Functional Carboxyl Groups in the Red Cell Anion Exchange Protein

*Modification with an Impermeant Carbodiimide*

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**Abstract** Anion exchange in human red blood cell membranes was inactivated using the impermeant carbodiimide 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide (EAC). The inactivation time course was biphasic: at 30 mM EAC, ~50% of the exchange capacity was inactivated within ~15 min; this was followed by a phase in which irreversible exchange inactivation was ~100-fold slower. The rate and extent of inactivation was enhanced in the presence of the nucleophile tyrosine ethyl ester (TEE), suggesting that the inactivation is the result of carboxyl group modification. Inactivation (to a maximum of 10% residual exchange activity) was also enhanced by the reversible inhibitor of anion exchange 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) at concentrations that were 10³-10⁴ times higher than those necessary for inhibition of anion exchange. The extracellular binding site for stilbenedisulfonates is essentially intact after carbodiimide modification: the irreversible inhibitor of anion exchange 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) eliminated (most of) the residual exchange activity; DNDS inhibited the residual (DIDS-sensitive) Cl⁻ at concentrations similar to those that inhibit Cl⁻ exchange of unmodified membranes: and Cl⁻ efflux is activated by extracellular Cl⁻, with half-maximal activation at ~3 mM Cl⁻, which is similar to the value for unmodified membranes. But the residual anion exchange function after maximum inactivation is insensitive to changes of extra- and intracellular pH between pH 5 and 7. The titratable group with a pKₐ of ~5.4, which must be deprotonated for normal function of the native anion exchanger, thus appears to be lost after EAC modification.

**Introduction** The major component of anion movement across red blood cell membranes is the tightly coupled 1:1 exchange mediated by the integral membrane protein, band 3.

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also named capnophorin (Wieth and Bjerrum, 1983). For a recent review, see Passow (1986). The amino acid sequence of the murine form of the exchange protein has been deduced from cDNA sequencing (Kopito and Lodish, 1985), and structural models for the organization of the exchanger have been proposed (Kopito and Lodish, 1985; Jay and Cantley, 1986; Passow, 1986). The detailed molecular mechanism for the selective and rapid exchange of anions is not known, although salt bridges between positively charged arginyl (guanidinium) residues and negatively charged residues (possibly carboxylates) may be essential components of the extracellular anion recognition site (Macara and Cantley, 1981; Wieth, 1981; Brock et al., 1983).

Initial evidence for the possible functional roles of arginyl and carboxyl residues was obtained from studies of the effects of extracellular pH on the exchange function. Titration of the extracellular solution in the alkaline range provided evidence for a critical group with a $pK_a \approx 12$ in 165 mM chloride, and it was suggested that an arginyl residue was involved (Wieth and Bjerrum, 1982). Further support for this hypothesis was obtained in experiments where the exchange system was covalently modified with group-specific reagents. Arginyl residues in proteins can be modified irreversibly by reaction with phenylglyoxal (PG) (Takahashi, 1968; Riordan, 1979), and at least one functionally essential arginyl residue was found at the extracellular anion recognition site using chemical modification with PG (Wieth et al., 1982b; Bjerrum et al., 1983).

Titration of the extracellular solution in the acid pH range provided evidence for a critical group, or groups, with a $pK_a \approx 5.5$ (Wieth et al., 1982a). The $pK_a$ of the acid branch of the titration function is fairly independent of temperature, suggesting that the critical group(s) has a low ionization enthalpy. Hence, among amino acid side chains with $pK_a$ values in this pH range, carboxyl groups are likely candidates (e.g., Edsall and Wyman, 1958). Carboxyl residues in proteins can be irreversibly modified by carbodiimides (Hoare and Koshland, 1967; Carraway and Koshland, 1972; Lundblad and Noyes, 1984), and we report here on the use of chemical modification with a water-soluble, membrane-impermeable carbodiimide to search for functionally important carboxyl residues in the anion exchange protein.

Previously, several attempts have been made to identify functionally essential carboxylates in the anion exchange protein using water-soluble carbodiimides. Deuticke (1977) reported that sulfate permeability was irreversibly inactivated by reacting red cells with 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC), but the details were not published. Reaction with EDC likewise led to an irreversible inactivation of phosphate permeability (Craik and Reithmeier, 1984). We attempted to use EDC to inactivate $Cl^-$ exchange, but in our hands EDC treatment of red cells was accompanied by massive hemolysis, presumably because the reagent, in its unprotonated form is a fairly nonpolar tertiary amine and thus permeant and able to modify intracellular as well as extracellular carboxyl groups. To investigate the functional effects of modifying only carboxyl groups that are accessible from the extracellular solution, we reacted red cell membranes with a quaternary alkylammonium carbodiimide, 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide (EAC) (Sheehan et al., 1961; George and Borders, 1979), which we found could not cross the red cell membrane (see below). Our results support the hypothesis that one or
more extracellularly exposed carboxyl groups in the exchange protein are important for the normal anion exchange. Preliminary reports of some of this work have been presented (Wieth et al., 1982a; Andersen et al., 1983).

**MATERIALS AND METHODS**

**Materials**

Radioactive isotopes. $^{36}$Cl as NaCl or KCl, specific activity (sp. act.) 500 µCi/mmol, and carrier-free $[^{35}S]$sulfate in dilute HCl were from AEK (Risør, Denmark); $[^3H]$inulin, sp. act. 900 mCi/mmol, was from Radiochemical Centre (Amersham, England); $[^{14}C]CH_3I$, sp. act. 40 mCi/mmol, and L-ring-2,6-$[^3H]$tyrosine, 34.7 Ci/mmol, were from New England Nuclear (Boston, MA). L-$[^3H]$Tyrosine ethyl ester (L-$[^3H]$TEE) hydrochloride, sp. act. 0.2 mCi/mmol, was synthesized from L-$[^3H]$tyrosine and absolute ethanol as described elsewhere (Borders et al., 1984); and $[^{14}C]$EAC, sp. act. 0.4 mCi/mmol, was synthesized as described below.

Chemical reagents. All solutions were prepared from reagent grade chemicals. Sodium 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) was from ICN K and K Laboratories Inc. (Stamford, CT). 4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) was prepared according to the method of Funder et al. (1978), phenylglyoxal (PG) was from Aldrich Chemical Co. (Milwaukee, WI), while TEE and glycine methyl ester hydrochloride (GME) were from Fluka (Basel, Switzerland). The buffer salts, 2-N-morpholinoethanesulfonic acid (MES), piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) were from Calbiochem-Behring Corp. (San Diego, CA). Albumin from bovine serum (fraction V, powder and crystalline), trypsin and chymotrypsin from bovine pancreas, phenylmethanesulfonyl fluoride (PMSF), EDC hydrochloride, and DL-dithiothreitol (DTT) were from Sigma Chemical Co. (St. Louis, MO). EAC (as the $I^-$ salt) was synthesized from EDC hydrochloride and methyl iodide (see below). Reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and molecular weight standards were from Biorad Labs (Richmond, CA). Lumasolve was from Lumac B.V. (Landgraaff, The Netherlands). Commassie brilliant blue R 250 was from Merck, Sharp and Dohme (West Point, PA).

Synthesis of $[^{14}C]$EAC. A typical synthesis is described. 384 mg (2.00 mmol) EDC hydrochloride was added to a vigorously stirred mixture of 5 ml 80% saturated aqueous Na$_2$CO$_3$ and 3 ml ether. After 1 min, stirring was discontinued and the ether layer was removed and dried over CaSO$_4$. The aqueous layer was extracted with three additional 1-ml aliquots of ether, and all ether extracts were combined and dried over CaSO$_4$. The ether solution of EDC free base was filtered into a vigorously stirred solution of 75 µl (171 mg, 1.20 mmol) CH$_3$I plus $\sim$250 µCi $[^{14}C]CH_3I$ in 3 ml anhydrous ether in a 12-ml cylindrical vial. The vial was sealed and covered with aluminum foil to exclude light, and the mixture was stirred at room temperature for 17 h. The white crystalline solid that formed was isolated by vacuum filtration, recrystallized by dissolution in 2 ml dry acetone followed by trituration with 6 ml anhydrous ether. It was then isolated by filtration and dried to a constant weight in a vacuum desiccator. Yield: 188 mg (53%); m.p., 94.5–95.5°C.
For the synthesis of cold EAC, the same procedure was used on a 5X scale, except that a 1.5-fold mole excess of CH₃I over EDC free base was used.

Methods

Preparation of red cell membranes. Resealed human red cell membranes containing 165 mM KCl, 2 mM TRIS, 0.25 mM EDTA, and 0.25 mM EGTA, were prepared as described by Funder and Wieth (1976) and Bjerrum et al. (1983).

Electrolyte solutions. Chemical modification of intact red blood cells and resealed red cell membranes with EAC was carried out at 38°C, usually in 165 mM KCl buffered with 10 mM MES. At [DNDS] >2 mM, NaCl was used because of the higher solubility of Na₂DNDS. When present, DNDS and TEE were dissolved in the modification solution, which was titrated to pH 5.8 before starting the modification. To minimize degradation of EAC in the slightly acidic reaction solution, EAC was added as a dry powder immediately before the addition of cells or resealed membranes. This caused no pH change.

The EAC reaction was stopped by diluting the membrane or cell suspension into an alkaline solution at 0°C. Two stopping solutions were used. Solution I contained 165 mM KCl plus 4 mM TRIS, and the pH was adjusted such that the addition of 1 vol of the reaction mixture to 10 vol of stopping solution resulted in a pH > 9. Solution II contained 165 mM KCl plus 10 mM PIPES, pH 7.5. The modification was stopped by adding 1 vol of the reaction mixture to 10 vol of the stopping solution. Solutions I and II were equally efficient in interrupting the EAC modification, and the inactivation kinetics were biphasic when either was used.

The efflux solution contained 165 mM KCl, 2 mM K₂HPO₄, and was titrated to the desired pH (usually 7.3) at 0°C. The same solution, plus 1 mM EDTA, was used for washing the resealed membranes after reaction with EAC. Reacted cells or membranes were washed thrice at pH 7.3, 0°C, and in most experiments they were stored overnight in the same solution containing 0.5% albumin before measuring the 36Cl⁻ efflux. (In experiments where 36Cl⁻ exchange was measured before and after overnight storage, there was a slight enhancement in the degree of exchange inactivation.)

EAC modification. Two different procedures were used to react intact cells and resealed membranes with EAC. Except where noted, the modifications were done at pH ~6.0.

In method A, resealed membranes with an intracellular pH of ~7.3 (0°C) were packed in unbuffered KCl to a cytocrit of ~80% by centrifugation for 15 min at 44,000 g (Bjerrum et al., 1983). 2–6 ml of packed membranes were reacted in a one- to threefold larger volume of modification solution. The final [EAC], in millimolar, was calculated as [EAC] = a·1,000/[MW·(S + M)·(1 − C₁·Cₘ·M/(C₁·S + Cₘ·M))], where a denotes mg EAC added, MW is the molecular weight of EAC (297 g/mol), S and M denote the volume (in milliliters) of the modification solution and packed membranes, respectively, C₁ is the corrected cytocrit of the resealed membranes, and C₁ and Cₘ are the total solute concentrations in the modification and resealed membrane solution before mixing. At appropriate time intervals, 0.5–12 min after mixing, 0.5–1.5-ml aliquots were withdrawn from the well-stirred reaction suspension and mixed with the stopping solution. During the 30–60-min reac-
tion, the pH of the reaction medium increased ~0.3 units. The modified membranes were washed and stored as described above.

In method B, intact cells or resealed membranes were titrated to an intracellular pH of ~6.0 at 38°C to avoid pH changes during the modification. Intact cells were titrated with CO₂ to pH 6.6 in an unbuffered solution at 0°C. They were then washed thrice, with a 10-min equilibration period at 38°C, in 165 mM KCl plus 10 mM MES, pH 6.2 (0°C). (The final intracellular pH at 38°C was ~6.0 when measured after hemolysis in distilled water.) Resealed membranes were titrated to pH 6.2 (0°C) by washing thrice in the same solution. Between each centrifugation, the suspension equilibrated for 5 min at room temperature. The cells or resealed membranes were then packed and used for modification as described for procedure A. The pH increase during modification was usually <0.1 pH units over 30 min. When cells or resealed membranes were modified using method B, the intracellular pH was raised to ~7.3 before the Cl⁻ exchange flux was determined. This was done by three washes in the efflux solution with a 10-min equilibration at 38°C, at an extracellular pH of 7.4.

Cl⁻ exchange flux. EAC-modified cells or resealed membranes were washed once in the efflux solution and used for efflux experiments at 0°C as described by Dalmark and Wieth (1972). Cells labeled with [¹⁴C]EAC were divided into two portions, one for determination of Cl⁻ efflux, the other for determination of membrane-bound EAC.

The rate coefficient for Cl⁻ efflux, k, was determined as described by Wieth and Bjerrum (1982). The cell (or resealed membrane) volume varied by <10% after 30 min of modification by EAC, which was followed by incubation in the stopping solution. The EAC-induced inactivation, as a percent of the control flux, could thus be expressed as 100·(k₀ - k_{EAC})/k₀, where the subscripts “0” and “EAC” denote rate coefficients for control and inactivated cells (or membranes), respectively.

SDS-PAGE. The localization of [¹⁴C]EAC or [³H]TEE in the membrane proteins was examined by SDS-PAGE (Bjerrum et al., 1983), using the discontinuous buffer system described by Laemmli (1970). Each sample was run in duplicate. One strip was stained for protein while the second was cut into 2.5-mm slices that were counted for ¹⁴C or ³H. The recovery of radioactivity was 90 ± 7% (n = 18).

Labeling with [¹⁴C]EAC or [³H]TEE. A 3-ml aliquot of packed resealed membranes was labeled with [¹⁴C]EAC or [³H]TEE in 3–4 ml modification solution using method B. Nonspecific labeling was reduced by treating the membranes with trypsin (normally before the chemical modification). After stopping the EAC reaction, the membranes were washed thrice in an efflux solution containing 1% albumin plus 1 mM EDTA, and stored overnight at 0°C. This reduced [¹⁴C]EAC binding at band 3 insignificantly. There was, likewise, only a little reduction in [¹⁴C]EAC labeling if membranes were washed extensively at 38°C, indicating that only a small amount of [¹⁴C]EAC is physically adsorbed. In contrast, incorporation of [³H]TEE was reduced by incubating membranes with albumin plus EDTA. This reduction was temperature and pH dependent, indicating that part of the [³H]TEE is physically adsorbed. SDS-PAGE of [³H]TEE-labeled membranes revealed a large amount of radioactivity that migrated at the position of the phospholipids. It was this component that was reduced by treatment with albumin plus EDTA.
Treatment with trypsin and chymotrypsin. Resealed membranes, 20–50% cytocrit, were incubated with 0.5–1 mg/ml enzyme in the efflux solution (pH 7.3, 38°C) for 1 h. The incubation was interrupted by washing the membranes thrice in the same solution, with 0.2 mM PMSF in the last wash. When treated with trypsin before EAC modification, the membranes were modified using method B. After treatment with trypsin the anion exchange protein was partially cleaved to a 60-kD fragment. Similar degradation fragments have been reported after treatment with extracellular chymotrypsin (Cabantchik and Rothstein, 1974b) or with extracellular trypsin at low ionic strength (Jenkins and Tanner, 1977). Given the extensive incubation with trypsin, our results can be explained by a direct action of trypsin or by a small chymotrypsin contamination of the trypsin.

Determination of bound [14C]EAC or [3H]TEE. The amount of membrane-bound EAC or TEE was calculated from the reagent sp. act. and the amount of radioactivity associated with extensively washed membranes using procedures described by Bjerrum et al. (1983). Membrane concentrations were determined by cell counting, or quantitative analysis of membrane proteins (Lowry et al., 1951), using the conversion factor 5.25 × 10⁻¹⁵ g protein/membrane (Bjerrum et al., 1983).

Treatment of resealed membranes with DIDS or PG. This was done as described by Wieth et al., (1982b) and Bjerrum et al. (1983) for DIDS and PG, respectively.

Membrane permeability to EAC and TEE. The (im)permeability to [14C]EAC was determined in influx experiments with a cytocrit of ~50%. The loss of (extracellular) [14C]EAC from a solution containing 10 mM EAC was undetectable after 60 min of incubation at 38°C, pH 7.3. This indicates that resealed membranes are impermeable to EAC, and, additionally, that the amount of EAC that became covalently attached to membranes during EAC modification was insignificant compared with the total amount present. The impermeability to [14C]EAC was confirmed by SDS-PAGE of the [14C]EAC-labeled membranes, which showed that intracellular proteins such as spectrin were not measurably labeled (see Figs. 10 and 11).

Membrane permeability to TEE was also determined in influx experiments. At 38°C, pH 7.3, [3H]TEE equilibrated across resealed membrane with a half-time of <10 s.

RESULTS

Kinetics of EAC-induced Inactivation of Anion Exchange

Incubation of red cell membranes with EAC leads to irreversible inactivation of the anion exchange function. The time course for irreversible inactivation of membranes reacted with 26 mM EAC at pH 6 is illustrated in Fig. 1. There was a rapid initial phase of inactivation during the first 10–15 min, during which the exchange function decreased to ~50% of the control value. This was followed by a slow phase, where the rate of inactivation was ~100-fold smaller. Similar results were obtained with intact cells, results not shown. EAC is somewhat labile in aqueous solution, especially at acid pH (C. L. Borders, Jr., unpublished results), and the biphasic time course could result from a decrease in [EAC]. This is not the case. If resealed membranes were reacted with EAC for 32 min, such that the exchange function was ~50% of control, then washed free of reagent and exposed to a fresh but otherwise
identical EAC solution, the inactivation proceeded at a rate comparable to that of the slow phase (results not shown).

These experiments, as well as others in which reacted membranes were stored for >18 h at 0°C with no reversal of inactivation, demonstrate that the modification is irreversible. As an additional test of irreversibility, resealed membranes were reacted with EAC, washed free of reagent, and incubated at 38°C and pH 7.4 for 30 or 60 min before determining the $^{36}$Cl$^-$ efflux. The extent of inactivation after this
incubation was within 5% of that determined before the 38°C incubation step (results not shown).

The rate of inactivation of anion exchange varied as a function of [EAC]. The major effect of increasing the [EAC] was to accelerate the rapid phase of inactivation. A second effect was to increase the fraction of the exchange capacity that was inactivated during the rapid phase from ~30% at 12 mM EAC to ~75% at 80 mM EAC. The rate of the slow phase was not affected by changes in [EAC]. Fig. 2 shows effects of changes in [EAC] on the inactivation kinetics (cf. Fig. 1). When resealed membranes that had been reacted with EAC to ~50% inactivation were exposed to a fresh EAC solution with an increased [EAC], there was additional rapid inactivation. A similar result was obtained if the [EAC] was increased during the modification by the addition of solid EAC. In either case, the "maximal" rapid inactivation approaches that observed when the membranes are reacted at the same high [EAC] from the beginning. Irrespective of the kinetic complexities implied by the biphasic inactivation time course, the extent of inactivation in the rapid phase depends only on the final [EAC].

In contrast, if [EAC] in the reaction solution was diluted 180-fold with an EAC-free solution (from 36–0.2 mM) after an 8-min resection, and the reaction was continued for 20 to 60 min before quenching it at pH 7.5 and 0°C, the extent of the inactivation was unaffected; at 8 min (before dilution), the exchange was inactivated to ~45% of control, 20 and 60 min after dilution, the inactivation was ~45% and ~50%, respectively. The reaction between EAC and the anion exchange protein is irreversible.

Inactivation of sulfate permeability by EAC also had a biphasic time course (results not shown) similar to that for inactivation of Cl⁻ exchange. In these experiments, intact cells were reacted with EAC in a 165-mM KCl solution. After the reaction was quenched, the cells were incubated in 2 mM SO₄²⁻ and loaded with [³⁵S]SO₄²⁻. Sulfate efflux was determined in a medium containing 165 mM KCl, 2

![Figure 3](image-url)
mM K$_2$SO$_4$, at pH 7.3 and 0°C. With 16 mM EAC, 35% of the exchange capacity was inactivated after 8 min, and 50% inactivation was reached after 18 min; these results are nearly identical to those observed when Cl$^-$ efflux was determined (results not shown). The similar time courses for inactivation of Cl$^-$ and SO$_4^{2-}$ fluxes suggest that the modification affects the permeation of mono- and divalent anions to the same extent.

Carbodiimide-induced modifications of carboxyl groups are sometimes accelerated by the addition of nucleophiles (Lundblad and Noyes, 1984). Inactivation of anion exchange by EAC was accelerated twofold by the addition of 40 mM of the nucleophile TEE (Fig. 3). The addition of 40 mM TEE had two effects: the rapid phase of the inactivation was accelerated twofold, and the extent of inactivation after 30 min was increased from ~50 to ~70%. This nucleophile's effect is consistent with the inactivation being due to modification of carboxyl group(s). Different nucleophiles affect the inactivation rate differently, however, as modification in the presence of 50–75 mM GME decreased the inactivation rate by 30–50% (results not shown). The possible origin of this selective effect of different nucleophiles is discussed below.

**Effects of pH, Substrate Anions, and Reversible Anion Exchange Inhibitors**

To examine the molecular basis for the EAC-induced inactivation of anion exchange, we studied the effects of pH, substrate anions, and reversible inhibitors on the inactivation rate.

Water-soluble carbodiimides react with the protonated form of a carboxyl group (see Discussion). One would therefore expect that the inactivation rate would decrease as the pH is increased. This was the case: the rapid phase of inactivation of Cl$^-$ exchange by 30 mM EAC in 165 mM KCl, at 38°C, was threefold slower at pH 7 ($k = 0.04 \pm 0.02$ min$^{-1}$, mean ± SD) than at pH 6 ($k = 0.13 \pm 0.02$ min$^{-1}$) (results not shown). If we were modifying the group(s) that underlie the acid branch of the titration function, the reaction should have been slowed about eightfold. That the observed decrease was less than expected may reflect that we modified at least two different groups in the exchange protein (see Discussion).

To examine whether EAC modified a group involved in anion binding, we compared the rate of the rapid inactivation phase in I$^-$ and SO$_4^{2-}$ solutions with that in a Cl$^-$ solution ($k = 0.13 \pm 0.02$ min$^{-1}$). Both I$^-$ and SO$_4^{2-}$ bind with higher affinity than Cl$^-$ to the anion exchanger (e.g., Knauf, 1979; Milanick and Gunn, 1982). Thus, one might expect that they would protect against inactivation if a carboxyl group at the anion binding site were being modified. The somewhat surprising results were that modification was slowed threefold ($k = 0.05 \pm 0.01$ min$^{-1}$) when the KCl was replaced by 110 mM K$_2$SO$_4$, but increased twofold ($k = 0.29 \pm 0.03$ min$^{-1}$) when KCl was replaced by 165 mM KI (results not shown). The protective effect of sulfate could indeed suggest that EAC modifies a carboxyl group in the exofacial anion binding site. But the I$^-$-induced enhancement of the inactivation rate strongly suggests the exchange inactivation is not due to modification of a carboxyl group at the halide binding site. To examine this question further, we determined the effects of DNDS on the EAC-induced inactivation of anion exchange.

DNDS, a reversible competitive inhibitor of anion exchange (Fröhlich, 1982), is
believed to bind at the extracellular anion binding site. In 165 mM Cl\(^-\), DNDS inhibits Cl\(^-\) exchange with half-maximal inhibition at 5 mM (Fröhlich, 1982). At this [DNDS], there was no effect on the rate or extent of the EAC-induced inactivation of anion exchange (results not shown). But when the modification was carried out in the presence of 2 mM DNDS, a concentration that reversibly inhibits ~99.8% of the exchange function, the rate and extent of inactivation increased, Fig. 4a. In the presence of DNDS, the inactivation time courses were similar in the absence and

![Diagram](image-url)

**FIGURE 4.** Effect of DNDS on the time course of anion exchange inactivation by EAC. (a) Resealed membranes (O, □, △, x) were modified using method A, intact cells (●) were modified using method B; both experiments were done in 165 mM KCl, plus 2 mM DNDS. The final [EAC] was 13 mM (O), 30 mM (△, □), 48 mM (plus 28 mM TEE) (x), and 50 mM (●). (b) Repeated EAC modification of resealed membranes in the presence of DNDS. Resealed membranes were modified using method B in 165 mM KCl and 2 mM DNDS. The final [EAC] was 30 mM (●). A membrane sample was isolated after 8 min, at the arrow, washed free of reagents, stored overnight at 0°C, and subjected to a second treatment (x) under the same conditions as before. The abscissa denotes the total reaction time. (c) Addition of DNDS during inactivation with EAC. Resealed membranes were modified using method B, 165 mM KCl. The final [EAC] was ~30 mM. The figure shows results from two experiments: controls before addition of DNDS are denoted by △ and x; in one experiment (●), 2 mM DNDS was added 5 min into the reaction (at arrow A); in the other, (□), the membranes were reacted for 20 min in the absence of DNDS, at which time they were collected (at arrow B), washed free of reagent, and subjected to a second reaction with the same concentration of EAC in the presence of 2 mM DNDS. The abscissa denotes the total time of reaction with EAC.
The EAC-induced anion exchange inactivation in the presence of DNDS was irreversible for > 18 h at 0°C. Further, if the reaction was quenched before full inactivation was reached, a second incubation with the same concentrations of EAC plus DNDS had no additional effect on the rate and extent of the inactivation (Fig. 4b). The continued inactivation had the same time course as that for membranes that were reacted without interruption. When DNDS was added to the reaction solution during inactivation with EAC alone, the rate and extent of inactivation was enhanced, even when DNDS was added during the slow phase of inactivation (Fig. 4c). The maximal inactivation is comparable to that obtained when DNDS was present from the beginning of the experiment. As [DNDS] was increased, the inactivation was accelerated and the time course became approximately exponential at [DNDS] ≥ 20 mM (Fig. 5). At 50 mM DNDS, the same maximum extent of inactivation was obtained with either 8 or 28 mM EAC (Fig. 5). The rate of inactivation, however, was directly proportional to [EAC]. Under these conditions, the second order inactivation rate constant for EAC is ~35 M⁻¹·min⁻¹.

When modifying with EAC in the presence of DNDS, the pH dependence of the inactivation rate was larger than that seen in the absence of DNDS. At 12 mM EAC plus 60 mM DNDS, the fast phase of the inactivation was about sevenfold slower at pH 7 than at pH 6, ~0.1 min⁻¹ vs. ~0.7 min⁻¹, (results not shown). This result is consistent with the modification of carboxyl groups with an apparent pKₐ of ~5.5. In contrast to inactivation with EAC alone, SO₄²⁻ provided no protection in the
presence of DNDS; when modifying in 110 mM K$_2$SO$_4^-$ plus 2 mM DNDS (and 28 mM EAC), 55% of the exchange capacity was inactivated after 2 min and 75% inactivation was reached after 16 min, cf. the results in Fig. 4a.

**Titration of the Exchange Function in EAC-modified Membranes**

Titration curves of the Cl$^-$ exchange function in resealed membranes after extensive modification by EAC under varying conditions are shown in Fig. 6. Fig. 6a depicts results obtained when the extracellular pH was varied at a constant intracellular pH of ~7.3. For completeness, results between pH 3 and 13 are illustrated. We focus on the results at pH ≤ 8. The top curve, taken from Wieth et al. (1982a), illustrates the titratable exchange function for unreacted resealed membranes with an apparent pK$_a$ of ~5.4. The bottom curve shows results for cells that have been reacted with EAC plus DNDS, in the absence or presence of TEE, to ~90% inactivation. The striking feature is that the acid branch of the extracellular titration curve was abolished. After maximal inactivation with EAC alone (to ~40% residual
flux) (middle curve), the residual flux remained (partially) titratable in the acid pH range.

In unreacted membranes, anion exchange varies as a function of intra- as well as extracellular pH. When titrating the exchange function with $pH_i = pH_o$ (between pH 5.3 and 10), anion exchange is activated by deprotonating a group with a $pK_a$ of $\sim 6$ (Funder and Wieth, 1976). This group seems to be located near the intracellular surface of the anion exchanger (Wieth et al., 1980). Surprisingly, maximal inactivation by EAC (in the presence of DNDS) removes not only the extracellular but also the intracellular pH dependence of the exchange function ($5.6 \leq pH \leq 8$) (Fig. 6b).

The Residual Anion Flux

After extensive modification with EAC in the presence of DNDS, the residual $Cl^-$ flux varied but little with the reaction conditions used; after 30 min of incubation with 50 mM EAC plus 1.6 mM DNDS the residual $Cl^-$ flux was $10 \pm 1\%$ of the control. A similar degree of inactivation by EAC was seen at [DNDS] up to 50 mM. To characterize the exchange activity that remains after reacting red cell membranes with EAC in the absence or presence of DNDS, we examined the relation between residual exchange and covalent DIDS binding of EAC-inactivated resealed membranes (Fig. 7). Fig. 7a shows DIDS binding to control membranes, and to membranes that were modified by EAC in the absence of DNDS to $40 \pm 5\%$ of the
control exchange capacity. In control membranes (solid symbols), there is a linear relation between the amount of DIDS that is added to the membrane suspension and the Cl⁻ exchange that remains after covalent DIDS binding (Cabantchik and Rothstein, 1974a; Wieth et al., 1982c). Extrapolating to 100% inactivation of Cl⁻ exchange, 11 × 10⁵ DIDS molecules are bound per membrane. In the EAC-modified membranes the residual exchange activity also decreased as the number of added DIDS molecules was increased, but the relation between the remaining exchange activity and the number of added DIDS molecules was nonlinear. At less than ~6 × 10⁵ DIDS/membrane, there was a linear relation between the number of added DIDS molecules and the exchange activity that remained after covalent DIDS binding. At higher DIDS levels the exchange activity was higher than would be expected from a linear extrapolation of the results at ~6 × 10⁵ DIDS/membrane. The extrapolated intercept for complete inhibition is ~8 × 10⁵ DIDS/membrane.

![Figure 8](image-url)

**FIGURE 8.** Cl⁻ activation and DNDS inhibition of Cl⁻ efflux in EAC-modified red cell membranes. Resealed membranes were modified using method B in 165 mM KCl, 60 mM EAC, and 2 mM DNDS, to a residual Cl⁻ permeability that was 11% (●) or 10% (▲) of the exchange capacity in unmodified membranes. The Cl⁻ efflux was corrected for a DNDS-insensitive component (5.8 pmol · cm⁻² · s⁻¹ [●], 6.8 pmol · cm⁻² · s⁻¹ [▲]) that was observed when 2 mM DNDS was added to the efflux solution. The straight lines denote least-square linear regressions for the results. (a) [Cl⁻]₀ activation of Cl⁻ efflux at pH 8.0. The results are plotted in a Lineweaver-Burke plot. [Cl⁻]₀ was varied by isosmolar substitution of the standard efflux solution by a solution containing 25 mM K₆ citrate plus 200 mM sucrose. The [Cl⁻]₀ activation was half-maximal at 2.7 mM. (b) DNDS inhibition of Cl⁻ efflux at pH 7.3. The [DNDS] was varied by the addition of aliquots of a freshly prepared stock solution (10 mM) to the efflux solution. The DNDS inhibition was half-maximal at 7 μM.

and ~90% of the residual Cl⁻ exchange was inhibited by covalent binding of ~8 × 10⁵ DIDS/membrane. The remaining 10% is inhibited by binding an additional ~4 × 10⁵ DIDS/membrane. It appears that reaction with EAC alone divided the anion exchangers into two populations. In any case, since both populations are sensitive to DIDS, the extracellular anion binding site was left essentially intact after EAC modification.

Fig. 7 b shows results obtained after reacting with EAC, in the presence of DNDS, to ~10% of the control exchange capacity. At ~5 × 10⁵ DIDS/membrane, there was
no reduction of Cl\textsuperscript- flux, presumably because of nonspecific DIDS binding to "high affinity" sites not involved in anion exchange. Between $5 \times 10^5$ and $15 \times 10^5$ DIDS/membrane, there was a linear decrease in Cl\textsuperscript- flux, suggestive of covalent DIDS binding to a single population of modified exchangers. At $\approx 15 \times 10$ DIDS/membrane there was little or no additional inhibition of the Cl\textsuperscript- flux. This DIDS-insensitive flux, $\approx 2.5\%$ of the control exchange flux, was not studied further. These results suggest that the residual Cl\textsuperscript- flux in membranes that were maximally inactivated with EAC in the presence of DNDS results from a reduced flux ($\approx 10\%$ of the control value) through each anion exchanger, rather than from a normal flux through $\approx 10\%$ of unmodified exchangers.

The relation between [Cl\textsuperscript-]\textsubscript{o} and Cl\textsuperscript- efflux, and the (reversible) flux inhibition by DNDS, in resealed membranes that had been reacted to $\approx 10\%$ of the control exchange capacity with EAC in the presence of DNDS is shown in Fig. 8. The flux measurements were done with [Cl\textsuperscript-]\textsubscript{i} = 165 mM. For the [Cl\textsuperscript-]\textsubscript{o} activation (Fig. 8 a) the Cl\textsuperscript- efflux was half-maximal at $\approx 3$ mM Cl\textsuperscript-, which is similar to the value (3 mM) obtained by Gunn and Fröhlich (1979). For the DNDS inhibition of Cl\textsuperscript- efflux (Fig. 8 b), the inhibition was half-maximal at $\approx 7 \mu$M DNDS (at a [Cl\textsuperscript-]\textsubscript{o} of 165 mM), which is similar to the value (5 $\mu$M) obtained by Fröhlich (1982). These results indicate, again, that the extracellular anion binding site is left essentially intact after extensive modification by EAC.

**Interaction between EAC, DIDS, and PG**

In red cell membranes, the anion exchangers exist as dimers (Steck, 1974) or oligomers of dimers (Weinstein et al., 1980). The biphasic time course of the EAC-induced inactivation (Fig. 1) could thus result because one monomer in a dimer loses its reactivity when its neighbour has reacted with EAC (Wieth et al., 1982a). But the separation between the fast and slow components varies with the following experimental conditions: changes in [EAC] (Figs. 1 and 2), an addition of nucleophile (Fig. 3), and an addition of DNDS (Figs. 4 and 5). To obtain more decisive evidence as to whether there are interactions among exchange protein promoters with respect to their reaction with EAC, resealed membranes were partially inactivated with DIDS or PG to between 46 and $15\%$ of the control exchange activity before reacting with EAC. In this way we produce a population of anion exchangers in which only one promoter in a dimeric assembly should be able to react with EAC. The kinetics of EAC inactivation of anion exchange in PG- or DIDS-treated membranes were nevertheless indistinguishable from the kinetics observed with untreated membranes (Fig. 9). The complex kinetics of the EAC-induced inactivation of anion exchange cannot result from intermolecular interactions between promoters, but appear to result from intramolecular interactions among several carboxyl groups that can be modified by EAC.

**Correlation of Anion Exchange Inactivation with Protein Modification**

To study the relation between anion exchange inactivation and the covalent modification of the exchange protein, the incorporation of [14C]EAC and [3H]TEE was examined as a function of residual exchange activity under various conditions. When resealed membranes were reacted with [14C]EAC in the absence of DNDS
FIGURE 9. EAC-induced inactivation of anion exchange in red cell membranes that had been reacted with DIDS or PG. Resealed membranes were modified using method A in 165 mM KCl and 30 mM EAC. After addition of the membrane suspension, the [EAC] was 26 mM. (a) Pretreatment with DIDS. Before reaction with EAC, the membranes were reacted with DIDS to different levels of Cl⁻ exchange inactivation. The symbols denote control membranes (A, O), membranes that were DIDS-inactivated to 46% (●), 21% (■), and 15% of control flux (▲). (b) Pretreatment with PG. Before reaction with EAC, the membranes were reacted with PG to 18% of the control flux. The symbols denote control membranes (Δ, ○), and PG pretreated membranes (●).

AND WITHOUT PRIOR TRYPsin TREATMENT, ~15 × 10⁶ EAC molecules were incorporated per membrane when the exchange was reduced to ~50% of the control (Fig. 10). The modification became more specific when the cells were treated with trypsin before exposure to EAC (in the absence of DNDS), where 50% inactivation

FIGURE 10. Anion exchange inactivation as a function of [¹⁴C]EAC incorporation into resealed membranes. The ordinate denotes the Cl⁻ exchange as a percent of the control flux. Resealed membranes were modified using method B. In all but one experiment, the reaction solution was 165 mM KCl plus 30 mM EAC in the absence (x, ■) or presence (O, ▲) of 2 mM DNDS. In the remaining experiment (●) the reaction solution was 100 mM NaCl, 12 mM EAC, and 60 mM DNDS. Each different symbol denotes the results from a separate experiment. The different points denote results obtained with membranes sampled at different times. In all but one experiment (x), the membranes were treated with trypsin before the EAC modification. To estimate the stoichiometry under the most selective conditions (●), the initial rate of EAC incorporation is extrapolated to maximal inactivation, 10% residual flux, to give a value of ~2.5 × 10⁶ EAC/membrane.
occurred when \( \sim 10 \times 10^6 \) EAC molecules were incorporated per membrane. Much higher selectivity was obtained when trypsin-treated membranes were reacted with EAC in the presence of DNDS; at 60 mM DNDS, and 23 mM EAC, 50% inactivation occurred when \( \sim 1.0 \times 10^6 \) EAC molecules were incorporated per membrane.

**Figure 11.** Incorporation of \(^{14}\text{C}\)EAC into the proteins of trypsin-treated membranes. Resealed membranes were exposed to extracellular trypsin and then modified using method B in 100 mM NaCl, 12 mM \(^{14}\text{C}\)EAC, and 60 mM DNDS. The final [EAC] and [DNDS] were \( \sim 10 \) and \( \sim 50 \) mM, respectively. (a) The top of the figure depicts Coomassie blue-stained gels of membrane proteins from membranes reacted for 30 s (140 \( \mu \)g protein applied) and 240 s (180 \( \mu \)g protein applied) before quenching. (Molecular weight standards [in kilodaltons] are indicated.) The bottom of the figure depicts the radioactivity in gel slices corresponding to the different bands: (○) after 30 s modification (210 \( \mu \)g protein applied); (△) after 240 s modification (270 \( \mu \)g protein applied). Total EAC incorporation after 30 s was \( 0.59 \times 10^6 \) molecules/membrane (with 22% in band 3 and the 60-kD tryptic or contaminant chymotryptic degradation fragment, see text); after 240 s the incorporation was \( 2.6 \times 10^6 \) molecules/membrane (with 25% in band 3 and the 60-kD degradation fragment). (b) Correlation between \(^{14}\text{C}\)EAC incorporation and exchange inactivation. The \(^{14}\text{C}\)EAC-modified samples include the two samples from part a plus samples quenched at intermediate times. The ordinate denotes Cl\(^-\) efflux as a percentage of the exchange in unreacted controls. (○) denotes total uptake into the membranes, (○) denotes uptake into band 3 and the 60-kD degradation fragment.

The number of EAC molecules incorporated into the anion exchange protein (band 3) was examined by SDS-PAGE of membranes treated with trypsin before the modification. This treatment improves the selectivity of the labeling in band 3 by removing PAS-1 proteins from the 100-kD region, where the exchange protein is
found (Steck, 1972). Fig. 11 shows the distribution of [14C]EAC incorporated into membrane proteins when cells were reacted with EAC plus DNDS for 30 or 240 s.

15–20% of the radioactivity was located in the band 3 region (Fig. 11 a); the rest was located mainly in band 4.5, a region that contains the glycoproteins as well as a 60-kD degradation product of the anion exchange protein (see Methods). The labeled cleavage fragment can be seen in Fig. 11 a as a sharp peak to the left of the band 4.5 region (see also Fig. 12). Approximately 10% of the total radioactivity was located at the position of the 60-kD fragment. The relation between anion exchange inactivation and EAC incorporation (into band 3 plus the 60-kD fragment at band 4.5) is illustrated in Fig. 11 b (open symbols). Extrapolation to maximal inactivation, suggests that about one molecule of EAC was incorporated per anion exchange protein.

Chymotryptic cleavage removes most radioactivity from the 95-kD region of the gel and shifts it to the 60-kD region, which indicates that EAC is primarily incorporated into the 60-kD chymotryptic fragment (Fig. 12). A comparison of the [14C]EAC distribution pattern before and after chymotrypsin digestion shows that the [14C] activity increases at the 60-kD fragment that is produced by the trypsin treatment.

The relation between anion exchange inactivation and incorporation of [3H]TEE is illustrated in Fig. 13. The [3H]TEE distribution was dominated by its incorporation into the membrane phospholipids, the peak at the right in Fig. 13 a. In addition, there was substantial incorporation into band 3 as well as band 4.5. The rela-
FIGURE 13. Incorporation of $[^3]$HTEE into the proteins of trypsinized membranes. Resealed membranes were modified using method B in 155 mM KCl, 36 mM EAC, and 60 mM $[^3]$HTEE. The final [EAC] and [TEE] were ~30 and ~33 mM, respectively. (a) The top of the figure depicts Coomassie blue–stained gels of membrane proteins from membranes pretreated with extracellular trypsin and then reacted for 2 and 8 min with EAC/TEE before quenching. The bottom of the figure depicts the radioactivity in gel slices corresponding to the different protein bands: (●) after 2 min modification, (△) after 8 min modification. Total TEE incorporation after 2 min was $15 \times 10^6$ molecules/membrane (with 2% in band 3); after 8 min it was $18 \times 10^6$ molecules/membrane (with 2% in band 3). (b) Correlation between TEE incorporation and exchange inactivation. The points denote the two samples shown in a, plus other similarly treated samples. The ordinate denotes Cl$^-$ efflux of modified membranes as a percent of the exchange in unreacted controls. The abscissa represents the uptake of TEE into band 3 of resealed membranes treated with trypsin before (●), as well as after (△, ○) inactivation. For each experiment, the extrapolated intercept for full (i.e., 90%) inactivation was determined. The line was drawn based on the average intercept: $0.9 \pm 0.2 \times 10^6$ molecules of TEE/membrane.

**DISCUSSION**

Exposing red cell membranes to EAC leads to a time-dependent irreversible inactivation of anion exchange. The time course of inactivation is biphasic; the rate and extent of the initial rapid phase depends on [EAC]. DNDS accelerates and extends
the initial phase such that the time course of inactivation is monophasic to a limit of ~90% inactivation. After maximal inactivation in the presence of DNDS, the ~10% residual Cl⁻ efflux no longer titrates between pH 4 and 8. We believe the results are most consistent with the modification of exofacial carboxyl groups that, although not at the external anion binding site, must be deprotonated for normal anion exchange function.

**Reaction of Water-soluble Carbodiimides with Carboxyl Groups in Proteins**

Water-soluble carbodiimides are the most commonly used reagents for the chemical modification of carboxyl groups in proteins (Lundblad and Noyes, 1984). This mod-

![Figure 14. Reaction paths for the modification of carboxyl groups by water-soluble carbodiimides. The initial reaction is between a protonated carboxyl group and carbodiimide (R₁⁻N=C=N-R₂) to form a protonated (and activated) carbodiimide and a nucleophilic carboxylate anion. The carboxylate can then attack the activated carbodiimide to give a labile O-acylisourea adduct, which can react further by several pathways. (Top pathway) H₂O can displace the substituted O-acylisourea to regenerate the free carboxyl group and an urea, in which case the carboxyl group is able to react again. (Middle pathway) An amine nucleophile (R₃⁻NH₂) can displace the substituted O-acylisourea to generate the urea and an irreversibly modified carboxyl group, where the amino group of the nucleophile is bound to the carbonyl group in an amide linkage. The nucleophile is often the (unionized) amino group of an added amino acid ester, but could also be a nucleophilic side chain of the protein, such as the ε-NH₂ group in a lysyl residue, in which case an intra- or interchain cross-link would form. (Bottom pathway) The O-isoacylurea can undergo an intramolecular O-to-N acyl shift to form a stable N-acylurea.

modification is enhanced at a more acidic pH (Lundblad and Noyes, 1984, and references therein), and it has recently been proposed that a protonated carboxyl group may catalyze its own selective modification (Chan et al., 1988). Fig. 14 shows possible reactions for the modification of a protonated protein carboxyl group by a water-soluble carbodiimide.

**Irreversible Carbodiimide-induced Inactivation of Anion Exchange**

Is a Result of Carboxyl Group Modification

After the appearance of our original reports on the effect of EAC on anion exchange in red cell membranes (Wieth et al., 1982a; Andersen et al., 1983), it was
reported that the EAC-induced inactivation of red cell anion exchange was reversible (Craik and Reithmeier, 1985). This is in conflict with our findings (Fig. 1). But Craik and Reithmeier used different reaction conditions and stopping solutions. We note, in particular, that they used phosphate or citrate to buffer their solutions and that EAC can react with either. It is therefore not clear how their results should be reconciled with our finding that the EAC-induced inactivation is irreversible. Craik and Reithmeier (1985) found, for example, that the EAC-induced inactivation was irreversible only when the modification was carried out in citrate-containing solutions and that the inactivation was accelerated at more alkaline pH. These were the results of modifying lysyl ε-NH₂ groups by carbodiimide-activated citrate (Wemer and Reithmeier, 1988).

The twofold acceleration of anion exchange inactivation by the nucleophile TEE (Fig. 3), strongly suggests that inactivation by EAC is due to the modification of functionally important carboxyl groups. The magnitude of the acceleration by TEE is comparable to that found by Pho et al. (1977) in their modification of an essential glutamyl residue in yeast hexokinase. A different nucleophile, GME, did not accelerate anion exchange inactivation. Pho et al. (1977) again observed a similar difference between TEE and GME. Perhaps the more hydrophobic TEE concentrates in nonpolar regions before the covalent trapping of the activated carboxyl group.

Anion exchange inactivation could be a direct result of the carboxyl group modification, with the activated carboxyl being converted to an N-acylurea, or it could result from internal cross-linking of an activated carboxyl group with the ε-NH₂ group of a nearby lysyl residue (cf. Pedemonte and Kaplan, 1986). Indeed, carbodiimides are used to cross-link low molecular weight soluble proteins, such as cytochrome c, to their binding proteins (Millett et al., 1982; Waldmeyer and Bosshard, 1985; Vieira et al., 1986). But the TEE-induced acceleration of the inactivation of anion exchange (Fig. 3) indicates that the most likely cause of the anion exchange inactivation by EAC is the conversion of a carboxyl group or groups to an N-acylurea, not cross-linking. In addition, the modifications were done at pH 6.0, which would disfavor the deprotonation of lysyl residues that might be candidates for cross-linking, and at an ionic strength approaching 0.2 M, which seems to disfavor cross-linking in systems otherwise predisposed to do so (Waldmeyer and Bosshard, 1985; Vieira et al., 1986).

The Biphasic Inactivation Kinetics

When membranes were reacted with EAC alone, the inactivation time course was biphasic (Fig. 1). This kinetic result suggests that we modified at least two classes of carboxyl groups: a class of functional groups, whose modification is associated with ~90% inactivation of total anion exchange (Fig. 7), and a class of protective groups, whose modification greatly slows subsequent modification of the functional groups. Both classes of reactive carboxyl groups reside on each protomer (Fig. 9). There is, in fact, clear protein-chemical evidence that we modify at least two classes of carboxyl groups (see below). But this will not, in itself, account for the complex inactivation kinetics because the inactivation is irreversible and the partition between the rapidly and slowly inactivating fractions is affected by increasing the [EAC], either during an initial inactivation or in a subsequent inactivation (Fig. 2), while decreas-
The [EAC] has no effect. In addition, DNDS increases the rate and extent of the rapid phase (Fig. 4) such that the time course is approximately exponential at the highest [DNDS] (Fig. 5). To account for these findings, a more elaborate scheme is needed.

First, we note that the accelerative effect of DNDS requires $10^3$–$10^4$ times greater [DNDS] than is necessary to inhibit anion exchange across red cell membranes (Fröhlich, 1982). The DNDS effect on inactivation cannot therefore be related to its conventional inhibitory action. A more plausible explanation is that the acceleration results from DNDS binding to (low affinity) "sites" other than its inhibitory site, which causes conformational changes in the protein that alter the relative accessibility of functional and protective carboxyl groups.

One plausible explanation for the complex inactivation kinetics is that [EAC] and [DNDS], because they are amphipathic, adsorb at the membrane-solution interface where they alter the environment of the exchange protein and, thereby, the equilibrium distribution of the protein among different conformational states (cf. Sawyer et al., 1988). If these states have different relative reactivities of the functional and protective carboxyl groups, an increase in the [EAC] or [DNDS], during the initial reaction or in a subsequent reaction, would establish a new equilibrium among these reactive states and thus alter the inactivation progress curve. Since inactivation is due to covalent modification of the exchange protein, a decrease in [EAC] during the reaction cannot increase the exchange activity.

These putative functional and protective carboxyl groups would be expected to have different pKs and different sensitivities to added nucleophile. It is not surprising therefore that the pH dependence of the EAC-induced inactivation is relatively low when modifying in the absence of DNDS, and that the pH-dependence is more pronounced when modifying in the presence of DNDS. That GME exerts a modest protective effect may reflect that the modification of the protective group(s) is accelerated by GME while the modification of the functional group is unaffected. That TEE does not accelerate the inactivation in the presence of DNDS could result because the increased accessibility of the reactive carboxyl groups makes the O-acylisourea adduct less susceptible to attack by the hydrophobic nucleophile.

The Modified Carboxyl Groups Are Outside the Extracellular Anion Binding Site

The large discrepancy between the [DNDS] necessary to inhibit anion exchange and enhance the EAC-induced inactivation rate indicates that the carboxyl groups we modify with EAC in the presence of DNDS are outside the extracellular anion recognition site (see above). This suggestion is supported by the finding that the residual anion exchange can be irreversibly inactivated by the binding of almost stoichiometric amounts of DIDS (Fig. 7). DIDS binds with high selectivity to the extracellular anion recognition site of red cells (e.g., Passow, 1986). That the anion exchange function retains its sensitivity to covalent inhibition by DIDS suggests, therefore, that the anion recognition site is fairly unperturbed by carboxyl modification. This conclusion was also reached by examining the $[Cl^-]_o$ activation of $Cl^-$ efflux or the sensitivity to DNDS in maximally inactivated red cell membranes (Fig. 8). Most likely, inactivation results from modification of carboxyl group(s) that are not an integral part of the extracellular anion binding site.
Localization of the Modified Carboxyl Group(s)

When the relation between exchange inactivation and protein modification is examined under the most selective conditions, the exchange inactivation is an approximately linear function of the incorporation of either $^{14}$C]EAC (Figs. 10 and 11) or $^3H]$TEE (Fig. 13). In both cases, full (i.e., 90%) inactivation occurs when about one molecule or label was incorporated per exchange protein. The $^{14}$C]EAC label was localized by subjecting the membranes to proteolytic cleavage, and the label was incorporated into the NH$_2$-terminal 60-kD chymotryptic fragment of the protein (Fig. 12). It was previously reported that $^3H]$TEE was incorporated into the COOH-terminal 35-kD chymotryptic fragment (Bjerrum, 1983). That the labels incorporate into both chymotryptic fragments shows that EAC can modify at least two carboxyl groups in the protein. Either labeled group could be functionally important.

It appears that the functional carboxyl group(s) that can be modified by EAC are different from the two groups that are modified by Woodward's reagent K (Jennings and Anderson, 1987; Jennings and Al-Rhaiyal, 1988), because modification of the latter groups is inhibited by stilbenedisulfonates. There is evidence for several classes of functional carboxyl groups in the anion exchange protein, both within and outside the extracellular anion binding site.

The Residual Anion Permeability and Functional Implications

When the anion transport protein is reacted with EAC at high [DNDS], we find that the inactivation proceeds as a first order process (Fig. 5) to produce an apparently homogenous population of modified exchange proteins (Fig. 7 b). The residual anion permeability is ~10% of that of the exchange capacity of the native molecules, and at least 75% of this residual anion permeability reflects anion exchange mediated by the exchange proteins (Fig. 7 b). We do not know whether the anion permeability that remains after covalent DIDS binding to the EAC-inactivated membranes results from nonspecific membrane leaks or whether it represents residual (possibly conductive) anion translocation mediated by the modified exchangers.

The basic architecture of the extracellular anion recognition site seems unaffected by the carboxyl group modification by EAC (see above). Nevertheless, after maximal modification at high [DNDS], there is no titratable exchange function between pH 4 and 8 (Fig. 6 a). This suggests that we have modified a group or groups that underlie the acid limb of the titration curve of the normal monovalent anion exchange function. The modification abolishes not only the extracellular titration, but also the dependence of the exchange function on intracellular pH, which ranges between 5.5 and 8 (Fig. 6 b). Jennings and Al-Rhaiyal (1988) also found that the intracellular pH dependence of sulfate exchange was abolished (between pH 6.5 and 7.6) after modification of extracellular carboxyl groups with Woodward's reagent K. But EAC and Woodward's reagent K seem to modify different (sets of) carboxyl groups (see above). It is thus unlikely that the abolition of the intracellular titration function occurs because the titratable group itself has been modified. The changes in the intracellular titration function may thus result from conformational changes in the anion exchanger, consequent to the extracellular carboxyl group.
modification, which alters the pK of functionally important group(s) at the intracellular side of the exchanger. Further, since the EAC-modified carboxyl groups appear to be outside the extracellular anion binding site of the exchanger, it appears that the group(s) that underlie the acid branch of the extracellular titration function are only allosterically coupled to this site. While this was not our original working hypothesis (cf. Wieth et al., 1982a, c), we find it to be the most parsimonious interpretation of the results. We also note that the concept of allosteric coupling is supported by the finding of a synchrony between anion and proton movement through the anion exchanger (Milanick and Gunn, 1986).

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REFERENCES

Andersen, O. S., P. J. Bjerrum, C. L. Borders, Jr., T. Broda, and J. O. Wieth. 1983. Essential carboxyl groups in the anion exchange protein of human red blood cell membranes. *Biophysical Journal.* 41:164a. (Abstr.)

Bjerrum, P. J. 1983. Identification and location of amino acid residues essential for anion transport in red cell membranes. In Structure and Function of Membrane Proteins. E. Quagliariello and F. Palmieri, editors. Elsevier Science Publishing Co., Inc., Amsterdam. 107-115.

Bjerrum, P. J., J. O. Wieth, and C. L. Borders, Jr. 1983. Selective phenylglyoxalation of functionally essential arginyl residues in the erythrocyte anion transport protein. *Journal of General Physiology.* 81:453-484.

Borders, C. L., Jr., D. M. Blech, and K. D. McElvany. 1984. L-Amino acid ethyl ester hydrochlorides: derivatives for qualitative organic analysis. *Journal of Chemical Education.* 61:814-815.

Brock, C. J., M. J. A. Tanner, and C. Kempf. 1983. The human erythrocyte anion transport protein. Partial amino acid sequence, conformation and possible molecular mechanism for anion exchange. *Biochemical Journal.* 213:577-586.

Cabantchik, Z. I., and A. Rothstein. 1974a. Membrane proteins related to anion permeability of human and red blood cells. I. Localization of disulfonic stilbene binding sites in proteins involved in permeating. *Journal of Membrane Biology.* 15:207-226.

Cabantchik, Z. I., and A. Rothstein. 1974b. Membrane proteins related to anion permeability of human red blood cells. II. Effects of proteolytic enzymes on disulfonic stilbene sites of surface proteins. *Journal of Membrane Biology.* 15:227-248.

Carraway, K. L., and D. E. Koshland, Jr. 1972. Carbodiimide modification of proteins. *Methods in Enzymology.* 25:616-623.

Chan, V. W. F., A. M. Jorgensen, and C. L. Borders, Jr. 1988. Inactivation of bovine thrombin by water-soluble carbodiimidates: the essential carboxyl group has a pKₐ of 5.51. *Biochemical and Biophysical Research Communications.* 151:709-716.

Craik, J. D., and R. Reithmeier. 1984. Inhibition of phosphate transport in human erythrocytes by water-soluble carbodiimidates. *Biochimica et Biophysica Acta.* 778:424-434.
Craik, J. D., and R. Reithmeier. 1985. Reversible and irreversible inhibition of phosphate transport in human erythrocytes by membrane impermeant carbodiimides. *Journal of Biological Chemistry*. 260:2404–2408.

Dalmark, M., and J. O. Wieth. 1972. Temperature dependence of chloride, bromide, iodide, thiocyanate and salicylate transport in human red cells. *Journal of Physiology*. 224:583–610.

Deuticke, B. 1977. Properties and structural basis of simple diffusion pathways in the erythrocyte membrane. *Review of Physiology, Biochemistry and Pharmacology*. 78:1–97.

Edsall, J. T., and J. Wyman. 1958. Biophysical Chemistry. Vol. 1. Academic Press, Inc., New York. p. 452.

Fröhlich, O. 1982. The external anion binding site of the human erythrocyte anion transporter: DNDS binding and competition with chloride. *Journal of Membrane Biology*. 65:111–123.

Funder, J., D. C. Tosteson, and J. O. Wieth. 1978. Effects of bicarbonate on lithium transport in human red cells. *Journal of General Physiology*. 71:721–746.

Funder, J., and J. O. Wieth. 1976. Chloride transport in human erythrocytes and ghosts: a quantitative comparison. *Journal of Physiology*. 262:679–698.

George, A. L., Jr., and C. L. Borders, Jr. 1979. Essential carboxyl residues in yeast enolase. *Biochemical and Biophysical Research Communications*. 87:59–65.

Gunn, R. B., and O. Fröhlich. 1979. Asymmetry in the mechanism for anion exchange in human red blood cell membranes. *Journal of General Physiology*. 74:351–374.

Hoare, D. G., and D. E. Koshland, Jr. 1967. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. *Journal of Biological Chemistry*. 242:2447–2453.

Jay, D., and L. Cantley. 1986. Structural aspects of the red cell anion exchange protein. *Annual Review of Biochemistry*. 55:511–538.

Jenkins, R. E., and M. J. A. Tanner. 1977. Ionic-strength-dependent changes in the structure of the major protein of the human erythrocyte membrane. *Biochemical Journal*. 161:131–138.

Jennings, M. L., and M. P. Anderson. 1987. Chemical modification and labeling of glutamate residues at the stilbenesulfonate site of human red blood cell band 3 protein. *Journal of Biological Chemistry*. 262:1691–1697.

Jennings, M. L., and S. Al-Rhaiyel. 1988. Modification of a carboxyl group that appears to cross the permeability barrier in the red blood cell anion transporter. *Journal of General Physiology*. 92:161–178.

Knauf, P. A. 1979. Erythrocyte anion exchange and the band 3 protein: transport kinetics and molecular structure. *Current Topics in Membranes and Transport*. 12:249–363.

Kopito, R. R., and H. F. Lodish. 1985. Primary structure and transmembrane orientation of the murine anion exchange protein. *Nature*. 316:234–238.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227:680–685.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*. 193:265–275.

Lundblad, R. L., and C. M. Noyes. 1984. Chemical reagents for protein modification. Vol. II. CRC Press, Inc., Boca Raton, FL. 105–121.

Macara, I. G., and L. C. Cantley. 1981. Interactions between transport inhibitors at the anion binding site of the band 3 dimer. *Biochemistry*. 20:5095–5105.

Milanick, M. A., and R. B. Gunn. 1982. Proton-sulfate co-transport: mechanism of H+ and sulfate addition to the chloride transporter of human red blood cells. *Journal of General Physiology*. 79:87–113.

Milanick, M. A., and R. B. Gunn. 1986. Proton inhibition of chloride exchange: asynchrony of band 3 proton and anion transport sites. *American Journal of Physiology*. 250:C955–C969.
Millett, F., V. Darley-Usmar, and R. A. Capaldi. 1982. Cytochrome c is cross-linked to subunit II of cytochrome c oxidase by a water-soluble carbodiimide. Biochemistry. 21:3857–3862.

Passow, H. 1986. Molecular aspects of band 3 protein-mediated anion transport across the red blood cell membrane. Review of Physiology, Biochemistry, and Pharmacology. 103:62–203.

Pedemonte, C. H., and J. H. Kaplan. 1986. Carbodiimide inactivation of Na,K-ATPase. A consequence of internal cross-linking and not carboxyl group modification. Journal of Biological Chemistry. 261:3632–3639.

Pho, D. B., C. Roustan, A. N. T. Tot, and L.-A. Pradel. 1977. Evidence for essential glutemyl residue in yeast hexokinase. Biochemistry. 16:4533–4537.

Riordan, J. F. 1979. Arginyl residues and anion binding sites in proteins. Molecular and Cellular Biochemistry. 26:71–92.

Sawyer, D. B., O. S. Andersen, and R. E. Koeppe II. 1988. Nonionic detergents increase the frequency of gramicidin mini channels. Biophysical Journal. 53:329a. (Abstr.)

Sheehan, J. C., P. A. Cruickshank, and G. L. Boshart. 1961. A convenient synthesis of watersoluble carbodiimides. Journal of Organic Chemistry. 26:2525–2528.

Stock, T. L. 1974. The organization of proteins in the human red blood cell membrane. Journal of Cell Biology. 62:1–19.

Takahashi, K. 1968. The reaction of phenylglyoxal with arginine residues in proteins. Journal of Biological Chemistry. 243:6171–6179.

Vieira, B., M. Davidson, D. Knaff, and F. Millett. 1986. The use of a water-soluble carbodiimide to study the interaction between Chromatium vinosum flavocytochrome c-552 and cytochrome c. Biochimica et Biophysica Acta. 848:131–136.

Waldmeyer, B., and H. R. Bosshard. 1985. Structure of an electron transfer complex. I. Covalent cross-linking of cytochrome c peroxidase and cytochrome c. Journal of Biological Chemistry. 260:5184–5190.

Weinstein, R. S., J. K. Khodada, and T. L. Steck. 1980. The mechanisms of inhibition of anion exchange in human erythrocytes by 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide. Biochimica et Biophysica Acta. 942:19–32.

Wieth, J. O. 1981. Exchange diffusion of ions in artificial and biological membranes. In Sixth School on Biophysics of Membrane Transport, School Proceedings. Vol. 2. Publishing Company of Wroclaw Agricultural University, Wroclaw, Poland. 59–88.

Wieth, J. O., O. S. Andersen, J. Brahm, P. J. Bjerrum, and C. L. Borders, Jr. 1982a. Chloride-bicarbonate exchange in red blood cells. Philosophical Transactions of the Royal Society of London. B299:383–399.

Wieth, J. O., P. J. Bjerrum, and C. L. Borders, Jr. 1982b. Irreversible inactivation of red cell chloride exchange with phenylglyoxal, an arginine-specific reagent. Journal of General Physiology. 79:283–312.

Wieth, J. O., P. J. Bjerrum, J. Brahm, and O. S. Andersen. 1982c. The anion transport protein of the red cell membrane. A zipper mechanism of anion exchange. Tokai Journal of Experimental and Clinical Medicine. 7(Suppl.):91–101.

Wieth, J. O., and P. J. Bjerrum. 1982. Titration of transport and modifier sites in the red cell anion transport system. Journal of General Physiology. 79:255–282.
Wieth, J. O., and P. J. Bjerrum. 1983. Transport and modifier sites in capnophorin, the anion transport protein of the erythrocyte membrane. *In Structure and Function of Membrane Proteins*. E. Quagliariello and F. Palmieri, editors. Elsevier Science Publishing Co., Inc., Amsterdam. 95–106.

Wieth, J. O., J. Brahm, and J. Funder. 1980. Transport and interactions of anions and proteins in the red blood cell membrane. *Annals of the New York Academy of Sciences*. 341:394–418.