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Three different actions of phenylglyoxal on band 3 protein-mediated anion transport across the red blood cell membrane

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Abstract

Phenylglyoxalation of the red blood cell membrane leads to three superimposed effects on band 3 protein-mediated anion equilibrium exchange as measured by means of radiosulfate: (1) a shift of the curve relating transport activity to pH towards lower pH values, possibly in combination with an increase of the maximal transport activity. This is accompanied by effect (2), the abolishment of a chloride-stimulated component of anion transport seen at low pH values. Effect (3) consists of inhibition of anion equilibrium exchange. Effect (1) prevails when phenylglyoxalation is performed at low concentrations of PG and low pH, while effect (3) predominates when exposure to PG is executed at high pH and high concentration of PG. Effect (1) is associated with a decrease of the K_i values for inhibition and binding of the reversibly acting stilbene disulfonates DNDS and DBDS. The inhibition observed as a consequence of effect (3) is linearly related to a decrease of the capacity of band 3 to combine with the stilbene disulfonate DBDS. The results are interpreted on the assumption that PG is capable of reacting with two or possibly three distinct binding sites in band 3. Reaction with one of them leads to effect (1) and, perhaps, to effect (2); reaction with the other to effect (3). The latter is possibly due to modification of Arg 730, which is homologous to Arg 748 in mouse band 3. Site-directed mutagenesis of this arginine residue showed that it is required for band 3-mediated anion transport.

Keywords: Sulfate transport; AE1; Erythrocyte membrane; Arginine

1. Introduction

In the study of structure–function relationships in the anion transporter of the red cell membrane (AE1, band 3 protein), site-directed mutagenesis has proven to be a useful tool for the identification of the nature and location of functionally important amino acid residues in the peptide sequence (summarized by Passow et al. [1]). For the planing of the mutations, previous knowledge of the nature and site of action of transport-modifying agents is helpful. In the context of our work on the effects of site-directed mutagenesis of certain arginine residues ([26]; reviewed in Ref. [2]) we have reexamined some of the previously published work on the modification of band 3 protein-mediated anion transport by the arginine-specific reagent phenylglyoxal.

Abbreviations: DBDS, 4,4′-dibenzoyl stilbene-2,2′-disulfonate; DNDS, 4,4′-dinitro stilbene-2,2′-disulfonate; H_2DIDS, 4,4′-diisothiocyano stilbene disulfonate; EDTA, ethylenediamine tetraacetic acid; PG, phenylglyoxal

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Phenylglyoxal is a well known inhibitor of enzymes with anionic substrates [3]. Although the action of the reagent is not strictly confined to arginine residues, in many instances, its inhibitory effects could be unequivocally attributed to phenylglyoxalation of arginines. It is assumed, therefore, that the inhibition of band 3 protein-mediated anion exchange across the red blood cell membrane is also due to a modification of one or several arginine residues (summarized most recently by Refs. [4–6]).

The functional significance of at least one arginine residue has recently been confirmed by the observation that mutagenetic substitution of Arg 748 in mouse band 3 by an uncharged amino acid residue leads to inhibition of chloride transport (Karbach et al., unpublished observations; see also Ref. [2]). However, the relationships between Arg 748 and the amino acid residue(s) responsible for the effects of PG are still unresolved.

In the native band 3 protein at least a dozen arginine residues can be modified by PG. Choosing suitable conditions, however, the selectivity of binding can be augmented. At low chloride concentration, low internal and high external pH, complete inhibition of transport is achieved, when the band 3 protein is labeled with PG at a stoichiometrical ratio of 2:1, suggesting the modification of no more than one single arginine residue per band 3 molecule [7]. In the presence of more elevated chloride concentrations, at least two arginines are labeled and, after incubation at low pH inside and outside the cells, a third arginine may become modified (for reviews see Refs. [4,5])

The functional significance of the arginine residue(s) involved in anion transport is still unknown. It has been strongly suggested by Zaki [6,8] and extensively discussed by Wieth and coworkers [7,9–11] that the positive charge of an arginine residue is essential for substrate binding to the transfer site of the band 3 protein. This claim is supported by measurements of Cl^- binding by means of $^{35}Cl^-$-NMR. Shami et al. [12] and Aranibar (Thesis, Frankfurt 1992; [2]) observed a decrease of $^{35}Cl^-$ binding over the alkaline pH range, where anion exchange becomes inhibited parallel to the deprotonation of a dissociable group with a pK of about 11–12, which has been attributed to an arginine residue [4,5,9]. Moreover, Falke et al. [13] found a release of $^{35}Cl^-$ upon modification of band 3 in situ by phenylglyoxal, suggesting a loss of the capacity for substrate binding.

Although the effects of pH and phenylglyoxal indicate a participation of one or several arginine residues in anion binding, it is not yet clear whether or not this represents a direct involvement in complex formation with the anion. Hamasaki et al. [14] described a release of Cl^- upon modification of band 3 with diethylpyrucarbonate, a histidine reactive reagent. This effect is clearly allosteric and thus modification of band 3 by phenylglyoxal may also produce a change of substrate binding by combination with an amino acid residue that is not directly involved in anion binding.

In the present paper we try to answer two questions. First, we wish to establish whether phenylglyoxalation of one single amino acid residue suffices to explain the effects seen on anion transport, or if additional effects are involved that had escaped observation in the past. Second, we wish to contribute to the elucidation of the role of the phenylglyoxal-reactive amino acid residue(s) in anion transport and to clarify whether they are directly involved in substrate binding, or if they play a more indirect role by maintaining the tertiary structure of the substrate binding site in a functional state.

Our data show that phenylglyoxalation produces at least three clearly distinguishable effects on anion transport, which involve the modification of two or more functionally distinct amino acid residues. Modification of one of them leads to inhibition, while that of the other(s) leaves transport intact but alters its pH dependence and susceptibility to inhibition by stilbene disulfonates, and abolishes its activation by chloride at low pH, suggesting that at least a second residue is allosterically linked to the substrate binding site. We conclude that our present level of knowledge does not yet permit to decide whether or not anyone of the observed effects can be attributed to phenylglyoxalation of an arginine residue that is involved in substrate binding.

2. Materials and methods

Rh^+ human blood was obtained from the Blood Bank of the Red Cross and used within about one

week after withdrawal. After centrifugation the blood plasma and buffy coat were removed and the cells were washed three times in a medium containing 108 mM Na_2SO_4 and 20 mM EDTA (pH 7.4). After the last wash, the red cells were subdivided into two portions. One portion was resuspended at pH 8.25 in the SO_4^{2-} medium described above without further additions. The other portion was resuspended at the same pH of 8.25 in the same medium containing 7.5 mM phenylglyoxal (Sigma). After 45 min of incubation at 37°C, the cells were washed three times in the SO_4^{2-} medium (pH 7.4), and hemolyzed at 0°C by 1:50 dilution in a hypotonic medium containing 4 mM $MgSO_4$ and sufficient acetic acid to establish in the hemolyzate pH 6.0 [15]. After 5 min at 0°C, sufficient Na_2SO_4, NaCl, and EDTA were added to yield an EDTA concentration of 20 mM and the desired concentrations of SO_4^{2-}, Cl^- and H^+. After resealing at 37°C in the presence of $^{35}SO_4$ for 60 min, the ghosts were washed at 0°C to remove external $^{35}SO_4$ and finally resuspended at a hematocrit of 2% at 30°C to measure $^{35}SO_4$ efflux. Rate constants were determined by fitting the data to a single exponential. When required, appropriate corrections for differences of ghost volume were applied.

DBDS binding was measured in resealed white ghosts made from untreated or phenylglyoxalated red blood cells. The ghosts were prepared by hemolysis on a Sepharose column equilibrated with 20 mM EDTA (pH 6.0) at 0°C as described by Wood [16]. DBDS fluorescence was measured after suspension of the resealed ghosts (0.12 mg/ml) in 160 mM NaCl buffered with 20 mM EDTA to the desired pH. Excitation was at 352 nm, emission at 425 nm.

For the interpretation of the anion flux measurements obtained after phenylglyoxalation of red blood cell membranes, it seemed useful to know more about the stability and pK values of the PG derivative of arginine residues. To obtain this information we synthesized the PG derivative of *N*-acetylarginine as described by Takahashi [17]. The UV spectrum of the purified product (the elementary analysis yielded for the ratio C:N:H the values: 56.3:10.9:6.0, expected values: 55.2:11.7:5.7) was measured immediately after solution of the agent in 20 mM EDTA (pH 8.0). Three hours later the spectrum was unaltered. After 18 h the spectral peak was broadened and its height reduced by some 15%.

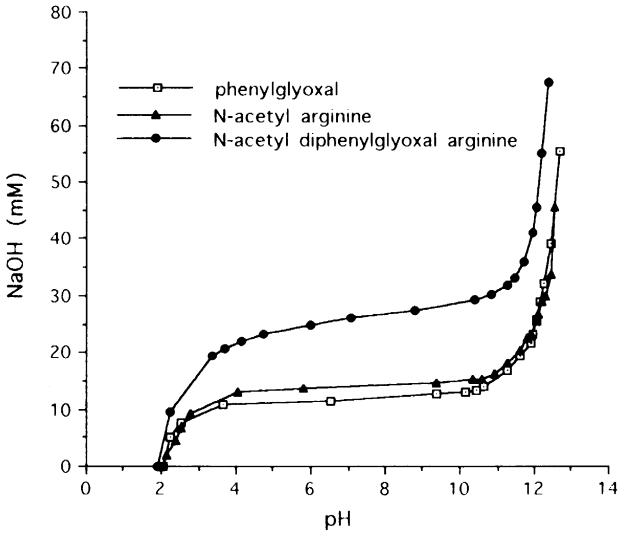


Fig. 1. Acid-base titration of phenylglyoxal, *N*-acetyl arginine and its di-PG derivative. Ordinate: NaOH added, mM. Abscissa: pH.

After we had reassured ourselves about the stability of the synthesized PG derivative, we subjected the synthetic derivative to acid base titration. The results are independent of whether the titration was initiated at pH 3 and went up to pH 11 or vice versa. We found that the curve of the Di-PG-*N*-acetyl arginine was essentially similar to the curve for the unmodified *N*-acetyl arginine, except that instead of one carboxylic acid residue, a second acidic residue with similar pK value was titrated (Fig. 1). This is presumably due to the fact that the second PG residue, after reaction with the 1:1 arginine-PG adduct to form the final 1:2 product, is capable of undergoing keto-enol tautomery. This should give rise to an additional titratable group which is deprotonated above pH 3.5 and thus conveys one additional negative charge to the di-PG-derivative. This is associated with a reduction of the Coulombic force, which is further accentuated by the fact that the single positive charge is distributed over a larger volume than in the unmodified compound. From the point of view of the present paper, it is most significant that, over the pH range covered in our experiments, the degree of protonation of the arginines remains nearly unaltered after phenylglyoxalation even though in the immediate vicinity of the guanidinium group, a negative charge is established.

3. Results

3.1. After phenylglyoxalation under standard conditions, the observed inhibition of band 3-mediated anion transport depends on the composition of the medium in which transport is measured

In resealed ghosts made from red blood cells that had been phenylglyoxalated under standard conditions for 60 min, sulfate equilibrium exchange is inhibited. The magnitude of the inhibition depends on the ionic composition and pH at which equilibrium exchange is measured. In the presence of 1.0 mM sulfate and a large excess of chloride (80 mM), inhibition at pH 6.0 and 7.4 is rather similar and was found to amount to $75 \pm 3\%$ (standard error of mean, $n = 8$). When the flux measurements are performed in a medium containing 108 mM sulfate and no chloride, inhibition at pH 7.4 is still $72 \pm 5\%$ ($n = 17$), but at pH 6.0, inhibition is reduced to $44 \pm 7\%$ ($n = 3$). The difference between the inhibition at the two pH values becomes more pronounced when the sulfate concentration is lowered to 1.0 mM. At pH 7.4, inhibition now amounts to about $71 \pm 3\%$ ($n = 17$), at pH 6.0 to no more than $19 \pm 1\%$ ($n = 8$). It should be noted that at least some of these results are averages of many experiments. In individual experiments, the deviations from the average behavior may be considerable.

Possibly, the large difference observed at 1.0 mM Na_2SO_4 could reflect a dissociation or reorganisation at low pH and ionic strength of the adduct of PG and the arginine residue(s) responsible for the inhibition of anion exchange. However, this explanation can be ruled out. When ghosts made from phenylglyoxalated red cells are first exposed to pH 6.0 – where the inhibition remains insignificant – and are then titrated to pH 7.4, inhibition becomes apparent. This inhibition is indistinguishable from the inhibition seen in a control that had not been subjected to an incubation period at low pH (data not shown).

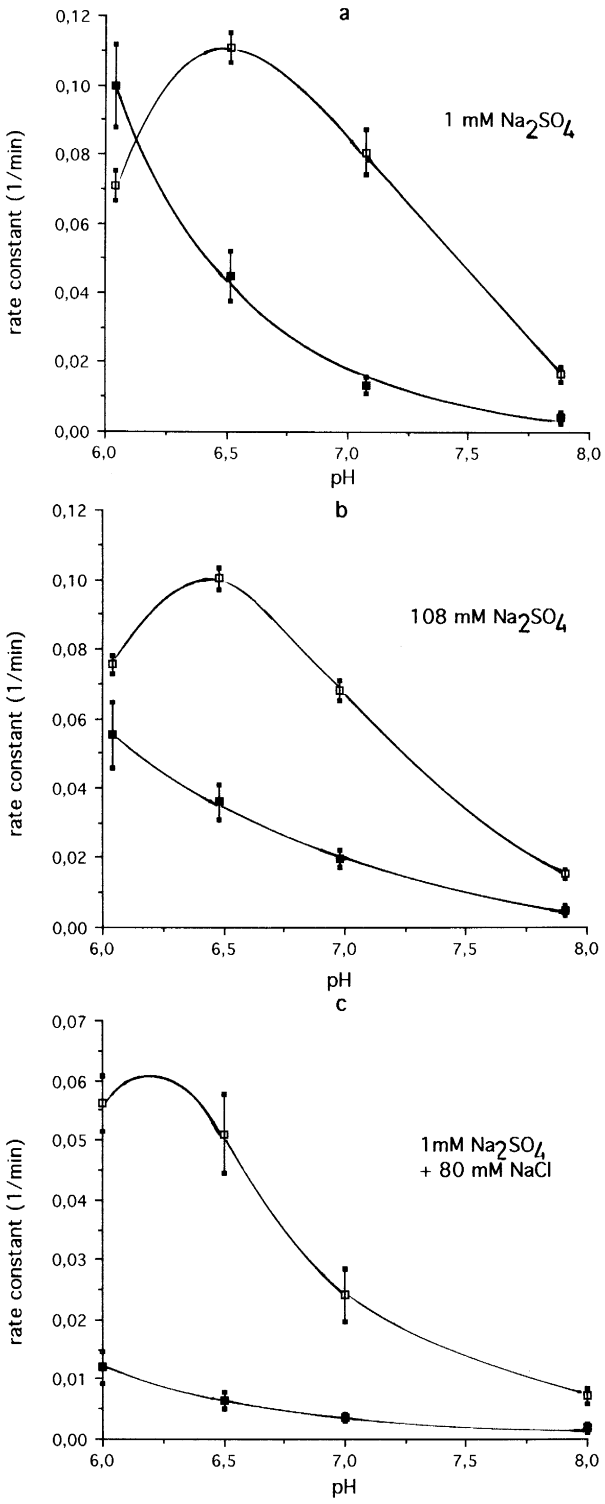
The results described above are also pertinent with respect to the work of Bjerrum [4], who noted that after termination of phenylglyoxalation the anion transport system may undergo spontaneously partial recovery upon incubation at 37°C. This recovery was prevented when, prior to the flux measurements, the phenylglyoxalated ghosts were incubated in the absence of PG at 0°C for 20 h. Bjerrum performed phenylglyoxalation under conditions (sucrose-citrate medium, pH 10.25 in the external medium, 25°C, 60 s) which differed considerably from those employed in the present work (sulfate medium, pH 8.25, 37°C, 45–60 min). Nevertheless, it seemed useful to explore whether or not incubation at 0°C of the ghosts made from the phenylglyoxalated red blood cells would yield results different from those described here. For this purpose we incubated the ghosts for 20 h at 0°C, pH 7.4, prior to the flux measurements at pH 6.0 and pH 7.4 and compared the results of these measurements with those obtained in ghosts made from the same PG-treated red cells which had not been incubated at 0°C but had been used immediately after resealing for the flux measurements. The results were indistinguishable. In eight different experiments we found that the ratio between the fluxes measured immediately after resealing and the fluxes measured 20 h thereafter was 1.0 ± 0.17 (S.D.). This confirms that under our conditions of phenylglyoxalation, a stable product is formed, and hence that there is no reason to assume that the effects observed are influenced by time-dependent variation of the properties of the modified transport protein.

In summary, it is clear that the modification of band 3 with PG prior to the flux measurements is in fact irreversible and survives unaltered under the extremes of the experimental conditions used in the present work. The data presented above show, therefore, that phenylglyoxalation does not necessarily destroy the capacity of the band 3 protein to mediate sulfate equilibrium exchange. Under the conditions chosen in the present experiments, it leads at least to a change of the pH dependence of the anion transport activity which is modulated by the ionic composition of the medium.

3.2. Band 3-mediated anion transport measured after irreversible modification by PG under standard conditions; effect of variation of pH, sulfate, and chloride concentration

A more complete picture of the pH dependence of SO_4^{2-} equilibrium exchange in ghosts made from untreated and phenylglyoxalated red blood cells is shown in Fig. 2. Regardless of whether the measurements of the equilibrium exchange were performed at

1.0 mM or 108 mM Na_2SO_4 in medium and cells, the controls show the well known maximum around pH 6.5. In the ghosts made from the phenylglyoxalated red blood cells, the curves are shifted to lower pH values, such that with decreasing pH the SO_4^{2-} flux increases dramatically relative to the control ghosts. This effect is most pronounced at 1.0 mM sodium sulfate in the medium where, at pH 6.0, we observe fluxes that may exceed the fluxes in the controls (Fig. 2a). In the presence of 108 mM sodium sulfate, the effect of decreasing the pH is quenched (Fig. 2b). This is even more pronounced if, in place of sodium sulfate, NaCl is present during the flux measurements (Fig. 2c).



The curves derived from measurements with ghosts made from phenylglyoxalated red blood cells do not represent simple parallel shifts of the curves obtained with the controls. In the ghosts made from PG-treated red cells, the ascent at low pH seems less steep than in the controls and it remains unclear whether or not the fluxes in the modified ghosts reach a maximum, similar to the maximum in the controls. Nevertheless, it is clear that one of the actions of PG consists in shifting the pK value of a transport-activating site to a lower value and that the extent of this shift is modulated by the ion composition of the medium in which the flux measurements are performed.

The influence of the ion composition of the medium was further characterized by investigating the dependence of band 3-mediated transport on substrate concentration in ghosts made from untreated and PG-treated red cells.

The study of the dependence of SO_4^{2-} transport on SO_4^{2-} concentration seemed interesting, since this would provide a tentative answer to the question whether or not the shift of the pH dependence of transport primarily reflects a change of the capacity of the transfer site to combine with its substrate, or if

Fig. 2. pH-dependence of sulfate equilibrium exchange in resealed ghosts made from untreated red cells (open squares) and from red cells treated with 7.5 mM PG (filled squares) under standard conditions (see legend to Table 1) for 45 min (rather than 60 min as in the data presented in the text). Resealing and flux measurements in 20 mM EDTA and either (a) 1 mM Na_2SO_4, or (b) 108 mM Na_2SO_4, or (c) 1 mM sodium sulfate plus 80 mM NaCl at the pH values indicated on the abscissas. Ordinates: rate constant for sulfate efflux, min^{-1}. The difference between the effects of PG at pH 6.0 shown in Fig. 1a and those described in the text are essentially due to the different times of exposure to the agent prior to ghosting and flux measurements.

it essentially represents a change of the turnover rate of the anion-loaded transfer site.

Fig. 3 shows that the functional relationship between the rate constants for transport and substrate concentration are rather similar in ghosts made from untreated and PG-treated red blood cells. Perhaps there exists a small difference which consists in a diminution of the self stimulation at low sulfate concentration first described by Schnell and Besl [18].

The results obtained at pH 7.4 are somewhat difficult to interpret. The absolute values of the transport rates at 1 mM sulfate (equated to 100% in Fig. 3) were reduced in the ghosts made from the PG-treated red blood cells to about 30% of the rate in the ghosts made from untreated red cells. Some of these 30% represent transport by band 3 molecules that may not have been modified. Their contribution to the measured flux may dominate the picture. This problem does not exist for the data pertaining to pH 6.0, where no inhibition by PG was observed and hence the absolute values at 100% for ghosts made from untreated and PG-treated red cells are equal. Obviously, under these latter conditions most of the transport is accomplished by the modified transport protein. Hence the similarity of the concentration dependence of the rate constants for SO_4^{2-} equilibrium exchange in ghosts made from untreated and phenylglyoxalated red cells suggests that, at least when measured at pH 6.0, the apparent affinity for sulfate binding to the transfer site remained essentially unaltered by phenylglyoxalation.

Chloride and sulfate compete for the same substrate binding site in the band 3 protein [19]. Fig. 4 illustrates the effect of Cl^- on sulfate transport in ghosts made from untreated and phenylglyoxalated red cells. Fig. 4b shows that in ghosts made from untreated red cells, at pH 7.4, we observe a monotonic decrease of sulfate transport with increasing chloride concentration, as expected for competition between chloride and sulfate. At pH 6.0, the curve relating transport to chloride concentration measured in the same ghosts passes through a maximum (Fig. 4a). Obviously, the protonation of the transporter leads to the appearance of a binding site for chloride that acts like a transport-promoting modifier site. The transport promoting effect is only seen at low sulfate and chloride concentrations. At more elevated chloride concentrations, the inhibition by competition between chloride and sulfate becomes more important and obscures the transport-promoting effect.

The effect described disappears in the presence of

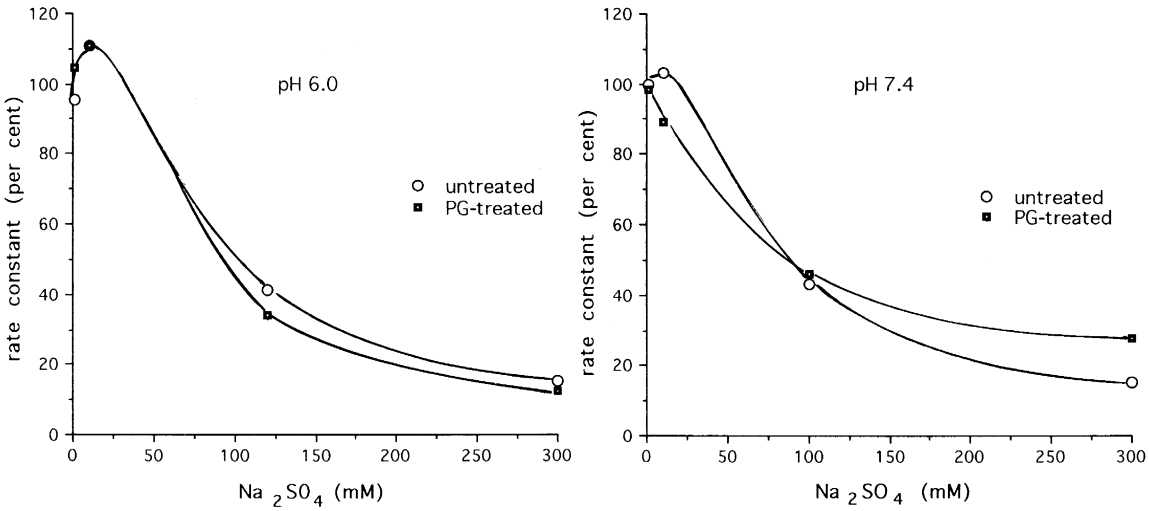
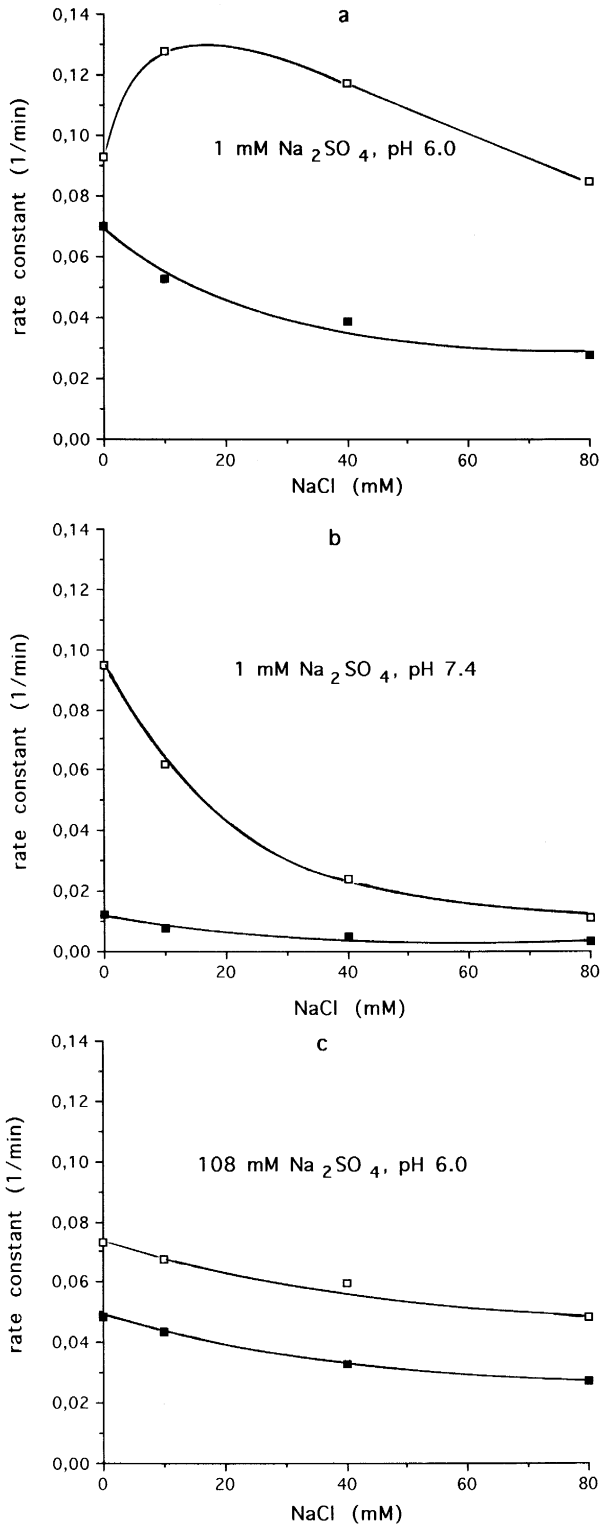


Fig. 3. Dependence of sulfate equilibrium exchange on sulfate concentration in resealed ghosts made from untreated red cells and from red cells treated with 7.5 mM PG under standard conditions for 60 min. Resealing and flux measurements were made in media containing 20 mM EDTA, pH 6 (left panel) or pH 7.4 (right panel) and the sulfate concentrations (mM) indicated on the abscissa. Ordinate: rate constant for sulfate efflux, as a percent of the flux at 1.0 mM Na_2SO_4.



a large excess of sulfate. When the chloride concentration is raised in the presence of a fixed back ground concentration of sulfate of 108 mM (Fig. 4c), activation can no longer be observed. Apparently, sulfate is able to combine with the protonated site, and to assume the transport-promoting role of chloride with the result that the additional chloride that displaces some of the sulfate, is no longer able to cause additional activation.

Phenylglyoxalation abolishes the accelerating effect of increasing the chloride concentration at the low, constant sulfate concentration of 1.0 mM seen at pH 6.0 (Fig. 4a). This is different from the enhancement of transport seen when ghosts made from PG-treated red cells are exposed to media of decreasing pH and low and constant ion concentration, e.g., in 1 mM sodium sulfate (cf. Fig. 2a). Thus, the observations described so far show that phenylglyoxalation causes at least two distinct effects: Abolishment of a pH-dependent activation of anion transport by anion binding and a shift of the enhancement of anion transport by protons to lower pH values.

3.3. Band 3-mediated anion transport measured in standard media after previous treatment with varying concentrations of PG

The flux measurements described up to this point had been performed in media of varying ion composition and pH with ghosts prepared from red blood cells that had been phenylglyoxalated under standard conditions, i.e., at a fixed pH, a fixed concentration of phenylglyoxal, and (if not expressly stated otherwise) at a fixed length of time. In the experiments described below, prior to ghosting, the red blood cells had been exposed to varying PG concentrations, ranging from zero to twelve mM. The result of the flux measurements in the ghosts derived from these PG-treated red blood cells depended on both the condi-

Fig. 4. (a–c) Effect of increasing chloride concentrations on sulfate equilibrium exchange in ghosts made from untreated red cells (open squares) and red cells treated with 7.5 mM PG under standard conditions (filled squares). Ordinate: rate constants of sulfate equilibrium exchange at the fixed sulfate concentrations and pH values indicated in the individual panels and the chloride concentrations represented on the abscissas.

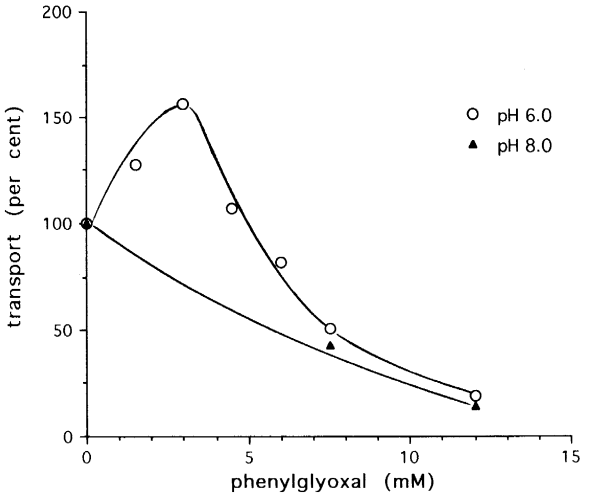


Fig. 5. Rate constants of sulfate flux measured in ghosts made from red blood cells phenylglyoxalated at pH 8.25 for 60 min under standard conditions at the PG concentrations indicated on the abscissa. Ordinate: transport rate as a percent of the rate measured in ghosts made from untreated control cells. The absolute values of the rate constants at PG = 0 were 0.052 min^{-1} at pH 6.0 and 0.007 min^{-1} at pH 8.0. The flux measurements were performed in a medium containing 1 mM sodium sulfate, 80 mM NaCl, and 20 mM EDTA.

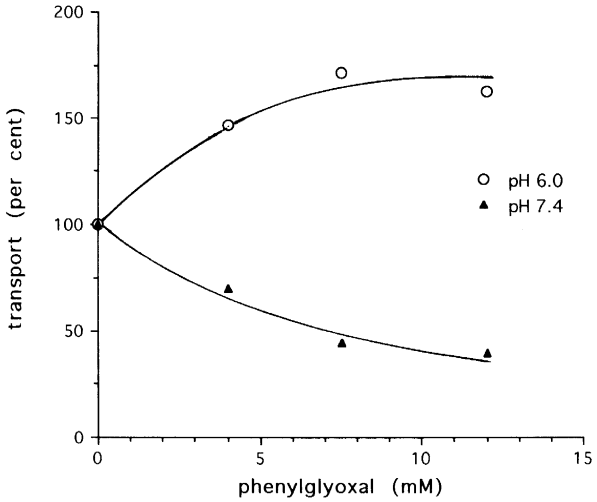


Fig. 6. Sulfate flux measured in ghosts made from red blood cells phenylglyoxalated at pH 6.5 under standard conditions at the PG concentrations indicated on the abscissa. Ordinate: rate constant of sulfate flux as a percent of the flux measured in a medium consisting of 1 mM sodium sulfate, no chloride, and 20 mM EDTA. pH as indicated at the curves.

tions under which the red cells had been modified with PG and the conditions under which the fluxes were measured in the ghosts made from these red blood cells.

When the red blood cells had been phenylglyoxalated at pH 8.25, i.e., at the same pH as in the experiments described above, we found that the effects on anion transport measured in the ghosts made from these cells depend on the pH at which the flux measurements were performed. If measured at pH 7.4 or higher (e.g., at pH 8.0 as in Fig. 5), the anion exchange rate monotonically decreases with increasing PG concentration used for the modification of the cells from which the ghosts had been made. When the flux measurements are executed in the same ghosts at pH 6, the exchange rate first increases beyond the value measured in the control ghosts derived from untreated red cells. It passes through a maximum in ghosts made from red cells exposed to PG at a concentration of about 3 mM and then

Table 1
Inhibition of SO_4^{2-} equilibrium exchange by DNDS in ghosts made from phenylglyoxalated red blood cells

Na_2SO_4	[PG] = 0 mM			[PG] = 7.5 mM			$\frac{K_i(PG)}{K_i(control)}$
mM	K_i mM	±S.D.: mM	Residual flux per cent	K_i mM	±S.D.: mM	Residual flux per cent	
1.0	1.60			0.27			4.1 ± 1.70.440.1010.3 ± 2.50.28
108.0	3.19			0.59			8.3 ± 0.70.800.2013.3 ± 2.50.25

Red blood cells were preincubated in 108 mM Na_2SO_4, 20 mM EDTA (pH 8.25) without or with 7.5 mM phenylglyoxal at 37°C for 1 h. Measurements of sulfate equilibrium exchange were made with ghosts derived from the preincubated cells which had been resealed in media containing 20 mM EDTA (pH 6.0) and either 1.0 or 108 mM Na_2SO_4. K_i values were derived from measuring the effect of increasing concentrations of DNDS on sulfate flux, as shown in Fig. 7. Each K_i value represents the mean of five independent determinations.

K_i = DNDS concentration producing 50% inhibition. Residual flux, flux at maximal inhibition by DNDS.

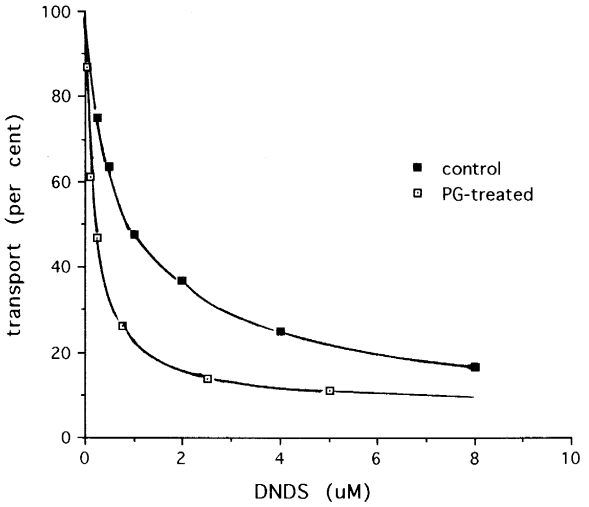


Fig. 7. Effect of DNDS on sulfate equilibrium exchange in resealed ghosts made from untreated red cells and red cells treated with 7.5 mM PG for 45 min under standard conditions. Resealing and flux measurements were performed in media containing 1 mM Na_2SO_4 and 20 mM EDTA (pH 6.0). Ordinate: rate constant for SO_4^{2-} efflux as a percent of rate constant in the absence of DNDS. Abscissa: DNDS concentration (μM). The drawn lines were fitted according to the equation: rate constant = $A/(K_i + [DNDS]) + B$, where A and B represent constants, the latter indicating the residual flux at maximal inhibition by DNDS; K_i = DNDS concentration at which inhibition is half maximal. Upper curve: ghosts made from untreated red cells (control). $K_i = 1.55$ μM, $B = 7.5\%$. Lower curve: ghosts made from PG-treated red cells. $K_i = 0.34$ μM, $B = 7.5\%$.

decreases steeply with further increasing PG concentration (Fig. 5).

When ghosts are used that had been prepared from red cells exposed to the varying PG concentrations at pH 6.5, we find again that their behavior depends on

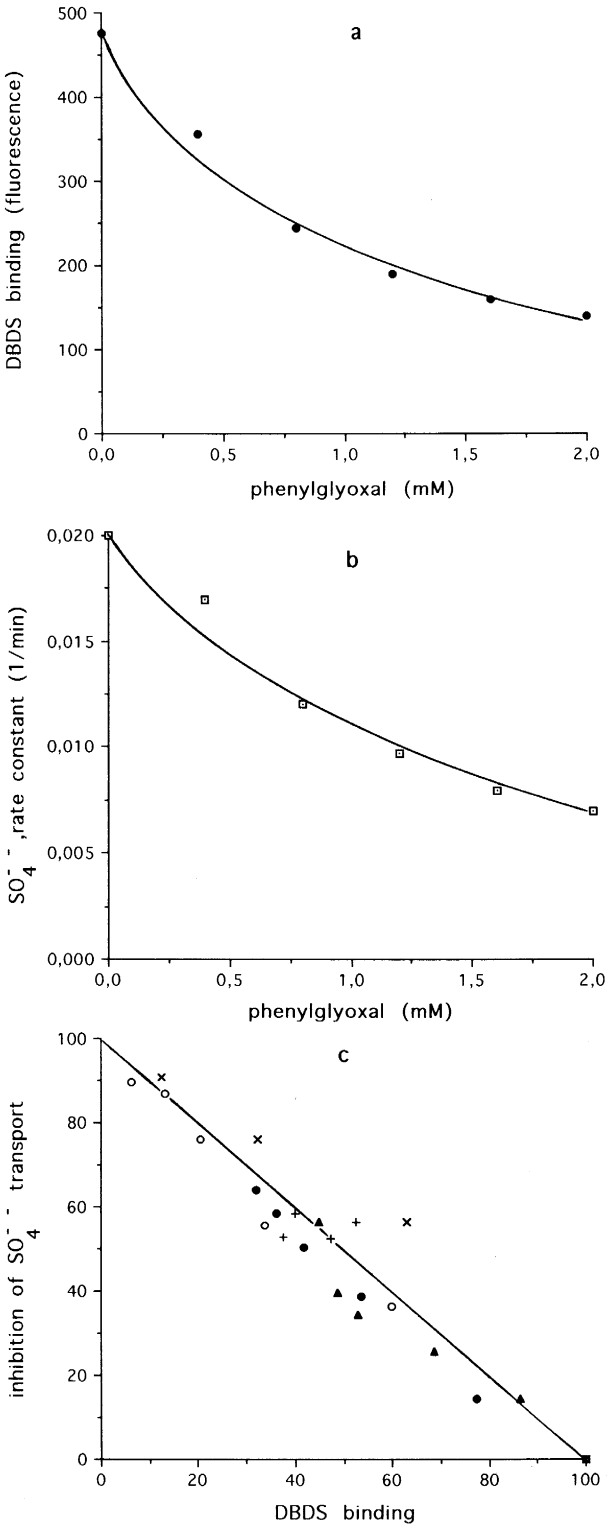


Fig. 8. Effect of phenylglyoxalation of the red cell membrane on DBDS binding to the band 3 protein and the rate of sulfate equilibrium exchange. Resealed white ghosts prepared by the method of Wood [16] had been phenylglyoxalated at a range of PG concentrations (abscissa) in a medium containing 160 mM NaCl, 1 mM sodium sulfate, 20 mM EDTA (pH 8.2) at 37°C for 1 h. Each batch of modified ghosts was subdivided into two, one for measuring DBDS binding (by fluorescence, a), the other for measuring transport (b). DBDS binding represents the difference between binding in the absence and presence of a large excess of H_2DIDS (20 μM) added to a saturating concentration of DBDS in the medium (20 μM). In c, the data represented in a and b pertaining to corresponding PG concentrations are plotted against one another (solid circles). The other symbols refer to additional experiments of the same type. Data from Ref. [30].

the pH at which the flux measurements are performed. At pH 6.0, the flux shows a monotonic increase with PG concentration until, around 8 mM PG, a plateau (or a flat maximum) is reached. When the same ghosts are subjected to the flux measurements at pH 7.4, a monotonic decrease is observed (Fig. 6).

3.4. Transport inhibition by and binding of stilbene disulfonates after phenylglyoxalation of band 3

An important criterion for the specificity of the effects of PG with respect to its site of action is the susceptibility of the transport to inhibition by the band 3 specific stilbene disulfonates. We studied, therefore, the effects of two reversibly binding stilbene disulfonates, DNDS and DBDS, on ghosts made from phenylglyoxalated red cells.

In a first set of experiments, we compared the concentration dependence of inhibition by the reversibly binding stilbene disulfonate DNDS in ghosts made from untreated and phenylglyoxalated red cells at pH 6.0, i.e., at a pH value where the transport inhibition by PG seen at pH 7.4 is greatly reduced. Fig. 7 shows that sulfate transport mediated by the phenylglyoxalated band 3 protein can be successfully inhibited by the stilbene disulfonate. Table 1 summarizes the results obtained and indicates that the residual flux at high DNDS concentrations in the modified ghosts amounts to 10–15% and hence is only slightly higher than the residual flux in the controls. Thus, the susceptibility to inhibition by DNDS is fully preserved in the modified ghosts. Most surprising was the additional observation that the DNDS concentration that produces half maximal inhibition (K_i) decreases in ghosts made from phenylglyoxalated red cells to about $1/4$ the value in the control. This finding was the same at 1 mM or 108 mM sulfate in ghosts and medium. In a second set of experiments we compared the effects of phenylglyoxalation on transport and stilbene disulfonate binding. The latter was determined using the fluorescent DBDS, which exhibits a considerable fluorescence enhancement upon binding in a hydrophobic environment. It was observed that the inhibition of sulfate equilibrium exchange as measured at pH 7.4 was linearly related to a decrease of DBDS binding capacity, as determined by displacement of DBDS from band 3 by phenylglyoxalation (Fig. 8c).

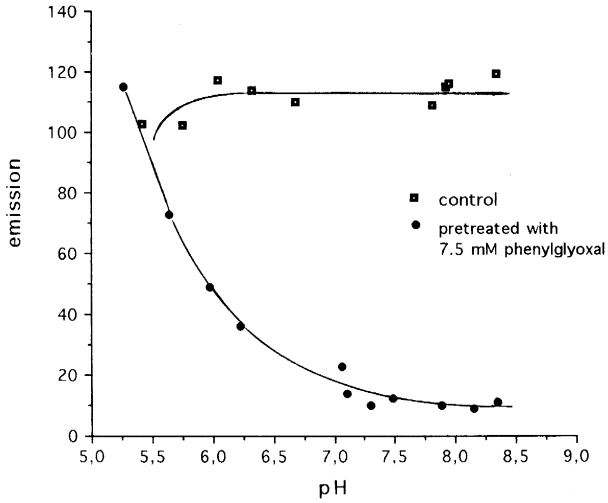


Fig. 9. DBDS binding to white ghosts made from untreated and phenylglyoxalated (7.5 mM PG, in 108 mM sodium sulfate, 20 mM EDTA (pH 8.25), 37°C, for 1 h) red cells. The ghosts were suspended in media containing 3 μM DBDS, 1 mM sodium sulfate, and 20 mM EDTA adjusted to the pH values indicated on the abscissa. Fluorescence (ordinate) represents the difference of the readings before and after addition of 20 μM H_2DIDS to the suspension.

In ghosts made from PG-treated red cells, above pH 7, the binding of DBDS is independent of pH. Below pH 7, DBDS binding increases, indicating an increase of DNDS affinity that is associated with the recovery of the transport activity described above. This behavior contrasts with that of ghosts made from untreated red cells, in which under these conditions DBDS binding tends to decrease (Fig. 9). This effect deserves further study. Here it is reported simply to demonstrate that the modification of transport by phenylglyoxalation also affects stilbene disulfonate binding.

4. Discussion

4.1. Three functionally distinct binding sites for phenylglyoxal

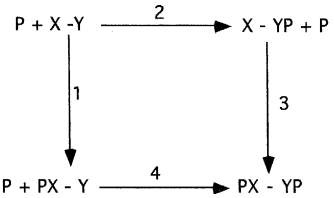
In the ghosts made from PG-treated red cells three superimposed effects on band 3-mediated anion exchange were observed: (1) a change of pH depen-

dence of transport (Fig. 2); (2) an abolishment of the stimulation of sulfate equilibrium exchange by chloride seen at low pH and low sulfate concentration (Fig. 4a); and (3) an inhibition. Effect (1) is accompanied by an increase of the affinity to band 3 of the reversibly binding, transport inhibiting stilbene disulfonates DNDS and DBDS (Fig. 7, Fig. 9). This confirms that the enhancement of anion transport associated with this effect is not significantly influenced by unspecific leakage. Our data do not permit to decide whether or not effects (1) and (2) are due to phenylglyoxalation of the same single site, or if two distinct sites are involved.

This uncertainty is largely due to the fact that the nature of the increase of sulfate flux caused by the increase of chloride concentration at low pH and low sulfate concentration has not been explored in detail. From the work of Hautmann and Schnell [20] on Cl transport, and Schnell and Besl [18] on sulfate and phosphate transport, it is known that the dependence of anion flux on anion concentration shows some cooperativity (see also Fig. 3 in the present paper). Perhaps, chloride binding to an activating, allosteric site (a 'modifier site') that in Schnell's experiments becomes occupied when either chloride or sulfate concentration is raised, causes the enhancement of sulfate transport by chloride. The effect disappears after PG treatment and at chloride concentrations high enough to inhibit sulfate transport by competitive replacement of sulfate by chloride at the substrate binding site of band 3.

Modifying the intact red blood cells at varying PG concentrations at the fixed pH of 8.25 and measuring anion flux in the resealed ghosts made from these cells at a fixed ionic composition at pH 6.0 in the medium reveals a diphasic effect of PG: an enhancement of sulfate transport up to about 1.7-fold at 3.0 mM PG and an inhibition at higher PG concentrations (Fig. 5). This indicates that PG reacts with two functionally distinct sites in band 3. The maximum disappears and is replaced by a monotonic decrease of transport when the flux measurements are performed in the same ghosts at pH 8. This shows that the accelerating site is activated by lowering the pH. Both activation and inhibition are seen when, prior to ghosting, the exposure of the red cells to PG is done at pH 6.5 (Fig. 6).

The assumption that the two dominating effects (1) and (3) are due to modification of at least two distinct sites in the band 3 protein implies that, depending on the occupancy of these sites with PG, four different states may exist, each of which could mediate the anion exchange at a different rate. The maximum in the curve relating transport measured in the ghosts to the PG concentration used to modify the red cell membrane prior to the flux measurements (Fig. 5) indicates that the site (designated X) responsible for the shift of the pH dependence and the concomitant enhancement of transport is more susceptible to phenylglyoxalation than the inhibitory site (designated Y). The further observation that, at high PG concentrations, the rate of transport assumes much lower values than in the ghosts made from untreated red cells shows that modification of Y leads to inhibition of transport even if the accelerating site X is phenylglyoxalated (Hypothesis I). This would give rise to four different transport rates that are associated with the four possible permutations of PG binding to the sites X and Y. The forms X–Y, PX–Y, X–YP, and PX–YP (where PX and YP represent, respectively, sites X and Y after phenylglyoxalation) would be associated with enhancement (PX–Y) or inhibition (X–YP and PX–YP) relative to the unmodified transport protein (X–Y). These inferences from our observations are schematically represented in the following reaction diagram:



Alternatively, one could assume that both singly phenylglyoxalated forms, PX–Y as well as X–YP, contribute to the observed enhancement of transport while transport is inhibited only if both X and Y are phenylglyoxalated, i.e., when the transporter exists in the form PX–YP (Hypothesis II). Our experiments do not permit to decide between hypothesis I and II. However, from the work of Wieth et al. [7] it is known that after phenylglyoxalation at low chloride concentration, low internal and high external pH, the modification of a single arginine residue with a p*K*

of about 12 suffices to inhibit anion transport. If the inhibition observed in our work and that of Zaki [6] is due to the modification of the inhibitory site with the pK of about 12 described by Wieth et al. [7], Hypothesis I would be true. Below, we shall focus entirely on Hypothesis I.

The relative amounts of the four forms in the population depend among other things on pH, anion composition of the medium and the PG concentration used for phenylglyoxalation of X–Y in the intact red blood cells. The transport activity in the ghosts derived from these cells represents the sum of the contributions of each one of the four forms in the total population of band 3 molecules. The transport activity of each one of these forms is also a function of pH and ionic composition of the medium in which the flux measurements were performed. In other words, pH and ionic composition of the medium affect both the modification produced during exposure of band 3 to PG and the transport activity measured after completion of the irreversible modification of the band 3 protein.

At the upper end of the pH range covered, the sum of the transport activities of all modified forms is lower than that of the unmodified molecules. At the lower end of the range the transport activity may be equal to or even higher than that of the unmodified transport molecules. It appears, therefore, that the main effect of the formation of PX–Y (Hypothesis I) consists of a more or less parallel shift of the pH dependence of the transport process to lower pH values. Thus, each one of the four phenylglyoxalated forms may exist in a protonated and a deprotonated form, and a substrate anion (e.g., sulfate, chloride) may or may not be bound to each one of these forms. The mass law constants for the various reactions may depend on the orientation of the substrate binding site towards the outside or inside. Our data suffice to recognize these essential variables as governing a complex situation. They are, however, inadequate for a quantitative evaluation. We abstain, therefore, from the presentation of a mathematical description.

4.2. Molecular basis of the effects of phenylglyoxalation on band 3-mediated anion transport

The acid-base titration curves of *N*-acetyl arginine and its di-PG-derivative are rather similar, except that the PG derivative carries one negative charge more than the untreated arginine residue. Thus, at least over the limited pH range covered in our experiments (pH 6.0–8.0), the changes of pH dependence of anion transport by phenylglyoxalation cannot be related to a change of the pK of the guanidinium residue by the formation of its di-PG-derivative. An allosteric effect seems to be involved.

Although the present paper shows that PG reacts with at least two, possibly three different, functionally significant sites of band 3, it was not yet possible to identify any one of them. Studies of Zaki and Julien [21,22] and Bjerrum [23] with radioactively labeled PG suggest that inhibition of transport is related to the binding of 2–4 PG molecules. This would be equivalent to the modification of a minimum of one and a maximum of six arginine residues, depending on whether one assumes a 1:1 or 2:1 stoichiometry of PG to arginine.

The best characterized effects are those studied by Wieth et al. [10] and Bjerrum [4]. During exposure to PG at pH values in the range 9–12.5, they maintained the pH inside the red cell ghosts near pH 7.2, i.e., at a value where reaction of PG with inward-facing amino acid residues is unlikely. Under these conditions inhibition is produced within a few minutes by the binding of no more than two PG residues to the outward-facing surface of the chymotryptic 36 kDa fragment of the hydrophobic domain of band 3 [23]. It was further shown that the dependence of the rate of irreversible modification was a function of pH and Cl concentration in the external medium. The rate of irreversible modification could be described by a single pK value. The numerical value was indistinguishable from that of the pH dependence of anion transport at alkaline pH in unmodified red cells, indicating the functional significance of the phenylglyoxalated arginine residue [9]. In the absence of external Cl, the pK value was about 10.2 and hence about two pK units lower than one would expect for the titration of the guanidinium group of an arginine residue in an aqueous medium. Assuming a 1:2 stoichiometry for the modification reaction, the Danish workers concluded that the effects of PG as measured under their conditions are due to the phenylglyoxalation of one single arginine residue [23].

Although there is little doubt that this arginine residue is of functional significance, it is not yet

clear, what its physiological function could be. The dependence of the pK on the external Cl concentration was originally explained by simple electrostatic shielding of the PG binding site [10]. A later reinvestigation by Bjerrum [24] revealed, however, that the effect did not only depend on external Cl concentration but was also affected by the Cl concentration gradient across the membrane. According to the ping-pong model of band 3-mediated anion transport, this suggests that the rate of reaction of PG depends on the orientation of the transfer site with respect to the inner or outer membrane surface. It remains open, however, whether or not this effect indicates a modification of the transfer site itself or that of a modifier site whose susceptibility to PG depends on the orientation of the transfer site.

The experimental conditions used in our work differed from those of Bjerrum and his associates and were more similar to those of Zaki and associates. In many of their experiments, the latter authors exposed the red cell membrane to PG in the absence of chloride or other penetrating anion species at somewhat lower pH values (pH 8.5–9.5) than the Danish workers (pH 9–12), i.e., at pH values even further below the 'normal' pK of an arginine residue. However, the time of exposure to PG was more than an order of magnitude longer and may have compensated at least in part for the decreased sensitivity of the arginine to phenylglyoxalation. In our experiments we exposed the red cells to PG at pH 8.25 in chloride-free medium that contained sulfate as the predominant anion species. Under these conditions this anion affords less protection than Cl against the reaction of PG with the transport protein [21,22] and virtually none under the conditions of Wieth et al. [7]. It should be noted that Zaki and Julian [21,22] resealed their ghosts in hypertonic sulfate media. This makes the ghosts shrink, which leads to an overestimate of the rate constants for sulfate transport and hence also to an overestimate of the protective action of sulfate which further increases with increasing sulfate concentration.

At the lower pH values used by Zaki and associates and in the present paper, a variety of glycolytic enzymes with anionic substrates are susceptible to inhibition by PG [25]. Under these conditions only very few amongst the many arginines in these enzymes are modified. This indicates that the dissociation constant of the guanidinium groups of arginine residues that interact with the active center of these enzymes is abnormally low, supposedly because the groups are located in an environment of low DK and high positive charge density, which facilitates the dissociation of the proton from the guanidinium residue of arginine.

An instructive example for the abnormal behavior of a functionally important arginine represents the reaction of pancreatic ribonuclease A with PG studied by Takahashi as early as 1968 [17]. At complete inhibition only two out of four arginines are modified. From the X-ray structure of the ribonuclease A molecule it is known that a lysine residue located close to a PG susceptible arginine is required for catalytic activity. The electrostatic interactions between the two residues could lower their respective pK values. This would enhance the reaction of the arginine with PG which causes inhibition of the enzyme by altering the pK of the lysine residue at the active center. Takahashi also considers other possible interactions to explain the abnormal reactivity. Regardless of the final explanation, it is likely that the enhancement of band 3-mediated anion transport brought about after PG treatment at pH 6.5 (see Fig. 6 of the present paper) can only be explained on the assumption that the arginine residue involved is located in an environment different from that which is responsible for the inhibitory effects seen by Bjerrum and Zaki. A differentiation between the inhibitory effects seen by Bjerrum, Zaki and ourselves after phenylglyoxalation at more elevated pHs does not seem to be feasible without more systematic studies on the dependence of the rate of phenylglyoxalation on the pH and ion composition used in the various laboratories.

In an accompanying paper, which was considered incomplete by one of the referees because we had only investigated the effects of mutation of arginine residues 509 and 748 rather than those of all 12 conserved arginines in the transport domain of band 3, and which, on his advice alone, was rejected for this and many other reasons, we had shown that mutation of Arg 748 leads to inhibition of anion transport ([26]; preliminary report in Ref. [2]). We suspect that this residue is identical to the site of action of PG seen by Wieth, Bjerrum and coworkers. We have pointed out that this residue resides close to

His 752, which is capable of establishing a hydrogen bond with Glu 699. The formation of this hydrogen bond seems to be involved in the control of the pH dependence of anion exchange. It can be described by a single pK value of about 5.8–5.9, which is too low for a histidine residue and too high for a carboxyl group [2,27,28]. We believe that this is caused by the low dielectric constant in the membrane and the positive charge of the neighboring arginine residue. We proposed, therefore, that the mutation of Arg 748 to an uncharged amino acid residue leads to a shift of the pK of anion transport to a much higher value and hence to inhibition of transport. Phenylglyoxalation of Arg 748 would introduce a negative charge close to the positive charge of the guanidinium group and hence could produce a similarly indirect inhibitory action as the mutation. It may be noted that this interpretation is rather analogous to that of Takahashi [17] discussed above.

In previous publications, Zaki has suggested that the inhibition by PG of band 3-mediated anion transport is the result of the modification of one arginine residue that is essential for substrate binding (reviewed in Ref. [6]). Our results do not rule out the possibility that Arg 748 exerts such a function. However, they also do not lend any support for such idea. It is well known that treatment of band 3 with PG leads to a release of Cl [13]. However, this does not prove that the PG exerts its action at the transfer site itself. Diethylpyrocarbonate (a histidine specific reagent) also abolishes chloride binding to band 3 but by some clearly allosterical effect [14].

The reaction of PG with the accelerating binding site X is more difficult to assess than the reaction with the inhibitory binding site Y. This accelerating site becomes phenylglyoxalated at a lower pH than the standard pH of 8.25 used in most of our experiments. When red cells are treated with PG at pH 6.5 and the ghosts derived from them are subjected to flux measurements at either pH 6.0 or 7.4, one observes at the higher pH about 65 percent inhibition, at the lower pH an acceleration (Fig. 6). The acceleration increases with increasing PG concentration until a plateau (or flat maximum) is reached where the transport rate assumes about 170% of the control value. This indicates that under these conditions predominantly PX–Y is formed. The enhancement of transport suggests an allosteric interaction between this as yet unidentified amino acid residue and the substrate binding site. The decrease of the K_i value for stilbene disulfonate inhibition and binding shows that there exists also an allosteric relationship to the site of reversible stilbene disulfonate binding, which includes Lys 558 and Lys 869 in helices 5 and 13, respectively, as functionally important amino acid residues [1,29].

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