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Towards the localization of the essential arginine residues in the band 3 protein of human red blood cell membranes

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Abstract

The effects of 4-hydroxy-3-nitrophenylglyoxal (HNPG), on the binding of eosin-5-maleimide (EMA), and diethyl pyrocarbonate (DEPC) to the anion transport system in the human red blood cell membrane, have been investigated. HNPG is a reversibly binding, arginine-specific, anion transport competitive inhibitor, known to act on the anion binding site. The EMA reaction site is an external facing lysine residue (Lys-430) in the 17 kDa transmembrane segment. The DEPC reaction site is an intracellular histidine (His-819) in the 35 kDa fragment. The results show that inhibition of the transport system with EMA increases in presence of HNPG to about 2.3 times. This finding suggests a positive cooperativity between the HNPG and EMA binding site and give evidence that the essential arginine is either nearby or allosterically linked to Lys-430. The inhibition of the cells with DEPC was nearly unchanged or slightly decreased in the presence of 10 mM HNPG. These results suggest that the intracellular His-residue which reacts with DEPC is *not* a part of the transport pathway. Our experiments with 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) have shown that its affinity to the transport system does not change after pre-treatment with phenylglyoxal (PG). We also found that the binding of [^{14}C]phenylglyoxal (PG) to band 3 reduces significantly in presence of chloride. This is another evidence for the direct involvement of arginine residues in substrate binding.

Keywords: Anion transport; Erythrocyte membrane; Essential arginine; (Human)

1. Introduction

Studies on the interactions between two different types of anion transport inhibitors was one of the main results that led to the identification of band 3 (AE1) as the protein which is responsible for the exchange of anions in human red blood cell membranes [1]. Our further investigations have shown that the transport system can be inhibited by a large number of arginine-specific reagents [2–7]. We have also been able to show that the inhibition caused by the irreversibly acting reagent phenylglyoxal decreases significantly in the presence of the substrate anions chloride and sulfate. We also found that the loaded transporter is unable to react with PG [4]. Such results suggested the involvement of arginine residues in the binding of the substrate anions. These findings have been confirmed through the use of the reversible inhibitor 4-hydroxy-3-nitrophenylglyoxal (HNPG). This agent is found to act as a competitive inhibitor for both the substrate anions, chloride and sulfate [8].

Using [^{14}C]phenylglyoxal, it has been shown that complete inhibition of the transport system is accompanied by modification of about three arginine residues. Two thirds of the [^{14}C]phenylglyoxal was found on the 17 kDa transmembrane segment and one third on the other 35 kDa fragment [3,9]. These segments result after cleavage of band 3 with extracellular chymotrypsin followed by trypsin 'cleavage' from the cytoplasmic side [10,11]. Through structural activity studies with different phenylglyoxal derivatives and investigations on the chemical nature of the essential arginine residues, we have been able to suggest the important role of 4 arginine residues out of the 44 residues found in band 3, that may be involved in the anion transport process [6,12,13]. These arginines are Arg-490, -514, -518 on the 17 kDa and Arg-730 on the 35 kDa fragments [6,12]. In order to verify this hypothesis and to get more information about the localization of the essential

Abbreviations: PG, phenylglyoxal; HNPG, 4-hydroxy-3-nitrophenylglyoxal; EMA, eosin-5-maleimide; DEPC, diethyl pyrocarbonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate.

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arginine residues in the peptide chain, we studied the interaction between the arginine specific and competitive inhibitor HNPG and two anion transport inhibitors with well localized binding sites. The first one is eosin-5-maleimide (EMA) and the second one is diethyl pyrocarbonate (DEPC). Both of them inhibit the transport system in a noncompetitive fashion, and bind to different locations on the transmembrane segments of band 3.

EMA is known to react with Lys-430, an extracellular lysine residue on the 17 kDa fragment [14]. DEPC is a histidine oriented reagent known to react with an intracellular histidine on the 35 kDa fragment, most probably 819 [15,16]. We also investigated the inactivation of the transport system with the well studied stilbene derivative, 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), after modification of the cells with phenylglyoxal. Also the effect of chloride ions on [^{14}C]phenylglyoxal binding to band 3 and its fragments has been investigated.

2. Experimental procedures

2.1. Materials

Human RH^+ blood was obtained from the blood bank in Frankfurt am Main, and stored at 4°C in acid/citrate/dextrose buffer. The cells were used within 4 days of storage. 4-Hydroxy-3-nitrophenylglyoxal (HNPG) was synthesized according to [8], eosin-5-maleimide was purchased from Molecular Probes, USA. Diethyl pyrocarbonate (DEPC) was from Sigma and DNDS was obtained from Aldrich. Phenylglyoxal (pure) was obtained from Serva, [^{14}C]phenylglyoxal (15.1 mCi/mmol) from Amersham. Hepes was obtained from Calbiochem and all other buffer substances were from Merck, Darmstadt.

2.2. Methods

All the experiments presented in this paper were done with resealed human red blood cell ghosts. Resealed ghosts were prepared essentially as in [1]. Red cells were hemolysed at 0°C at a cell: medium ratio of 1:20 in a medium containing 4 mM Mg_2SO_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 (SM) containing (mM): 200 sucrose, 27 gluconate, 25 citrate, 5 Hepes and 1 Na_2SO_4. In the experiments done in chloride medium, sucrose was partialy replaced by chloride ions at the concentration indicated in the figures.

The reaction of the resealed ghosts with EMA was done in the dark at pH 7.4 at the concentrations indicated in the figures. The incubation time was 45 min. The experiments with DEPC were done in SM pH 7.4 at 0°C. The concentration of DEPC is indicated in the figures. Flux measurements and calculations of the rate constants were done as described previously [1]. Calculation of the sulfate flux was done according to Schnell [17]. The kinetic data were fitted with a least-squares method by a non-linear regression program. Transport is expressed as percent residual activity relative to a control value measured in the same medium and under the same conditions, but without the inhibitors.

In the experiments where the interaction between the reversibly binding inhibitors (HNPG and DNDS) and the irreversibly binding inhibitors EMA, DEPC and PG have been studied, the resealed ghosts were first incubated with the reversibly acting inhibitor for 10 min followed by incubation with the irreversible inhibitor under the conditions described above for each inhibitor.

The reversibly binding inhibitor was then removed by washing the cells twice with standard buffer (SM), containing 0,5% albumin, followed by further washes in standard buffer (SM) at 0°C. Then the cells were subjected to flux measurements. Polyacrylamide gel electrophoresis in SDS was carried out according to Laemmli [18] or Schägger et al. [19]. The slab gels were 1.5 mm thick. Determination of the [^{14}C]phenylglyoxal radioactivity in gel slices was done as described previously [3]. Protein concentrations were determined according to Peterson [20] (which is a modification of the method of Lowry et al. [21]).

3. Results

3.1. Interaction between the essential arginines and the binding site for eosin-5-maleimide

As described in Section 2 the resealed ghosts were first incubated with the reversibly acting competitive inhibitor, HNPG, for 10 min at a concentration of 5 or 10 mM. Eosin-5-maleimide (EMA) was then added from a stock solution to give a final concentration of either 10 or 25 μM. After an incubation time of 45 min the cells were washed as described in Section 2 to remove HNPG and the unreacted EMA. The modified ghosts were then resuspended in standard buffer and sulfate efflux was measured.

Fig. 1 shows that under our experimental conditions the inhibition of the transport system with 10 μM EMA was about $34 \pm 4\%$ (column 1). This inhibition increases to $71 \pm 8.6\%$ in the presence of 10 mM HNPG (column 2). Also at a concentration of 25 μM EMA, the inhibition of the transport system was $51 \pm 10\%$ (column 3). This inhibition increased to about $84 \pm 1.3\%$ in the presence of 10 mM HNPG (column 4).

In Fig. 2, one can see that chloride ions have no effect on the inhibition of sulfate exchange with EMA. This is in agreement with the earlier observations of Knauf et al. [22] who found that EMA is a non-competitive inhibitor of Cl^-

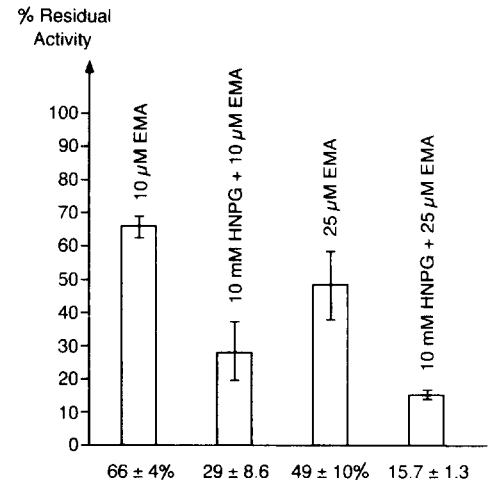

Fig. 1. Interaction between the HNPG binding site and the binding site for EMA. The ordinate represents the residual activity as percentage of a control value without EMA. The first and the third columns represent the effects of either 10 or 25 μM EMA on the sulfate flux. The second and fourth columns show the effect of HNPG when present in the incubation medium containing EMA.

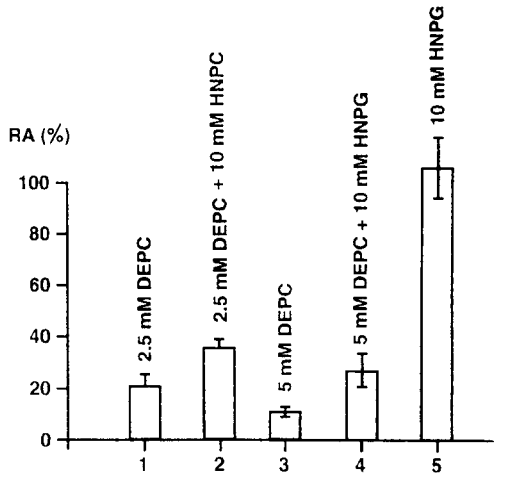

Fig. 3. Effect of HNPG on the inactivation of sulfate equilibrium exchange by DEPC. Ordinate: The rate constant of $^{35}SO_4^{2-}$ exchange in percent of a control value in the same medium as in the columns without inhibitor. Resealed ghosts were exposed to either 2.5 or 5.0 mM DEPC. Column 1 and 3 show the inactivation of the resealed ghosts with 2.5 and 5 mM DEPC. Column 2 and 4 represent the inactivation in presence of 10 mM HNPG. Column 5 represents the transport activity of control cells without DEPC after removing the reversibly binding inhibitor HNPG.

flux and that the site of reaction of EMA, Lys-430, is not at the anion transport site.

3.2. Interaction between the essential arginines and the histidine residue modified by DEPC

In this type of experiment, shown in Fig. 3, we have tried to determine if the anion transport site, known to react with HNPG, is near, or overlaps sterically or allosterically with the histidine residue, known to interact with DEPC and suggested to be His-819 [15,16]. The results show that at a concentration of 10 mM HNPG (a concentration under which all the anion binding sites are completely occupied by this reagent) the inactivation of the transport system with DEPC is *not* or *very slightly* (about 10–15%) changed. The concentration of DEPC in these experiments was either 2.5 or 5 mM (Fig. 3).

The results presented in the diagram are averages of at least six experiments.

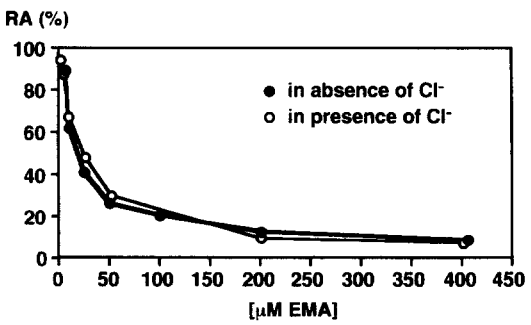

Fig. 2. Chloride ions do not interact with EMA binding site. The curves show the inactivation of the sulfate equilibrium exchange in resealed ghosts with EMA in standard medium (●), and in medium containing 100 mM Cl (○). Ordinate: rate of $^{35}SO_4^{2-}$ efflux in percent of a control value in the same medium as in (●) and (○) but without the inhibitor. Abscissa: EMA concentration (μM), temp. 37°C, pH 7.4, incubation time 45 min.

3.3. Effect of phenylglyoxalation of resealed ghosts on the inactivation of the transport system by DNDS

Resealed ghosts were first modified with 1.2 mM PG at pH 7.4 for 10 min at 37°C (under these conditions the transport is inhibited to about 30%). The excess of the inhibitor was removed by washing. The modified ghosts were then subjected to different concentrations of DNDS and sulfate efflux was measured. A control was run with ghosts without pre-treatment with phenylglyoxal, using the same concentration of DNDS. The results are shown in Fig. 4. One can see that pre-treatment of the cells with phenylglyoxal has no effect on the inactivation of the transport system with DNDS.

The value of the ID_{50}, i.e. the concentration of DNDS at which the transport system is half-inhibited, is about the same in both pre-treated and untreated cells, which was about 1.0 μM.

3.4. Chloride ions prevent the binding of [^{14}C]phenylglyoxal to band 3

In these experiments, the effect of chloride ions on the labelling of band 3 with [^{14}C]PG and its transmembrane fragments, 35 kDa and 17 kDa, has been investigated. These segments generate after extracellular chymotrypsin treatment of the resealed ghosts followed by treatment of

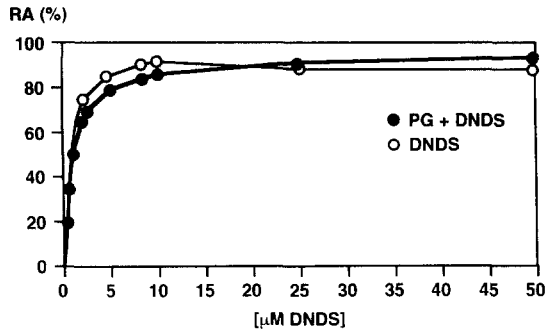

Fig. 4. Pretreatment of resealed ghosts with PG has no effect on the inactivation of sulfate flux by DNDS. The ordinate represents the rate of inhibition of sulfate equilibrium exchange in resealed ghosts, with DNDS, in untreated cells (○), and in resealed ghosts after pre-treatment with 1.2 mM PG for 10 min (●). Transport is expressed as percentage of the residual activity relative to a control value measured in the same medium with similar ghosts (either untreated or PG pretreated). Abscissa: DNDS concentration in μM.

the white ghosts with a low concentration of trypsin. Incubation of the resealed ghosts with [^{14}C]phenylglyoxal was done either in presence or absence of 100 mM chloride. The concentration of phenylglyoxal was either 2.5 mM or 1.25 mM. After an incubation time of ca. 30 min, the ghosts were washed to remove the unreacted reagent (Section 2). The ghost suspension was subdivided into two portions, one was used for flux measurements and the other one for gel electrophoresis. The amount of [^{14}C]phenylglyoxal binding to band 3 was calculated as described previously [3].

In the presence of 100 mM chloride ions the inhibition caused by 2.5 mM was reduced from 70% (column 1) to 10% (column 2) in comparison to a control value without inhibitor (Fig. 5). The effect of chloride ions on the capacity of band 3 to bind [^{14}C]PG has been calculated as previously described. In the absence of chloride ions, Fig. 5 (first column), the amount of [^{14}C]PG/mol band 3 was found to be 5.5 (about 2–3 arginine residues, since the

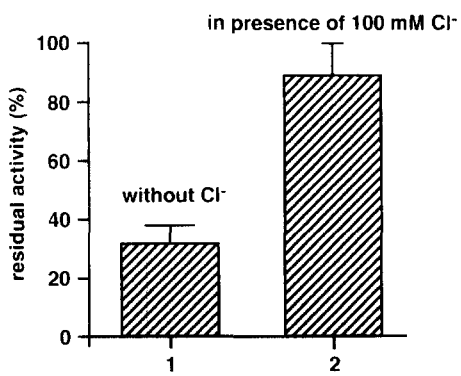

Fig. 5. Effect of chloride ions on phenylglyoxalation of resealed ghosts. Resealed ghosts were exposed to 2.5 mM phenylglyoxal for 45 min. The first column indicates the effect of phenylglyoxal. The second column represents the protective effect of chloride ions. Ordinate: The rate constant of $^{35}SO_4^{2-}$ exchange in percent of a control value in the same medium as in the experiments without inhibitors.

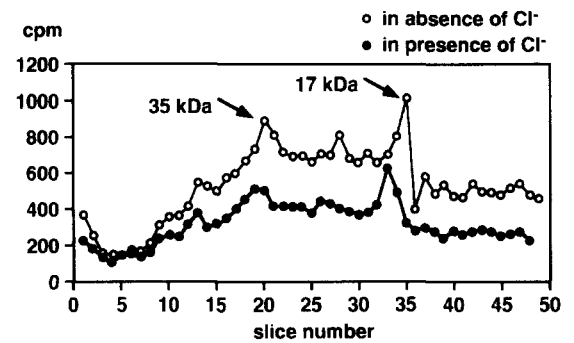

Fig. 6. Distribution of ^{14}C phenylglyoxal residues in the proteins of red blood cell membrane, in absence of chloride ions (upper curve), in presence of 100 mM chloride ions (lower curve).

stoichiometry of the binding of PG to arginine residues was found to be 2:1 [24]), the degree of inhibition of sulfate transport was about 70%. These results are in agreement with previous results [3], where we found that complete inhibition of the transport system is accompanied by the modification of about 2–3 arginine residues. In the presence of chloride ions (second column), the amount of [^{14}C]PG/mol band 3 was reduced to about 2.3 mol (about one arginine residue). The protection of the transport system was about 60%. These results show that 60% protection of the transport activity is accompanied by less labeling to about one arginine residue.

In Fig. 6 the upper curve represents the radioactivity profile of the transmembrane fragments, 35 kDa and the 17 kDa, in the absence of chloride ions, the lower curve in the presence of chloride ions. One can see that in the presence of chloride, there is a great reduction of the labeling of both fragments (30% reduction in the case of the 35 kDa fragment and 35% in the case of the 17 kDa fragment).

4. Discussion

4.1. The EMA binding site

It is clear from Fig. 1 that EMA is a good inhibitor for sulfate transport and its inactivation on the system is found to be concentration-dependent. Fig. 2 shows that chloride ions have no effect on the inhibition caused by EMA. These results confirm the results of Knauf et al. [22] who found that EMA does not act on the anion binding site. On the other hand, we found that the inhibition of the transport system with EMA increases in the presence of the competitive inhibitor HNPG. These results show that the helix that contains the essential Arg, must be near Lys-430 or allosterically linked to it, and can be explained in the following way: When the arginine specific reagent HNPG binds to the transport site, translocation of the anion cannot occur and the transporter may be fixed in a conformational state in which the substrate binding site is in the outward facing state. In this case the inhibitory binding site for

EMA, which is found to be on the extracellular site Lys-430 [14], may be near the transport channel (that may be composed of many adjacent helices) and more available when the transporter is fixed in the outward-facing conformation. In this case the inhibition caused by EMA increases. This is in agreement with Knauf and co-workers, who have shown that the EMA binding site does not overlap the transport site and suggested that EMA binds to a site that is affected by changes of the transport site conformation from the inward-facing to the outward-facing state and that EMA binds preferentially to the outward-facing state [22]. Our results may demonstrate that HNPG binds to the transport site and prevents translocation of the anion and keeps the transporter in the outside-facing conformation.

4.2. DEPC binding site

The results with DEPC are completely different. HNPG has little or nearly no effect on the inhibition caused by DEPC. This shows that there is no direct interaction between the transport site (HNPG site) and the histidine residue(s) which react(s) with DEPC. These results are similar to our previous results on the interaction between arginine-specific reagents and the stilbene disulfonate binding site, where we have been able to show, that after complete inhibition of the transport system with 2,3-butanedione, band-3 can still bind $^{3}H_2$DIDS nearly up to its total capacity [7].

Our results with DNDS have shown that the affinity of DNDS to the transporter does not change after pre-treatment of the cells with PG. The DNDS concentration required to produce 50% inhibition of sulfate equilibrium exchange is the same in the unmodified and PG modified cells, which is about 1 μM (this value is near the value (0.9 μM) found by Barzilay and Cabantchik in 1979 [23]) This may confirm our previous results that PG either completely inhibits the transporter or not at all (all or non-behavior) and that the binding of PG to band 3 does not produce major conformational changes to the monomer molecule and its nearest neighbor. These results are contrary to the findings of Wieth and his co-workers [25] who found that the affinity of DIDS decreases, or its binding is prevented after treatment of the cells with PG under their experimental conditions (at very high extracellular pH). They reported also that under such experimental conditions the reversible inhibition with phenylglyoxal is rather uninfluenced by changes in the extracellular chloride concentrations. They were also *not* able to show that the modifiable residue can be recruited to the inside or the outside of the membrane barrier [26]. Under such experimental conditions phenylglyoxal may not bind to the anion binding site, but seems to bind to a site which interacts with the DIDS binding site and is not affected by the substrate anion chloride (for example — an extracellular lysine residue).

The binding of [^{14}C]phenylglyoxal to band 3 is reduced significantly in the presence of the substrate anion chloride (Fig. 6). This is another indication for the direct participation of the arginine residues in the anion binding site. The reduction is found in both the 17 kDa and the 35 kDa fragment. These results are in agreement with our previous results [3] and support our hypothesis (Zaki [12,13]) that Arg-490, in the 17 kDa fragment and/or Arg-730 in the 35 kDa fragment, may be present in the transport pathway. Site-directed mutagenesis of these residues are now being done and yields results which may support this hypothesis (Zaki and Passow and Karbach, et al., unpublished data).

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