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The mechanisms of inhibition of anion exchange in human erythrocytes by 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide

Pamela K. Werner and Reinhart A.F. Reithmeier

Department of Biochemistry, University of Alberta, Edmonton, Alberta (Canada)

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Treatment of human erythrocytes with the membrane-impermeant carbodiimide 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide (ETC) in citrate-buffered sucrose leads to irreversible inhibition of phosphate-chloride exchange. The level of transport inhibition produced was dependent on the concentration of citrate present during treatment, with a maximum of approx. 60% inhibition. [^{14}C]Citric acid was incorporated into Band 3 (M_r = 95 000) in proportion to the level of transport inhibition, reaching a maximum stoichiometry of 0.7 mol citrate per mol Band 3. The citrate label was localized to a 17 kDa transmembrane fragment of the Band 3 polypeptide. Citrate incorporation was prevented by the transport inhibitors 4,4′-diisothiocyano- and 4,4′-dinitrostilbene-2,2′-disulfonate. ETC plus citrate treatment also dramatically reduced the covalent labeling of Band 3 by [^{3}H]4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonate ($^3H_2$DIDS). Noncovalent binding of stilbene disulfonates to modified Band 3 was retained, but with reduced affinity. We propose that the inhibition of anion exchange in this case is due to carbodiimide-activated citrate modification of a lysine residue in the stilbenedisulfonate binding site, forming a citrate-lysine adduct that has altered transport function. The evidence is consistent with the hypothesis that the modified residue may be Lys *a*, the lysine residue involved in the covalent reaction with H_2DIDS. Treatment of erythrocytes with ETC in the absence of citrate resulted in inhibition of anion exchange that reversed upon prolonged incubation. This reversal was prevented by treatment in the presence of hydrophobic nucleophiles, including phenylalanine ethyl ester. Thus, inhibition of anion exchange by ETC in the absence of citrate appears to involve modification of a protein carboxyl residue(s) such that both the carbodiimide- and the nucleophile-adduct result in inhibition.

Introduction

The 95 kDa integral membrane protein of human erythrocytes, known as Band 3 protein, functions to facilitate the exchange of anions across the membrane (for a recent review, see Ref. 1). The amino-terminal cytoplasmic domain (M_r = 41 000) appears not to be necessary for the trans-

Abbreviations: CH35, the C-terminal 35 kDa chymotryptic fragment of Band 3; CH60, the N-terminal 60 kDa chymotryptic fragment; H_2DIDS, 1,2-(2,2′-disulfo-4,4′-diisothiocyano)diphenylethane, and $^3H_2$DIDS is used to represent [^{3}H]dihydroDIDS; ETC, 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; BADS, 4-benzamido-4′-aminostilbene-2,2′-disulfonate; PBS, phosphate-buffered saline (150 mM NaCl, 5 mM sodium phosphate (pH 7.4)); SDS, sodium dodecyl sulfate; Pipes, 1,4-piperazinediethanesulfonic acid; PheOEt, phenylalanine ethyl ester.

Correspondence (present address): R.A.F. Reithmeier, MRC Group in Membrane Biology, Room 7307, Medical Sciences Building, Department of Medicine, University of Toronto, Toronto, Ontario, M5S 1A8, Canada.

port function of Band 3 [2,3], while the carboxyl terminal domain ($M_r = 55\,000$) spans the membrane bilayer at least eight times [1,4] and contains the segments required for anion exchange [3]. The publication of the sequence of the murine anion exchange protein, determined from the sequence of a cloned cDNA [5], has allowed comparison of the many results from proteolysis and chemical modification, and has resulted in several models of folding of the protein [6,7]. Identification by chemical modification of several different amino acid residues essential to or involved in anion exchange has made possible investigations of the relationships between structure and function in the human protein. Evidence for the involvement of arginine residues, believed to contribute to the anion binding site [8], is substantial [1,9]. At least two lysine residues (denoted Lys *a* and Lys *b*), although apparently not directly involved in anion translocation [10], have been defined by their reactivity with several inhibitors of anion exchange, most notably H_2DIDS [1].

The involvement of carboxyl residues in the anion exchange function was suggested by the pH dependence of exchange flux [11–13], in which deprotonation of a group with an apparent pK_a of 5 on the outside of cells was shown to be required for monovalent anion exchange, and protonation of a group of similar pK_a was shown to activate Band 3-mediated sulfate influx [13]. The value and low temperature dependence of the pK_a indicated the titratable group is likely a carboxyl group [15]. The postulated involvement of carboxyl residues is supported by chemical modification studies, using carbodiimides as carboxyl-selective reagents to inhibit transport [14–20]. Using another carboxyl reagent, Woodward's Reagent K, Jennings and Anderson [21] recently provided evidence that modification of at least two glutamate residues results in transport inhibition.

The previous work from our laboratory [20] indicated that the characteristics of the inhibition by the membrane-impermeant carbodiimide ETC * appeared to be sensitive to the reaction medium: reaction in citrate-buffered sucrose producing irreversible inhibition, and reaction in phosphate-buffered saline producing reversible inhibition (i.e., it remains after washing away the ETC, but reverses upon prolonged incubation of 60 min or longer). The differing inhibition characteristics were proposed to be due to the difference in orientation of the anion binding site of the protein in the two reaction media [20]. However, it was not determined which residues are modified to result in inhibition of anion exchange, or what accounts for the difference in inhibition in the two conditions. The work described herein shows that the inhibition in the two media appears to arise by different mechanisms. The inhibition produced by ETC in citrate-containing medium is due to carbodiimide activation of citrate and subsequent modification of a residue (possibly lysine *a*) in Band 3. The inhibition produced in saline involves modification of an essential carboxyl residue(s), either directly by ETC, or by subsequent displacement of the carbodiimide by a hydrophobic nucleophile (A preliminary report of this work has been published: Werner, P.K. and Reithmeier, R.A.F. (1987) Biophys. J. 51, 513a).

Materials

Carrier free [^{32}P]phosphate and [1,5-^{14}C]citric acid (56.9 mCi/mmol) were obtained from New England Nuclear; 1,2-Ditritio-1,2-(2,2′-disulfo-4,4′-diisothiocyano)diphenylethane ([^{3}H]H_2DIDS, at 202 mCi/mmol) was obtained from Research Development Corporation, Hospital for Sick Children, Toronto. DIDS and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were from Pierce Chemical Company, Rockford Illinois, and the amino acid ethyl esters were from Sigma, except nitrotyrosine ethyl ester, which was synthesized from L-nitrotyrosine [22]. BADS was synthesized according to Kotaki et al. [23] as previously described [24]. 1-Ethyl-3-[3-(trimethylammonio)propyl]carbodiimide iodide (ETC) was prepared from the free base of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide by the method of Sheehan et al. [25], as described previously [20]. Outdated human red cells were kindly donated by the Canadian Red Cross. All other chemicals were reagent grade or better from standard suppliers.

* This is an alternate nomenclature for that sometimes used for the same compound, 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide (EAC).

Methods

Preparation of erythrocytes. Red cells were washed four times with 10–15 volumes of 0.9% w/v NaCl at 4°C to remove storage medium and white cells. If chloride-free medium was required, the cells were then washed three times with 10 volumes of citrate-buffered sucrose (28.5 mM sodium citrate (pH 7.0), 205.3 mM sucrose).

Treatments of cells. Treatment with ETC was done in one of several media, usually citrate-buffered sucrose or phosphate-buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, at pH 7.4 unless stated otherwise). ETC was added, by dilution from a freshly dissolved solution in the same buffer, to cells at 50% suspension in the stated buffer, and the suspension incubated at 37°C for 5 min (or the stated time). For example, the usual conditions for treatment in citrate-buffered sucrose were as follows: a 50% suspension of cells in 28.5 mM citrate, 205.3 mM sucrose, at pH 6.8, and 37°C was exposed to 4.5 mM ETC for 5 min. In all cases, after the incubation with ETC the cells were then washed twice with ice-cold treatment media containing 0.5% bovine serum albumin and three times in the buffer to be used for the next incubation or for transport measurements. The process from the end of ETC exposure to the start of the transport measurement generally took about 15 min. Treatment of cells with α-chymotrypsin was done at 1 mg/ml enzyme for 1 h at 37°C at a 25% suspension of cells in PBS.

Transport measurements. The exchange of extracellular phosphate for intracellular chloride was measured by following the loss of [^{32}P]phosphate from the supernatant of a 50% hematocrit suspension of chloride-loaded cells in 28.5 mM citrate-buffered sucrose (pH 7.4) as described previously [26,19,20]. Inhibition of transport was estimated from the change in half time for the initial loss of radioactivity from the extracellular medium.

$$\text{Inhibition (\%)} = (1 - (t_{1/2}\ \text{control}/t_{1/2}\ \text{treated})) \times 100\%.$$

Preparations of erythrocyte membranes. Following post-treatment washing, cells were lysed in ice-cold 5 mM sodium phosphate (pH 8), containing 0.1 mM phenylmethylsulfonyl fluoride [27], and washed extensively with the same buffer. An exception is the $^3H_2$DIDS crosslinking experiments, in which ghost preparation was done at pH 7.0. For the labeling experiments with [^{14}C]citrate and $^3H_2$DIDS, aliquots of a diluted suspension of membranes were taken for scintillation counting, protein determination, and gel electrophoresis.

[^{14}C]Citrate labeling. A stock of citrate-buffered sucrose was prepared by dissolving lyophilized [1,5-^{14}C]citric acid in 5 mM citrate, 285 mM sucrose (pH 7), resulting in a specific radioactivity of 197300 dpm/nmol citrate (89.7 nCi/nmol). Erythrocytes washed in 5 mM citrate buffered sucrose were suspended at 50% suspension in the [^{14}C]citrate buffered sucrose and treated with 4.5 mM ETC as described above. Treatments in media containing 1 mM and 28.5 mM citrate were done similarly, at specific radioactivities determined from the treatment supernatant of 107000 and 19000 dpm/nmol, for 1 mM and 28.5 mM citrate, respectively.

Covalent $^3H_2$DIDS labeling. Solid $^3H_2$DIDS (202 mCi/mmol) was dissolved in PBS (pH 7.0) at a concentration of 240 μM, and was stored in aliquots at −20°C. Cells washed in PBS were treated at 25% suspension with 15–50 μM $^3H_2$DIDS, without dilution with nonradioactive H_2DIDS, for 90 min at 37°C [28]. For bifunctional reaction the treatment was done in 150 mM $NaHCO_3$ (pH 9.5) for 90 min at 37°C [29]. The cells were then washed three times with PBS containing 0.5% fatty acid free bovine serum albumin, then with buffer without added albumin.

Noncovalent binding of $^3H_2$DIDS. The reversible binding of $^3H_2$DIDS to ghost membranes were performed under conditions similar to those described by Shami et al. [28], with the special precautions to reduce covalent binding by using a low temperature (4°C) and short times of exposure (5 min or less). Measurements were similar to those described for [^{14}C]BADS binding [30]. The covalent component of the binding was estimated by thorough washing of the membranes remaining after removal of aliquots for the noncovalent measurements, and processing as described above for covalent $^3H_2$DIDS labeling. A measurement of the nonspecific component of the noncovalent binding was attempted by using membranes that had been treated with 20 μM unlabeled DIDS,

or by inclusion of 27 μM unlabeled DIDS in the incubation with $^3H_2$DIDS.

BADS binding. Binding of BADS to Band 3 in ghost membranes by fluorescence enhancement titration was performed as previously described [24,30], using an emission wavelength of 450 nm and excitation wavelengths of 340 nm or 280 nm. Binding parameters were estimated from double-reciprocal plots of the data.

[^{32}P]PBS treatment. Erythrocytes in 0.9% NaCl were added to an equal volume of PBS containing [^{32}P]phosphate (110 nCi/nmol), then 18 mM ETC was added and the mixture incubated at 37 °C for 5 min. At this level, for example, if 0.1 mol P_i per mol Band 3 were incorporated, approximately 1000 dpm per 20 μg membrane protein should be detected.

Nucleophile treatment. The nucleophiles, in stock solutions of 150 mM of the chloride salt in distilled H_2O, were added to cells at a 50% suspension in PBS to achieve a final concentration of approximately 50 mM (based on extracellular volume) and a pH of 6.8. After pre-incubation for 5 min at 37 °C, 18 mM ETC was added, reacted for the desired time (2 to 120 min), and the cells were then washed 3 times with ice cold PBS containing 0.5% bovine serum albumin before washing with transport buffer.

Analytical techniques. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [31] and protein bands stained with Coomassie blue. Densitometry of Coomassie stained gels was performed on a Joyce-Loebl Chromoscan 3. The protein concentration was determined according to Lowry et al. [32], in the presence of 1% SDS, using bovine serum albumin as a standard. Fluorography involved pretreatment of the gel with Enlighten enhancer (New England Nuclear), and exposure of the dried gel at −70 °C to Kodak X-Omat X-ray film. Calculation of the distribution of radioactivity in gels was done either by densitometry of a fluorogram prepared using pre-flashed film, or by scintillation counting of gel slices that had been hydrolyzed by addition of 0.4 ml H_2O_2 and heating for 16 h at 50 °C. Scintillation counting was performed in a Beckman LS7800 scintillation counter with an external standard method of quench correction.

Results

Inhibition in citrate-containing media

Treatment of erythrocytes in citrate-buffered sucrose with the carbodiimide ETC resulted in irreversible inhibition of anion exchange. The possible pathways by which a carbodiimide could react with protein carboxyl residues have been well summarized elsewhere [33–35]. The possibility that the irreversible inhibition of anion exchange is caused by stable incorporation of the carbodiimide into the protein, such as by base-catalyzed rearrangement of the *O*-acylisourea adduct, has been ruled out by the lack of incorporation of [^{14}C]ETC into Band 3 [20]. Another possibility that has been suggested [20] is that a crosslink between the activated carboxyl and a nucleophilic group in a protein may be formed. We have no evidence that this occurs, and have observed no crosslinked products among the 4 kDa pepsin-generated fragments [36] of Band 3 (data not shown). An alternate reaction that could lead to irreversible inhibition is that of an exogenous ETC-activated carboxyl reacting with an essential lysine side chain (or other nucleophile) in Band 3. This possibility is explored in the following sections.

Involvement of citrate in the inhibition by ETC. A potential exogenous carboxyl is the buffering ion, citrate, present during treatment with ETC. To test whether citrate was involved in the inhibition of anion exchange by ETC, the dependence of the level of inhibition produced on the citrate concentration in the reaction medium was measured. Treatment was performed using the same concentration of ETC (4.5 mM) in a series of citrate-buffered sucrose solutions of increasing citrate concentration. For the measurement at zero citrate concentration, modification was carried out in citrate-free media, substituting a non-carboxyl buffer (Pipes was chosen as a suitable buffer since it contains no groups activatable by ETC, no potential nucleophilic groups, and is not expected to be transported by the anion transporter). When treatment of cells with 4.5 mM ETC (for 5 min at 37 °C) was carried out in Pipes-buffered sucrose (30 mM Pipes (pH 6.3), 220 mM sucrose), and transport measured in the same buffer, the result-

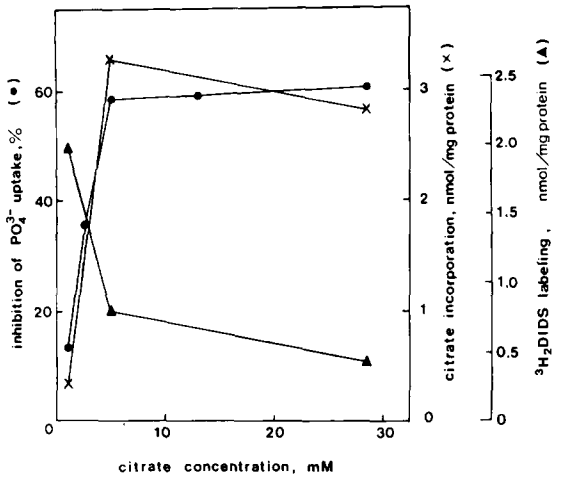

Fig. 1. Variation of (●) inhibition of phosphate uptake, (×) [^{14}C]citrate incorporation, and (▲) $^3H_2$DIDS labeling with the citrate concentration present during ETC treatment. Treatment of cells with 4.5 mM ETC was done as described in Methods except in a series of citrate-buffered sucrose solutions of citrate concentration varying from 1 to 28.5 mM. The calculated osmolarity was maintained by addition of extra sucrose in these solutions. After ETC treatment, the cells were washed, and transport was measured in 28.5 mM citrate, 205.3 mM sucrose. The points for inhibition of phosphate uptake for each concentration represent an average of from 2 to 7 determinations. [^{14}C]Citrate incorporation and $^3H_2$DIDS labeling were measured as described in Fig. 2 and in Methods, but on cells that had been treated with ETC in citrate-buffered sucrose containing 1, 5, or 28.5 mM citrate. For citrate incorporation each point represents a single determination. For $^3H_2$DIDS labeling the points represent 3, 2 and 7 determinations, respectively, for 1, 5 and 28.5 mM citrate. Note: The $^3H_2$DIDS labeling of untreated cells in this experiment was 2.13 nmol/mg. In the case of $^3H_2$DIDS labeling of cells treated with ETC in 5 mM citrate the inhibition of transport was only 48% rather than the average of 57%.

ing level of inhibition was 12% (average of four determinations). With increasing citrate concentrations the resulting inhibition increased from an average of 13% in 1 mM citrate to an average of approximately 60% in 5 mM citrate (Fig. 1). Little additional increase in inhibition was observed with citrate concentrations up to 28.5 mM. At concentrations of citrate above 4.5 mM the concentration of ETC available to generate the activated complex might become limiting. However, attempts to increase the inhibition by using higher concentrations of ETC (up to 30 mM) rarely produced higher than 60% inhibition. The level of 60% inhibition of anion transport is in very close agreement with the values obtained by Craik and Reithmeier in 28.5 mM citrate-buffered sucrose. Thus, it appears that the production of inhibition of anion transport (above 12%) is dependent on the presence of citrate.

Incorporation of citrate into Band 3. If the dependence of transport inhibition on the presence of citrate is due to the reactions suggested above, in which ETC activates citrate and the complex then reacts with a nucleophile in Band 3 protein, then incorporation of citrate into Band 3 protein would be predicted. Inclusion of [^{14}C]citrate in the reaction medium during treatment of erythrocytes with ETC resulted in incorporation of [^{14}C]citrate into Band 3. The incorporation was dependent on the concentration of citrate present during treatment (Fig. 1). At a concentration of 1 mM citrate, incorporation of 0.33 nmol citrate/mg total membrane protein was achieved, at 5 mM citrate, incorporation was 3.3 nmol/mg, and at 28.5 mM citrate the value was 2.8 nmol/mg. For the treatment in 5 mM citrate the fraction of the total radioactivity in the membranes that is associated with Band 3 was approx. 0.52 (determined from a gel identical to lane 1 in Fig. 2). Some radioactivity (9–15% of total) was associated with a band that migrated just below Band 3 (Fig. 2B, lane 1), which most likely corresponds to glycophorin *. Correction of the amount of citrate incorporated into membranes for the fraction in Band 3 gives values of 0.17, 1.72 and 1.46 nmol/mg membrane protein, for 1, 5 and 28.5 mM citrate, respectively. Assuming Band 3 represents 25% of the membrane protein, the stoichiometry of citrate labeling is 0.07, 0.7, and 0.6 mol citrate/mol Band 3, respectively. Apparently, even at the highest levels, incorporation of [^{14}C]citrate does not represent modification of all molecules of Band 3.

The effect of occupation of the stilbenedisulfonate binding site on the labeling produced by ETC plus 5 mM [^{14}C]citrate is shown in Fig. 3.

* The position is that expected for glycophorin, as confirmed by Stains-All staining of an identical gel. In addition, chymotrypsin treatment of cells is known to remove an extracellular fragment of glycophorin, leaving a membrane-bound portion that migrates at about 68 kDa [37]. Our results indicate this treatment removed the label in the region of glycophorin (Fig. 2B, lane 2).

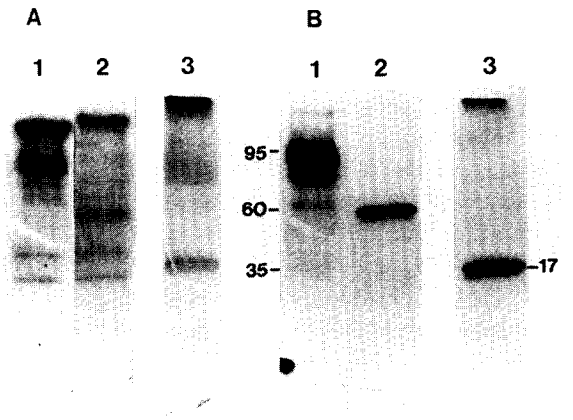

Fig. 2. Incorporation of [^{14}C]citrate into erythrocyte membrane proteins during treatment with ETC. Erythrocytes were treated with ETC (4.5 mM) in 5 mM [1,5-^{14}C]citrate, 285 mM sucrose, the cells were washed, and ghost membranes were prepared (in sample 2, after proteolysis). (A) SDS polyacrylamide gel electrophoresis of ghost membranes from the ETC plus [^{14}C]citrate treatment. Samples are: 1, no proteolysis; 2, α-chymotrypsin-treated cells; 3, α-chymotrypsin-treated ghosts, stripped with 2 mM EDTA (pH 12). Lanes 1 and 2 are from a gel containing 10% acrylamide, while lane 3 is from a separate gel containing 15% acrylamide. (B) Fluorogram of the gel lanes shown in (A). The numbered bands indicated represent the well characterized Band 3 fragments mentioned in the text: 95 kDa intact Band 3, the 60 kDa and 35 kDa fragments in chymotrypsin treated cells, and the 17 kDa fragment in chymotrypsin treated ghosts. The band in lane 3 (designated 17 kDa) co-migrated with a similar band from $^{3}H_2$DIDS labeled cells (not shown), which is known to represent the 17 kDa DIDS-binding chymotryptic fragment.

The presence of 50 μM DNDS during the ETC plus [^{14}C]citrate treatment reduced the [^{14}C]citrate labeling of Band 3 to 24% of that with no DNDS. Transport is inhibited by only 5% when cells were treated with ETC plus citrate in the presence of similar concentrations of DNDS [20], whereas that in cells treated with citrate without DNDS was inhibited by 57%. This shows that DNDS protects against both the irreversible inhibition of anion transport and the citrate labeling of Band 3 produced by treatment with ETC in citrate. Pre-treatment of cells with 10 μM DIDS, which should produce complete reaction of DIDS with Band 3, reduced the labeling of Band 3 by citrate to 5% that of the control.

The protection from citrate labeling by DNDS is not totally specific for Band 3. Glycophorin is also substantially protected from [^{14}C]citrate labeling by the presence of DNDS or DIDS. The protection of glycophorin is unexpected, and there are a number of possible explanations: glycophorin could be labeled on an internal site made accessible by Band 3-mediated entry of ETC-activated citrate, which would be inhibited by stilbenedisulfonates; or the site may be external and is in some way protected by stilbenedisulfonate binding to Band 3, or to glycophorin.

To determine whether the incorporated citrate was distributed throughout Band 3 protein, or localized to a small region, proteolytic fragmentation was performed. Fragmentation of Band 3 by chrymotrypsin treatment of the cells that had been treated in 5 mM [^{14}C]citrate indicated that most of the incorporated citrate is in the CH60 amino-terminal chymotryptic fragment (of the radioactivity in CH60 + CH35, that in CH60 accounted for about 90%), with a small amount (10% as calculated above) of radioactivity detecta-

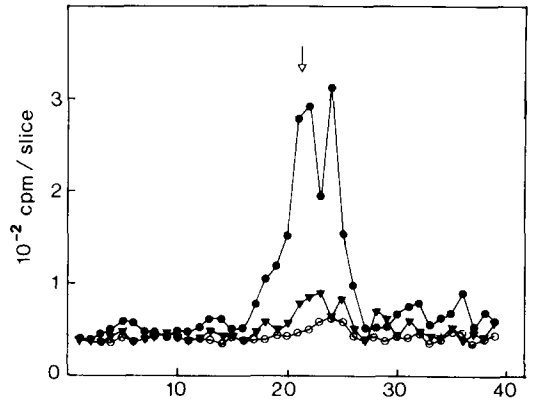

Fig. 3. Effect of stilbenedisulfonates on the incorporation of [^{14}C]citrate into erythrocyte membrane proteins. Erythrocytes were treated with ETC in 5 mM citrate, 285 mM sucrose, as in Fig. 2, in three batches, either with no other treatment (●), in the presence of 50 μM DNDS (▼), or after pre-treatment with 10 μM DIDS at pH 7.0 (○). After washes and membrane isolation, equal amounts of membrane protein from each preparation were run on an SDS-polyacrylamide gel containing 8% acrylamide. The arrow indicates the position of the stained Band 3. The transport activity of the ^{14}C labeled cells was not measured, but the inhibition produced by the ETC treatment alone should be near the average of 57% stated for 5 mM citrate in Fig. 1. The value in the presence of 50 μM DNDS should be similar to that determined previously [20], and the DIDS-treated would be fully inhibited.

ble in the CH35 fragment (Fig. 2B, lane 2). Upon further fragmentation of unsealed ghosts with chymotrypsin, a procedure that generates a transmembrane subfragment of 17 kDa from CH60, the majority of the radiolabel remained associated with the 17 kDa fragment (Fig. 2B, lane 3, from a separate gel).

The citrate concentration-dependent, stilbenedisulfonate-protectable, incorporation of [^{14}C]citrate into a small region of the protein, with a maximum stoichiometry of 0.7 mol/mol provides a direct demonstration that citrate modification of Band 3 is involved in the inhibition of anion exchange by ETC. Although the data above strongly suggest that citrate modification of Band 3 is involved in the production of inhibition of transport, and limit the modification to the 17 kDa chymotryptic fragment, they do not locate a specific site of citrate modification. To further investigate the effects of citrate modification of Band 3, the effect on interaction of Band 3 with stilbenedisulfonate inhibitors was studied.

Loss of $^3H_2$DIDS covalent labeling in ETC-treated cells. To result in incorporation of [^{14}C]citrate into the 17 kDa chymotryptic fragment of Band 3, an ETC-activated citrate could potentially react with any nucleophilic group in this fragment. The DIDS-reactive lysine residue, Lys *a*, is a notable candidate. Lys *a* (defined by its reactivity with the isothiocyanate derivatives of stilbenedisulfonates) is localized in the 17 kDa chymotryptic fragment [38,39], and most likely corresponds to either lysine 558 or 561 in the murine Band 3 sequence [5]. The reactivity of this residue to H_2DIDS would provide an indicator of whether it is affected by the citrate modification. Measurement of the $^3H_2$DIDS labeling of cells at pH 7.0, conditions where one end of DIDS reacts with Lys *a*, indicated that the citrate modification was accompanied by a reduction in the $^3H_2$DIDS covalent labeling of Band 3. $^3H_2$DIDS treatment of cells that had not been treated with ETC resulted in incorporation of 1.85 ($\pm$0.35) nmol $^3H_2$DIDS/mg membrane protein (six determinations). The $^3H_2$DIDS labeling in the membranes from cells that had been treated with ETC in 28.5 mM citrate-buffered sucrose (4.5 mM ETC, producing inhibition of anion transport of 59 ($\pm$4)% for six determinations) was 0.45 ($\pm$0.15) nmol/mg membrane protein. This represents a loss of $^3H_2$DIDS labeling of 1.4 nmol/mg membrane protein, or a 75% loss of sites. Again, as with the incorporation of citrate, the alteration responsible for the observed loss of $^3H_2$DIDS labeling does not occur in all molecules of Band 3.

In addition, the $^3H_2$DIDS treatment was sufficient to increase the level of inhibition of anion exchange in ETC plus citrate-treated cells from 60% to greater than 95% (data not shown). This sensitivity of the residual transport in citrate-modified cells demonstrates that even the reduced level of covalent reaction with $^3H_2$DIDS in these cells is enough to fully inhibit anion transport. This suggests the possibilities that either the citrate-modified Band 3 is fully inactive, or any residual transport by the modified transporters is able to be inhibited by $^3H_2$DIDS covalently bound to 25% of the Band 3 molecules (see Discussion).

The $^3H_2$DIDS labeling results are confirmed and extended in a similar experiment in which cells were first treated with chymotrypsin before treatment with $^3H_2$DIDS. ETC plus citrate treatment (in 28.5 mM citrate) resulted in an 80% decrease in $^3H_2$DIDS labeling of the CH60 fragment, but very little decrease in the (already low) labeling of the CH35 fragment at pH 7.0 (Fig. 4B, panel c). In addition, most of the chymotrypsin-cleaved Band 3 molecules that are labeled with $^3H_2$DIDS in ETC-treated cells are able to regenerate a 95 kDa species (Fig. 4B, panel d). These results indicate that the treatment results in a preferential loss of the $^3H_2$DIDS reaction with Lys *a*, but does not abolish the crosslinking function of Lys *b*. Also, there was no quantitative labeling of CH35 by $^3H_2$DIDS at pH 9.5 in ETC plus citrate-treated cells, indicating the treatment does not promote monofunctional reaction of $^3H_2$DIDS with Lys *b* even at pH 9.5.

In addition, the level of $^3H_2$DIDS labeling of Band 3 was inversely dependent on the citrate concentration in the ETC reaction medium (Fig. 1). At concentrations of citrate producing submaximal and maximal inhibition (1 mM and 28.5 mM, respectively) the $^3H_2$DIDS labeling stoichiometries represent a loss of $^3H_2$DIDS labeling of 0.14 nmol/mg and 1.4 nmol/mg membrane protein, respectively. Fig. 1 illustrates the relationship between the citrate concentration-dependen-

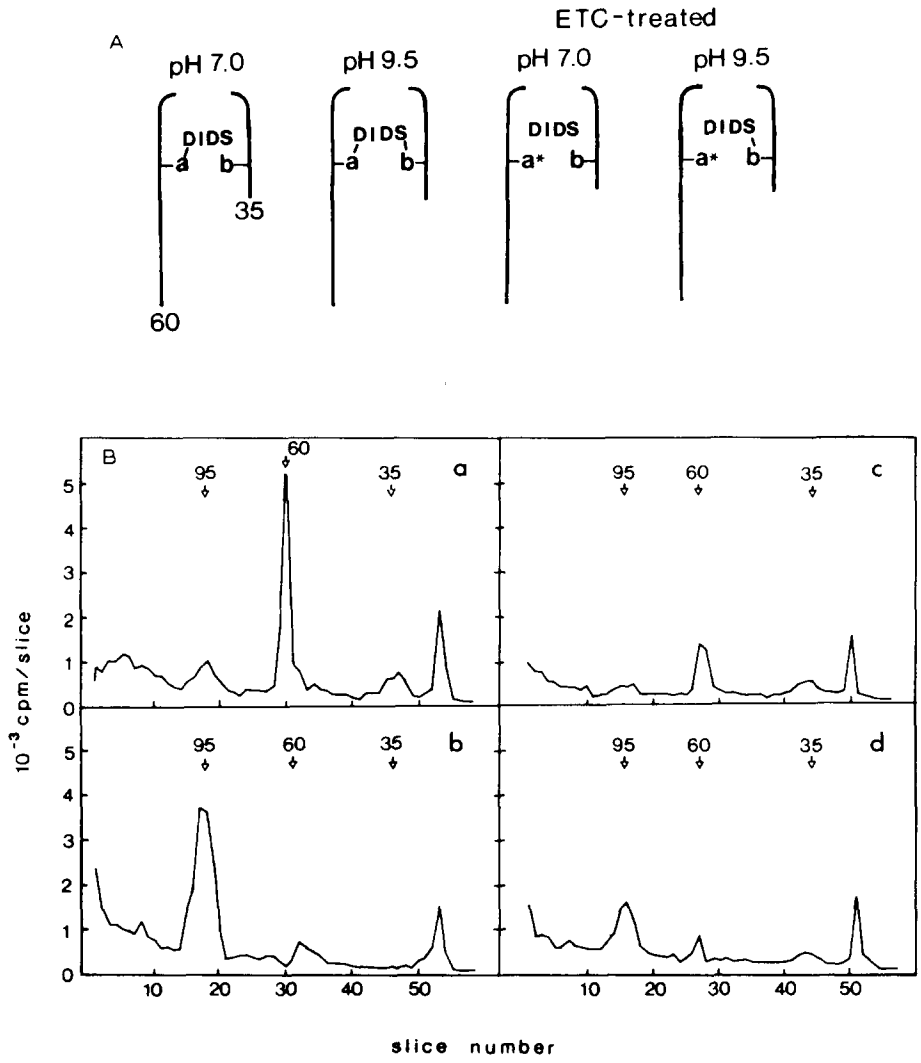

Fig. 4. Effect of modification with ETC in citrate on the $^3H_2$DIDS labeling and crosslinking of the 60 kDa and 35 kDa chymotryptic fragments. Aliquots of ETC plus citrate treated cells and of untreated cells were digested with α-chymotrypsin and each was divided into two portions which were then treated with 30 μM $^3H_2$DIDS at either pH 7.0 or pH 9.5. (A) Cartoon of the situations expected in each of the four cases. Letters represent Lys *a* (a) and Lys *b* (b) in the 60 kDa and 35 kDa fragments, respectively. The cartoons for the ETC-treated cases represent only that fraction of the protein which has been modified by citrate, with the modification assumed to be of Lys *a* (designated as an a*). Because of this assumption, the pair of cartoon figures on the right-hand side are only to be regarded as a hypothetical view presented to aid in understanding the results of the $^3H_2$DIDS labeling shown in (B). (B) Profiles of the radioactivity in the $^3H_2$DIDS labeled membranes after separation by SDS-polyacrylamide gel electrophoresis. Equal amounts of ghost protein from each treatment were applied to a gel containing 10% acrylamide. Small arrows indicate the position of the crosslinked, 60 kDa and 35 kDa fragments, and the four panels represent: (a) no ETC, pH 7.0; (b) no ETC pH 9.5; (c) ETC-treated, pH 7.0; (d) ETC-treated, pH 9.5.

cies of citrate modification, $^3H_2$DIDS labeling, and inhibition of transport. Clearly, with increasing citrate concentrations both the inhibition of anion exchange and the incorporation of [^{14}C]citrate into Band 3 increase and reach a maximum. The H_2DIDS labeling of Band 3 decreases with increasing citrate concentration, with a similar concentration dependence as the other effects.

Noncovalent binding of stilbenedisulfonates. Measurement of noncovalent binding of stilbenedisulfonates was carried out to determine whether

the loss of $^3H_2$DIDS labeling in ETC-treated cells was due to some alteration of noncovalent binding of stilbenedisulfonates, which could reduce the subsequent covalent reaction. Measurement of noncovalent binding of $^3H_2$DIDS, the same stilbenedisulfonate that was used to measure covalent labeling, was performed under conditions in which the covalent reaction of the isothiocyano groups is suppressed, while the noncovalent association still occurs [41]. The data suggested that the affinity of noncovalent binding of $^3H_2$DIDS by ghosts from ETC-treated cells is reduced compared to untreated membranes, but the presence of a large component of nonspecific binding (also present in previous work [41]) made it impossible to estimate the exact affinities or number of sites involved in the specific stilbenedisulfonate binding. Attempts at measurement of the nonspecific component, and correction for it, were not successful. In addition, the measurements often were complicated by a substantial covalent component of the binding, although cautions noted in Lepke's work were heeded.

For these reasons, the binding of another stilbenedisulfonate inhibitor, BADS, was measured, the important feature being that this analog can bind only noncovalently since it lacks the reactive isothiocyano groups of $^3H_2$DIDS. Measurement of binding of BADS to Band 3 by fluorescence enhancement titration (Fig. 5) indicated no substantial loss of maximum binding in ETC-treated membranes. The maximum fluorescence intensity attained in a titration of BADS binding to ghost membranes from ETC-treated cells ($F_{max} = 37.0$) was comparable to that of untreated membranes ($F_{max} = 42.5$). Inasmuch as the maximum fluorescence intensity due to BADS binding per mg of ghost protein can provide a relative measure of the number of BADS binding sites, this indicates that the number of BADS binding sites is not altered by ETC treatment. However, the BADS concentration required to produce half-maximal fluorescence enhancement ($C_{1/2}$) was 2-fold higher in ETC-treated membranes ($C_{1/2} = 12$ μM, compared to 6 μM in untreated), indicating ETC treatment decreased the affinity of BADS binding to Band 3 2-fold. A similar titration done at an excitation wavelength of 280 nm, in which case the fluorescence enhancement is due to resonance energy transfer from tryptophan residues near the BADS binding site to bound BADS [24], produced similar results: a comparable number of sites for the two preparations, and a twofold reduction of the apparent affinity of binding for ETC-treated membranes (data not shown).

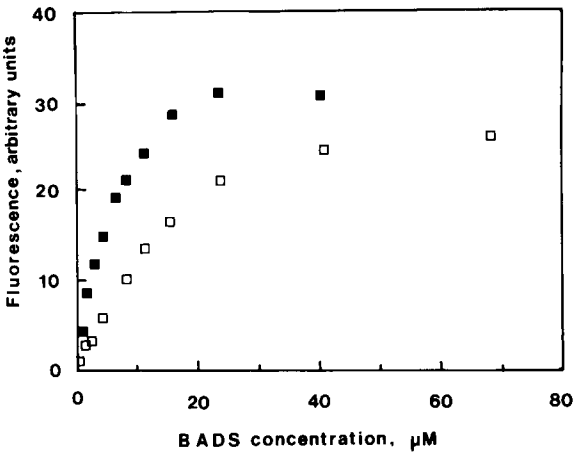

Fig. 5. Effect of ETC in citrate treatment on the binding of BADS to Band 3. The binding of BADS to Band 3 in membranes from untreated (■) and ETC plus citrate-treated (□) cells was measured by titration of the enhancement of BADS fluorescence that occurs upon binding to the hydrophobic stilbenedisulfonate site on Band 3. The data shown are for $\lambda_{ex} = 340$ nm and $\lambda_{em} = 450$ nm, and approximately the same amount of total membrane protein was used for each.

It is clear that the covalent attachment of $^3H_2$DIDS to Lys *a* is prevented to the same extent that citrate modification of Band 3 occurs. Because the noncovalent binding of stilbenedisulfonates to the modified protein is not eliminated, but occurs with 2-fold reduced affinity, and the labeling experiments were done with an excess of $^3H_2$DIDS (15 to 50 μM), it seems likely that the reduction in $^3H_2$DIDS labeling may be due to a blocking of the groups with which the isothiocyanates react. This will be considered further in the Discussion.

Inhibition in the absence of citrate

ETC treatment of intact erythrocytes in the absence of citrate, for example, in phosphate-buffered saline, or Pipes-buffered sucrose (as mentioned in the previous section), resulted in inhibition of anion exchange. The inhibition produced

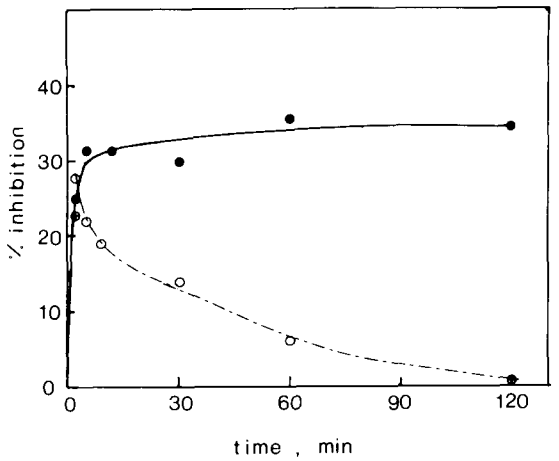

Fig. 6. Time course of inhibition of anion exchange by treatment with ETC in phosphate-buffered saline (PBS) in the presence and absence of added nucleophile. Treatment of erythrocytes in PBS, at a suspension pH of 6.8, with 18 mM ETC was carried out for the designated time, either in the absence (○, ⊕) or the presence (●) of 50 mM phenylalanine ethyl ester (PheOEt). The cells were washed in PBS with 0.5% bovine serum albumin (3×), then with 28.5 mM citrate-buffered sucrose, in preparation for the transport measurement. Data points from Ref. 20 are included to illustrate the usual time course in the absence of added nucleophile (○), which was confirmed (⊕) by measurement at the first and last times.

in phosphate-buffered saline remained after the ETC was removed by several washes but, unlike the inhibition produced in citrate-buffered sucrose, reversed over a period of 60 min incubation in PBS (see Fig. 6, without nucleophile). This reversal occurred with a similar time course whether ETC was present over the entire time period, or only during the first five minutes, but occurred more consistently under the latter conditions. The extent of reversal was dependent upon the ETC concentration, with little reversal occurring at ETC concentrations above 30 mM. It is possible that the reversal of inhibition with time could be produced by hydrolysis of an ETC-carboxyl intermediate by water.

It was established that buffer ions are not involved in the inhibition in phosphate-buffered saline, as was the case in citrate-containing media. There are suggestions in the literature that phosphate can be activated by carbodiimides in aqueous media and that cautious attention should be paid to the effect of such side reactions [42]. Because treatment with ETC was usually performed in phosphate buffered saline (5 mM P_i) we wanted to rule out reaction with phosphate. Treatment of erythrocytes with ETC in [^{32}P]PBS (5 min or 60 min incubation) resulted in no incorporation of ^{32}P into the isolated membranes or Band 3 (data not shown). Thus, it is unlikely that a mechanism of inhibition involving reaction of activated phosphate with Band 3 occurs.

Addition of exogenous nucleophile stabilizes inhibition. One approach to obtaining labeling and identification of the carboxyl residue(s) that are modified and are thus implicated as important for transport function is by addition of an exogenous nucleophile (in high enough concentration to compete with water). Carbodiimide-coupled incorporation of nucleophile has been used in many instances in other systems (see, for example, Refs. 33, 34). Reaction of intact cells with ETC was done in the presence of taurine and a series of amino acid ethyl esters in which the α-carboxyl is esterified to prevent activation by ETC, and the α-NH_2 is free to act as a nucleophile. Treatment of cells with the nucleophiles alone did not cause inhibition. At short times of treatment (5 min), for all nucleophiles tested, inhibition after treatment with ETC plus nucleophile was not substantially enhanced compared to reaction without exogenous nucleophile. However, for some nucleophiles, inhibition remained after treatment for longer times (up to 120 min). The time course of inhibition by ETC in PBS, with and without the added nucleophile PheOEt, is shown in Fig. 6. Without added nucleophile the inhibition reversed almost fully by 60 min. With added PheOEt (50 mM) inhibition was clearly stabilized, remaining at the level (35% inhibition) that is reached within 2 min of reaction for up to 120 min.

Table I lists the level of inhibition, relative to control, remaining after a 60 min incubation with ETC plus various nucleophiles. The ability to afford stabilization of the inhibition to reversal appeared to be related to the hydrophobicity of the nucleophile. Note that glycine ethyl ester did not stabilize, in agreement with what Craik and Reithmeier found previously [20]. The observation of stabilization of inhibition by addition of a nucleophile further supports that the reaction of ETC in citrate-free media is with a carboxyl re-

TABLE I

INHIBITION OF ANION EXCHANGE REMAINING AFTER 60 MINUTES' TREATMENT WITH ETC IN PBS IN THE PRESENCE OF ADDED NUCLEOPHILE

The nucleophiles used included a series of amino acid esters (abbreviated AAOEt). A nucleophile concentration of 50 mM was used, and did not cause inhibition without added ETC, provided washes, as outlined in Methods, preceded the transport measurement. Treatment was as described in Fig. 6, but for 60 min. The % inhibition is relative to that for ETC alone after 60 min.

Nucleophile	% inhibition at 60 min	Number of determinations
Taurine	0	2
GlyOEt	3	2
AlaOEt	0	2
LeuOEt	16	1
NTyrOEt	12	1
TyrOEt	17	2
PheOEt	35	6; S.D. = 7.3

sidue, since it is only this adduct that can be stabilized by addition of a nucleophile [33,34]. Further, it appears that modification of the carboxyl group(s), either by the carbodiimide isourea intermediate, or by the formation of an amide with an exogenous nucleophile, alone is enough to cause inhibition of anion exchange.

Discussion

Inhibition in citrate-containing media

The incorporation of [^{14}C]citrate into Band 3, (1), the loss of $^{3}H_2$DIDS covalent labeling of Band 3 (2), and inhibition of anion exchange (3) all show a similar dependence on the citrate concentration present during ETC treatment. This correlation supports the following mode of inhibition by ETC in citrate containing media: ETC activates a carboxyl group in citrate, and the activated citrate then modifies a reactive nucleophilic residue in Band 3 (perhaps a lysine residue), thus forming a citrate-amino acid adduct that has altered transport function. In addition to sharing similar citrate concentration-dependencies, the effects of ETC treatment are also stilbenedisulfonate-protectable. The presence of 50 μM DNDS prevents to a similar extent both the inhibition of transport [20] and the [^{14}C]citrate labeling of Band 3. Further support for the proposed mechanism comes from the close correlation between the percentage inhibition of anion exchange and the stoichiometries of both citrate incorporation and loss of $^{3}H_2$DIDS labeling. Incorporation of 0.7 mol citrate/mol Band 3, mostly localized in the 17 kDa Lys *a*-containing fragment, and loss of 75% of the covalent reaction of Lys *a* with $^{3}H_2$DIDS, occur with treatment that results in 60% inhibition of transport.

The comparable stoichiometries of citrate incorporation, loss of $^{3}H_2$DIDS labeling and level of inhibition also suggest that the citrate-modified protein is completely inactive under the assay conditions. The residual transport is presumably due to unmodified Band 3, as was indicated by its sensitivity to covalently bound $^{3}H_2$DIDS. The fact that about 50% inhibition occurs at neutral pH suggests that modification of one half of the Band 3 dimer with citrate precludes reaction of the other half. More complete inhibition has been obtained at alkaline pH [20], however this may be due to modification of other residues. Since little inhibition of anion exchange is obtained at a citrate concentration (1 mM) that produces low citrate incorporation and little loss of $^{3}H_2$DIDS labeling, the citrate modification of Band 3 may very well account for most of the inhibition of anion exchange by ETC in citrate media. However, the existence of a small component of the inhibition due to ETC reaction with Band 3 protein carboxyl residues cannot be entirely ruled out since exclusion of citrate from the reaction media did not always result in absence of inhibition. Lower levels of incorporation of [^{14}C]citrate into the fragment CH35 and some loss of crosslinking function associated with Lys *b* were also observed, but our data do not show whether or not this modification contributes to transport inhibition, beyond the determination that the stoichiometries of these effects are much lower than the observed level of inhibition of transport.

The proposed mode of inhibition in citrate-containing media can accomodate the major results described by Craik and Reithmeier [20]. (1) Their finding of no incorporation of [^{3}H]ETC is not at all surprising, since nucleophilic attack on the activated carboxyl should produce free carbodiimide urea. In fact, it is still evidence for lack of

incorporation of ETC into tyrosine or cysteine residues and lack of a component of reaction with Band 3 carboxyls resulting in base-catalyzed rearrangement. (2) The pH dependence of inhibition, namely, increasing inhibition with increasing extracellular pH, might be expected for a reaction scheme involving nucleophilic attack of a lysine residue, the deprotonated form of which acts as a nucleophile. (3) Protection from inhibition by the presence of DNDS (42.5 μM), and our companion result of protection from citrate labeling by DNDS, suggest that the modification is of a residue within the stilbenedisulfonate binding site.

We hypothesize that the site of citrate modification in Band 3 may be the particularly reactive lysine residue, lysine *a*. Lys *a* was first defined by its covalent reaction, at neutral pH, with the isothiocyanate derivatives of stilbenedisulfonates, most notably H_2DIDS and DIDS [1]. The exact residue corresponding to Lys a is not known, even though a DIDS-reactive peptide has been actively pursued for many years [1,36,38]. The amino acid sequence deduced from the cDNA for murine Band 3 has two lysine residues in the appropriate fragment, Lys-558 and Lys-561 [6]. Both the localization of the incorporated [^{14}C]citrate to the 17 kDa chymotrypsin fragment (which also contains the lysine residue thought to correspond to Lys *a*), and the loss of H_2DIDS reaction after ETC-treatment in citrate are consistent with the proposal that the modified residue in Band 3 is Lys *a*. It is important to recall that the evidence for a direct modification of the residue with which H_2DIDS reacts lies in the loss of covalent $^3H_2$DIDS labeling without loss of noncovalent binding of stilbenedisulfonates. The protection from labeling of Band 3 by citrate in the presence of DNDS could then be understood by considering the demonstrated lack of accessibility of Lys *a* to chemical modification when a stilbenedisulfonate such as DNDS is bound [43,44,10]. If our proposal that modifications of Lys *a* is correct, then the effects of this modification can potentially further define the functional role of te Lys *a* residue. Several different chemical modifications of Lys *a* have been described [1,10,44–48]. The proposed citrate-modified Lys *a* would clearly be different from the previous modifications. The effects of citrate modification of Band 3 on transport and inhibitor binding are consistent with the data from other modifications of the Lys *a* residue, and would potentially clarify the conclusion from the results of bis(sulfosuccinimidyl)-suberate (BSSS) modification [10] that the charge on Lys *a* (rather than on either Lys *a* or Lys *b*) is important in some part of the exchange function.

It is conceivable that the [^{14}C]citrate label in the 17 kDa fragment is in a stilbenedisulfonate-protectable nucleophilic residue other than Lys *a*, and that this change would alter noncovalent stilbenedisulfonate binding enough that covalent reaction of $^3H_2$DIDS would be impaired (that is, it would still bind, but to a slightly different region). We consider this possibility less likely, and note that the same criteria have been met as were used previously to define modifications of Lys *a*. Regardless of which residue in Band 3 is modified by citrate, though, it is still an almost stoichiometric modification, within the stilbenedisulfonate binding site, that inhibits anion exchange. Unambiguous identification of the citrate-modified residue awaits isolation of the appropriate peptide. At present we are attempting to purify a small citrate-labeled peptide suitable for sequencing, by separations in detergent solutions or organic solvents.

Inhibition in the absence of citrate

Contrary to the previous assumption–that inhibition of anion exchange by ETC in the two buffers, citrate-buffered sucrose and PBS, involves carbodiimide activation of the same residue and in which the subsequent inhibition mechanism is dependent on the conformation of the protein [20]–the modes of inhibition produced in the two conditions appear to be different. Unlike the inhibition produced by ETC in the presence of citrate, the inhibition in saline appears to be due to the modification of protein carboxyl residues. Our results of reversible inhibition that is stabilized by the addition of certain nucleophiles are consistent with the modification of a carboxyl residue(s) that is important in anion exchange, in which either the carbodiimide-carboxyl intermediate or the nucleophile adduct produces inhibition (based on known carbodiimide chemistry, it is only a carboxyl adduct which can be affected by addition of a nucleophile). The identification

of the particular residues that are modified by ETC in saline is still unknown. We can speculate that the region of the protein surrounding these residues is hydrophobic since the more hydrophobic nucleophiles tested provided stabilization, while the more hydrophilic ones (including taurine) did not. We are attempting to label Band 3 with [^{14}C]PheOEt to localize and identify the modified carboxyl(s).

It remains unclear whether the mode of inhibition that we report for treatment in citrate-free media (e.g., PBS, or Pipes-buffered sucrose) is the same as that reported by Wieth and co-workers. Since in Wieth's work the treatment with 30 mM ETC was performed in 165 mM KCl, 5 mM 4-morpholineethanesulfonic acid, at pH 6, the results should be comparable to those for our treatments with 18 mM ETC in PBS (pH 6.8). In fact, we can confirm their results of the production of about 35% inhibition with ETC plus a hydrophobic nucleophile, (though not with the same one). Differences between their findings and ours are: (1) they do not report any evidence of decay of the initially 'irreversible' inhibition in the absence of added nucleophile, as we have observed; (2) they observed an accelerated reaction rate in the presence of tyrosine ethyl ester, which we did not distinguish; and (3) they observed an apparent loss of covalent DIDS binding, while we observed no reduction in H_2DIDS labeling after treatment with ETC in the absence of citrate (data not shown).

We can only attempt to understand these differences by noting that there are small differences in the treatment conditions: they 'stopped' their reaction by the addition of a cold solution containing tris(hydroxymethyl)amino methane (pH 9) (a potential nucleophile); and the pH difference of 0.8 pH units, or the difference in ETC concentration, may be significant. Both of these differences may make the results less comparable, especially regarding reversal. Additionally, they measured transport activity by Cl^--Cl^- exchange (as efflux), and we measured P_i-limited influx into Cl^--loaded cells. This difference is perhaps the most significant, since it is possible that a modification may have different effects on P_i and Cl^- transport, or on the inward and outward steps of the transport. Alteration of Band 3 protein by limited proteolysis with papain is known to have different effects on the outward and inward rate constants of the transport [49]. The relationship between their tentative location, using tyrosine ethyl ester, of the labeled residue to the 35 kDa fragment [18] and our work can be considered only after the discrepancies in the characteristics of the inhibition are clarified. It will be interesting to find what residues are involved and examine their role in the anion exchange process.

Using a different method for labeling carboxyl groups, Woodward's Reagent K followed by $NaBH_4$, Jennings' group recently reported that the modification of at least two glutamate residues near the stilbenedisulfonate site results in greater than 90% inhibition of chloride-bromide exchange [21]. As Jennings and Anderson point out, it is possible that this reagent reacts with a different set of carboxyl groups in Band 3 from those reactive with ETC.

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