Vol. 110, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 27, 1983 Pages 616-624

ANION TRANSPORT IN RED BLOOD CELLS AND ARGININE SPECIFIC REAGENTS

(1) EFFECT OF CHLORIDE AND SULFATE IONS ON PHENYLGLYOXAL SENSITIVE SITES IN THE RED BLOOD CELL MEMBRANE

L. Zaki

Max-Planck-Institut für Biophysik
6000 Frankfurt am Main 71, Fed. Rep. of Germany

Received October 18, 1982

Inhibition of anion transport by the arginine specific reagents phenylglyoxal and 1,2 cyclohexandione depends on the pH and anion concentration in the medium. At pH 8.0, chloride ions protect the transport system against inhibition by PG and 1,2 CHD, while sulfate ions do not protect (1).

In the present paper it is shown that at pH 6.5 and 7 both sulfate ions and chloride ions protect the transport system. The protection increases with increasing concentration of the two substrate ions.

INTRODUCTION Anion transport across red blood cells is mediated by an integral membrane protein with a molecular weight of 95,000 daltons, the so-called band 3 protein. This conclusion was mainly based on the fact that the binding sites of a number of covalently binding inhibitors of anion transport are located on this protein (2,3,4). Detailed studies with $^{3}H_2$DIDS have shown that the site for covalent binding of this potent and selective inhibitor of anion transport includes two lysine residues on the transport protein (5). The reactivity of one of these lysine residues is varied by variations of the concentrations of substrate anions and the addition of reversibly acting inhibitors of anion transport (6). Using the arginine specific reagents 1,2-cyclohexandione and phenylglyoxal, Zaki demonstrated

Abbreviations used in the paper:
PG = phenyglyoxal
H_2DIDS = 4.4'-diisothiocyano-dihydrostilbene-2.2'-disulfonate

0006-291X/83/020616-09$01.50/0

the participation of other functional amino acid residues, most probably arginines, in anion transport across the red blood cell membrane and it was also found that there is no stoichiometrical relationship between the inhibition caused by the two reagents and the capacity of band 3 to bind $^{3}H_2DIDS$ (1,7). Wieth et al. also found that chloride exchange in red cells can be inactivated by phenylglyoxal (8).

At pH 8.0, the reactivity of the binding sites for arginine specific reagents is reduced by chloride ions, but not by sulfate ions (1). The present paper deals in more detail with the influence of pH and substrate anion on the interactions of these reagents with the anion transporting site in the red blood cell membrane.

MATERIALS AND METHODS Human blood (ORh^+) from apparently healthy donors was obtained from the Red Cross in Frankfurt and stored at 4°C in acid/citrate/dextrose buffer for 2-4 days. After removal of plasma and buffy coat, the cells were washed three times in isotonic buffer. Resealed ghosts were prepared essentially as described previously (9). They were hemolyzed at a cell medium ratio of 1:20 in a medium containing 4 mM $MgSO_4$ and 1.45 mM acetic acid. 5 min after hemolysis sufficient EDTA was added to obtain a final concentration of 20 mM EDTA in the hemolysate. After centrifugation, the ghosts were resuspended and resealed in the different medium according to the type of experiment:

- Sulfate medium 1., 50 mM borate, 5 mM EDTA, 56 Na_2SO_4, pH 8.0
- Sulfate medium 2., 20 mM EDTA, 20 mM sucrose 10 mM NaH_2Po_4 with varying concentration of Na_2SO_4 at pH 6.5, 7.0 or 8.0.
- Chloride medium 1., 50 mM borate, 5 mM EDTA, 83.5 mM NaCl, 1 mM Na_2SO_4, pH 8.0.
- Chloride medium 2., 20 mM EDTA, 20 mM sucrose 10 mM NaH_2Po_4, 1 mM Na_2SO_4 with varying concentrations of NaCl at either pH 6.5, 7.0 or 8.0.

The reaction of the resealed ghosts with phenylglyoxal was performed at 37°C in either sulfate medium 1. or 2., or in chlo-

ride medium 1. or 2., as indicated in the figure legends. The hematocrit was 10%.

$^{35}SO_4$ equilibrium exchange was measured after removal of excess PG in the same medium that had been used for resealing and reaction with the inhibitors. The flux measurements were done as described previously (4).

Chemicals: Phenylglyoxal (pure) was obtained from Serva, Heidelberg. All buffer substances were obtained from Merck, Darmstadt.

RESULTS

Inhibition of sulfate equilibrium exchange in resealed ghosts at various concentrations of PG in SO_4^{--} and Cl^- media at pH 8.0

In Fig. 1, the upper curve represents the residual transport after treatment with PG in SO_4^{--}-containing medium 1.; the lower curve shows the effect in chloride-containing medium 2.

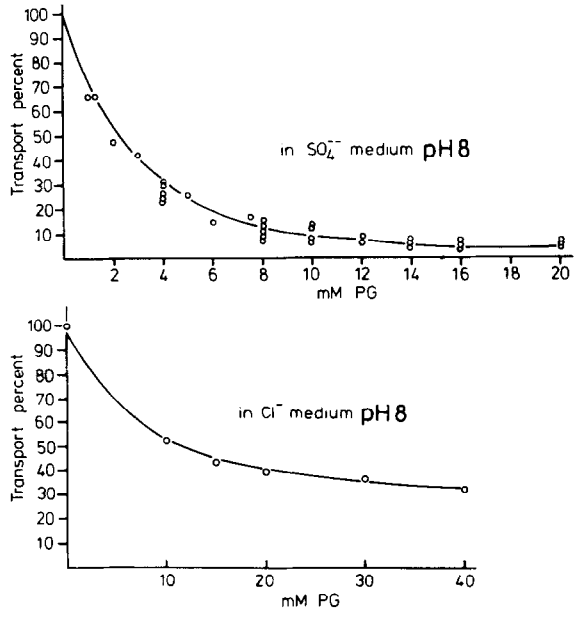

Fig. 1 Ordinate: penetration rate in % of control value without phenylglyoxal. Abscissa: concentration of phenylglyoxal in mM. Temp. 37°C pH 8.0 time of reaction 60'. Upper curve in SO_4^{--} medium 1, lower curve in Cl^- medium 1.

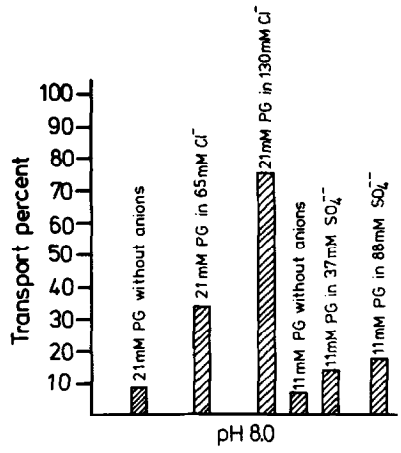

Fig.2 In Fig. 2, Column 1 indicates the residual activity after incubation of the cells with 21 mM of PG in 1 mM sulfate buffer ("without anions") Columns 2 and 3 represent the residual activity in the presence of 65 and 130 mM Cl ions, respectively. Columns 4, 5 and 6 represents the residual activity after pretreating the cells with 11 mM PG in the presence of 1 mM SO_4^{--} 37 mM SO_4^{--} and 88 mM SO_4^{--} ions respectively.

The experiments indicate that in the media which contain 83.5 mM Cl^-, the inhibition of sulfate efflux by 40 mM PG is about 70%, while in SO_4^{--} medium (56 mM) the degree of inhibition caused by the lower concentration of PG 10 mM is much higher and reaches about 90%.

Inhibition of sulfate equilibrium exchange in resealed ghosts by PG at various concentrations of SO_4^{--} and Cl^- ions at pH 8.0

The results represented in Fig. 2 show that the protection caused by chloride ions increases with increasing chloride ion concentration in the media, while SO_4^{--} ions exert little, if any, influence at this pH.

The effect of substrate anions on the rate of inhibition caused by PG at pH 6.5 and 7.0

Inhibition of sulfate efflux by 10 mM PG at pH 6.5 in the presence of Cl^- or SO_4^{--} ions is shown in Table 1.

Table 1

Effect of So_4^{--} and Cl^- ions on PG Inhibition at PH 6.5

Buffer contains	mM PG	Rate Coefficient of So_4^{--} selfExchange (^0k_s 10^{-2})	% ^0k_s
1 mM So_4^{--}	0	15.6 ± 0.62	100 % ± 3.9 %
" "	10	8.23 ± 0.98	52.75 % ± 11.9 %
88 mM So_4^{--}	0	27.3 ± 1.27	100 % ± 4.65 %
" "	10	29.1 ± 1.63	106 % ± 5.49 %
132 mM Cl^-	0	13.68 ± 0.58	100 % ± 4.23 %
" "	10	13.3 ± 1.07	97.2 % ± 8.04 %

In these experiments the cells were equilibriated and loaded with ^{35}S-sulfate in the various media during the resealing period prior to exposure to PG in the same media.

Inhibition is expressed as % decrease in the ^{35}S efflux produced by 10 mM PG relative to a control in the same medium without PG.

The experiments show that at pH 6.5, in contrast to the situation at pH 8.0, both Cl^- ions and SO_4^{--} ions protect the transporter against PG. Similar observations are made at pH 7.0 (Fig. 3). The results in this figure show the protective effect of Cl^- and SO_4^{--} ions against inhibition with PG at pH 7 and the increase of this protective effect with increasing concentration of the substrate anions in the medium.

Interaction between Cl^- ions and SO_4^{--} ions with the inhibitory PG binding site in resealed ghost at different pH's.

In these experiments the effect of SO_4^{--} on the chloride protection of the transporter at different pH's was examined.

At pH 8 the protection caused by 65 mM Cl^- does not change when increasing concentrations of SO_4^{--} are added to the chloride-containing medium (up to 160 mM SO_4^{--}). However, at pH 7.0 the

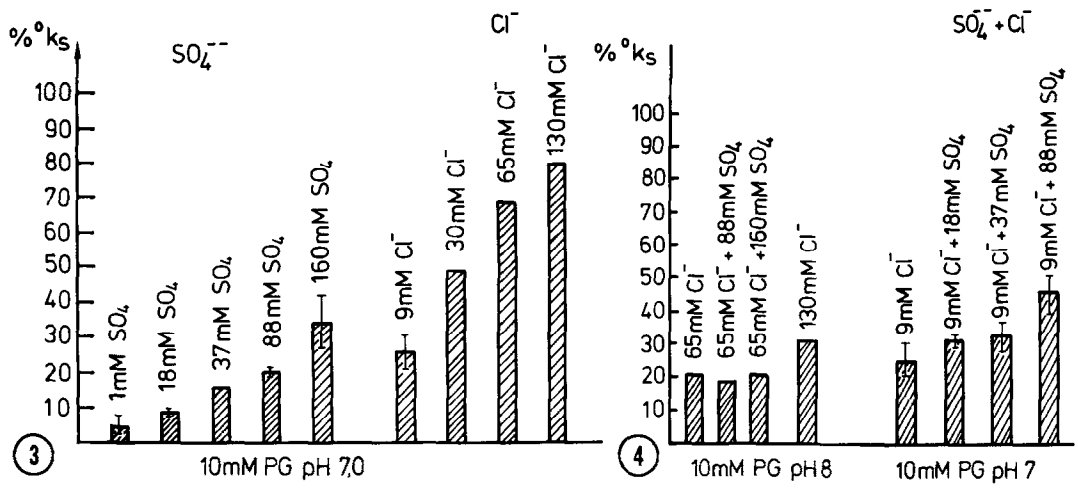

Fig.3 The results shown in Fig. 3 summarize the effect of Cl^- and SO_4^{--} ions on sulfate transport in resealed ghosts at pH 7. The efflux was measured after exposure to 10 mM PG at 7.0 for 45 min. at 37°C. The ghosts were equilibriated in the various media and loaded with ^{35}S-sulfate prior to exposure to PG. Transport is expressed as a percent of the residual activity relative to a control value without inhibitor. The results in this figure show the protective effect of Cl^- and SO_4 ions against ihibition with PG at pH 7 and that this protective effect increases with increasing the concentration of the substrate anions in the medium.

Fig. 4 Inhibitory effect of phenylglyoxal in the presence of Cl^- and SO_4^{--} ion at pH 8.0 and 7.0. Ordinate: penetration rate in % of control value in the same medium as in the columns without inhibitor. Experiments in columns 1-4 are done at pH 8.0 in the media indicated in the figure. Experiments in columns 5-8 are done at pH 7.0 in the medium indicated in the figure.

residual activity that remains after treatment with PG at 9 mM Cl^- increases with the increase of the concentration of SO_4^{--} ions in the chloride medium. The same results were found at pH 6.5 (not shown).

The results demonstrate that both SO_4^{--} and Cl^- ions have additive protective effects at low pH; however, at pH 8 only Cl^- ions but not SO_4^{--} ions are able to protect the PG binding sites.

DISCUSSION The pH dependence of the self-exchange of monovalent and divalent anions is quite different. Sulfate transport has a

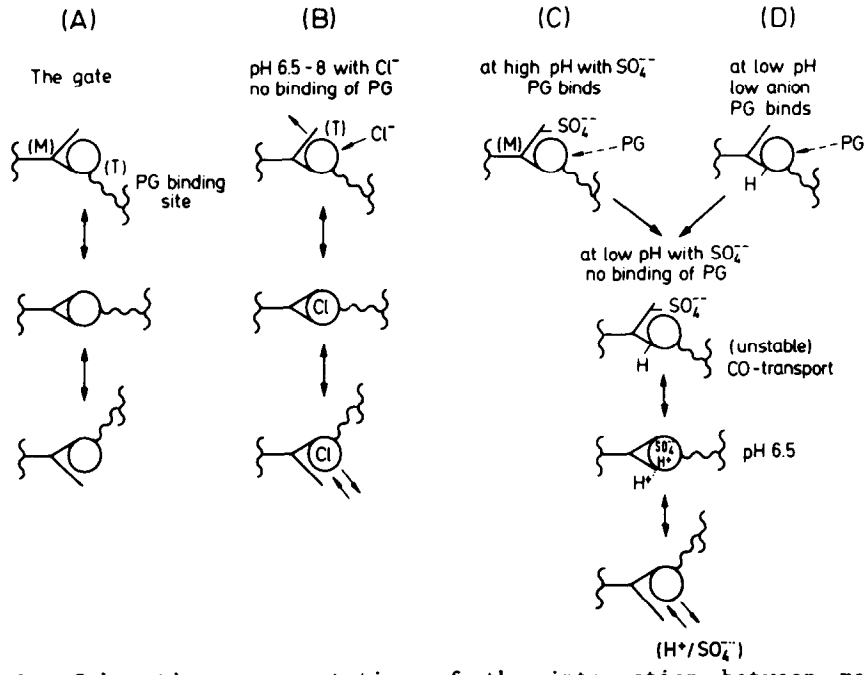

Fig. 5 Schematic representation of the interaction between monovalent, divalent anion and phenylglyoxal binding site (two step model).

maximum near pH 6.3 (10). This is supposed to be due to a superimposition of proton/SO_4^{--} co-transport (11) (which decreases with increasing pH) and an activation of the transport protein by deprotonation of a regulatory proton binding site. Chloride self-exchange, which is 10^5 times faster than sulfate exchange, increases with increasing pH until it reaches a plateau above pH 7.0 or a flat maximum (12).

On the basis of these views, the results can be interpreted as follows: at pH 6.5 and pH 7.0, where sulfate transport is near its maximum value and where the proton concentration in the medium is higher than at pH 8.0, proton binding to the transporter increases the affinity of SO_4^{--} binding about 9 times (13). Under these conditions the amount of SO_4^{--} ions which are capable of binding to the transfer site (called T) is high and can protect it against PG. At pH 8.0, where the transport of the SO_4^{--} ions are relatively slow (the amount of the protons available for

the co-transport is small) the number of the SO_4^{--} ions which bind to the transfer site is also small and unable to compete with PG for its binding site. However, one may assume the existence of a transitional binding site (M), to which divalent anions can be bound even at pH 8.0 until they are translocated through T. SO_4^{--} ions cannot protect T against PG at high pH because they are preferentially bound to M.

This can be demonstrated in the scheme represented in Fig. 5.

Schematic representation of the interaction between monovalent, divalent anion and the Phenylglyoxal binding site (two step model)

T + M represents the constitution of the anion gate (15) between different peptide chains (column A). T represents the "transfer site" (i.e. a group of amino acid residues involved in anion binding during the translocation process from outside to the inside and vice versa (cis/trans movement). Monovalent anions can bind directly to this site (T) (column B), which is also the binding site for PG.

M represents a "transition binding site" for divalent anions where they are deposited until they can be translocated through T. M may consist of several amino acid residues and may also contain the binding site for the proton necessary for the co-transport (column C). When a divalent anion and a proton are both present, co-transport through T can take place.

At high pH the fraction of T that is occupied by SO_4^{--} ions is relatively low; there is no protection against PG (column C). At low pH the fraction of T that is occupied is relatively high, T is protected against PG. In the absence of anions at low pH, PG can bind to T and the system is inhibited (column D).

The results support the hypothesis that the transport of both chloride ions and sulfate ions are mediated by the same transpor-

ter (9,14) and that they are in accordance with the sulfate-proton co-transport model (11,16,13). The results also support the general rule that arginine residues are the anion recognizing-sites in proteins and enzymes which have a negatively charged substrate or co-factors (17).

More studies are now being done to characterize these sites in more detail.

Acknowledgements

I thank Professor H. Passow for stimulating discussions and his comments on the manuscript, Dr. Phillip Wood and Dr. Wolfgang Schwarz for their comments on the manuscript and Mr. Thomas Julien for his cooperation during much of the experimental work.

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