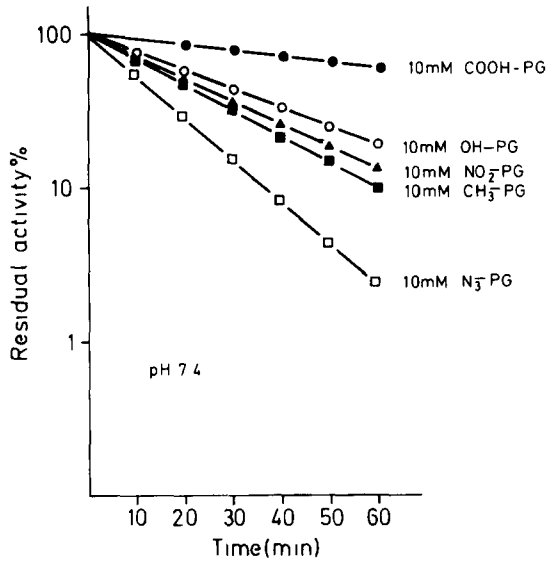

Fig 7 Comparison of the inactivation course of 10 mM of phenylglyoxal derivative on sulfate flux in resealed ghosts The data present in the figures are taken from experiments presented in the text

positively charged compound is unable to react with the essential arginine residue(s)

Comparison of the inhibitory activities of phenylglyoxal and its derivatives

Fig 7 shows the time course of inactivation of sulfate flux with the various PG derivatives The concentration of the inhibitors was the same in all experiments (10 mM) Incubation of the inhibitors with the resealed ghosts were done at pH 7 4, 37 °C, at the times indicated on the abscissa, aliquots were withdrawn, excess of the inhibitors was removed by washing and the residual activity of sulfate equilibrium exchange was measured Incubation with the N_3-PG was performed in the dark The most effective inhibitors were N_3-PG and PG followed by CH_3-PG, NO_2-PG, OH-PG, and COOH-PG The calculated half-time of inactivation of the various derivatives and the dissociation constant of the transport inhibitor complex (k_1) are presented in Table IV) Table IV lists $t_{1/2}$ and k_1 of PG and its different derivatives (calculated from experiments in previous figures) together with parameters which characterize their hydrophobic and electronic character Hydrophobic properties were determined by thin-layer partition chromatography according to Motais and Cousin [17] The R_M values are a measure of hydrophobility They are equivalent to π, the Hansch constant which is related to the free-energy change associated with the transfer of a substance from an aqueous phase to a lipophilic phase The electronic properties of the substituent were quantified by the Hammet constant σ The numerical values for the various substituents were obtained from the table published by Hansch [21]

When the logarithm of $1/t_{1/2}$ or log $1/K_1$ of PG and its various derivatives were plotted against either σ, the Hammett factor of the substituents of PG or the measured R_{M,H_2O} value, no correlations could be found

TABLE IV

Structure-activity relationship of phenylglyoxal derivatives

$t_{1/2}$ (min^{-1}), half-time of inactivation at cocentration of 10 mM of the inhibitors at pH 8 0 K_1, the dissociation constant of the transporter inhibitor complex calculated according to Ref 9 $R_{M\,H_2O}$, calculated as described in Materials and Methods σ, the numerical values for the various substituents were obtained from a table published by Hansch [21]

Substituent	$t_{1/2}$ (min^{-1}) (10 mM, pH 8 0)	K_1 (mM) (pH 8 0)	$R_{M\,H_2O}$	σ
PG	5 9	6 46	−0 47	0 00
CH_3-PG	10 2	1 92	−0 18	−0 17
NO_2-PG	13 3	34 50	−0 72	0 78
N_3-PG	5 9	5 50	−0 176	0 08
OH-PG	16 5	29 80	−1 47	−0 37
COOH-PG	59 9	-	−1 29	0 45
SO_3-PG	103 5	54 90	-	0 09

Interaction between the binding site of phenylglyoxal derivatives and the binding site of other reversible acting anion transport inhibitors

Fig 8 indicates that DNDS and flufenamate are able to protect the transport system against inhibition with

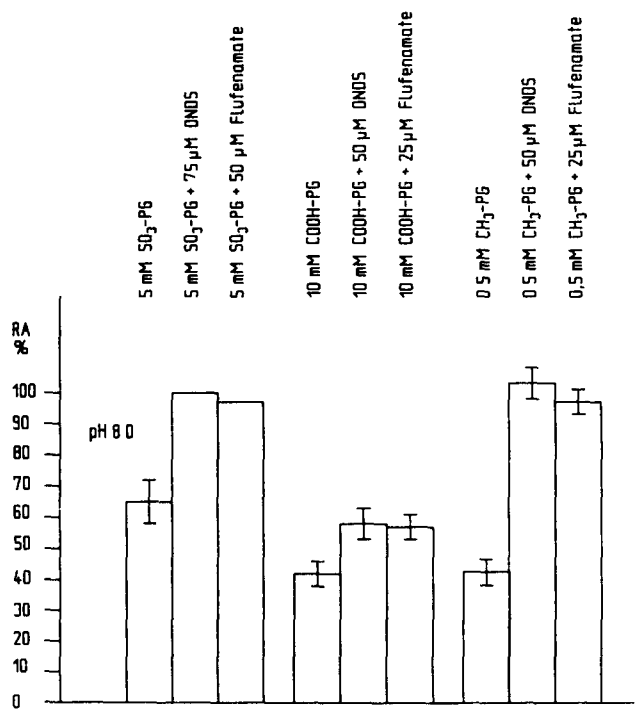

Fig 8 Effect of reversible acting anion transport inhibitors on the binding site of phenylglyoxal derivatives Resealed ghosts were exposed to CH_3-PG, COOH-PG and SO_3-PG in presence of either DNDS or flufenamate at the concentrations indicated in the figure at pH 8 0 (for experimental details se Ref 12) Ordinate The rate constant of $^{35}SO_4^{2-}$ exchange in percent of control value in the same medium without inhibitors

both the hydrophilic (COOH-PG, SO_3-PG) and the hydrophobic (CH_3-PG) derivatives of phenylglyoxal

Conclusion

The results presented in this paper show that the chemical properties of the binding site of these arginine-specific reagents differ from the properties of the binding sites of other anion transport inhibitors like the stilbene disulfonates and flufenamate

In the case of stilbene disulfonates Barzilay et al [22] have reported that the SO_3-group is essential and that the presence of electrophilic and hydrophobic moieties in the probe increases its inhibitory potency They also suggest that the binding site includes an electron donating residue This is not the case for the binding site of phenylglyoxal derivatives *p*-Methylphenylglyoxal with the CH_3 group which has electron-releasing character is more effective than the *p*-nitro derivative with the NO_2 group which has strong electron-attracting character In the case of flufenamate, Cousin and Motais [17] found that the presence of a carboxyl group is essential for inhibition This is not the case with the reagents used in this work The anionic derivatives of phenylglyoxal (OH-, COOH- and SO_3-PG) react irreversibly with the transport system with a rate which is 3–18-times slower than that of PG The results with *p*-azidophenylglyoxal show that the 17 kDa which is the transmembrane segment of the 60 kDa and 35 kDa cannot be cross-linked by N_3-PG Our findings also suggest that the essential arginine(s) which reacts with the glyoxal moiety is (are) not located within a distance of about 9 Å to other groups that are essential for anion transport

The positively charged PG derivative used is unable to inactivate the transport system This suggests that the essential arginine is not situated on the extracellular surface of the membrane, or that electrostatic forces in the vicinity prevent the reagent from reacting with the guanidino group(s)

Considering the chemical properties of these reagents and other results presented in this paper, one can say that the essential arginine(s) is (are) not a part of the stilbene sulfonate-binding site The existance of allosteric interactions between the binding sites for stilbene disulfonates, flufenamate, and the arginine reagents have been shown in the results presented in Fig 8 where it was found that DNDS and flufenamate are able to protect the transport system against phenylglyoxalation These results are in agreement with previous findings [11] On the other hand, after complete inhibition of the

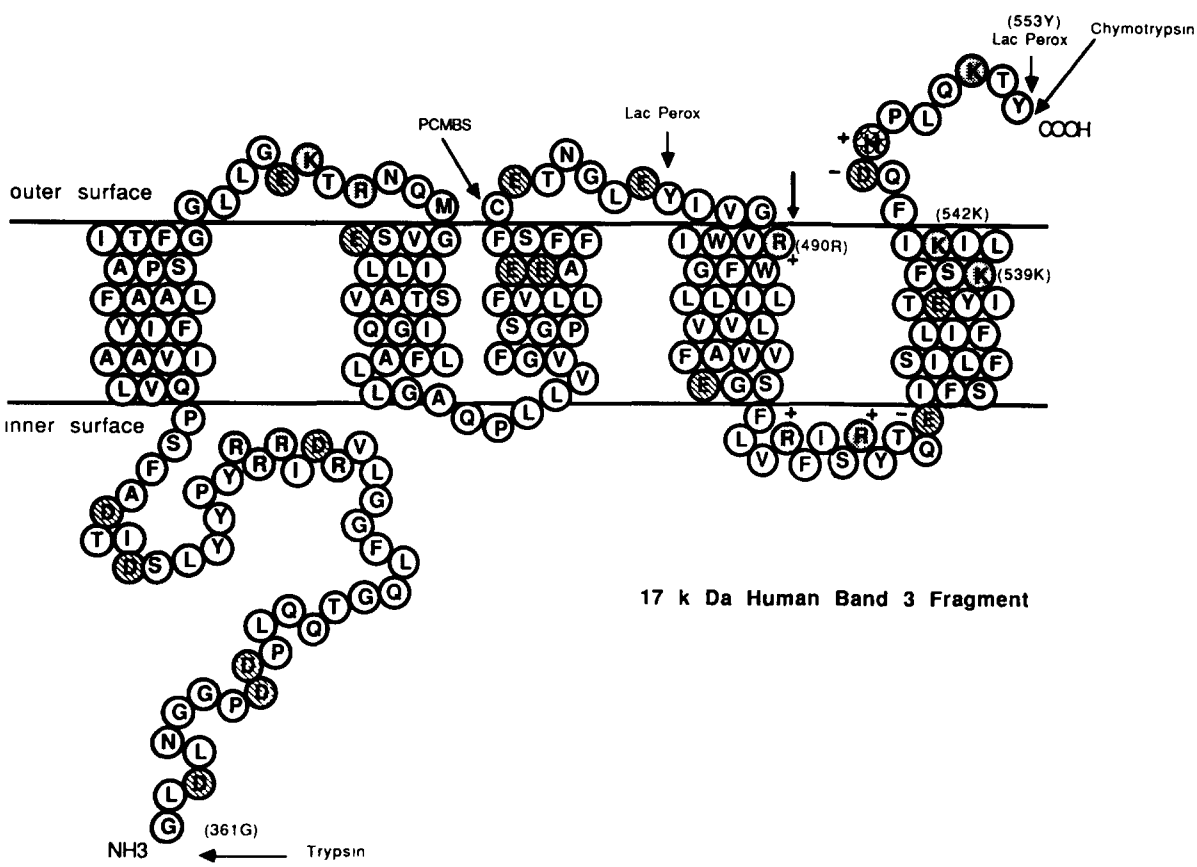

Fig 9 The figure presents the schematics orientation of the hydrophobic segments of the 17 kDa fragment of human band 3 in the membrane It is a modified presentation of the folding pattern suggested by Tanner [24]

transport system by arginine specific reagents, [$^3H_2$]DIDS can still bind to band 3 up to its total capacity [10,15]

These findings(and the results presented in this paper) suggest that the essential arginine may be located in a segment of the peptide chain which does not contain the stilbene disulfonate binding site

Recently, the complete amino acid sequence of human band 3 protein has been deduced from the cDNA sequence and it has been suggested that band 3 may contains up to 14 membrane spanning segments The binding site for H_2DIDS could be located in the 17 kDa segment at Lys-539 [23,24] In a previous paper, we have been able to show that complete inhibition of anion transport is accompanied by modification of two to three arginine residues, one or two of which are located in the 60 kDa fragment [8] We also found that the label is located in the 17 kDa fragment (unpublished results) An arginine residue which seems to be a possible candidate for the reaction with PG is Arg-490 The position of this arginine seems to be very characteristic It is in a hydrophobic region (link) containing two glycines Glycine residues are known to be α-helix breakers and allow relatively good rotational freedom to the peptide chain containing Arg-490 (Fig 9) These residues are conserved in murine band 3 [25] Arg-490 after binding to the substrate anion may be able to undergo conformational changes necessary for the transport process Such conformational changes have been proposed in the transition model [26], and the cascade model (Zaki, in press), where the binding of the substrate anion to band 3 protein is accompanied by structural changes that are necessary for the translocation of the substrate anion This is also in agreement with the results which suggest that the transport site is alternatively accessible from both sides of the membrane [27,28] The transmembrane segment with Arg-490 (as shown in Fig 9) may be allosterically linked to the segment containing the stilbene disulfonate binding site, which would be in agreement with our results [11]) From this position it can also interact with the charged groups Asp-546, His-547, Arg-514, Arg-518 These groups may serve to guide the substrate to or away from the transport site More studies are now being done to characterize these residues in more detail

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