Anion transport in red blood cells and arginine-specific reagents

The location of [^{14}C]phenylglyoxal binding sites in the anion transport protein in the membrane of human red cells

Laila Zaki

Max-Planck-Institut für Biophysik, 6000 Frankfurt am Main 71, FRG

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The reaction of phenylglyoxal, a reagent specific for arginine residues, with erythrocyte membrane at pH 7.4 results in complete inhibition of sulfate equilibrium exchange across human red cells. The inactivation was found to be concentration and time dependent. The binding sites of this reagent in the anion transport protein (band 3) under these conditions were determined by using [^{14}C]phenylglyoxal. The rate of incorporation of the radioactivity into band 3 gave a good correlation with the rate of inactivation. Under conditions where the transport is completely inhibited about 6 mol [^{14}C]phenylglyoxal are incorporated into 1 mol band 3. Treating the [^{14}C]phenylglyoxalated ghosts at different degrees of inactivation with extracellular chymotrypsin showed that about two-thirds of these binding sites are located on the 60 kDa fragment.

Anion transport *Band 3* *Arginine-specific reagent*

1. INTRODUCTION

Anion exchange across the red cell membrane is mediated by an integral membrane protein (band 3) 95 kDa. Its involvement was based on our finding that through differential labelling we were able to find a common binding site for the two anion transport inhibitors, SITS and DNFB [1,2]. The same conclusion was drawn in [3] using another anion transport inhibitor (DIDS). Detailed studies have shown that this common binding site is a certain lysine residue on the 60 kDa fragment of the band 3 protein [4]. Kinetic studies showed that this lysine residue does not participate in the substrate binding site [5]. On the other hand, several investigations have shown that many negatively charged substrates bind to proteins via electrostatic interactions with arginyl residues [6].

The role of arginyl residues in anion transport in the red blood cell membrane has been studied in [7–11]. It was found that sulfate exchange across the red cell membrane can be inhibited by the arginine-specific reagents 1,2-cyclohexanedione [7] and PG [8–11]. It was also shown that the site of action of these reagents is not identical to the covalent binding site of the anion transport inhibitors H_2DIDS, SITS and DNFB [7]. It was also found that chloride exchange in red cells can be inactivated by PG [12].

The involvement of the site of action of these arginine-specific reagents in the substrate binding sites has been shown in the finding that under conditions where SO_4^{2-} has its maximum transport capacity, it is able to protect the system against inhibition by the arginine-specific reagents. Cl^- is also able to provide protection [8,9].

Abbreviations: PG, phenylglyoxal; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; DNFB, 1-fluoro-2,4-dinitrobenzene

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2. MATERIALS AND METHODS

Human red blood cells from normal donors were obtained from the Blood Bank, Frankfurt, and stored at 4°C in acid–citrate–dextrose buffer for 4 days.

Resealed ghosts were prepared essentially as in [2]. Red cells were hemolysed at a cell : medium ratio of 1 : 20 in a medium containing 4 mM $MgSO_4$ and 1.45 mM acetic acid. Five 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 standard medium containing 200 mM sucrose, 25 mM gluconate, 25 mM citrate, 5 mM Hepes and 1 mM Na_2SO_4 (pH 7.4).

The reaction of the resealed ghosts with PG was performed in standard buffer at 37°C for 60 min or as indicated in fig. 5. $^{35}SO_4$ equilibrium ex- Excess PG was removed by washing 3 times with standard buffer (pH 7.4) containing 0.5% bovine serum albumin followed by washing 3 times with the same buffer without albumin.

In some experiments, the cells were incubated for 60 min at 37°C in standard buffer before the last washing.

Chymotrypsin treatment of the ghosts was carried out at concentrations of 1.0 mg chymotrypsin/ml suspension (pH 7.4) at 37°C for 45 min. The hematocrit of the suspension was 10%. The reaction was stopped by the addition of the chymotrypsin inhibitor PMSF to a final concentration of 2 mM. The treated cells were then washed twice with standard buffer containing 0.2 mM PMSF followed by washing twice with 0.5% albumin containing buffer and two further washes in standard buffer at 0°C. The untreated cells were washed in the same way.

Hemoglobin-free ghosts were isolated by hemolysis and washing the resealed ghosts in media containing 0.1% saponin, 10 mM KCl and 5 mM EDTA (pH 7.2). For measuring [^{14}C]PG binding the membranes were dissolved in 1 vol of 5% or 2% SDS, treated at 100°C for 5 min, and then diluted to a final concentration of 0.5% or 0.2% SDS.

Polyacrylamide (5%) gel electrophoresis was performed as in [2], using 7.5% polyacrylamide gels (ratio acrylamide : bisacrylamide 37 : 1) containing 0.1 M Na phosphate (pH 7.1) and 0.2% SDS. The electrode buffer contained the same concentrations of SDS and phosphates as in the gels. Determination of the radioactivity in the gels was done as in [2].

PG (pure) was obtained from Serva Heidelberg. [^{14}C]PG was either from CEA, Gif-sur-Yvette, (spec. act. 28–32 mCi/mmol) or from Amersham (spec. act. 15.1 mCi/mmol). All buffer substances except Hepes were obtained from Merck-Darmstadt. Hepes was obtained from Calbiochem Behring.

3. RESULTS

3.1. Inactivation of sulfate equilibrium exchange in erythrocyte membranes with phenylglyoxal at pH 7.4

Fig.1 shows the inhibition caused by various concentrations of the reagent at pH 7.4. This pH is within the range at which PG has been reported to have its highest selectivity for arginine residues [13]. The molar concentration required to produce 50% inhibition under these experimental conditions (fig.1) was found to be 1.2 mM.

In some experiments before measuring SO_4 efflux, the modified cells were incubated for 60 min at 37°C in the absence of the modifier followed by 3 washes. The degree of inhibition of the modified cells was the same, regardless of whether the exposure to PG was followed by incubation in the absence of the agent. This demon-

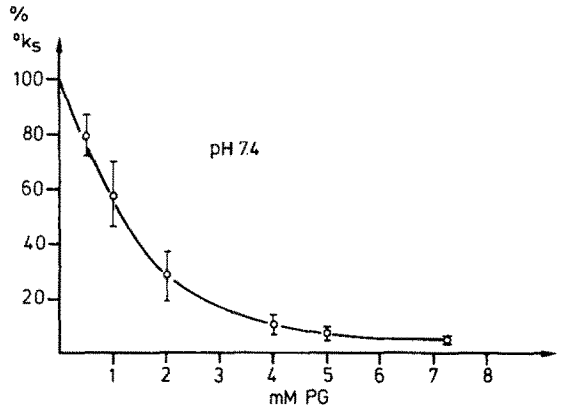


Fig.1. Inactivation of sulfate equilibrium exchange in resealed ghosts with PG at pH 7.4. Ordinate: penetration rate in % of control value without PG. Abcissa: concentration (mM) of PG; temperature 37°C, incubation time 60 min, pH 7.4.

strates that the inhibition of anion transport under our experimental conditions is irreversible.

3.2. [^{14}C]Phenylglyoxal binding to the erythrocyte membrane

After modification of the resealed ghosts at different [^{14}C]PG concentrations at a constant time and after the removal of unreacted reagent, the ghost suspension was subdivided into two portions: one was used for flux measurements and the other to determine the distribution of [^{14}C]PG binding in membrane proteins after subjecting them to SDS gel electrophoresis as described in section 2. Fig.2 shows the labeling profile of the various proteins after modification of cells at two different concentrations of PG. The upper curve represents the labeling profile of the membrane proteins after incubation in a medium containing 2.5 mM PG. Under these conditions, the degree of inhibition of the anion transport system was 84%. The lower curve represents the radioactivity profile of the membrane proteins after incubation in a medium containing 1.25 mM [^{14}C]PG. The inhibition degree of sulfate transport was 52%.

These results show that increasing the concentration of PG is accompanied by increasing amount of ^{14}C which is incorporated into the anion transport band 3 protein. (The binding to other membrane proteins is of no interest in anion transport [2].) Fig.3 shows the time-dependent incorporation of [^{14}C]PG into membrane proteins. It is also found that the extent of [^{14}C]PG incorporation into the anion transport protein (band 3) parallels the loss of sulfate exchange (fig.6). The upper curve represents the radioactivity profile after an incubation time of 41 min, the middle curve after 21 min, and the lower curve after 10 min. All the experiments were carried out at a constant concentration of the modifier (5 mM). The inhibition of sulfate efflux was 77, 44 and 11% respectively. Data points from various experiments show a linear relationship between incorporation of [^{14}C]PG into band 3 and the inhibition of sulfate exchange (fig.6a).

The radioactivity on the first slices, where there are no Coomassie blue-stainable bands, presumably corresponds to unreacted PG and can be extracted with albumin [14]. We also found that this activity disappears after alkali-stripping; therefore, it does not seem to be an aggregation product of integral proteins [15].

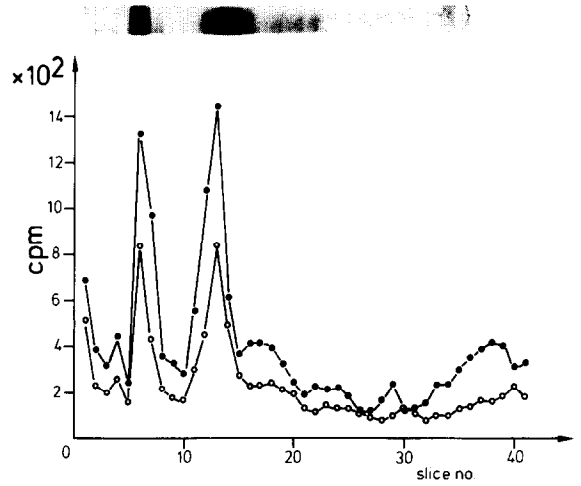


Fig.2. Distribution of [^{14}C]PG residues in the proteins of the red blood cell membrane at different degrees of inhibition of sulfate exchange. Concentration-dependent [^{14}C]PG incorporation. Upper curve: radioactivity profile after labeling in 2.5 mM PG in the medium. Lower curve: radioactivity profile at lower concentration of PG (1.25 mM). The inhibition degree of sulfate exchange was 84 and 52%, respectively.

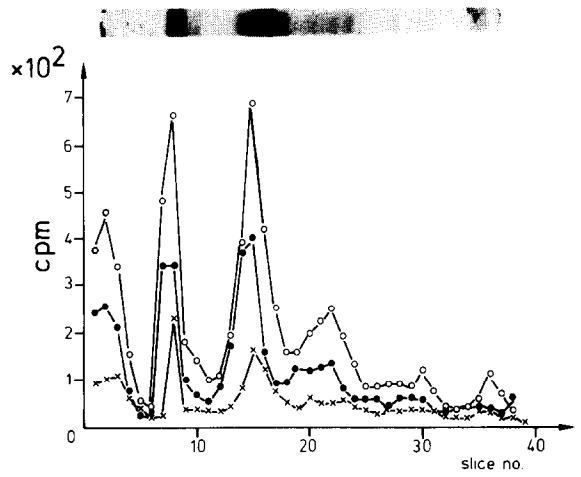


Fig.3. Distribution of [^{14}C]PG residues in the proteins of the red blood cell membrane at different degrees of inhibition of sulfate exchange. Time dependence [^{14}C]PG incorporation. Time of reaction: 41 min (upper curve), 21 min (middle curve) and 10 min (lower curve). The concentration of [^{14}C]PG in the medium was 5 mM; the reaction condition was as described in section 2. Sulfate exchange was inhibited by 77, 44 and 11%, respectively.

3.3. *Extracellular chymotryptic digestion of [^{14}C]PG-modified cells*

For further localization of the arginine residues that are involved in anion transport and that can be labelled by [^{14}C]PG, extracellular chymotrpysin was used. Extracellular chymotrypsin is known to cleave the integral membrane protein band 3 (anion transport protein) into 60 and 35 kDa fragments without inhibiting anion transport [16]. Fig.4 shows the protein pattern before and after digestion on either (a) 5% SDS gel electrophoresis or (b) gradient slab gel (4–30% acrylamide) with marker proteins of 94, 67, 43, 30, 21 and 14 kDa.

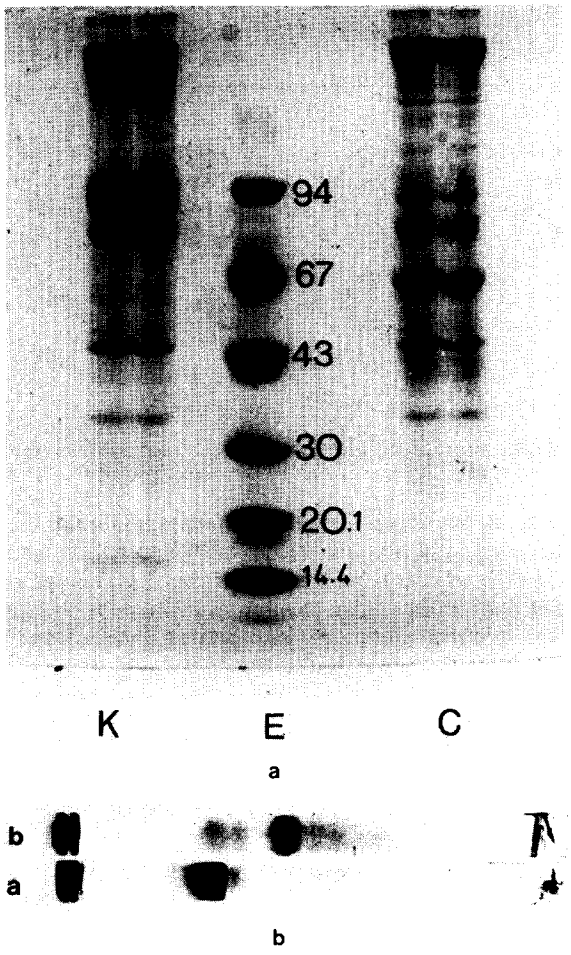


Fig.4. (a) a: polyacrylamide gradient slab gel (4–30% AA, 0.2% SDS) of extracellular chymotrypsin-digested and undigested PG-treated membrane protein. K, undigested PG-treated cells; C, chymotryptic digested PG-treated cells; E, marker proteins [94 kDa (phosphorylase *b*), 67 kDa (bovine serum albumin), 43 kDa (ovalbumin), 30 kDa (carbonic anhydrase), 20.1 kDa (soybean trypsin inhibitor), 14.4 kDa (lactalbumin)]. (b) PAGE (4.5–7.5%) (0.2% SDS): (a) [^{14}C]PG-treated cells. (b) [^{14}C]PG-treated cells after extracellular chymotrypsin treatment. The gels were always run in duplicate, one was stained and the other sliced and radioactivity in each slice determined as described in section 2.

After inactivation of anion transport to different extents with PG in resealed ghosts (either time-dependent or concentration-dependent inactivation) aliquots from the modified cells were taken and subjected to extracellular chymotrypsin treatment. The distribution of the radioactivity in the resulting 60 and 35 kDa fragments was studied. Fig.5 shows the results of experiments where the ghosts were inhibited to 52% of the original activity in fig.5a and 84% in fig.5b. Fig.5c shows the same result on PAGE gradient slab gel.

It was observed that under both conditions about 2/3 of the radioactivity originally bound to band 3 was now found in the 60 kDa fragment. In some experiments the gels were stained first and then the 60 kDa band was isolated and the radioactivity extracted and calculated. For calculating the radioactivity in the 35 kDa fragment, which stains poorly, we counted the radioactivity in the slices which correspond to the molecular mass markers protein in the region of 35 kDa.

Fig.6a shows a plot of relative transport activity *vs* mol [^{14}C]PG dimer per mol band 3. Fig.6b shows the distribution of [^{14}C]PG dimer in the 60 kDa fragment after chymotrypsin treatment in correlation to the relative transport activity. The data points from the various experiments are scattered around a straight line with 100% inhibition at a binding of about 2–3 [^{14}C]PG per mol band 3 (fig.6a). After the splitting of band 3 into its 60 and 35 kDa fragment, about 2/3 of the radioactivity was found on the 60 kDa fragment. The determination of label on the 35 kDa band is subject to a large error, since it is difficult to visualize on SDS gels (weakly stained) and because it overlaps with other bands. The data are shown in fig.6c.

Fig.7 shows an experiment in which the membrane sample from chymotrypsin-treated cells and undigested cells were treated with 0.1 M NaOH before electrophoresis. This treatment (stripping) releases most of the weakly associated membrane protein [15] making the integral membrane protein

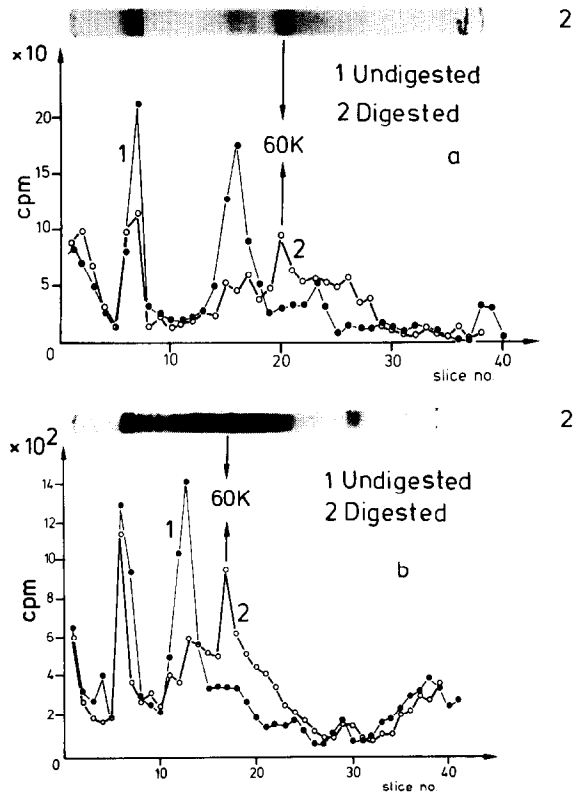


Fig.5. (a,b) Distribution of [^{14}C]PG residues after cleavage of the 95 kDa protein. It was found that most of the radioactivity which was located in the 95 kDa fragment was shifted to the 60 kDa fragment. The 35 kDa fragment was also labeled. Concentration of [^{14}C]PG in the media was 1.25 mM (a) and 2.5 mM (b). The incubation time was 45 min in standard buffer at pH 7.4. (c) Distribution of ^{14}C on PG gradient slab gel (4–30% AA, 0.2% SDS).

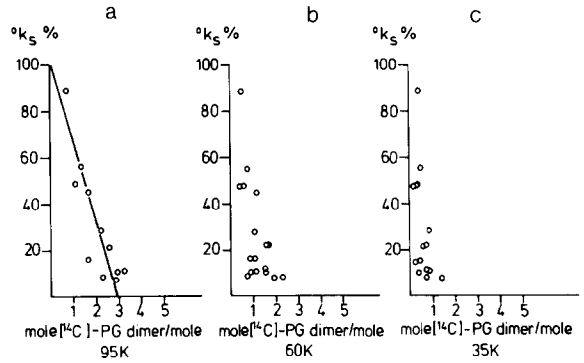
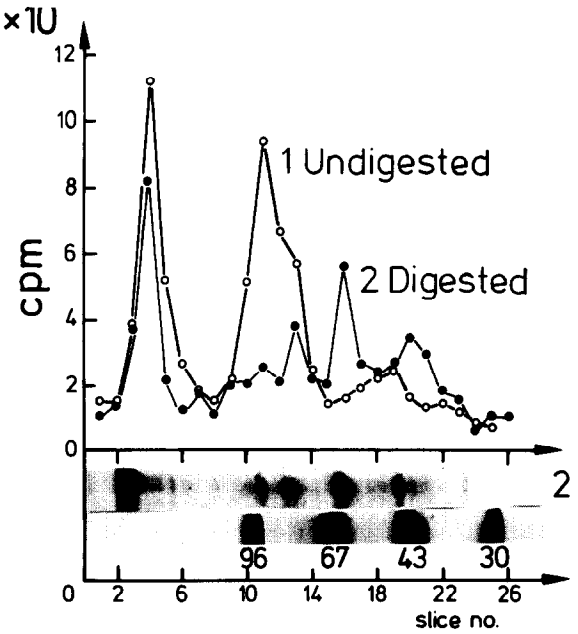


Fig.6. Relationship between sulfate equilibrium exchange and [^{14}C]PG binding to: (a) the protein in band 3; (b) the 60 kDa band (resulting from the cleavage of band 3 by extracellular chymotrypsin); (c) the 35 kDa band (determination of radioactivity was done a described in the text).



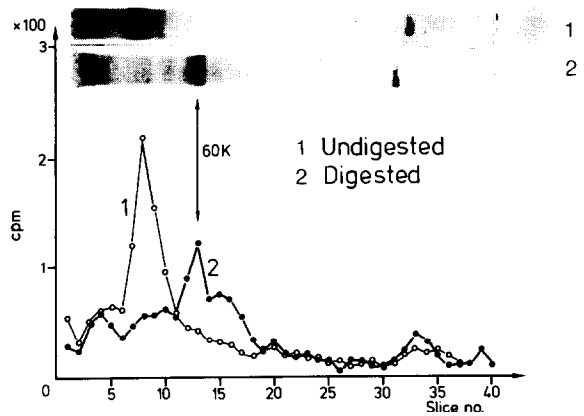


Fig.7. Distribution of [^{14}C]PG in membrane protein after extracellular chymotrypsin treatment and NaOH stripping. (Upper curve) Undigested cells; (Lower curve) chymotrypsin-digested cells. The results show that [^{14}C]PG residues are located on both the 60 and 35 kDa fragments.

(in this case the 60 and 35 kDa fragments) more apparent.

Fig.7 shows that a great amount of the radioactivity is located on the 60 kDa band.

4. DISCUSSION

Anion transport across red blood cells was inactivated by [^{14}C]PG at pH 7.4 in a time- and concentration-dependent manner. The rate of incorporation of radioactivity into the anion transport protein (band 3) gave a good correlation with the rate of inactivation. Although the inactivation is accelerated under alkaline conditions the experiments were performed at pH 7.4 since at this pH the side reaction with amino acids other than arginine has been reported to be negligible [17,18]. It has also been found that PG has a higher selectivity for arginine residues at pH between 7 and 8 [13].

Under our experimental conditions complete inactivation of anion transport is accompanied by binding of about 6 mol [^{14}C]PG per mol band 3. Since the stoichiometry of the binding of PG to arginyl residues is found to be 2:1 [19], the number of arginine residues modified by [^{14}C]PG is about 3 mol/mol band 3. A plot of relative transport activity mol [^{14}C]PG dimer/mol band 3 from various experiments gives data points scattered around a straight line with 100% inhibition at binding of about 2–3 mol PG dimer per mol band 3. After chymotryptic cleavage of band 3 into its 60 and 35 kDa fragments at different extents of inactivation with [^{14}C]PG most of the radioactivity was always found on the 60 kDa fragment. Data from different experiments seem to be scattered around a straight line with 100% inhibition at binding of about 1–2 mol PG dimer/per mol 60 kDa fragment. We also found some PG binding to the 35 kDa fragment, but due to the scatter of the data it is difficult to assign a stoichiometry between the inactivation of anion transport and PG binding to this fragment. The binding of PG to the 60 kDa fragment under our conditions contrasts with the finding of authors in [14] who found that PG binds exclusively to the extracellular site of the 35 kDa fragment under conditions where the cells were treated with PG at an extracellular pH of 10.5 and neutral pH inside the cells. Under these conditions, peptides exposed to the inner membrane surface are not phenylglyoxalated.

The most recent opinion concerning the arrangements of the integral membrane proteins in the lipid bilayer is that segments of amphipathic transmembrane secondary structure are linked by more hydrophilic regions and that the basic structure of the membrane-bound domain of the anion transport protein is a cluster of transmembrane helices. Authors in [20] described a cluster of 6 basic residues in fragment P5 located intracellularly. This fragment is known to be involved in transport activity. Three of the basic residues are arginine residues.

This type of environment would lead to a lowering of the pK_a of the arginines and make them reactive to PG under our experimental conditions where it is found that only 2–3 of the 45 arginine residues in band 3 protein can react with PG at pH 7.4 and cause complete inhibition of anion transport across the red cells. This is in agreement with authors in [21] who reported that the pK_a value of arginyl residues of the anion binding site is lower than that of other arginyl residues, providing an explanation for the observation of the remarkable selectivity of dicarbonyl reagents for arginyl residues involved in anion binding. The finding that $^{3}H_2$DIDS binding to resealed ghosts is almost unaffected after modification of the cells with PG [8] may support the idea that (under our conditions) PG binding sites which may be directly involved in transport activity (translocation of anions) are not located on the most exposed extracellular surface. Another finding which may support this hypothesis is that a non-penetrating PG derivative is not able to inhibit SO_4^{2-} exchange under conditions where phenylglyoxal causes complete inhibition (unpublished).

Our results cannot give an answer to the question as to whether only one, or more than one, of the 3 arginine residues that are labeled under the experimental conditions described here is involved in the transport mechanism. Also, I have not been able to clarify the existence of interaction between the 3 arginine residues. An explanation would be as follows: In the case where the transport is inhibited to 50%, we found about 1.5 mol labeled arginine molecules per mol 95 kDa protein. After digestion of the cells with extracellular chymotrypsin we found about one molecule labelled arginine per molecule 60 kDa fragment and about half of this number on the 35 kDa fragment. It is, there-

fore not clear whether the PG binding site (say, a) on the 60 kDa fragment is the one responsible for the 50% inhibition and another arginine residue needs to be labeled for complete inhibition; the latter would be on the 60 kDa fragment (b) or on the 35 kDa fragment (c). Another case is that either only (b) (binding site of PG on the 60 kDa fragment) or (c) (binding site of PG on the 35 kDa fragment) or both are the translocating sites but the binding to one or both can only happen when (a) is first labeled.

The possibility that complete inhibition is accompanied by modification of more than one arginine residue per band 3 protein is in agreement with the now generally accepted hypothesis of the participation of more than one amino acid residue in the mechanism of anion translocation along the exchange pathway in the red cell membrane. Studies for further identification of these sites are now underway.

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