Anion-Proton Cotransport through the Human Red Blood Cell Band 3 Protein

ROLE OF GLUTAMATE 881*

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(Received for publication, January 7, 1992)

The band 3 protein of the human red blood cell membrane contains a glutamate residue that must be protonated in order for divalent (SO₄)²⁻ anion transport to take place at an appreciable rate. The carboxyl side chain on this glutamate residue can be converted to the primary alcohol by treatment of intact cells with Woodward's reagent K (N-ethyl-5-phenylisoazolium 3'-sulfonate) followed by reductive cleavage with BH₄. Edman degradation of CNBr fragments from band 3 labeled in intact cells with Woodward's reagent K and ['²H]BH₄ showed that Glu881 is heavily labeled under conditions in which Cl⁻ exchange is inhibited, SO₄ exchange is accelerated, and Cl⁻ conductance is accelerated. No other glutamate residue in band 3 is detectably labeled under the conditions of these experiments, as demonstrated either by Edman degradation or by the lack of label in major known proteolytic fragments. It is concluded that Glu881 is the binding site for the H⁺ that is transported with SO₄ during band 3-catalyzed H⁺/SO₄ cotransport. This residue is conserved among all species of red cell band 3 (AE1) as well as the related proteins AE2 and AE3. Glu881 is the first amino acid residue in band 3 which has been identified as a binding site for a transported substrate (H⁺). The functional characteristics of this residue suggest that it lies within the transport pathway and can be alternately exposed to the intracellular and extracellular media.

Anion transport across the red blood cell membrane is catalyzed by a protein known as band 3 or AE1 (Passow, 1986; Kopito, 1990; Alper, 1991). Red cell band 3 is a dimer or tetramer of 95-kDa subunits, each of which has a single binding site for the stilbenedisulfonate inhibitors of anion transport (see Passow, 1986). The protein is a major component of a membrane that is easy to isolate; consequently, band 3 has been an important model system for the study of structure-function relations in a transport protein. The sequences of mouse (Kopito and Lodish, 1985), chicken (Kim et al., 1988), and human (Tanner et al., 1988; Lux et al., 1989) band 3 are known from the sequence of cloned cDNA, and all consist of two distinct domains of roughly equal size. The N-terminal cytoplasmic domain is water-soluble and is not essential for transport (Lepke and Passow, 1976; Grinstein et al., 1978). The function of the cytoplasmic domain is to serve as an attachment site for the membrane skeleton (Bennett and Stenbuck, 1980). The cytoplasmic domain of human band 3 also binds hemoglobin and glycolytic enzymes (see Low, 1986).

The C-terminal membrane domain is hydrophobic and consists of multiple membrane-embedded segments of polypeptide. The topography of the membrane domain is relatively well defined, on the basis of predictions from primary structure as well as direct experimental evidence. For example, both the N terminus (Steck et al., 1976) and C terminus (Lieberman and Reithmeier, 1988) are cytoplasmic, and several sites in the sequence have been localized to one or the other membrane surface (e.g. Jennings et al., 1986). Although more experimental data are available about band 3 topography than for most membrane proteins, structural models of band 3 are nonetheless primitive and do not yet provide detailed information about the mechanism of transport.

To understand the transport mechanism it will be necessary to know not only the three-dimensional structure of the protein but also to know the roles of individual amino acid residues in the transport process. Initial interest focused on amino groups, because DIDS¹ and H₂DIDS, potent inhibitors of anion transport, react covalently with amino groups (see Cabantchik and Rothstein, 1974; Rudloff et al., 1983; Passow, 1986). It has been known for years that a single bound H₂DIDS molecule can react covalently with two different amino groups to form a covalent intramolecular cross-link (Jennings and Passow, 1979). The most rapid covalent reaction with H₂DIDS has been recently shown by site-directed mutagenesis to be mouse Lys599/human Lys393 (Bartel et al., 1989a, 1989b), although DIDS appears to be able to react reasonably rapidly with other lysines (Garcia and Lodish, 1988). Functional expression of band 3 has been demonstrated that mouse Lys599 (human Lys393) is not necessary for anion transport (Bartel et al., 1989a, 1989b; Garcia and Lodish, 1989).

The extracellular pH dependence of anion exchange (Wiith and Bjerrum, 1982; Wieth and Brahms, 1985) as well as chemical modification with vicinal diketones (Bjerrum et al., 1983; Zaki, 1981; Julien et al., 1990) indicate that at least 1 arginine...
residue is essential for transport. The exact locations of the arginines are not known. There is also evidence that an inward-facing histidine residue has a role in anion transport (Matsuyama et al., 1985).

The present study concerns a carboxyl group that is believed to participate in the catalysis of anion transport. The extracellular pH dependence of monovalent anion exchange indicates that there is a carboxyl group that must be deprotonated for translocation to take place (Wiegh and Brahm, 1985). A carboxyl group with similar pK, must be prototnated for translocation of the divalent anion SO₄⁻ to occur at an appreciable rate (Milanick and Gunn, 1984). Moreover, a proton is cotransported stoichiometrically with SO₄⁻ during net Cl⁻-SO₄⁻ exchange (Jennings, 1976), suggesting that the unprotonated carboxyl group is translocated with Cl⁻ in one direction, and the protonated form of the carboxyl group is translocated in the other direction with SO₄⁻.

The translocation events for both Cl⁻ and H⁺/SO₄⁻ are, to a first approximation, electrically neutral, i.e., less than 0.1 charge is transported through the transmembrane electric field (see Jennings et al., 1990). Although the most important physiological mode of action of band 3 is monovalent (Cl⁻/HCO₃⁻) exchange rather than H⁺/SO₄⁻ cotransport, the binding site for H⁺ is nonetheless of interest because it is closely associated with the anion transport pathway.

We showed previously that it is possible to use Woodward's reagent K and BH₄⁻ to convert at least 1 glutamic acid residue in band 3 to the corresponding alcohol (Jennings and Anderson, 1987). The functional consequences of this treatment are consistent with the idea that a single glutamate residue is responsible for both the extracellular pH dependence of SO₄⁻ influx and the intracellular pH dependence of SO₄⁻ efflux (Jennings and Al-Rhaiyel, 1988); that is, the carboxyl group binds the cotransported proton from either side of the membrane and thus appears to be able to cross the permeability barrier.

The goal of the present work was to characterize further the effects of carboxyl group modification on the function of band 3 and to localize the important glutamate residue(s) in the primary sequence. The effects of graded Woodward's reagent K/BH₄⁻ treatment of intact cells are consistent with the idea that conversion of a single carboxyl group to the alcohol form has major functional effects: inhibition of monovalent anion exchange, stimulation of divalent anion exchange, and stimulation of monovalent anion conductance. Labeling experiments with [³H]BH₄⁻ show that the residue of interest is in the C-terminal 28-kDa papain fragment but not the C-terminal 20-kDa trypsin fragment. Further localization demonstrated that Glu⁶⁰⁰ is labeled and that no other glutamate residue is detectably modified. Glu⁶⁰¹, located at the end of a very hydrophobic and conserved sequence of 22 residues, is the first negatively charged amino acid residue in band 3 which has been shown to be of functional importance.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human blood was drawn from normal donors into EDTA and was stored at 4°C for at most 4 days before use. 5-Hydroxyornovaline was a generous gift from Dr. Craig Townsend (Johns Hopkins University). The detergent C₁₂E₅ was kindly provided by Dr. Shio Makino (Nagoya University). Woodward's reagent K (N-ethyl-β-phenylisoxazolium 3'-sulfonate), NaBH₄, CNBr, MOPS, trypsin, N-o-p-tosyl-L-lysine chloromethyl ketone, and carboxypeptidase Y were purchased from Sigma. [³H]NaBH₄ (400-600 mCi/mmol), RbCl, and Na₂35SO₄ were purchased from Du Pont-New England Nuclear. Chromatography grade papain, and carboxypeptidase P from Boehringer Mannheim; endo-β-galactosidase of Escherichia freundii was from Miles. Sequencing reagents were from EM Science or Applied Biosystems. All other buffers and salts were from Fisher.

**Modification of Red Cells—**For both the labeling and functional studies, the modification procedure was essentially identical to that used for transport studies published previously (Jennings and Al-Rhaiyel, 1988). Cells were washed three times in 150 mM KCl, 10 mM MOPS, pH 7.0, and a 20-40% suspension was chilled on ice. Woodward's reagent K was then added to a final concentration of 1-2 mM (as specified in the figure legends). After 10 min on ice, NaBH₄ or [³H]NaBH₄ was added. The BH₄⁻ concentration was equal to that of Woodward's reagent K for the transport studies. To avoid wasting "H, the [³H]BH₄⁻ concentration was half that of Woodward's reagent K in the labeling experiments. After 10 min on ice, the cells were centrifuged and washed twice in 10-20 volumes of 150 mM KCl, 10 mM MOPS, pH 7.

**Anion Exchange and Conductance—**The influx of SO₄⁻ into Cl⁻-loaded cells was measured as described previously (Jennings and Al-Rhaiyel, 1988) in a medium consisting of 100 mM K₂SO₄ buffered with 20 mM Tris/HEPES, pH 8, 20°C. The reason for measuring influx at high extracellular pH is that the stimulatory effect of Woodward's reagent K/BH₄⁻ treatment is large (several-fold) at alkaline pH because the basal flux is low.

The chloride conductance of the red cell membrane is mediated mainly by band 3 and can be estimated from the tracer efflux of Rb⁺ in the presence of a cation ionophore such as valinomycin or gramicidin (Knauf et al., 1983; Frohlich et al., 1985). Cells were loaded with 1 mCi/ml of Rb⁺ in 50 mM Tris/HEPES, pH 7.0, 20°C (see Jennings et al., 1986). N-methyl-D-glucamine is not transported by gramicidin. From the rate constant of "Rb⁺ efflux, the CI⁻ conductance was calculated from the constant field equation, assuming that the only appreciable conductances present are those for K⁺ and CI⁻.

**Labeling, Proteolysis, and Peptide Isolation—**Cells treated with Woodward's reagent K/BH₄⁻ as described above were washed at pH 6 with endo-β-galactosidase as described previously (Jennings et al., 1984). This enzyme removes much of the carbohydrate from band 3 (Mueller et al., 1979). For the experiment shown in Fig. 2, cells were treated with chymotrypsin (1 mg/ml), 1 h, 37°C, or chymotrypsin followed by papain (1 mg/ml), as described previously (Jennings et al., 1984). After inhibition of papain with 5 mM iodoacetamide, cells were washed and membranes prepared as described (Jennings et al., 1984). Trypsin treatment of unsealed membranes was in 30 mM NH₄, acetate, pH 7, 100 𝜇g trypsin/ml. Affinity purification of the C-terminal 35-kDa chymotryptic fragment (CH35) and 20-kDa tryptic fragment (TR20) was essentially as described previously (Jennings et al., 1986). SDS-polyacrylamide gel electrophoresis was on 6-15% or 6-18% linear gradients of polyacrylamide in the buffer system or Laemmli (1970).

For large scale preparation of labeled 35-kDa fragment, membranes were stripped of peripheral protein by washing once in 0.1 N NaOH (Stuck and Yu, 1973) and then washed once further in 5 mM NaHCO₃, before being solubilized by mixing with 10% SDS at 100°C and heating 2-3 min further at 100°C. The solubilized fragments were then fractionated on Sephadex G3 in 50 mM NH₄ acetate, 0.1% SDS.

To prepare fragments smaller than 20 kDa from intact membranes, the membranes were suspended in 150 mM KCl, 10 mM MOPS, plus 1 mg/ml papain and 2 mM cysteine and incubated at 37°C for 1 h. The digestion was terminated by the addition of 5 mM iodoacetamide, and the membranes were pelleted at 19,000 rpm for 15 min. The pellet was washed once in 150 mM NaHCO₃, before solubilization and Sephadex 6b chromatography in 50 mM NH₄ acetate, 0.1% SDS. Labeled hydrophobic peptides were extracted into organic solvent by vortexing with 4 volumes of 2:1 CHCl₃/MeOH. The lower phase was chromatographed on Sephadex LH-60 in 2:1 CHCl₃/MeOH containing 100 mM NH₄ acetate (added from a 5 M aqueous stock).

As an alternate approach to generating labeled peptides in situ, whole band 3 was purified from labeled cells by the same method as was used for the 35-kDa fragment (above). The protein was concentrated to about 1 mg/ml in 0.1% SDS by ultrafiltration (Amicon YM-30) and then digested with CNBr (20 mg/ml) in 100 mM HCl, 0.5% N-o-p-tosyl-L-lysine chloromethyl ketone, pH 2.0, 20°C, dark, room temperature, N₂ atmosphere, with fresh CNBr added after 24 h. The solution was then mixed with 4 volumes of 2:1 CHCl₃/MeOH, shaken vigorously, and...
the lower phase was dried and chromatographed on Sephadex LH20 as described above.

**Reversed Phase Chromatography of Hydrophobic Peptides**—The labeled material in the Sephadex LH20 fractions could not be purified further by conventional reversed phase HPLC in aqueous 0.1% trifluoroacetic acid and/or CH3CN solvent. The labeled peptides are so hydrophobic that they either do not dissolve in the HPLC solvent or do not elute from the reversed phase column even in 90–100% acetonitrile. After considerable trial and error, the following method was adopted for the reversed phase purification of labeled hydrophobic peptide. The method does not give high resolution chromatography, but the results are reproducible and give easily interpretable sequence of the peptide.

A short-chain column (Beckman Ultrapore C3) was used with a binary solvent system. Solvent A was 0.1% trifluoroacetic acid in H2O. Solvent B was 80% CH3CN, 20% tetrahydrofuran. The inclusion of tetrahydrofuran in the B solvent made it possible to elute peptides that would not come off the column in CH3CN alone. Dried LH20 fractions were taken up in 200 µl of 88% formic acid. Direct dissolution in HPLC buffers was not possible, but the samples could be diluted in 1 ml of the loading solvent (40% B) and injected onto the column. It was necessary to inject in large volumes because of the limited solubility of the peptides. The peptides were eluted in a 20–90% B linear gradient at room temperature. In some preparations, the reversed phase run was preceded by gel filtration on TSK G2000SW in 80% B.

**Protein Sequence Determination**—Primary structural analysis was performed using an Applied Biosystems 475A protein/peptide microsequencer with an on-line model 120A microbore PTH-derivative analyzer and a model 900A data processor (see Nagle et al. 1988). For determination of the H, part of each cycle was diverted to a fraction collector before injection onto the 120A analyzer. The main labeled CNBr peptide is very hydrophobic and tended to wash off the solid support. Spotting the sample on polyvinylidene difluoride did not improve the repetitive yield. For sequencing 25–30 cycles, the C terminus was covalently coupled to arylamine-derivatized polyvinylidene difluoride using N,N'-diethyl-3-(3-dimethylaminopropyl)carbodiimide (Millipore Sequelon AA attachment kit). The derivatization also couples carboxylate side chains, causing reduced yield of unmodified glutamates.

The PTH-derivative of 5-hydroxynorvaline was prepared by reacting 100 µg of free 5-hydroxynorvaline with 5 µl of phenylisothiocyanate in 4.5 ml of 50% pyridine at 50°C for 50 min. The solution was then dried under N2 and redissolved in 25% aqueous trifluoroacetic acid (Applied Biosystems reagent R) and heated at 55°C for 20 min under N2. The product was then dissolved in 20% CH3CN and purified by reversed phase HPLC.

**RESULTS**

The reactions of Woodward's reagent K and BH4 with carboxyl groups have been described previously (Jennings and Anderson, 1987). The active ester adduct (Dunn et al., 1974) can be reductively cleaved with BH4 to produce the alcohol corresponding to the original carboxylic acid. However, the active ester can also rearrange to form the N-acyl derivative; the rearrangement is favored by higher temperature and alkaline pH. Reduction of the N-acyl derivative with [3H]BH4 produces stable incorporation of H into the protein, without cleavage of the reagent from the protein (Jennings and Anderson, 1987). The experiments in this paper were all performed under conditions designed to minimize the formation of the N-acyl derivative (low temperature, short exposure, pH 6.5–7).

**Effects of Woodward's Reagent K/BH4 on Anion Exchange and Conductance**—We showed previously (Jennings and Al-Rhaiyel, 1988; Jennings et al., 1989) that treatment of intact cells with 2 mM Woodward's reagent K and BH4 at 0°C causes inhibition of monovalent cation (Cl–/Br–) exchange, acceleration of H2DIDS-sensitive Cl–/SO4 exchange, and acceleration of H2DIDS-sensitive Cl– conductance. The acceleration of Cl–/SO4 exchange takes place over the same Woodward's reagent K concentration range in which Cl–/Br– exchange is inhibited, suggesting that the same group could be responsible for both effects. Fig. 1 shows dose-response curves for the effect of Woodward's reagent K/BH4 on two different assays of band 3 function: Cl– conductance and SO4 influx at high extracellular pH. In both cases there is strong acceleration, and the accelerated transport is entirely inhibited by H2DIDS. As described previously, we believe that the acceleration of SO4 influx is a consequence of the conversion of the substrate anion binding region of band 3 to a charge state that is the equivalent of that attained by lowering the extracellular pH of unmodified cells (Jennings and Al-Rhaiyel, 1988). The acceleration of the Cl– conductance could be explained if the reactive carboxyl group normally provides negative charge that constitutes part of the barrier to conductive anion movement through band 3; removal of the charge on this group therefore increases conductance (see “Discussion”).

The data in Fig. 1 and those published previously (Jennings and Al-Rhaiyel, 1988) are consistent with the idea that the conversion of a single carboxyl group to an alcohol is responsible for all the functional effects. It is important to note that Woodward's reagent K alone (without BH4) does not have any accelerating effect on either Cl– conductance or Cl–/SO4 exchange; it is only the conversion of the carboxyl group to the alcohol which causes the acceleration. Therefore, the modified residue(s) responsible for the functional effects should be heavily labeled by treatment of cells with 1–2 mM Woodward's reagent K followed immediately by [3H]BH4 at 0°C.

**Labeling Pattern at 0°C**—Our original paper on the labeling of band 3 with Woodward's reagent K and BH4 showed that exposure of cells to Woodward's reagent K at 22°C, followed by BH4 at 0°C, causes incorporation of label into both the 60 kDa N-terminal (CH60) and CH35 chymotryptic fragments (Jennings and Anderson, 1987). If the Woodward's reagent K exposure is at 0°C, followed immediately by reduction, the labeling is more selective; CH35 is much more heavily labeled than CH60 (Fig. 2). Digestion of intact cells with papain shows that the labeled residues are on the C-terminal 28-kDa papain fragment (Fig. 2). This result is expected on the basis of the fact that the main modified residue is glutamate rather than aspartate (Jennings and Anderson, 1987); the material that is removed from CH35 to produce P28

![Fig. 1. Effect of various concentrations of Woodward's reagent K/BH4 on Cl– conductance (open symbols) and SO42– influx into Cl–-loaded cells (filled symbols). Cells were treated with the indicated concentration of Woodward's reagent K for 10 min at 0°C at pH 6.5–7.0, followed by BH4 reduction. Cells were then washed, and the transport parameters were measured as described under “Experimental Procedures.”](image-url)
contains no glutamates (Brock et al., 1983; Jennings et al., 1984).

Uncleaved Woodward’s Reagent K on the 60-kDa Fragment—The experiment in Fig. 2 demonstrates that the conversion of carboxyl groups to alcohols takes place almost exclusively on CH35. There is, however, significant uncleaved and unlabeled reagent on CH60 under these conditions. This can be demonstrated by treating cells as in Fig. 2, but with nonradioactive BH4, and then incubating 60 min at 22 °C to allow the uncleaved enol esters to rearrange to the N-acyl derivatives. Reduction of the N-acyl compounds with [3H]BH4 for 60 min at pH 9 causes much more incorporation of label into CH60 than into CH35 (not shown). Therefore, under the conditions of the functional studies (Fig. 1) and labeling (Fig. 2), a significant fraction (roughly 25%) of band 3 has reagent covalently bound and unlabeled on CH60. These copies of the protein are of no relevance in the present study because they are not labeled under our usual conditions and they are functionally invisible. As mentioned above, the acceleration of SO42− exchange and Cl− conductance requires reductive cleavage; reagent alone, without BH4, causes no acceleration. Uncleaved reagent may actually inhibit transport and account for the lower rates of transport at 2 mM versus 1 mM reagent (Fig. 1). The important point is that the site responsible for the acceleration of SO42− transport and Cl− conductance must have been reductively cleaved and labeled under the conditions used in Fig. 2. Therefore, the residue of interest is not on CH60.

Lack of Labeling of C-terminal 168 Residues—It is known that exposure of unsealed membranes to trypsin in 30 mM NH4 acetate cleaves human band 3 at Lys143, producing a fragment denoted TR20 which consists of the C-terminal 168 residues (Jennings et al., 1986). To determine whether this C-terminal fragment contains residues that are labeled by Woodward’s reagent K/BH4, intact cells were labeled and then treated with chymotrypsin as described above. Membranes were isolated and incubated with or without trypsin, washed, alkali stripped, and the membrane-bound fragments were solubilized and separated by SDS-polyacrylamide gel electrophoresis. Without trypsin, the counts are distributed in the broad band consisting of glycosylated CH35 (Fig. 3), as expected from the results Fig. 2. (The band is broad in this experiment because the cells were not treated with endo-β-galactosidase.)

Treatment of the unsealed membranes with trypsin at low ionic strength shifts most of the counts to a very broad zone, the leading edge of which has apparent molecular mass of 17 kDa. This zone represents a glycosylated fragment that very likely starts at Met609 and ends somewhere between Lys661 and Lys674. The C-terminal tryptic fragment TR20 that begins at Ala744 migrates as a sharp band located two slices slower than the leading edge of the labeled zone. The data in Fig. 3 indicate that there is little or no label in TR20.

To examine the issue of TR20 labeling more closely, membranes from the above preparation were solubilized in SDS, diluted in phosphate-buffered saline-Tween (150 mM NaCl, 10 mM sodium phosphate, pH 7.4, 0.05% Tween 20), and incubated with Sepharose 4B-immobilized monoclonal antibody IVF12, which binds to CH35 and TR20 (Jennings et al., 1986). The Sepharose was then washed, and bound peptides were eluted by resuspending the Sepharose in 1% SDS and centrifuging after 2 min of gentle agitation at 22 °C. Western blots (not shown) demonstrated that most of the C-terminal 20-kDa fragment TR20 is recovered in the final SDS supernatant, but there is no detectable labeling.

**Fig. 2.** Labeling of the C-terminal chymotryptic (CH35) and papain (P28) fragments by Woodward’s reagent K/[3H]BH4. Cells were treated with Woodward’s reagent K for 10 min at 0 °C followed by reduction with [3H]BH4, washed, and then treated with chymotrypsin (solid symbols) or chymotrypsin followed by papain (open symbols). Cells were then treated with endo-β-galactosidase, membranes were prepared, and protein was separated on a 6-15% polyacrylamide gel. Stained gel showed major unlabeled or weakly labeled bands, including spectrin (slices 8-12) and the N-terminal 60-kDa chymotryptic fragment (slices 26-28). Similar results, without deglycosylation, were published previously (Jennings et al., 1988).

**Fig. 3.** Labeling of glycosylated product of internal trypsin digestion of CH35. Cells were labeled as in Fig. 2, treated with chymotrypsin (no endo-β-galactosidase), and membranes were isolated and incubated with or without trypsin, 2 mM Woodward’s reagent K/BH4, intact cells were labeled and then treated with endo-β-galactosidase. Half the membranes were treated with 100 pg/ml of 10% SDS, diluted in 10 ml of phosphate-buffered saline-Tween, and incubated in for 1 h, 22 °C with 2 ml of Sepharose 4B coupled to monoclonal antibody IVF12. The Sepharose was centrifuged and washed twice in 10 ml of phosphate-buffered saline-Tween. The radioactivity in these first three supernatants is represented as a percent of the total initially present. Finally, bound peptides were eluted by resuspending the Sepharose in 1% SDS and centrifuging after 2 min of gentle agitation at 22 °C. Western blots (not shown) demonstrated that most of the C-terminal 20-kDa fragment TR20 is recovered in the final SDS supernatant, but there is no detectable labeling.

**Fig. 4.** Lack of labeling of immunopurified TR20. The membranes (400 µl) from the experiment in Fig. 3 were solubilized in 0.1 ml of 10% SDS, diluted in 10 ml of phosphate-buffered saline-TWEEN, and incubated in for 1 h, 22 °C with 2 ml of Sepharose 4B coupled to monoclonal antibody IVF12. The Sepharose was centrifuged and washed twice in 10 ml of phosphate-buffered saline-Tween. The radioactivity in these first three supernatants is represented as a percent of the total initially present. Finally, bound peptides were eluted by resuspending the Sepharose in 1% SDS and centrifuging after 2 min of gentle agitation at 22 °C. Western blots (not shown) demonstrated that most of the C-terminal 20-kDa fragment TR20 is recovered in the final SDS supernatant, but there is no detectable labeling.
right). Western blots (not shown) confirmed that TR20 was in fact bound and recovered in high yield from the immobilized antibody. We conclude that there is no detectable labeling of the C-terminal tryptic fragment that begins at Ala-744.

**Lack of Labeling of Glu**

The above experiments strongly indicate that the labeled residues are between Met (N terminus of CH35) and Lys<sup>143</sup>. The only glutamate residues in this part of human band 3 are in positions 658, 681, and 693 (Tanner et al., 1988; Lux et al., 1989). We attempted several times to prepare smaller fragments of isolated CH35 by treating with V8 protease from *Staphylococcus aureus*. The smallest labeled fragment that could be prepared has an apparent M<sub>r</sub> of 20,000 and N terminus at Met<sup>699</sup>, again consistent with the idea that the label is in position 658, 681, or 693.

Because cleavage of isolated 35-kDa fragment into well defined small peptides proved to be difficult, an alternative approach was used. Unsealed membranes were digested with papain in the hope of producing small hydrophobic fragments. Ramjesingh et al. (1984) and Falke et al. (1985) had shown that bilateral proteolysis of red cell membranes produces fragments of roughly 20–40 residues. In this kind of experiment, bilateral digestion of membranes with nonspecific enzymes can sometimes destroy even transmembrane fragments (Dumont et al., 1986). Fortunately under the digestion conditions used here, more than 90% of the <sup>3</sup>H remained with the membranes. Membranes were solubilized in SDS, and the peptides were chromatographed on Sepharose 6B in the presence of SDS, followed by Sephadex LH60 in CHCl<sub>3</sub>/MeOH, 2:1, 20 mM NH<sub>4</sub> acetate. The main labeled peak was then purified further by gel filtration on TSK G2000SW in 64% CH<sub>3</sub>CN, 16% tetrahydrofuran, 20% H<sub>2</sub>O, 0.02% trifluoroacetic acid. The resultant peptide was not absolutely pure, but an easily interpretable major sequence began at Ser<sup>687</sup>. Significantly, the second cycle gave a good yield of unmodified glutamic acid (Glu<sup>681</sup>), but no radioactivity was released with the PTH-derivatives in this cycle. The same peptide was sequenced in three separate preparations, and no radioactivity (<1 cpm/pmol) was released in the position of Glu<sup>681</sup>. Therefore, Glu<sup>681</sup> is not labeled by treatment of intact cells with Woodward's reagent K and [3H]BH<sub>3</sub>.  

**CNBr Peptides: Labeling of Glu**

The amino acid sequence of human band 3 (Tanner et al., 1988) indicates that there are methionine residues at positions 663, 664, and 696. Therefore, if either Glu<sup>681</sup> or Glu<sup>683</sup> is labeled, the label should appear in a 32-residue CNBr peptide bounded by Phe<sup>666</sup> and Met (homoserine)<sup>696</sup>. To try to isolate and sequence labeled CNBr fragments, whole band 3 from labeled cells was purified and treated with CNBr (Fig. 5). The majority of the radioactivity was associated with hydrophobic peptides that extracted into CHCl<sub>3</sub>/MeOH. The peptides eluted in a broad band from a C3 HPLC column. Edman degradation of fractions from the leading, middle, and trailing portions of the labeled peak all give an unambiguous major sequence that matches the known band 3 sequence starting at Phe<sup>666</sup>. Moreover, counts are released in cycle 17 (Fig. 6), i.e. in the position of Glu<sup>681</sup>. It is significant in cycle 17 contained a peak that eluted just after dimethylphenylthiourea (Fig. 7). A PTH-5-hydroxynorvaline standard elutes in the same position. Thus, both the radioactivity and PTH-5-hydroxynorvaline appear in cycle 17, exactly as expected if the Glu<sup>681</sup> side chain had been converted to the labeled alcohol.

The specific activity of the PTH-5-hydroxynorvaline is about 150 cpm/pmol (500 dpm/pmol). With an initial [3H] BH<sub>3</sub> specific activity of 500 mCi/mmol, the theoretical specific activity of 5-hydroxynorvaline should be 250 mCi/mmol (two of the four reducing BH<sub>3</sub> equivalents incorporated), or 550 dpm/pmol, which is essentially identical to that observed. This would suggest that there is very little kinetic isotope effect in the reductive cleavage of the enol ester adduct. However, there are numerous sources of systematic error in the specific activity determination, and a kinetic isotope effect of as much as 2-fold could have escaped detection.

**Lack of Labeling of Glu**

The Edman degradation in Fig. 6 was interpretable only to about cycle 23 (Leu<sup>68</sup>). In a
FIG. 7. Detection of the PTH-derivative of 5-hydroxyxnorvaline (PTH-HNV) in cycle 17 of Edman degradation of the labeled CNBr fragment. The first 15 min of the chromatogram of the PTH-derivatives for cycles 16 and 17 of Edman degradation of the sample from Fig. 6 are shown. The peak in the position of PTH-5-hydroxyxnorvaline is indicated. The PTH-Leu in cycle 16 elutes much later and is not shown. Full scale A270 is 0.004.

FIG. 8. Isolation of labeled peptides following carboxypeptidase Y digestion of the labeled CNBr peptide. As described under "Experimental Procedures" CPY produces an endoproteolytic clip in the labeled CNBr fragment, resulting in labeled peptides that elute from the C3 column at relatively low concentrations of organic solvent.

separate run, sequencing was carried out for 32 cycles, with no radioactivity released in the position of Glu. However, the yield of PTH-derivatives was very low (<1 pmol) after these many cycles, and it was not possible to draw conclusions about the extent of labeling of Glu.

To investigate in more detail the possibility that Glu is labeled, the CNBr peptide was treated with carboxypeptidases. Carboxypeptidase P digestion did not yield detectable amino acids, probably because enzyme is sensitive to detergents. Carboxypeptidase Y digestion was performed in the presence of 0.1% C6E9. The results were surprising. Instead of the expected homoserine (Met), Lys, etc., the digestion produced about 2 mol of leucine and 1 mol each of isoleucine and phenylalanine/mol of peptide. Inspection of the known sequence of band 3 suggests that carboxypeptidase Y could have made an endoproteolytic clip near Leu and then digested the new C terminus to release isoleucine, leucine, and phenylalanine. The peptides in the carboxypeptidase Y digest were separated by reversed phase HPLC. In sharp contrast to the whole Phe-Met-homoserine peptide, which elutes from a C3 column at 60–70% B, over half the counts in the CPY digest did not bind to the column at all in 40% B. The breakthrough fractions were dried and reloaded on the same column in 0% B and eluted with a 0–40% gradient. Several peptides were eluted, but one fraction was heavily labeled (Fig. 8).

Edman degradation of the main labeled fraction produced two PTH-derivatives in the first five cycles (Fig. 9). The second cycle contained PTH-5-hydroxyxnorvaline, and very little PTH-Glu. The third cycle contained mainly PTH-Ser. These results are exactly what would be expected if the labeled fraction consisted of a mixture of the following two peptides (both derived from the original CNBr peptide):

680
Leu-5-hydroxyxnorvaline-Ser-Gln-Ile-
688
Ile-Val-Ser-Lys-Pro-Glu-Arg-Lys-

The 3H was released almost entirely in the second cycle (Fig. 9, bottom), confirming that Glu has been modified to radiolabeled hydroxyxnorvaline. Another notable result in Fig. 9 is that there is no radioactivity (less than 15 cpm) but an easily measurable amount of PTH-Glu, released in cycle 6, i.e. in the position of Glu. Therefore, Glu is not detectably labeled.

DISCUSSION
The major new finding described here is that Glu of human red cell band 3 is labeled and converted into 5-hydroxyxnorvaline by treatment of intact cells with Woodward's reagent K and [3H]BH4 at neutral pH and 0 °C. Under the conditions of the modification, the pH dependence of SO4 transport is dramatically altered, indicating that the titratable acid group associated with H+/SO4 cotransport has been converted into a neutral species. These findings strongly indicate that Glu is the binding site for the H+ that is cotransported with SO4 during net Cl/SO4 exchange (Jennings and Al-Rhaiyel, 1988; Jennings et al., 1988). This is the first amino acid residue in band 3 which has been localized in

FIG. 9. The main labeled fraction (eluting at 13 min) from the preparation in Fig. 8 was applied to the sequenator in Polybrene (no covalent coupling) and subjected to Edman degradation. Two PTH-derivatives were produced in the first five cycles, indicating that the fraction consisted of a mixture of two peptides. See "Results."
the sequence and also shown to be closely associated with the transport pathway. Other residues, notably Lys465 and Lys679, have been shown to react cotransportively with charged reagents (Kawano et al., 1988; Bartel et al., 1989a, 1989b), but the inhibitors are rather bulky and may inhibit transport by a steric mechanism. The modification of Glu641 by Woodward’s reagent K/BH4 does not introduce steric bulk but nonetheless causes major effects on transport.

In addition to showing that Glu641 is labeled and converted into 5-hydroxynorvaline, we have shown that no other glutamate residue is detectably labeled. For example, the entire N-terminal chymotryptic fragment CH60 is labeled only very slightly under these conditions (Fig. 2). Some copies of CH60 contain uncleaved reagent, but the residue responsible for H+/SO42− cotransport is definitely not on CH60 (see above).

Similarly, the experiment in Figs. 3 and 6 rules out the C-terminal tryptic peptide TR20, leaving only Glu641, Glu640, and Glu643 as possible sites. Edman degradation showed that, of these, only Glu641 is labeled. We cannot place a precise upper limit on the extent of labeling of glutamates other than Glu641, but the stoichiometry is much less than 10% under conditions in which the stoichiometry of labeling of Glu640 is over 50%.

**Sidedness of Glu641**—Glutamate 681 is conserved among different species of AE1 and among the related proteins AE2 and AE3. Fig. 10 depicts the sequences of human, mouse, and chicken AE1, human AE2, and mouse AE3 in the vicinity of Glu641, which is preceded by a very hydrophobic stretch of 22 residues. It is probable that the N-terminal end of this sequence is extracellular for the following reasons. Papain treatment of unsealed ghosts at low ionic strength cleaves at Arg656, which is preceded by a very hydrophobic stretch of 22 residues, and there is only 1 serine and no other hydrophilic residue. Perhaps the hydrophobic sequence between Pro660 and Glu641 forms a pitched helix (with a kink at Pro660), which converges with the access channel of Glu641 near the middle of the membrane.

The sequence downstream from Glu641 is also quite well conserved but is less hydrophilic. The 15 residues starting with Ser460 could form part of the lining of an access channel leading to the inner surface of the membrane, ending with a cluster of three positive charges (and usually one negative charge). Positive charges could be part of the inward-facing mouth of the access channel. The exact positions of the lysines and arginines are not conserved (except Arg665), but all sequences have three closely spaced positive charges, suggesting an important role for the positive charges. Although it is attractive to speculate that these charges are near the mouth of an inward-facing access channel, the sidedness of these residues has not been established experimentally.

**Acknowledgment**—We are grateful to Pamela H. Fritz for assistance in the performance of many of these experiments.

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