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Inhibition of anion transport in the red blood cell membrane by anionic and non-anionic arginine-specific reagents

LAILA ZAKI
Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Stra ße 7, 6000 Frankfurt am Main 71, FRG

Abstract. Arginine specific reagents are found to be powerful inhibitors of anion exchange in the red blood cell membrane. Some of these inhibitors such as cyclohexandione, phenylglyoxal and 2, 3-butandione are found to produce their inhibition by interacting covalently with band 3. In contrast to the action of these compounds, the inhibition caused by the phenylglyoxal derivative 4-hydroxy-3-nitrophenyl-glyoxal has been found to be completly reversible. In extending the studies on the mode of action of these compounds on sulfate exchange and to get some more information about their binding site, the degree of inhibition caused by different phenylglyoxal derivatives which have a similar core but differ in their substituent groups have been compared. The interaction between the binding sites of these compounds and other anion transport inhibitors have also been studied.

Keywords. Red blood cell; anion transporter; arginine-specific reagents.

Introduction

During the last 20 years many important discoveries have been made in the field of membrane research. One of these important discoveries was the identification of band 3 protein as the protein which catalyses the exchange of anion across the red blood cell membrane (Cabantchik and Rothstein, 1974; Zaki *et al.,* 1975). The method which was used and which enabled to characterize this protein among the complex mixture of the membrane components was based on chemical modification. The interactions between two types of anion transport inhibitors have been studied. One of these inhibitors is 4-acetamido-4′-isothiocyano 2,2-stitbene disulfonate (SITS), which is a nonpenetrating, very specific anion transport inhibitor. The other one is flouro-2,4-dinitrobenzene (DNFB). The common covalent binding site of DNFB, SITS and 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS) is a lysine residue which is located in the 60 kDa fragment of band 3 protein (Brazilay *et al.,* 1979). Kinetic studies have shown that this lysine does not participate in the substrate binding site (Passow *et al.,* 1980). α-Dicarbonyl reagents which are known to react selectively with guanidyl residues in proteins are found to be potent inhibitors of sulfate exchange across the red blood cell membrane (Zaki, 1981, 1983,1984; Zaki and Julien, 1985; Julien and Zaki, 1987). Complete inhibition of anion-transport is accompanied by modification of 2 to 3 arginine residues (Zaki, 1984). The results with the reversible binding phenylglyoxal derivative 4-hydroxy-3-

Abbreviations used; SITS, 4-Acetamido-4′-isothiocyano-2,2′-disulfonicstilbene; DNFB, 2,4-dinitrofluoro-benzene; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; HNPG, 4-hydroxy-3-nitro-phenylglyoxal; PG, phenylglyoxal; OH-PG, *p*-hydroxyphenylglyoxal; N_3-PG, *p*-azidophenylglyoxal; NO_2-PG, *p*-nitrophenylglyoxal; COOH-PG, *p*-carboxyphenylglyoxal; CH_3-PG, *p*-methylphenylglyoxal; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate.

nitrophenylglyoxal (HNPG), suggest that these reagents are interacting with the substrate binding site (Julien and Zaki, 1988).

Materials and methods

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

Cells were hemolyzed at 0°C at a cell/medium ratio of 1 : 20 in medium containing 4 mM $MgSO_4$ and 1·45 mM acetic acid. Five minutes after hemolysis, sucrose, gluconate, citrate and HEPES were added from a concentrated stock solution to obtain a final concentration of 200 mM sucrose, 27 mM gluconate, 25 mM citrate and 5 mM HEPES in the hemolysate.

After centrifugation, the ghosts were resuspended and resealed in standard medium containing (mM) : 200 sucrose, 27 gluconate, 25 citrate, 5 HEPES and 1 Na_2SO_4. The pH was either 7·4 or 8. Modification of resealed ghosts was done with HNPG. The reaction of the resealed ghosts with HNPG was carried out at a hematocrit of 10% in standard medium at 37°C.

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

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

Phenylglyoxal was obtained from Serva. Phenylglyoxal (PG) derivative HNPG was synthesied according to Julien and Zaki (1988); other derivatives according to Julien *et al.* (1990). All other substances were from Merck, Darmstadt.

Results

Labelling pattern of resealed ghosts with DNFB in the presence and absence of SITS.

Treatment of the erythrocytes with [^{14}C] DNFB causes labelling of all the membrane protein and retreatment of the cells with 0·5 mM SITS caused protection of band 3 against dinitrophenylation figure 1.

Inactivation course of sulfated equilibrium exchange by 4-hydroxylphenylglyoxal and 4-methylphenylglyoxal

Figure 2 shows the inactivation course of sulfate equilibrium exchange by *p*-hydroxyphenylglyoxal (OH-PG) and *p*-azidophenylglyoxal (CH_3-PG) at pH 7·4. It also shows that the activity loss followed pseudo first-order kinetics and the degree of inhibition is dependent on reagent concentration.

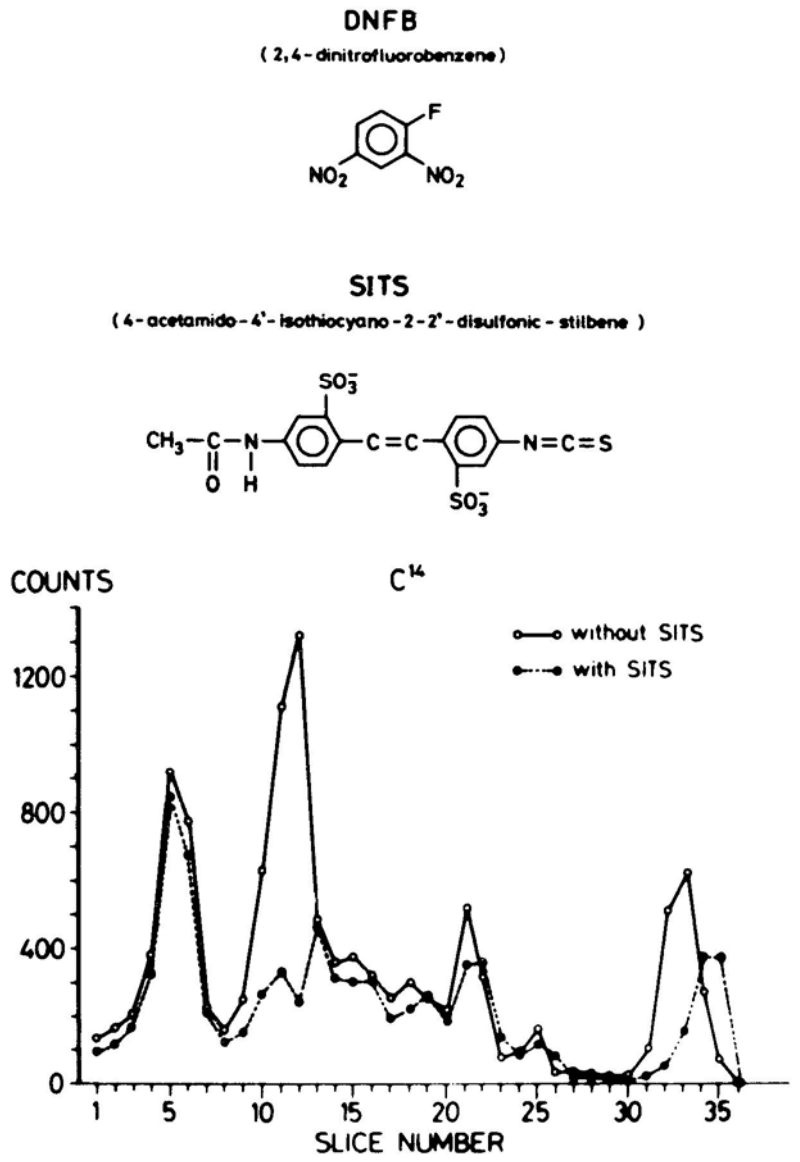

Figure 1. Labelling pattern of resealed ghosts with [^{14}C] DNFB in the absence (O) and in presence (●) of 0·5 mM SITS. Dinitrophenylation was done at pH 7·2 for 30 min at 25°C.

Inactivation of sulfate exchange in resealed ghosts by different derivatives of phenylglyoxal at pH 8

To evaluate the chemical properties of the binding site of arginine-specific reagents, different derivatives of phenylglyoxal which have a similar core but different substituent groups have been synthesised.

The degree of inhibition of the sulfate exchange by the various derivatives has

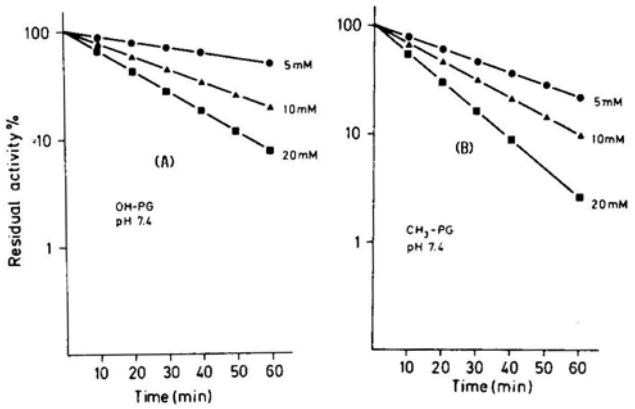

Figure 2. Kinetics of inactivation of sulfate exchange in resealed ghosts by OH-PG (A) and CH_3-PG (B). The ghosts were incubated with different concentrations of the reagent at pH 7·4. The transport activity was assayed in aliquots. The observed pseudo-first order rate constant values were calculated from the data presented.

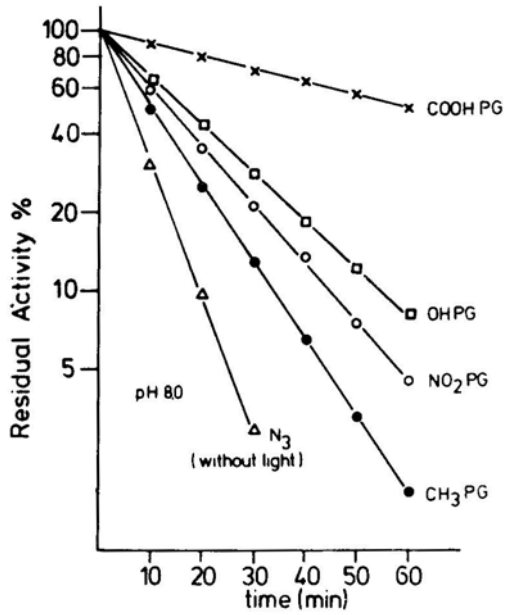

Figure 3. Time course of the inhibition of sulfate transport with differed PG derivatives, Resealed ghosts were modified with 10 mM of COOH-PG, OH-PG, NO_2-PG, CH_3-PG and N_3-PG.

been compared. Figure 3 presents the time course of inactivation of sulfate equilibrium exchange by *p*-carboxyphenylglyoxal (COOH-PG), OH-PG, N_3-PG and *P*-methylphenylglyoxal (CH_3-PG). The concentration of the inhibitors was 10 mM, and the pH of the reaction medium was 8. N_3-PG was found to be the most effective one.

The hydrophobic properties of the different PG derivatives were determined by

thin-layer partition chromatography according to Motais and Cousin (1976) as shown in figure 4.

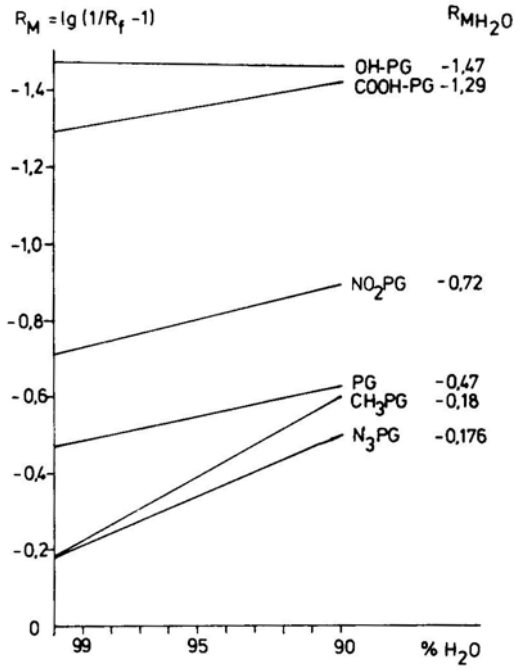

Figure 4. Lipophilic character of the PG derivatives. The lipophilic properties of the different PG derivatives were determined by thin-layer partition chromatography. Silica gel was the stationary phase, a mixture of water and acetic acid was the mobile phase. Ordinate the R_M values where R_M = log $(1/R_f - 1)$. Abscissa: % of H_2O in the mobile phase. The intercept of the straight lines with the ordinate yielded the R_M value for pure H_2O.

Under the experimental conditions used, the half time of inactivation of sulfate exchange by N_3-PG was found to be 5·9 min. This value is equal to the half time of inactivation of the system by PG.

These results show that the hydrophobic properties of these probes do not correlate with their inhibitory potency.

Effect of 4,4'*-dinitrostilbene-*2,2'*-disulfonate and flufenamate on 4-hydroxyphenylglyoxal binding site*

Resealed ghosts were first incubated with the reversible acting inhibitors (table 1).

Table 1. Effect of DNDS and Flufenemate on the rate of transport inactivation by OH-PG at pH 8.

Conditions	Residual activity (%)
5 mM OH-PG	33 ± 1·74
5 mM OH-PG in the presence of 50 μM DNDS	67 ± 4·80
5 mM OH-PG in the presence of 50 μM Fufenemate	76 ± 4·90

After 5 min of incubation OH-PG was added and the ghosts were further incubated for 45 min at 37°C. After removal of the reversible-acting inhibitors and the excess of PG derivative by washing, flux measurements were performed.

The results in table 1 show that the reversible-acting anion transport inhibitors are able to protect the transport system against inhibition with the phenylglyoxal derivative OH-PG.

Discussion

The present data show that both anionic and non-anionic PG derivatives are able to inhibit sulfate exchange across the red blood cells.

Previous results (Zaki and Julien, 1986) have shown that the binding site of these inhibitors is mutually exclusive to the binding site of PG and HNPG.

The structural activity studies show that neither the lipophilic nor the electronic properties of PG derivatives seem to play a role in their inhibitory potency. This is not the case found in the structural activity studies done with the stilbene disulfonate derivatives (Brazilay *et al.*, 1979). We have also been able to show that the binding of PG does not effect the $^{3}H_2$ DTDS binding to band 3.

These findings suggest that the essential arginine(s) in band 3 protein is not located in the segment of the peptide chain that contains the stilbene disulfonate binding site.

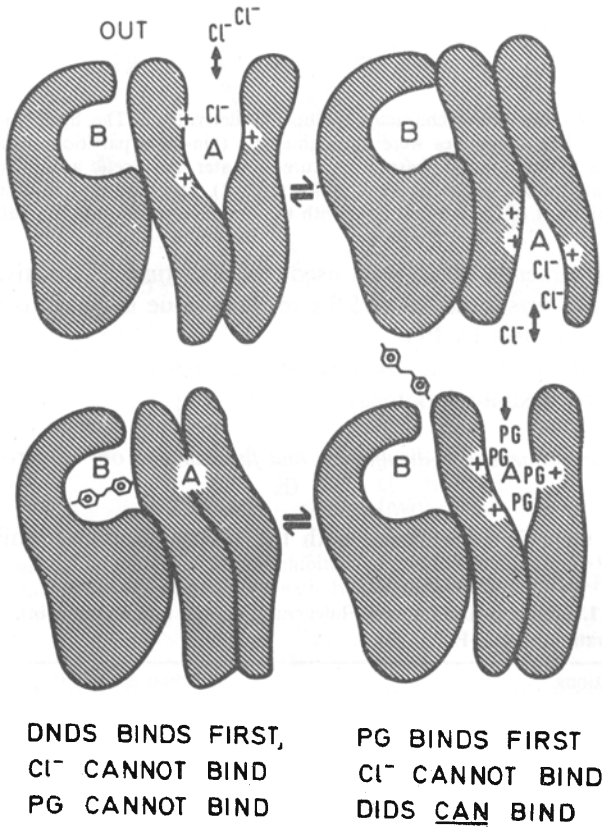

Figure 5. Cascade model.

On the other hand, allosteric interaction between the two binding sites is shown in table 1.

Cascade model

Figure 5 shows the interaction between the two binding sites and transportable anions. The anion transport site which reacts with PG or its derivatives (one or several arginine residues) may be located in a segment of band 3 designated A. The binding site of the stilbene disulfonate may be located in an adjacent transmembrane segment in a hydrophobic niche called B. Site A is likeley to exist in a cleft which is inaccessible to positively charged probes and less accessible to probes which are bulky and negatively charged. The rate of inactivation of sulfate flux with these latter reagents is much slower than with PG and other derivatives presented in this work.

The binding of the bulky and negatively charged stilbene derivative to site B causes a large conformational change in band 3 and its environment (Singer and Morrison, 1980). These changes may lead to the burial of the transport site and make it incapable of reacting with PG or with the substrate anion. When phenylglyoxalation of the resealed ghosts is done first, the stilbene disulfonate binding site remains essentially unchanged since it is known that PG reaction with protein causes no perturbation of their tertiary structure (Catherine and HSV, 1983). DNDS can still bind, but the substrate anion cannot bind.

In the presence of SO_4^{2-} or Cl^- the site A is loaded with the substrate anion and translocation takes place. This may be accompanied by changing the orientation of the transporter from facing outward to inward. This conformational change causes the DIDS-binding site to be buried. PG is also prevented from binding, hence the site is occupied by a substrate anion.

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