Selective Phenylglyoxalation of Functionally Essential Arginyl Residues in the Erythrocyte Anion Transport Protein

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ABSTRACT The red cell anion transport protein, band 3, can be selectively modified with phenylglyoxal, which modifies arginyl residues (arg) in proteins, usually with a phenylglyoxal:arg stoichiometry of 2:1. Indiscriminate modification of all arg in red cell membrane proteins occurred rapidly when both extra- and intracellular pH were above 10. Selective modification of extracellularly exposed arg was achieved when ghosts with a neutral or acid intracellular pH were treated with phenylglyoxal in an alkaline medium. The rate and specificity of modification depend on the extracellular chloride concentration. At 165 mM chloride maximum transport inactivation was accompanied by the binding of four phenylglyoxals per band 3 molecule. After removal of extracellular chloride, maximum transport inhibition was accompanied by the incorporation of two phenylglyoxals per band 3, which suggests that transport function is inactivated by the modification of a single arg. After cleavage of band 3 with extracellular chymotrypsin, [14C]phenylglyoxal was located almost exclusively in a 35,000-dalton peptide. In contrast, the primary covalent binding site of the isothiocyanostilbenedisulfonates is a lysyl residue in the second cleavage product, a 65,000-dalton fragment. This finding supports the view that the transport region of band 3 is composed of strands from both chymotryptic fragments. The binding of phenylglyoxal and the stilbene inhibitors interfered with each other. The rate of phenylglyoxal binding was reduced by a reversibly binding stilbenedisulfonate (DNDS), and covalent binding of [3H]DIDS to phenylglyoxal-modified membranes was strongly delayed. At DIDS concentrations below 10 μM, only 50% of the band 3 molecules were labeled with [3H]-DIDS during 90 min at 38°C, thereby demonstrating an interaction between binding of the two inhibitors to the protomers of the oligomeric band 3 molecules.

INTRODUCTION

Anion exchange through the red cell membrane is mediated by band 3, an integral transmembrane protein with a molecular weight of ~100,000. The...
most convincing evidence that the integrity of band 3 is essential for normal anion transport is that transport can be fully and selectively inactivated by covalent attachment of the site-specific reagent 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (Cabantchik and Rothstein, 1974a). Full inactivation is attained at an approximate 1:1 stoichiometry of DIDS to band 3 (Lepke et al., 1976; Ship et al., 1977), one of the isothiocyanate groups of DIDS that bind readily to a lysyl residue in the vicinity of the functional transport site (Ramjeesingh et al., 1981).

We have recently shown (Wieth and Bjerrum, 1982) that chloride exchange in resealed ghosts is dependent on the protonation of a group with an apparent pK of ~12 at 0°C and high chloride concentrations, and have suggested that this group might be an arginyl residue of the band 3 protein. We have also shown (Wieth et al., 1982b) that chloride exchange is irreversibly inactivated by phenylglyoxal, a group-specific reagent that is highly selective for the modification of arginyl residues in proteins (Takahashi, 1968). These data suggest that positively charged arginyl residues are critical functional components of the anion transport system of red cell membranes.

We report here that under the proper conditions inactivation of anion transport by phenylglyoxal can be made highly selective. Maximum transport inactivation in red blood cell ghosts is achieved by incorporation of an amount of phenylglyoxal that should modify ~1 arginyl residue per band 3 molecule. Significantly, most of the [14C]phenylglyoxal is incorporated into a major chymotryptic peptide other than DIDS, the 35,000-dalton chymotryptic fragment. Preliminary reports of this work have previously been published (Bjerrum et al., 1982a, b).

MATERIALS AND METHODS

Radioactive Isotopes

[14C]Phenylglyoxal, sp act 25–35 mCi/mmol, was obtained from CEA, Gif-Sur-Yvette, France, and was kept at −20°C under nitrogen atmosphere in methanol. The radiochemical purity, checked by radiogas chromatography and thin-layer radiochromatography, was reported to be 97–99%. 36Cl as NaCl, sp act 500 μCi/mmol, and [3H]inulin, sp act 900 mCi/mmol, were purchased from the Radiochemical Centre, Amersham, England. [3H]DIDS, with sp act 30–60 mCi/mmol, was prepared as described by Ship et al. (1977). The absorption spectrum of this [3H]DIDS was identical with the spectrum of unlabeled DIDS and showed that almost all of the DIDS molecules were in the trans configuration (Fröhlich and Gunn, 1980). Full inhibition of anion transport with this preparation of [3H]DIDS was obtained after binding of ~0.95 × 10⁶ DIDS molecules per ghost independent of the dilution of the [3H]DIDS with unlabeled DIDS (Wieth et al., 1982c).

Chemical Reagents

All media were prepared from reagent grade chemicals. Phenylglyoxal was purchased from EGA-Chemie, Steinheim-Albuch, Federal Republic of Germany, and DNDS (4,4'-dinitrostilbene-2,2'-disulfonic acid) from ICN K&K Laboratories Inc., Plainview, NY, and Pfaltz & Bauer Inc., Stamford, CT. DIDS was prepared according to the method of Funder et al. (1978). The buffers CHES [2-(N-cyclohexylamino)ethane-
sulfonic acid, }pK_a 9.5 at 20°C, and CAPS [3-(N-cyclohexylamino)-1-propanesulfonic acid], }pK_a 10.4 at 20°C, were from Calbiochem-Behring Corp., Switzerland. Albumin from bovine serum (fraction V powder), α-chymotrypsin from bovine pancreas, and PMSF (phenylmethanesulfonyl fluoride) were from Sigma Chemical Co., St. Louis, MO.

Reagents for Sodium Dodecyl Sulfate Gel Electrophoresis (SDS-PAGE)

Acrylamide and bisacrylamide were from Eastman Kodak Co., Rochester, NY. Protein standards for SDS-PAGE (molecular weights from 14,300 to 94,000) included: lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase b. All were from Bio-Rad Laboratories, Richmond, CA. DTT (dithiothreitol), Temed (N,N,N′,N′-tetramethylethylenediamine), and bromphenol blue were from Sigma Chemical Co. Lumasolve was from Lumac B. V., The Netherlands, and Coomassie Brilliant Blue R 250 was from Merck Sharp & Dohme, Darmstadt.

Electrolyte Media

Treatment of ghosts with phenylglyoxal was carried out in one of the following two media: (A) 165 mM KCl, buffered with 2.5 mM CAPS or 5 mM CHES; (B) 25 mM potassium citrate and 200 mM sucrose, buffered with 2.5 mM CAPS or 5 mM CHES. Phenylglyoxal was dissolved in the thermostatted medium and titrated to the pH of the experiments with 1 M KOH immediately before addition of the cells.

The stopper solution used for interrupting the phenylglyoxal reaction contained 165 mM KCl, 4 mM KH_2PO_4, and 2 mM EDTA (ethylene-diaminetetraacetic acid). The pH was titrated so that the mixing of 1 vol of the cell suspension with 4 vol of the stopper solution resulted in a pH of 7.2–7.4 at 0°C, stopping the chemical reaction instantaneously and completely according to our previous results (Wieth et al., 1982b).

The flux medium contained 165 mM KCl, 2 mM KH_2PO_4, and 1 mM EDTA, and was titrated to pH 7.3 at 0°C. This medium was also used for washing the resealed ghosts. Bovine albumin (0.5–1%) was added as indicated in the text when the medium was used for extracting adsorbed phenylglyoxal from the resealed ghosts.

Ghost Preparation

The resealed ghosts containing 165 mM KCl, 2 mM Tris, and 0.2–0.5 mM EDTA were prepared as described by Funder and Wieth (1976). EDTA was added with the reversal solution to reduce Ca^{2+}-activated protein degradation and aggregation of membrane proteins during the 45-min resealing at 38°C (Bjerrum et al., 1981).

Hemoglobin-depleted white membranes, prepared by the method of Dodge et al. (1963), were used in one of the experiments shown in Fig. 1. The white ghosts were used for labeling all protein fractions of the membrane. We thereby avoided the binding of hemoglobin to the membrane, which takes place when pink ghosts are phenylglyoxalated at an alkaline intracellular pH. The white ghosts were washed at 0°C in 10 mM KCl, 2 mM phosphate, and 1 mM EDTA, pH 7.3, before addition of reversal solution (1 vol of 2 M KCl, 25 mM CAPS, and 1 mM EDTA to 11 vol of the membrane suspension). Partial resealing was achieved by incubating the ghosts for 30 min at 38°C. The intracellular pH was subsequently raised to 10.3 at 38°C by washing the ghosts repeatedly in the cold with 165 mM KCl and 2.5 mM CAPS.

Labeling of Ghosts with [14C]Phenylglyoxal

Experiments were performed to determine the relationship between transport inhibition and phenylglyoxal binding. One sample of the resealed ghosts was saved for
control measurement of chloride exchange in untreated ghosts. Another portion was packed (at 44,000 g, 15 min) to a cytocrit of 85–90%, as determined by measurements of the \([3H]\)inulin space in the centrifuged sample, and used for labeling with \([14C]\)-phenylglyoxal. Two different procedures were used for modifying the ghost membranes: method A was used to determine phenylglyoxal binding to whole resealed ghosts; method B was used to label the membrane proteins at a higher specific activity for subsequent SDS-PAGE analysis.

By method A, 2–6 ml of packed ghosts with an intracellular pH of 6.7 at 25°C (initial cytocrit 85–90%) were treated with phenylglyoxal in 3 or 4 vol of an alkaline, well-buffered electrolyte medium as described by Wieth et al. (1982a). The medium contained 20 mM labeled phenylglyoxal, and the final concentration was 15–16 mM after addition of ghosts. The specific activity of the \([14C]\)phenylglyoxal for these experiments was 0.03–0.06 mCi/mmol. Temperature, pH, and electrolyte composition in the individual experiments are stated in the legends to the figures and tables. Samples (2–5 ml) were withdrawn from the well-stirred reaction suspension at appropriate time intervals, and the covalent phenylglyoxal reaction was interrupted by injecting the alkaline cell suspension into 4 vol of an ice-cold acidic stopper solution, thus lowering the pH of the suspension to 7.2–7.4 at 0°C. For a typical experiment one could obtain five to eight samples of ghosts with varying degrees of membrane labeling and transport inhibition. In method B, the ghosts were exposed to phenylglyoxal at a higher specific activity (0.5–3 mCi/mmol) when labeling was performed for subsequent SDS-PAGE, and only one cell sample was processed at a time. Packed ghosts (0.5 ml) were injected into 1.5 ml of the alkaline medium normally containing 20 mM labeled phenylglyoxal. The chemical reaction was stopped after the appropriate time (1–40 s) by diluting the suspension with 8 ml of the ice-cold acidic stopper solution. The two labeling procedures gave identical results with respect to the inhibition of anion transport as a function of phenylglyoxal binding. In experiments performed with method A, the pseudo-first-order rate coefficient of transport inactivation was determined by linear regression analysis of the data as described by Wieth et al., 1982a. In the experiments performed with method B, only a single data point was obtained in each experiment, and the variation of the rate of inactivation was, accordingly, somewhat larger from one experiment to another.

To avoid the modification of arginyl residues exposed to the intracellular phase, it is essential that the intracellular pH not become alkaline during the exposure to phenylglyoxal. As discussed by Wieth et al. (1982a), the membrane permeability to hydroxide ions is sufficiently low to achieve this during brief exposures.

Binding of \([14C]\)phenylglyoxal to all protein fractions of red cell membranes was examined in one of the experiments shown in Fig. 1 by treating white ghosts at pH 10.3 in the absence of a transmembrane pH gradient. Phenylglyoxal permeates the membrane readily (Wieth et al., 1982a), and endofacial membrane proteins are modified rapidly when the intracellular pH is alkaline. The ghosts were labeled with \([14C]\)phenylglyoxal as described in the legend of Fig. 1. Adsorbed phenylglyoxal was removed by washing and the concentration of ghosts in the analyzed samples was calculated from the protein content (vide infra).

**Removal of Adsorbed Phenylglyoxal**

To remove phenylglyoxal that had adsorbed to but not reacted covalently with membrane proteins, it was necessary to wash the labeled ghosts with an albumin-containing electrolyte solution. After addition of stopper solution, the ghosts were
washed twice in the efflux medium containing 0.5% bovine albumin and subsequently resuspended in the same medium and stored over night at 0°C in the presence of 1% albumin. The following day the ghosts were washed twice in flux medium containing 0.5% albumin and then twice in an albumin-free flux medium. Next, the ghosts were incubated 30 min at 38°C. By this procedure we were able to remove all noncovalently bound phenylglyoxal from the membranes (cf. Fig. 2). In early experiments where the incubation at 38°C was not included, the removal of adsorbed phenylglyoxal was less complete, as in the experiments of Fig. 5. The degree of inactivation of chloride transport in a given sample was found to be unaffected by the removal of adsorbed phenylglyoxal or the prolonged periods of incubation, which shows that the adsorbed fraction does not interfere with anion transport and that the observed inactivation of anion transport is not readily reversible.

**Treatment of Ghosts with α-Chymotrypsin**

Band 3 can be split into two fragments of ~65,000 and ~35,000 daltons by treatment with extracellular chymotrypsin (Cabantchik and Rothstein, 1974b). Enzymatic treatment of resealed ghosts was carried out to determine the location of phenylglyoxal binding. The digestion was performed during the 30-min incubation at 38°C after albumin extraction of adsorbed reagent (see Removal of Adsorbed Phenylglyoxal). The suspension contained 0.5 mg α-chymotrypsin/ml (pH 8.0 at 38°C) at a cytocrit of 5–10%, and the reaction was stopped by the addition of the chymotrypsin inhibitor PMSF to a final concentration of 1 mM, followed by two washings in flux medium containing 0.2 mM PMSF.

**Determination of the Chloride Exchange Flux**

The ghosts were washed in the flux medium after removal of adsorbed phenylglyoxal. The samples were divided in two, one for the determination of chloride transport capacity and the other for the determination of membrane-bound [14C]phenylglyoxal. The ghosts used for flux determination were resuspended in the flux medium at a cytocrit of 30%, labeled with 36Cl (0.3–0.6 μCi/ml suspension) at 5–15°C sufficiently long to attain isotope equilibration, and packed in slender nylon tubes for efflux experiments carried out at 0°C, pH 7.3, by the Millipore-Swinnex technique (Dalmark and Wieth, 1972). The inactivation of anion transport capacity followed pseudo-first-order kinetics during exposure to phenylglyoxal as previously described by Wieth et al. (1982b), and the degree of inactivation was calculated from the fractional decrease of transport capacity and expressed as a percent of the rate of chloride efflux of the untreated control sample.

**Determination of the Membrane Binding of Phenylglyoxal**

Membrane binding of phenylglyoxal was calculated from the specific activity of the reagent (counts per minute per millimole) and the determination of the 14C activity of the extensively washed ghost membranes. The labeled resealed ghosts were hemolyzed at 0°C in 20 vol of a solution containing 10 mM phosphate and 1 mM EDTA, pH 7.4. The ghosts were washed in this buffer at 0°C until they appeared creamy white. The ghosts were finally resuspended to a concentration of 1–2 × 10⁹ ghosts/ml and samples of 50–250 μl were counted by liquid scintillation. Identical results were obtained whether or not the ghosts were dissolved with Lumasolve (Lumac B. V.) before counting. The amount of membrane material processed was determined by two independent methods, cell counting and quantitative analysis of the membrane protein.
**Counting of Ghosts**

The unsealed ghosts were counted in a model DN Coulter Counter (Coulter Electronics Inc., Hialeah, FL). The suspension was diluted 50-fold in the 10-mM phosphate buffer, 1 mM EDTA, pH 7.4 at 0°C, and a sample of the primary dilution was counted after further dilution (1:101) in 154 mM NaCl. The electrical resistance of the unsealed membranes was sufficient for the particle counting. This was confirmed by counting samples before and after resealing of the ghost membranes.

**Protein Analysis**

Protein content of the ghosts was determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin and l-tyrosine as standards. Ghosts suspended in the 10-mM phosphate buffer were diluted 1:5 with 0.1 M NaOH. The EDTA concentration in the final reaction mixture was ~8 μM, 10-fold lower than the EDTA concentrations that have been found to affect protein determination (Ji, 1973; Peterson, 1979). By correlating protein analysis to cell counting we found an average content of $5.25 \times 10^{-10}$ mg protein/ghost (SEM $0.04 \times 10^{-10}$, n = 265). This value should be compared with the results of Dodge et al. (1963) ($6 \times 10^{-10}$ mg/ghost), Weed et al. (1963) ($5.6 \times 10^{-10}$ mg/ghost), and Lepke et al. (1976) ($5.1 \times 10^{-10}$ mg/ghost).

**Treatment of Ghosts with [3H]DIDS**

Phenylglyoxal-treated ghosts and untreated ghosts were incubated in the flux medium (25% cytocrit), pH 7.0-7.2 at 38°C, with [3H]DIDS. Samples of the suspensions were withdrawn at appropriate times after initiation of the reaction, and the covalent binding of DIDS was stopped by dilution into 15 vol of ice-cold medium containing 0.5% bovine albumin. The samples were washed once at 0°C in medium containing 0.5% albumin and once in medium without albumin to remove unreacted [3H]DIDS. The ghosts were then washed at 0°C in 20 vol of a solution containing 10 mM phosphate and 1 mM EDTA, pH 7.4, until they appeared creamy white. [3H]DIDS activity was determined after solubilization of 100 μl samples (1-2 \times 10^9 ghosts/ml) in 600 μl Lumasolve.

**SDS-PAGE**

The binding of [14C]phenylglyoxal to the erythrocyte membrane proteins were examined by SDS-PAGE. The methods used were either the standard procedure described by Fairbanks et al. (1971) or gradient electrophoresis using the discontinuous buffer system described by Laemmli (1970).

Ghost membranes (2-5 \times 10^9 ghosts/ml in 10 mM phosphate and 1 mM EDTA, pH 7.4) were solubilized in 1 vol of a solution containing 2% SDS, 20% glycerol, 5 mM EDTA, 50 mM Tris, and 25 mM DTT, pH 8.8. Polyacrylamide gels (5 or 7.5%) for standard electrophoresis were prepared as described by Fairbanks et al. (1971). Samples of the SDS-solubilized membrane proteins were run in duplicate at 12°C using either a Pharmacia model GE-4 Electrophoresis Apparatus (Pharmacia Diagnostics, Piscataway, NJ) or a Bio-Rad Protean Slab Cell Apparatus (Bio-Rad Laboratories). The electrophoresis was performed with a voltage gradient of 6-10 V/cm. The electrophoresis was stopped after 1.5-3 h when the tracking dye (bromphenol blue) reached the bottom of the gel.

Gradient gel electrophoresis, in combination with the discontinuous buffer system described by Laemmli (1970), gives an improved protein separation with sharper bands in the whole molecular mass-range from 10 to 200 kilodaltons. A linear gradient
slab gel (12–25% polyacrylamide) was prepared in a Bio-Rad Protean Slab Cell Apparatus in dim light. A degassed solution containing 25% polyacrylamide and 0.20% bisacrylamide was mixed with a degassed solution containing 12% polyacrylamide and 0.067% bisacrylamide using a linear gradient mixer. The fractions of bisacrylamide chosen gave a homogeneous gradient gel with respect to its swelling and shrinkage properties. The gradient gel solution containing 0.8 M Tris, 0.2% SDS, 0.02% Temed, 2 μg/ml riboflavin, and 0.0035% ammonium persulfate, adjusted to pH 8.8 at 20°C with HCl, was covered by isobutanol and photopolymerized for 20 min with neon light (TL 20W/55; Philips Electronic Instruments, Inc., Mahwah, NJ). A stacking gel was added on top of the gradient gel after washing away the isobutanol with separation buffer. The stacking gel contained 5% polyacrylamide, 0.19% bisacrylamide, 0.26 M Tris, 0.2% SDS, 0.1% Temed, and 4 μg/ml riboflavin, and was adjusted with HCl to pH 7.25 at 20°C. The gel solution also contained 0.075% ammonium persulfate to promote polymerization. Samples of SDS-solubilized membrane proteins and the molecular weight standards were applied to the gel with a 50–250-μl Hamilton syringe (Hamilton Co., Reno, NV). The electrode buffer (pH 9.2 at 20°C) contained 0.15 M glycine, 0.19 M Tris, and 0.15% SDS. The electrophoresis was done at 20°C. A voltage gradient of 2 V/cm was applied for ~2 h until the sample front had passed the stacking gel; thereafter, the electrophoresis was performed with a gradient of ~12 V/cm.

Each sample was run in duplicate. Immediately after electrophoresis the gel slab was cut into two strips. One strip was stained for protein band localization with 0.1% Coomassie Brilliant Blue in 25% 2-propanol/10% acetic acid for at least 20 h with gentle stirring. Destaining was performed in 40% methanol/10% acetic acid for 2–6 h, followed by further destaining in 10% glycerol/10% acetic acid for several days. The duplicate strip was cut into 2.5-mm slices using a manual gel slicer, and the slices were transferred to 20-ml low-potassium glass vials and digested with 600 μl Lumasolve for at least 20 h at room temperature. Scintillation fluid was added and the 14C activity of the samples was determined within 1–3% counting accuracy using a liquid scintillation counter. The recovery of 14C activity in the gels was found to be 90% (SD 7, n = 18) of the applied activity.

**RESULTS**

Previous kinetic studies of the inactivation of anion transport that accompanies the irreversible binding of phenylglyoxal to red cell membranes showed that inactivation is a pseudo–first-order process and thus is probably due to a single chemical event (Wieth et al., 1982b). We found that the rate of irreversible inactivation is a function of phenylglyoxal concentration and depends on the extra- and intracellular pH, the maintenance of a pH gradient across the membrane during modification, and the extracellular anion concentration. In the present work we have studied the stoichiometry of the phenylglyoxal binding that accompanies inactivation. By varying reaction conditions according to our previous results, we have attempted to increase the specificity of the membrane modification, with the aim of maximizing transport inactivation while minimizing the number of modified arginyl residues in the membrane.

**The Role of Extra- and Intracellular pH**

The reaction of phenylglyoxal with arginine apparently involves the neutral guanidinium form of the arginyl side-chain (Cheung and Fonda, 1979a; Wieth...
et al., 1982b); the reaction rate therefore increases at higher pH values. In the present work we settled on an extracellular pH of 10.3 for our phenylglyoxal binding studies. At lower pH values the rate of reaction is slowed by a factor of 10 for a one-unit pH decrease. At higher pH values we observed an even more rapid transport inactivation than at pH 10.3, but the cells have a tendency to become leaky and eventually to lyse. Fig. 1 shows the binding of phenylglyoxal to ghosts in the absence and presence of a pH gradient across the membrane. In white ghosts with an extra- and intracellular pH of 10.3 (cf. Methods), a rapid binding of phenylglyoxal occurs at 16 mM phenylglyoxal, and $120 \times 10^6$ phenylglyoxal molecules are bound per ghost in ~20 s. After 5 min, $\sim 350 \times 10^6$ phenylglyoxal molecules are bound per cell, or a sufficient number to modify all arginyl residues of the membrane proteins. When resealed ghosts with an initial intracellular pH of 6.4 and an extracellular pH of 10.3 are reacted with 16 mM phenylglyoxal, the membranes bind only $\sim 10 \times 10^6$ phenylglyoxals/cell after 20 s, and during the first 2 min phenylglyoxal proceeds at a much slower rate than when the intracellular pH is alkaline. After 2 min of exposure, however, the rate of phenylglyoxal incorporation increases dramatically to a value similar to that seen in ghosts without a pH gradient across the membrane.

The localization of phenylglyoxal in the membrane proteins of ghosts that were modified at an intra- and extracellular pH of 10.3 was examined by SDS-PAGE. The results showed that all membrane proteins are labeled when both extra- and intracellular pH are alkaline. The ghosts were only reacted a short time (5–15 s) because prolonged treatment with phenylglyoxal under these conditions induces irreversible aggregation in all protein fractions. Approximately one-third of the reagent was found in spectrin (bands 1 and 2), and another third was localized in band 3, while the remaining labeled reagent was distributed in all other protein bands roughly in proportion to their content in the membrane proteins (data not shown).

The results of Fig. 1 underline the importance of maintaining a neutral or acid intracellular pH in the search for conditions most conducive to selective modification of essential arginine residues in band 3. The subsequent labeling experiments were carried out with ghosts initially having a neutral intracel-

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1 The membrane proteins of a red cell contain $\sim 120-180 \times 10^6$ arginyl residues. This range is calculated from the following data. Band 3 contributes $\sim 50 \times 10^6$ residues per cell considering the reported values of 44 arginyl residues per band 3 molecule (Steck et al., 1978) and of $1.16 \times 10^6$ band 3 molecules per cell (Fairbanks et al., 1971). Spectrin comprises almost 30% of the membrane protein (Jones and Nickson, 1981), which by our determination was $5.2 \times 10^{-12}$ g/ghost (cf. Methods). The 6.4 mol percent arginyl residues of spectrin (Marchesi, 1979) thus contribute $\sim 40 \times 10^6$ residues per ghost. Spectrin plus band 3 constitute 50% of the total membrane protein (range 49–54% according to Jones and Nickson, 1981). If the arginine content of other membrane proteins is similar to that of band 3 (5.2 mol percent) and of spectrin (6.4 mol percent), the total membrane protein contains $180 \times 10^6$ arginyl residues/cell. The low estimate of $120 \times 10^6$ residues assumes that spectrin and band 3 have a higher arginine content than other membrane proteins. Considering a reported fractional arginine content of 4.5% for the total membrane protein material (Rosenberg and Guidotti, 1968), the average arginine mole fraction may be as low as 3.2% in proteins other than spectrin and band 3, and the membrane content of arginine amounts to $120 \times 10^6$ copies/cell.
lular pH. The membranes were exposed briefly to [14C]phenylglyoxal in alkaline media in an attempt to modify selectively only those groups exposed to the exofacial side.

Modification of Arginyl Residues in the Presence of 165 mM KCl

The rate of irreversible transport inactivation by phenylglyoxal is strongly dependent on the anion composition in the extracellular medium (Wieth et al., 1982b). The significance of this anion dependence was examined by determining the relationship between irreversible inactivation of transport and the binding of [14C]phenylglyoxal to the cell membranes both in a 165-mM KCl medium and at low extracellular chloride concentrations.

![Graph showing the number of phenylglyoxal molecules bound per cell as a function of the duration of phenylglyoxal treatment](image)

**Figure 1.** The number of phenylglyoxal molecules bound per cell as a function of the duration of phenylglyoxal treatment of (○) leaky white ghosts with an intracellular pH of 10.3 and (●) resealed pink ghosts with an initial intracellular pH of 6.4. Modification medium: 165 mM KCl, 2.5 mM CAPS, and 16 mM phenylglyoxal, pH 10.3, 38°C. Further explanation is given in the text.

Fig. 2 relates the inactivation of chloride self-exchange to the binding of [14C]phenylglyoxal to resealed ghosts under conditions where ghosts with an initially neutral intracellular pH were exposed to phenylglyoxal in the 165 mM KCl medium. One series of experiments (open symbols) was performed without removing nonspecifically adsorbed phenylglyoxal, while in a second set of experiments (closed symbols) nonspecifically adsorbed reagent was removed by washing with albumin. The results in both series were obtained with a ghost preparation that was divided in halves. The rate of transport inactivation was determined on one half as previously described (Wieth et al., 1982b), and the number of phenylglyoxal molecules incorporated was deter-
mined on the second sample under exactly the same conditions. As shown in
Fig. 2, there is a linear relationship between the phenylglyoxal content of the
membranes and the inactivation of transport capacity, and the slopes derived
from the two different experimental protocols are identical. The extrapolation
back to 100% transport activity shows that \( \sim 2 \times 10^6 \) phenylglyoxals/cell are
readily removed by the albumin adsorption procedure. The location of the
adsorbed [\(^{14}\text{C}\)]phenylglyoxal in SDS-electrophoretograms shows that the frac-
tion of phenylglyoxal that can be extracted with albumin is located in the
beginning of the gel, which corresponds to the first slices. This activity
presumably corresponds to unreacted phenylglyoxal, for when a sample of
[\(^{14}\text{C}\)]phenylglyoxal was run under identical conditions, the activity was located
in the same slices of the gel. It therefore appears that these molecules are
loosely adsorbed to membrane components and are desorbed by SDS. It also
appears from the results of Fig. 2 that the \( 2 \times 10^6 \) phenylglyoxal molecules
that are loosely associated with the membrane do not cause any irreversible
inhibition of anion transport. The data indicate that maximum inhibition of

![Figure 2](image-url)

**Figure 2.** Transport inactivation as a function of [\(^{14}\text{C}\)]phenylglyoxal binding
to resealed ghosts reacted from 1 to 40 s with 15 mM phenylglyoxal in the 165
mM KCl medium, pH 10.3, 38°C. The ordinate indicates the chloride self-
exchange flux in percent of an untreated control sample. Closed symbols show
results obtained after washing the ghosts with an albumin-containing medium
to remove noncovalently bound phenylglyoxal. Results obtained without albu-
min washing are indicated by the open symbols. The results shown by open and
closed circles were obtained with the same batch of ghosts and the pseudo–first-
order rate constant for transport inactivation was 0.16 s\(^{-1}\) (SD 0.006). The
sample indicated by \( \downarrow \) was used for the SDS-PAGE shown in Fig. 3. Maximum
inhibition of chloride exchange (88% in these experiments) was accompanied by
the binding of \( \sim 9 \times 10^6 \) phenylglyoxals/cell.
chloride exchange is accompanied by the binding of \( \sim 9 \times 10^6 \) phenylglyoxals/cell. We have previously noted that even after maximum irreversible inactivation of anion flux by phenylglyoxal, an average residual flux amounting to \( \sim 10\% \) that of the unmodified ghosts always remains (Wieth et al., 1982b). In the experiment shown in Fig. 2, this residual flux is attained on the covalent binding of \( \sim 9 \times 10^6 \) molecules of phenylglyoxal per cell (\textit{vide supra}). Further inhibition (to 98–99\%) can be attained if, subsequent to the first phenylglyoxal treatment, the cells are washed free of reagent at room temperature and later subjected to a second phenylglyoxal treatment (Wieth et al., 1982a). The number of band 3 molecules that are refractory to phenylglyoxal during the first exposure amounts to \( \sim 10\% \) of the band 3 proteins, for the residual flux can be completely abolished by the binding of only \( \sim 100,000 \) DIDS molecules/ghost (Wieth et al., 1982b).

To localize the \(^{14} \text{C}\)phenylglyoxal in the individual membrane proteins, modified ghosts were subjected to SDS-PAGE. Fig. 3 shows the distribution of membrane-bound \(^{14} \text{C}\)phenylglyoxal of a sample of ghosts that had been previously reacted such that the flux was 51\% of the control flux. Band 3 gives a rather diffuse band on SDS gels, ostensibly because of microheterogeneity in its carbohydrate component (Yu and Steck, 1975; Golovtchenko-Matsuboto and Osawa, 1980). In the gel shown in Fig. 3, it encompasses gel slices 7–12, and 54\% of the total \(^{14} \text{C}\) label is included in this fraction. The spectrin region, slices 2–4, contains very little activity. Some of this activity is presumably unreacted phenylglyoxal that has not been completely extracted by albumin (\textit{vide supra}). Small amounts of radioactivity were found that correspond to the regions of bands 5–6 and band 7 and to the very small amount of hemoglobin bound to the membranes. The recovery of radioactivity in the experiment of Fig. 3 was 86\% of the total activity applied to the gel.

By running SDS gels of ghosts that had been reacted to various extents with \(^{14} \text{C}\)phenylglyoxal, we were able to determine the number of reagent molecules incorporated into band 3 and correlate this with the extent of transport inactivation (Fig. 4). We did this by first determining the total number of reagent molecules incorporated into the cell at each level of inactivation (as in Fig. 2) and then calculating the fraction of the total radioactivity that was incorporated into band 3 after SDS electrophoresis (as in Fig. 3). Maximum inhibition of chloride flux in ghosts is attained when 4–5 \( \times 10^6 \) molecules of phenylglyoxal are bound to the band 3 proteins of a single cell. Accepting as a reasonable estimate a value of about one million copies of band 3 per cell (Fairbanks et al., 1971), the data in Fig. 4 suggest that maximum inhibition of chloride flux is obtained after the incorporation of four to five phenylglyoxal molecules per band 3.

\textit{Modification of Arginyl Residues at Low Extracellular Chloride Concentrations}

Fig. 5 shows the binding of \(^{14} \text{C}\)phenylglyoxal to resealed ghosts at 25°C when ghosts with an intracellular pH of 6.7 were treated at pH 10.3 with 16 mM phenylglyoxal in the sucrose-citrate medium containing 5 mM KCl. The rate of transport inactivation is dramatically increased on going from 16\%
extracellular KCl to 5 mM KCl (Wieth et al., 1982b). To compensate for this increased rate, the experiment of Fig. 5 was performed at 25°C. The pseudo-first-order rate constant for transport inactivation under these conditions was 0.19 s⁻¹, which corresponds to a half-time of inactivation of 3.6 s. The sample marked 1 had incorporated ~2 × 10⁶ phenylglyoxals/cell, and chloride exchange had been reduced to 40% of the native flux. The sample marked 2 contained ~3.8 × 10⁶ phenylglyoxals/cell and had only 14% of the control transport. All subsequent samples had the same residual flux activity (~9% of the native flux), but phenylglyoxal incorporation continued to increase long after maximum flux inhibition was obtained.

The data of Fig. 5 suggest that inactivation of anion exchange in ghosts is more selective at low extracellular KCl concentrations than it is at 165 mM KCl. To pursue this further, we studied the relation between transport inhibition and phenylglyoxal binding (Fig. 6). The inactivation process is indeed much more selective, for maximum flux inhibition (to ~9% of the native flux)
control flux) was attained after $2.3 \times 10^6$ molecules of phenylglyoxal were incorporated per cell. Extrapolation of the data of Fig. 6 back to 100% flux shows that very little noncovalently adsorbed phenylglyoxal remains when the modified cells are extracted with albumin. The data also show that once the residual flux is attained, further incorporation of $[^{14}\text{C}]$phenylglyoxal does not reduce the exchange activity further.

In Fig. 7 we directly compare the rate of phenylglyoxal uptake at high (A) and low (B) chloride concentrations. The two series of experiments were made at different temperatures, such that the pseudo-first-order rate constants for inactivation were comparable, $\sim 0.15$ s$^{-1}$ at both 165 and 8 mM KCl. To facilitate the comparison between the two sets of data directly to the inactivation of anion transport activity, the incorporation of $[^{14}\text{C}]$phenylglyoxal in Fig. 7 is expressed as a function of the number of half-times of transport inactivation. One can directly compare the number of phenylglyoxal molecules incorporated into the ghosts at different KCl concentrations with the extent of transport inactivation. For example, the exposure of ghosts to phenylglyoxal for a single half-time causes 50% reduction of the inhibitable flux. This inactivation is accompanied by the binding of $\sim 10^6$ phenylglyoxal molecules/ghost in the low chloride medium (lower line, Fig. 7). The corresponding uptake to obtain the same degree of inactivation in the 165 mM
FIGURE 5. Time course of phenylglyoxal binding to resealed ghosts treated with 16 mM phenylglyoxal in the sucrose-citrate medium containing 5 mM KCl, pH 10.3, 25°C. The points marked 1 and 2 are commented upon in the text.

FIGURE 6. Anion transport inactivation as a function of phenylglyoxal binding to resealed ghosts treated with 15 mM phenylglyoxal in the sucrose-citrate medium containing 8 mM KCl, pH 10.3, 25°C. One half of the modified ghosts were used for determination of [14C]phenylglyoxal incorporation and the second half were used to determine the chloride transport. The ordinate indicates the chloride self-exchange flux as a percent of an untreated control sample. The different symbols show results from experiments with seven different ghost preparations, all of which were treated with albumin to remove adsorbed phenylglyoxal. The pseudo-first-order rate constant for transport inactivation, calculated from the results of the seven experiments, was 0.15 s⁻¹ (SD 0.02).
FIGURE 7. Comparison of the rate of phenylglyoxal uptake during phenylglyoxalation in 165 mM KCl (upper curve: A) and in the sucrose-citrate medium (lower curve: B). The time scale on the abscissa indicates the number of half-times of chloride transport inactivation. For example, 50% of the inhibitable chloride exchange flux will be inactivated after one half-time period and 75% after two periods. The three ghost samples indicated by the different symbols in A were treated with 15–18 mM phenylglyoxal in the 165 mM KCl medium at 38°C and pH 10.3. The five ghost preparations shown by the five different symbols in line B were phenylglyoxalated in the sucrose-citrate medium in the presence of 5–8 mM KCl at pH 10.3, 25°C, and phenylglyoxal concentration 15–16 mM. By using a lower temperature at the low chloride concentration, the rates of chloride transport inactivation became comparable in all experiments with half-times of transport inactivation of $\sim 4$ s. The figure illustrates the considerable increase of selectivity obtained when phenylglyoxalation is carried out in a low chloride medium.

KCl medium is $\sim 4 \times 10^6$ molecules/cell. After five half-times, the inhibitable flux will be reduced by 97%. This degree of inactivation is obtained on binding $\sim 4 \times 10^6$ and $9 \times 10^6$ phenylglyoxals/ghost in 8 and 165 mM KCl, respectively. The data of Fig. 7 again show that inactivation is much more selective at low extracellular chloride concentration than at high chloride concentrations.
The distribution of [14C]phenylglyoxal in the various protein fractions of ghosts labeled in a low KCl medium was determined by SDS-PAGE (Fig. 8). The ghosts used for this experiment had been exposed for 7.6 s to 15 mM phenylglyoxal at 25°C and pH 10.3 in the sucrose-citrate medium. The inhibited flux was 50% of the native flux and the total binding of phenylglyoxal was $1.40 \times 10^6$ molecules/ghost. The recovery of radioactivity after slicing of the gels was in this instance 95%. Fig. 8 shows the distribution of

**Figure 8.** The distribution of [14C]phenylglyoxal in red cell membrane proteins after labeling in the sucrose-citrate medium in the presence of 8 mM KCl, 25°C, pH 10.3, phenylglyoxal 15 mM. Membrane proteins (70 μg) were separated by electrophoresis on a 12–25% SDS-polyacrylamide gradient gel. The stained gel and the calibrating gel described in the Methods section are shown in the upper part of the figure. The stacking gel, indicated by "s," contains 2% of the total activity in the gel. The band 3 region contained ~60% of the total radioactivity. Total membrane binding was $1.4 \times 10^6$ phenylglyoxal molecules/ghost, and chloride transport capacity was 50% of the control sample. 95% of the total radioactivity was recovered in the sliced gel.

[14C]phenylglyoxal in the membrane proteins, with 57% of the total activity found in the band 3 region (gel slices 8–13). The amount of radioactivity in band 3 in this experiment corresponded to the binding of ~7.9 $\times 10^6$ phenylglyoxal molecules/ghost. Besides a small amount of 14C corresponding to the location of the phospholipids (slices 41–48), four minor fractions of radioactivity were found in sections of the gel other than band 3. Some activity was found in the spectrin region (slices 3–5), which contained the
nonreacted phenylglyoxal that was not extracted by albumin. Minor amounts of radioactivity were located in the band 4.5 region (the “shoulder” of band 3 slices 14–20), and in the region of band 5–6 (slices 21–22) and in band 7 (slice 25). After “alkali stripping” of the membranes (Steck and Yu, 1973), the peripheral membrane proteins were removed with the same efficiency from phenylglyoxal-modified and intact membranes, but there were still significant amounts of radioactivity in the three regions corresponding to the “shoulder” of band 3 and the regions of bands 5–6 and 7. We suspect that this residual activity is located in integral membrane proteins that expose peptide segments to the extracellular environment, as do glycophorin and other integral glycoproteins of the membrane.

Fig. 9 shows the correlation between incorporation of [14C]phenylglyoxal into band 3 and the inhibition of chloride exchange when ghosts are modified in a low chloride medium. Maximum inactivation of chloride exchange is attained when $1.6 \times 10^6$ molecules of phenylglyoxal are bound to the band 3 molecules of a single cell. This corresponds to a minimum of 800,000 arginines modified per cell or ~1 arginine per band 3, assuming a phenylglyoxal: arginine stoichiometry of 2:1.

The data of Figs. 6–9 strongly suggest that one can selectively label band 3 with concomitant inactivation of anion transport in a low chloride medium. We therefore conducted an experiment in which resealed ghosts were reacted for approximately one half-time at 25°C and pH 10.3 with 15 mM labeled phenylglyoxal in a sucrose-citrate medium containing 8 mM KCl. The flux activity of these cells was ~50% that of untreated ghosts. We divided the labeled cells into two portions. On one portion, the chloride flux was determined and the distribution of [14C]phenylglyoxal was determined by SDS-PAGE as described above. The second portion was treated with extracellular chymotrypsin in a manner that cleaves band 3 into two fragments, one of ~65,000 daltons and a second of 35,000 daltons (Cabantchik and Rothstein, 1974b). This chymotrypsin-treated portion was then subjected to SDS electrophoresis and 14C counting as previously described.

Fig. 10 shows that in the modified ghosts that had not been treated with chymotrypsin (open circles), most of the [14C]phenylglyoxal is incorporated into band 3. Labeling of proteins other than band 3 is similar to that shown in Fig. 8. Fig. 10 also shows that in the chymotrypsin-treated sample (closed circles) the ~100,000-dalton band 3 has largely disappeared and two new bands of ~65,000 and 35,000 daltons were generated. Nearly all of the [14C]phenylglyoxal was incorporated into the 35,000-dalton fragment, which suggests that this fragment contains the arginyl residue(s) essential for anion transport function.

**Effect of DNDS on Phenylglyoxal Binding**

The rate of irreversible transport inactivation by phenylglyoxal depends on the anionic composition of the extracellular medium (Wieth et al., 1982b). At any fixed temperature, pH, and phenylglyoxal concentration, the maximal rate of inactivation was found in the chloride-free sucrose-citrate medium.
The rate of inactivation was decreased either by raising the concentration of KCl or by adding extracellular DNDS up to 2 mM to the extracellular medium. The effects of chloride and DNDS were not additive, as the rate of irreversible inactivation in 165 mM KCl was not reduced further by the addition of 2 mM DNDS (Wieth et al., 1982b, Table V). These results thus suggest that the effects of chloride and DNDS are only quantitatively different.

The relation between inactivation of chloride transport and phenylglyoxal binding in a sucrose-citrate medium containing 2 mM DNDS is illustrated in Fig. 11. As shown, we found a stoichiometric relationship between transport

![Graph](https://example.com/graph.png)

**Figure 9.** Inactivation of chloride self-exchange as a function of the binding of phenylglyoxal to band 3 of a red cell membrane. The ghosts were labeled in the presence of 15 mM phenylglyoxal in the sucrose-citrate medium containing 8 mM KCl, pH 10.3, 25°C. The amount of [14C]phenylglyoxal located in band 3 was determined by SDS-PAGE as in Fig. 8. The different symbols show results from experiments with seven different ghost preparations. The pseudo-first-order rate coefficient of transport inactivation, calculated from the experiments, was 0.15 s⁻¹ (SD 0.02).

inactivation and reagent binding that is indistinguishable from that seen in the chloride medium (Fig. 2). The number of phenylglyoxal molecules bound in band 3 at maximum transport inactivation, 4-5 × 10⁸ molecules/ghost, is identical with that found in the presence of 165 mM KCl (Fig. 4). These results therefore lend strong support for our previous suggestion (Wieth et al., 1982b) that chloride and DNDS influence the rate of transport inactivation by an identical mechanism, which may be to influence the protonation of an essential arginyl side-chain that only reacts with phenylglyoxal when it is in the deprotonated, uncharged state.
Covalent Binding of DIDS to Intact and Phenylglyoxalated Membranes

The results of Fig. 11 show that the reversible binding of stilbenedisulfonates interacts directly or indirectly with the state of the modifiable arginyl residues to affect their rate of phenylglyoxalation. We have therefore carried out experiments to investigate whether the rate of covalent binding of $^{3}H$-labeled DIDS is changed in phenylglyoxal-modified ghosts.

**Figure 10.** Distribution of $[^{14}C]$phenylglyoxal in red cell membrane proteins before (○) and after (●) chymotryptic cleavage of band 3. Labeling was performed in the sucrose-citrate medium containing 15 mM phenylglyoxal in 8 mM KCl, pH 10.3, 25°C. The membrane proteins (70 μg) were separated by electrophoresis on 12-25% SDS-polyacrylamide gradient gels. The two stained gels before (○) and after (●) treatment with chymotrypsin are shown in the upper part of the figure. The stacking gels, indicated by “s,” contained <2% of the total activity of the gels. The figure shows that the major part of the radioactive phenylglyoxal is located in the 35,000-dalton chymotryptic fragment of band 3. The inhibition of chloride self-exchange in the ghost preparation was 51%. The recoveries of radioactivity in the sliced gels before and after enzymatic treatment were 95 and 97%, respectively.

Fig. 12 compares the covalent binding of DIDS with native and with phenylglyoxal-treated red cell membranes at pH 7.0, 38°C. In the experiments of Fig. 12A we used an initial DIDS concentration of 9 μM, exposing the ghosts to $1.2 \times 10^{6}$ DIDS molecules/cell. In the intact membranes (closed circles, Fig. 12A), binding of $0.9 \times 10^{6}$ DIDS molecules had taken place in 10 min, and the binding was accompanied by almost complete inhibition of
transport (>99%). The number of DIDS-binding sites per cell is a little lower than the one usually reported. This is in agreement with our recent observation that the number of DIDS molecules causing complete transport inhibition is reduced to $9 \times 10^5$ when the cells are incubated as briefly as possible and the excess DIDS adsorbed in the membranes is carefully removed by albumin washing (Wieth et al., 1982c). Binding of DIDS to phenylglyoxal-treated membranes with a residual anion transport of 7.4% was much slower and leveled off after the labeling of 490,000 sites/cell, which corresponds to ~50% of the specific DIDS-binding sites in the red cell membrane. The DIDS concentration in the medium was still 4–5 $\mu$M after 90 min, when the reaction had attained 50% site occupancy. We have ascertained that the nonreacted DIDS molecules recovered from the supernatant are still able to bind irreversibly and inhibit anion transport in intact red cells. In five experiments it was found that an average of 68% (range 48–90%) of the DIDS molecules were still reactive by these criteria when used for treating a second cell sample. Therefore, it seems that half of the phenylglyoxalated band 3 molecules are unable to react covalently with DIDS under the conditions used in Fig. 12A. We arrive at the 50% estimate in the following way: 7.4% of the band 3

![Figure 11. Inactivation of chloride self-exchange as a function of the binding of phenylglyoxal to the band 3 of a red cell membrane.](image-url)
molecules (66,000), which are responsible for the residual flux after phenylglyoxal treatment, bind DIDS readily (Wieth et al., 1982b); if 50% of the remaining 834,000 band 3 molecules react with DIDS, total DIDS binding amounts to 66,000 + 417,000 = 483,000, in agreement with the result shown in Fig. 12A.

**FIGURE 12.** Binding of [3H]DIDS to native and phenylglyoxal-treated ghosts as a function of time. The ghosts were treated with phenylglyoxal in the sucrose-citrate medium, pH 10, 3, 25°C, to maximal inhibition. The ghosts were washed thrice in the flux medium and stored overnight at 0°C, then reacted at 38°C with [3H]DIDS. Part A shows the binding at a low concentration of DIDS (9 μM, 1.2 × 10⁶ DIDS molecules per ghost, cytocrit 29%, pH 7.2): (O) ghosts treated with 18 mM phenylglyoxal (residual transport 7.4%), (●) untreated control. Part B shows binding at a higher DIDS concentration (69 μM, 11 × 10⁶ DIDS molecules per ghost, cytocrit 28%, pH 7.2): (□) ghosts treated with 15 mM phenylglyoxal (residual transport 8.1%), (●) untreated control. At both high and low DIDS concentrations, maximal DIDS inhibition of chloride transport in the control ghosts was achieved after binding of 0.9–1.0 × 10⁶ DIDS molecules per ghost.

The binding of DIDS occurred at a slower rate in the phenylglyoxalated membranes. From the initial slopes of the binding curves in Fig. 12A, we calculated that the initial rate of DIDS binding to the phenylglyoxal-treated membranes was 12-fold slower than the initial rate of binding to native membranes (3.5 × 10⁴ vs. 4.2 × 10⁵ molecules/cell·min). The site of covalent DIDS binding in phenylglyoxalated membranes is likely to be identical with that in intact membranes. This was shown by examining the location of
[3H]DIDS after chymotryptic cleavage of band 3 from membranes that had been labeled under the conditions used in Fig. 12A. Almost all the [3H]DIDS activity was located in the 65,000 chymotryptic fragment, as is expected, when DIDS labeling is carried out at a neutral pH (Jennings and Passow, 1979).

The decreased rate of DIDS binding after phenylglyoxal modification may be due in part to a decreased affinity for the reversible binding of DIDS that precedes covalent labeling. This can be inferred from the experiments illustrated in Fig. 12B, which were performed with a high concentration of DIDS (69 µM, initially corresponding to $11 \times 10^6$ DIDS molecules/cell). The initial rate of DIDS binding to native membranes was comparable to that shown in Fig. 12A, presumably because reversible binding preceding the covalent reaction is almost complete at DIDS concentrations of both 9 and 69 µM. Fig. 12B shows that the binding in phenylglyoxalated membranes is moderately slower but continues above the value of 500,000 sites/cell (open squares), which suggests that all DIDS binding sites in the membrane are reactive when the DIDS concentration is sufficiently high. It must be noted that this conclusion rests on the assumption that nonspecific binding of DIDS to red cell membranes is not increased after phenylglyoxal treatment.

**DISCUSSION**

We have recently shown (Borders et al., 1981; Wieth et al., 1982a) that the chloride exchange system of the red cell membrane can be irreversibly inactivated by phenylglyoxal. The kinetics of transport inactivation are consistent with the specific reaction of arginyl residues with this reagent (Takahashi, 1968). This gives strong support to the notion that positively charged arginyl residues are critical for normal function of the exchange diffusion mechanism. The present study shows that phenylglyoxal modification of anion transport can be made highly specific by a proper choice of reaction conditions. Binding of the reagent takes place in a segment of band 3 (Fig. 10) that is different from that containing the primary site for covalent attachment of the stilbenedisulfonates (Cabantchik and Rothstein, 1974b; Ramjeesingh et al., 1980, 1981). It should be noted that the cross-linking of the two chymotryptic fragments with bifunctional stilbenes, which was observed by Jennings and Passow (1979), is insignificant when cells are treated with DIDS at a neutral pH.

**Reaction of Arginyl Residues with Phenylglyoxal**

Phenylglyoxal is highly but not absolutely selective for the modification of arginyl residues in proteins. The reported rate of reaction with lysyl residues is slower by orders of magnitude (Cheung and Fonda, 1979a). It has been established that positively charged guanidino groups have a widespread biological role as important components of anion binding sites in many proteins (Riordan et al., 1977). Many enzymes and other proteins contain a single particularly reactive arginyl guanidino group located at their anion recognition site (Riordan, 1979). Takahashi (1968) reported that phenylglyoxal reacts with the guanidino group of arginine in a 2:1 stoichiometry. A
probable reaction scheme is presented in Fig. 13. Recent reports (Cheung and Fonda, 1979a; Wieth et al., 1982b) suggest that the nonionized guanidine free base (Fig. 13, I) reacts with phenylglyoxal to form an initial 1:1 complex (II). This reaction appears to be rate limiting for the overall reaction. Structure II may then react rapidly with a second equivalent of phenylglyoxal to form a 2:1 complex (III) (Takahashi, 1968), either by protonation-reaction pathway 2ab or by reaction-protonation pathway 3ab. Most of the reports on the use of phenylglyoxal to modify essential arginyl residues at enzyme active sites have either substantiated or assumed that phenylglyoxalation occurs with the 2:1 stoichiometry shown in Fig. 13 (Riordan, 1979). In some studies, however, a 1:1 stoichiometry has been reported (Borders and Riordan, 1975; Werber et al., 1975; Kazarinoff and Snell, 1976; Philips et al., 1979; Vandenbunder et al., 1981). These 1:1 stoichiometries have usually been found under conditions of low phenylglyoxal concentration (Borders and Riordan, 1975) or in the presence of borate buffer (Werber et al., 1975; Kazarinoff and Snell, 1976; Vandenbunder et al., 1981). Borate can presumably complex the cis-diol of the 1:1 adduct (II or IV, Fig. 13) and prevent the reaction of the second equivalent of phenylglyoxal. Borate is known to stabilize the 1:1 adducts formed by the reaction of the arginyl guanidino group with both 2,3-butane-dione (Riordan, 1973) and 1,2-cyclohexanedione (Patthy and Smith, 1975), so stabilization of a 1:1 phenylglyoxal-arginine adduct is a likely possibility when borate is present in the phenylglyoxalation medium.

Selective Modification of the Anion Transport System

The maintenance of a neutral intracellular pH during the reaction of membranes with phenylglyoxal is a critical factor for obtaining selective modification of arginyl residues in the band 3 protein of erythrocyte ghosts. The red cell membrane contains \( \sim 150 \times 10^6 \) arginyl residues/cell. Most, if not all, can be labeled with phenylglyoxal. Fig. 1 shows that \( \sim 300 \times 10^6 \) phenylglyoxal molecules/cell are bound during the first 2 min of reaction when the pH is 10.3 on both sides of the membrane. Such indiscriminate protein modification is prevented when the intracellular pH is neutral. The lower curve of Fig. 1 shows that only a minor fraction of the membrane arginyl residues senses the
alkaline extracellular pH and reacts during the first 2 min. The subsequent abrupt increase in the rate of phenylglyoxalation suggests that the pH gradient breaks down after a few minutes, presumably because the integrity of some of the arginyl residues exposed to the extracellular phase is essential for the maintenance of an intact permeability barrier to protons and other monovalent cations, as proposed previously (Wieth et al., 1982b).

**Stoichiometry between Phenylglyoxal Binding and Transport Inactivation**

Modification of red cell membranes with arginine-specific reagents inactivates the transport capacities for both chloride (Borders et al., 1981; Wieth et al., 1982b) and sulfate (Zaki, 1981, 1982). The question arises whether the transport inhibition is a result of an unspecific “denaturation” of band 3 accompanying the chemical modification, or whether it indicates that one or a few arginyl residues are essential for the transport function of the protein molecule.

We have previously shown (Wieth and Bjerrum, 1982) that chloride exchange depends on the protonated form of one or more titratable groups having an apparent pK of ~12 at chloride concentrations above 100 mM. The titratable groups with the high pK did not appear to be related to the halide-binding modifier site (Dalmark, 1976), which was titrated with a significantly lower pK. The subsequent demonstration of a number of striking similarities between the properties of the transport regulating titratable groups with a high pK and its chemical reactivity towards phenylglyoxal made us conclude that interactions between arginyl residues in band 3 and the transported anions are critical for normal transport function (Wieth et al., 1982b). We now demonstrate that only a few of the 44 arginyl residues in band 3 need to be considered essential for anion transport. In a medium containing 165 mM KCl, maximal inactivation correlates with the incorporation of ~9 X 10⁶ phenylglyoxals/cell (Fig. 2) or with incorporation of 4.5 X 10⁶ phenylglyoxals into the approximately one million copies of band 3 in a single cell (Fig. 4). Modification under the conditions used in our experiments should favor a 2:1 phenylglyoxal:arginine stoichiometry, for we use relatively high concentrations of phenylglyoxal and avoid the use of borate buffers *(vide supra)*. Maximal inactivation of transport in 165 mM KCl should thus be due to modification of only 2 out of a total of 44 arginines per molecule of band 3. If, instead, the stoichiometry is 1:1 under these conditions, an upper limit of four arginines per band 3 would be modified on maximal inactivation.

Modification becomes even more selective when the reaction is carried out at a low extracellular KCl concentration, where maximal inactivation can be achieved by incorporation of only 2.3 X 10⁶ phenylglyoxals/cell (Fig. 6) or 1.6 X 10⁶ phenylglyoxals in the band 3 proteins of a single cell (Fig. 9). These data suggest that inactivation at low extracellular KCl is due to the modification of maximally two, and presumably only one, arginine per molecule of band 3. The high selectivity of the reaction is illustrated in Fig. 7, where a given degree of transport inhibition is obtained after binding of less phenyl-
glyoxal at low extracellular chloride concentrations than at high concentrations.

Maximum inactivation of erythrocyte anion transport can thus, by proper choice of the reaction conditions, be obtained after modifying <1% of the total arginyl residues in the membrane proteins of a single cell (Figs. 6 and 9). This selectivity can be compared to the remarkable selectivity of phenylglyoxal for modification of only the functionally essential arginyl residues at enzyme active sites, as many enzymes can be fully inactivated by phenylglyoxal after modification of a single arginyl residue per enzyme protomer or subunit (Borders and Riordan, 1975; Berghäuser, 1975; Armstrong et al., 1976; Kantrowitz and Lipscomb, 1976; Philips et al., 1979; Cheung and Fonda, 1979b; Borders and Johansen, 1980a, b; Vandenbunder et al., 1981). Phenylglyoxal is not an "affinity" reagent or an "active-site-directed" reagent, for most studies using this reagent show that rates of inactivation of enzymes are first order in phenylglyoxal. This is also the case in the inactivation of red cell anion transport (Wieth et al., 1982b). It thus appears that the arginyl residue(s) essential in anion transport possesses the same high reactivity that has been found with those arginyl residues that are critical components of numerous enzyme active sites (Borders and Riordan, 1975; Borders et al., 1979). This high degree of selectivity must reflect that the essential arginyl residues in both the enzymes and the anion transport protein are more reactive than the remaining arginines, possibly because they are located in a relatively hydrophobic environment or in a region with a positive surface potential that favors the phenylglyoxal reaction at a given pH by lowering the pK of the reactive guanidine group. We have recently argued that a positive surface potential seems to influence the apparent pK of the titratable group(s) regulating the membrane transport function by presenting evidence that the concentration of chloride affects the pK, which determines the transport function (Wieth and Bjerrum, 1982). The same effect of chloride on the degree of protonation is believed to determine the rate of transport inactivation by phenylglyoxal at a given extracellular pH (Wieth et al., 1982b).

Localization of Phenylglyoxal Binding in Band 3

By chymotryptic cleavage of band 3 (Fig. 10), we found that almost all [14C]phenylglyoxal bound to band 3 under the most specific conditions of reaction was recovered in the 35,000-dalton fragment. The radioactive labeling pattern was similar when membranes were phenylglyoxalated in the presence of 165 mM chloride (data not shown). This shows that the 35,000-dalton segment contains at least two highly reactive arginyl residues and that at least one of these is involved in transport function. The primary covalent attachment site for DIDS, a lysyl residue of the 65,000-dalton fragment (Ramjeesingh et al., 1980, 1981; Passow et al., 1980a), resides in a different primary segment of band 3. The results of our previous work (Wieth et al., 1982b) and of Fig. 11 clearly show that DNDS interferes with the rate of phenylglyoxalation.
These findings add support to the conclusion that the anion binding region of band 3 is composed of peptide segments derived from both the 35,000- and 65,000-dalton chymotryptic cleavage fragments of band 3, an idea that has its origin in the observation that the two fragments can be covalently cross-linked by DIDS at alkaline pH values (Jennings and Passow, 1979).

Further evidence for the interaction between the DIDS binding site and the site for phenylglyoxal binding was found by comparing the rates and degrees of DIDS binding with normal and phenylglyoxal-modified red cell membranes (Fig. 12). We have observed that the rate of covalent DIDS binding in intact membranes at pH 7.0 is independent of DIDS concentration as long as this is high enough to ensure saturation by reversible binding, which in the presence of 150 mM chloride occurs with a half-saturation constant of <0.2 μM (Funder et al., 1978). However, the rate of DIDS binding was reduced in phenylglyoxalated membranes at both high and low DIDS concentration (Figs. 12A and B), which suggests that the affinity for reversible binding of DIDS is decreased in phenylglyoxalated cells. The uptake of DIDS at low concentration (9 μM, or ~1 DIDS per band 3 at the cytocrit of the experiment) showed additionally that DIDS incorporation leveled off at a total binding of $5 \times 10^5$ molecules/cell in spite of the fact that the extracellular medium still contained reactive DIDS at the end of the labeling period. 50% of the sites in the phenylglyoxal-treated membranes must thus have a much lower affinity for reversible DIDS binding than the other half of the sites.

It is not possible at this time to decide to what extent the reduced affinity for DIDS is due to modification of charges (arginyl residues) that contribute to the reversible binding of the sulfonate groups, to allosterically induced conformational changes, or to steric hindrance caused by the covalent binding of the phenylglyoxal molecules. It seems likely that the differing affinities of the two populations of the DIDS binding sites (Fig. 12A) may be related to the occurrence of band 3 in the membrane matrix as dimers or higher oligomers (Steck, 1972; Weinstein et al., 1980; Dorst and Schubert, 1979). The interaction of phenylglyoxal binding with DIDS labeling is further evidence of the site-to-site interactions between binding of inhibitor molecules to band 3, as first observed with some particularly bulky stilbenediisulfonates (Dix et al., 1979; Macara and Cantley, 1981a, b). Along the same line, we have recently shown that membrane modification with a nonpermeating carbodiimide inactivates only 50% of the anion transport capacity and reduces covalent DIDS binding by 50%, presumably because modification of one band 3 protomer prevents the carbodiimide modification of the second, which still can bind DIDS covalently (Wieth et al., 1982a). Thus, there is increasing evidence that interactions between the protomers of band 3 are discernible by means of inhibitor molecules that bind covalently in the extracellularly exposed anion binding region of the transport protein. The results of Figs. 12A and B show that the interprotomeric interaction after arginyl modification is particularly conspicuous at low DIDS concentrations. This is in agreement with the report by Zaki (1982) that the $\text{H}_2\text{DIDS} [1,2\text{-bis(4-isothiocyanato-2-sulfophenyl)}\text{ethane or 4,4'\text{-diisothiocyanodihydrostilbene-2,2'-disulfonic acid}}$]
binding capacity is not significantly reduced when one uses relatively high concentrations of H$_2$-DIDS in the medium, whereas covalent H$_2$-DIDS binding to cyclohexanediene-modified membranes was reduced by 50% when the initial H$_2$-DIDS concentration was 10 µM (Zaki, 1981).

**Implications for the Structure of the External Anion Binding Region**

Our results supplement previous evidence which suggested that polypeptide strands of the 65,000- and 35,000-dalton chymotryptic cleavage fragments contribute to form the external anion binding region of the anion exchange system (Jennings and Passow, 1979; Kempf et al., 1981). Although the results of Fig. 9 make it clear that transport function can be inactivated by the binding of phenylglyoxal sufficient to modify a single arginyl residue in band 3, we find it premature to conclude that anion recognition at the external side of the membrane involves one arginyl residue of the protein only. In the literature on anion exchange diffusion in red cells, it is often assumed that the transport process is facilitated by the recognition and binding of an anion by a single functional group in the transport protein. Anion binding is assumed to induce a conformational change exposing this “binding site” to the trans side of the permeability barrier. This concept, which has an attractive similarity to the mobile carrier model of exchange diffusion, is likely to represent an oversimplification. Studies using chemical probes and enzymatic cleavage have made it quite clear that the functional transport region, the transport site in a wider term, is composed by a number of amino acid side-chains, donated by different segments of the band 3 protein (Passow et al., 1980b). Our concept of an anion binding site is based on the general model of a substrate-binding enzyme active site, as revealed by x-ray crystallographic studies of many enzymes, where many different residues act in concert in substrate binding and in the conformational changes accompanying the subsequent catalytic event. The modification of any one of a number of different residues may thus be sufficient to fully inactivate the enzyme, but that single residue is not, of itself, the active site. The external anion binding region of band 3 may be composed in a similar fashion.

We cannot exclude that the effect of phenylglyoxalation is allosteric, but we do not find it likely in view of the functional properties of the modifiable group(s) (Wieth and Bjerrum, 1982; Wieth et al., 1982b). It must be noted that it is not necessary to assume that an anion binding “site” is alternately exposed to the extra- and intracellular phases. The so-called ping-pong kinetics of anion exchange (Gunn and Fröhlich, 1979; Jennings, 1982) can be accounted for by a zipper-like array of salt bridges, exposing different anion binding regions at the two sides of the membrane (Wieth et al., 1982c). Extracellularly exposed arginine side-chains might thus provide a positively charged slide guiding anions to the exchange mechanism, similar to the arrangement of guanidino groups proposed to lead substrate anions to the active site of carboxypeptidase A (Nakagawa and Umezawa, 1978).

The chemical reactivity of an arginyl residue is determined by its state of protonation, because phenylglyoxal reacts preferentially with the electrically
neutral guanidino base. Because the rate of inactivation is fastest in the absence of transportable anions in the extracellular medium, we assume as a working hypothesis that the anion effect on the rate of transport inactivation by phenylglyoxal is due to the shielding of a positive interfacial surface potential at the anion binding region (Wieth et al., 1982b). The surface potential increases when the transportable anions are removed from the extracellular side of the membrane, and the apparent pK of the groups in the anion binding region is lowered because the electrostatic work of protonation increases. If two guanidino groups are located close to another at the exofacial anion binding site, only one will be labeled during a brief exposure to phenylglyoxal if the pK of the nonreacted group "jumps" to its high intrinsic value when the electrostatic interaction between the two groups is abolished by modification of the first. This may explain why we find that the reaction rate of a single arginyl residue is accelerated in the absence of external chloride, whereas inactivation of anion transport seems to be accompanied by the modification of two arginyl residues in the 35,000-dalton segment of band 3 when the chloride concentration is sufficiently high to prevent the presumed electrostatic interaction between the positively charged groups (Wieth and Bjerrum, 1982). Present evidence does not permit a final conclusion with regard to the number of guanidino groups involved in chloride binding at the external side of the membrane.

We do not know whether the four phenylglyoxal molecules that are bound in band 3, when transport is inactivated in the presence of chloride (Fig. 4), are located in two arginyl residues, or whether, for example, the second pair of phenylglyoxal molecules are randomly incorporated into a larger number of slowly reacting arginyl residues that are accessible from the extracellular medium. The question cannot be settled until it becomes possible to determine the location of the modified arginyl residues in the primary amino acid sequence. So far, the selectivity of the chemical modification is illustrated by the fact that none of the ~25 arginyl residues in the 65,000-dalton segment of band 3 are modified during transport inactivation, and that the essential residue(s) are located in the 35,000-dalton fragment, which contains a total of 16–18 arginyl residues (Steck et al., 1978).

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