

Modification of a Carboxyl Group That Appears to Cross the Permeability Barrier in the Red Blood Cell Anion Transporter

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ABSTRACT A recently developed method for converting protein carboxyl groups to alcohols has been used to examine the functional role of carboxyl groups in the red blood cell inorganic anion-transport protein (band 3). A major goal of the work was to investigate the carboxyl group that is protonated during the proton-sulfate cotransport that takes place during net chloride-sulfate exchange. Three kinds of evidence indicate that the chemical modification (Woodward's reagent K followed by borohydride) converts this carboxyl to an alcohol. First, monovalent anion exchange is inhibited irreversibly. Second, the modification stimulates sulfate influx into chloride-loaded cells and nearly eliminates the extracellular pH dependence of the sulfate influx. (The stimulated sulfate influx in the modified cells is inhibitable by stilbenedisulfonate.) Third, the proton influx normally associated with chloride-sulfate exchange is inhibited by the modification. These results would all be expected if the titratable carboxyl group were converted into the untitratable, neutral alcohol. In addition to altering the extracellular pH dependence of sulfate influx, the chemical modification removes the intracellular pH dependence of sulfate efflux. The modification is performed under conditions in which the reagent does not cross the permeability barrier. The large effect on the intracellular pH dependence of sulfate transport suggests that a single carboxyl group can at different times be in contact with the aqueous medium on each side of the permeability barrier.

INTRODUCTION

The major transmembrane protein of the human red blood cell is known as band 3 or capnophorin and catalyzes anion exchange across the membrane (see Passow, 1986, for a recent comprehensive review). The protein has molecular weight 95,000 and consists of two domains. The NH₂-terminal 43 kD is a water-soluble cytoplasmic domain that serves as an attachment site for the membrane skeleton (Bennett and Stenbuck, 1980). The COOH-terminal 52-kD domain is hydrophobically associated with the membrane and catalyzes a one-for-one chloride-bicarbonate exchange,

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which is one of the steps in CO_2 transport by the blood (see Wieth et al., 1982a). The abundance of band 3 and the relative ease of carrying out transport studies in red cells has allowed the structure and function of this protein to be studied in more detail than has been possible for most transport proteins.

The sequence of mouse band 3 has been deduced from the sequence of the cloned cDNA (Kopito and Lodish, 1985). The sequence of the membrane domain is consistent with a model containing as many as 12 membrane-spanning segments. Direct studies employing chemical labeling and *in situ* proteolysis have provided evidence that there are at least eight segments of polypeptide that cross the permeability barrier (see Jennings et al., 1986). Circular dichroism measurements indicate that the membrane-spanning segments are largely α -helical (Oikawa et al., 1985).

The anion transport catalyzed by band 3 is almost entirely an obligatory one-for-one exchange (see Knauf, 1979; Fröhlich and Gunn, 1986). Although the kinetics of band 3-mediated anion exchange are complex, there is widespread agreement that the mechanism involves a cycle of conformational changes that alter the access of a bound anion to each of the two aqueous solutions (Knauf, 1979; Fröhlich and Gunn, 1986; Passow, 1986). The simplest such mechanism is one in which there are two interconvertible conformations of the protein, denoted the inward-facing and the outward-facing states. The inward-facing state binds an intracellular anion, which is then transported outward by way of a conformational change in the anion-protein complex. The anion is then released, and the cycle is completed when an extracellular anion is transported inward by the reverse conformational change. The details of the kinetics of anion exchange require a considerably more complex model, including binding events that are responsible for substrate inhibition (Dalmak, 1975; Knauf, 1979).

Several different physical pictures of the anion-exchange process are possible. For example, the protein may form an aqueous channel that passes most of the way across the membrane. In this view, an unpaired anion moves by diffusional jumps to a thin permeability barrier, and a local conformational change allows the anion to cross the barrier or (equivalently) causes the position of the barrier to move (Cabantchik et al., 1978; Gunn, 1978). An alternative physical picture of the permeation pathway does not contain a thin permeability barrier separating aqueous channels; rather, the barrier separating the aqueous compartments is thick (more than a few angstroms) and is spanned by an array of paired charges. In this physical model of the transport, the anion movement would take place by way of a succession of exchanges of a transported anion for a carboxyl group in an ion pair with a positive charge. This kind of model has been termed a "molecular zipper" and was proposed independently by two laboratories five years ago (Brock et al., 1983; Wieth and Bjerrum, 1983).

In order to gain information at the molecular level about the mechanism of ion transport, it is necessary to identify amino acid residues that are involved in the transport process. For band 3, it is known that arginine residues have an important role in anion exchange (Zaki, 1981; Wieth et al., 1982b; Bjerrum et al., 1983), although the number, location, and exact functions of the essential arginines are not known. There are at least two amino groups at the stilbenedisulfonate site (Jennings and Passow, 1979; Passow, 1986), but the amino groups do not appear to be abso-

lutely essential for anion translocation (Jennings et al., 1985). The presence of at least one carboxyl group in the transport pathway is suggested by the extracellular pH dependence of chloride-chloride and chloride-sulfate exchange (Wieth et al., 1982a; Milanick and Gunn, 1982, 1984). Protonation of a group of pK_a 5.0–5.2 inhibits chloride-chloride exchange; protonation of a group of similar pK_a stimulates sulfate influx. These reciprocal effects on monovalent and divalent anion exchange are consistent with the idea that protonation of the carboxyl group converts the transporter from a monovalent anion transporter to one that transports divalent anions at an appreciable rate.

We recently demonstrated that it is possible, using Woodward's reagent K and BH_4 , to convert exofacial carboxyl groups in band 3 to primary alcohols (Jennings and Anderson, 1987). Only glutamate carboxyl groups were converted to alcohols; there was no detectable conversion of aspartate carboxyls. The present article examines the functional consequences of this chemical modification. We show that conversion of one or more extracellular carboxyl groups to alcohols inhibits monovalent anion exchange but stimulates stilbenedisulfonate-sensitive sulfate transport, measured either as sulfate-sulfate exchange or chloride-sulfate exchange. Although chloride-sulfate exchange is accelerated by the modification, the proton influx normally associated with this exchange is inhibited. This indicates that the modified carboxyl groups include the one that is normally protonated during proton-sulfate cotransport into the cells. The chemical modification is performed under conditions in which only extracellular groups are modified, but the modification has a major effect on the intracellular pH dependence of sulfate-sulfate exchange. This is evidence for the existence of a glutamate residue that can cross the permeability barrier in band 3.

MATERIALS AND METHODS

Materials

Human red blood cells (EDTA anticoagulant) were obtained from the Lipid Research Laboratory of the University of Iowa, Iowa City, IA, or from Dr. Claude Benedict of the University of Texas Medical Branch, Galveston, TX, and stored at 4°C as packed cells for at most 4 d. Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium 3'-sulfonate), NaBH_4 , and MOPS were purchased from Sigma Chemical Co. (St. Louis, MO). [^3H]NaBH₄ (319 mCi/mmol) and [^{35}S] H_2SO_4 were purchased from New England Nuclear (Boston, MA); [^{36}Cl]HCl was from ICN (Irvine, CA). H₂DIDS was prepared as described previously (Jennings et al., 1984). All other buffers, salts, and reagents were from Fisher Scientific Co. (Pittsburgh, PA).

Modification of Red Cells

Cells were washed three times in 150 mM KCl, 10 mM MOPS, pH 7.0, and resuspended at 10% hematocrit in the same medium. The suspension was chilled on ice, and once the temperature was <2°C, solid Woodward's reagent K was added to a final concentration of 2 mM unless otherwise specified. After 10 min on ice, NaBH_4 was added to a final concentration of 2 mM from a 1-M stock solution that had been freshly prepared. After another 5 min on ice, a second addition of NaBH_4 was made. To keep the extracellular pH from rising above 7 during the BH_4 reduction, 10 mM MOPS acid was added between the two BH_4 additions. 5

min after the second BH_4 addition, the cells were washed twice in 20–40 vol of 150 mM KCl, 10 mM MOPS, pH 7.

Monovalent Anion Exchange

Control or modified cells were loaded with ^{36}Cl by incubating them for 5 min at 22°C, 50% hematocrit, in 150 mM KCl, 10 mM MOPS, pH 7, plus 1 μCi $^{36}\text{Cl}/\text{ml}$. The suspension was then chilled, centrifuged, and the rate of efflux of tracer into 150 mM KBr, 10 mM MOPS, pH 7, at 0°C was measured as described previously (Jennings et al., 1985).

Net Exchange of Chloride for Sulfate

Net sulfate influx into chloride-loaded cells was measured as described previously (Jennings, 1980), except that the medium was 100 mM K_2SO_4 , buffered with 10 mM MOPS (pH 6.5–7) or 20 mM Tris (pH 7.5–8). The tracer measurements were carried out in an air atmosphere; we had previously shown (Jennings, M. L., unpublished) that the initial sulfate influx is not affected by the presence of atmospheric CO_2 , as long as the extracellular pH is buffered sufficiently. (The chloride efflux under the same conditions is, of course, accelerated by atmospheric CO_2 , but our measurements were of sulfate influx.) For estimates of the proton influx associated with the chloride-sulfate exchange, atmospheric CO_2 and HCO_3^- were removed by bubbling the flux medium with nitrogen for 30 min. The packed cells were depleted of CO_2 immediately before the influx measurement by blowing humidified nitrogen over the cells until the hemoglobin was visibly deoxygenated.

Tracer Sulfate Efflux

After the Woodward's reagent K/ BH_4 treatment (in 150 mM KCl, 10 mM MOPS, pH 7, as above), cells were washed three times in 20 vol of 100 mM K_2SO_4 , 10 mM MOPS, pH 7. Before each centrifugation, the suspension was incubated for 10 min at 37°C to allow the chloride to leave the cells and the sulfate to enter. For experiments in which intracellular pH was varied, cells were suspended in 20 vol of unbuffered 110 mM K_2SO_4 , and the suspension was titrated with NaOH or H_2SO_4 to the desired equilibrium extracellular pH. Because of the slower rate of pH equilibration in the sulfate medium than in a chloride medium, the titrations required ~30 min. The cells were then centrifuged and resuspended in 2 vol of unbuffered 110 mM K_2SO_4 plus 10 μCi $[^{35}\text{S}]\text{O}_4/\text{ml}$. The suspension was incubated for 30–90 min at 37°C for tracer loading. After the tracer loading, the extracellular pH of the 30% suspension was measured. The intracellular pH was calculated from measured extracellular pH of the unbuffered loading medium, using the relationship $\text{pH}_i = \text{pH}_e + 0.5 \log([\text{SO}_4]_i/[\text{SO}_4]_e)$. The efflux of tracer was measured in 100 mM K_2SO_4 , 10 mM MOPS, pH 7, 20°C, after two washes at 0–2°C in unbuffered 100 mM K_2SO_4 to remove extracellular tracer. The flux medium had been bubbled with nitrogen for at least 20 min, and the experiment was conducted under nitrogen. The unidirectional efflux (micromoles per milliliter cells per minute) was calculated from the initial rate (per minute) of tracer efflux times the sulfate content of the cells.

Permeation of the Reagent

Two different methods were used to estimate the rate of penetration of reagent. The first was a direct permeation measurement of the hydrolyzed reagent (see legend to Fig. 8). The second was an estimate of the amount of hemoglobin labeled by incubating red cells with Woodward's reagent K and $[^3\text{H}]\text{BH}_4$ under the same conditions used in the functional studies. After the treatment, cells were washed as for the flux measurements and then lysed in 40 vol of ice-cold 5 mM NaHCO_3 . The membranes were pelleted at 35,000 g for 15 min and the

supernatant was dialyzed against distilled water in an Amicon (Lexington, MA) ultrafiltration cell with a YM10 membrane. To estimate the extent of labeling of hemoglobin if the membrane were not present, a 10% suspension of cells in KCl/MOPS was lysed by freezing and thawing, and the membranes were pelleted. The supernatant was then labeled and subsequently dialyzed exactly as above.

RESULTS

Conversion of Carboxyl Groups to Alcohols

The reactions of Woodward's reagent K with carboxyl groups are summarized in Fig. 1. The first identifiable adduct that forms is the active ester (Dunn et al., 1974). At alkaline pH or elevated temperatures, the active ester can rearrange to the *N*-acyl derivative (Woodward and Olofson, 1966). We previously showed that, if borohy-

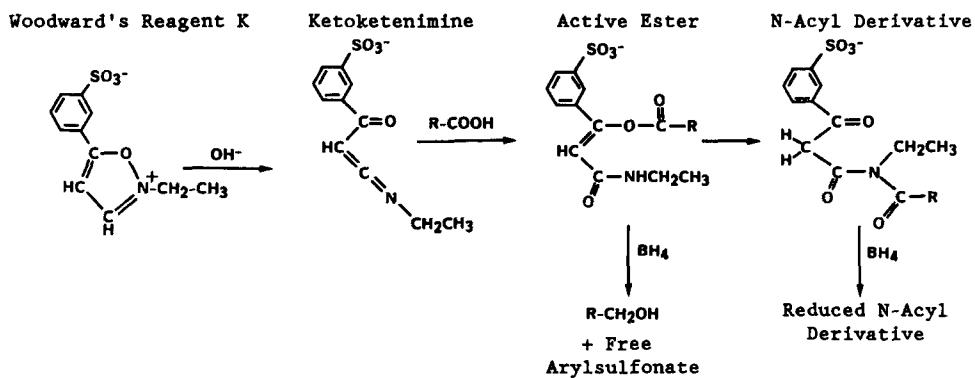


FIGURE 1. Reactions of Woodward's reagent K with carboxyl groups. Following the base-catalyzed ring-opening of the original isoxazolium compound (see Dunn et al., 1974), the ketoketenimine can react with R-COOH to form the active enol ester. BH₄ addition converts the ester to the primary alcohol (Jennings and Anderson, 1987) plus free arylsulfonate. If the *O*- to *N*-acyl shift (Woodward and Olofson, 1966) takes place before BH₄ addition, the BH₄ reduces the *N*-acyl derivative, leaving arylsulfonic acid attached to the protein (Jennings and Anderson, 1987). We have not determined the structure of the reduced arylsulfonate, but presumably at least one of the carbonyls is reduced by BH₄.

dride is added before the *O*- to *N*-acyl shift takes place, the ester is reductively cleaved, leaving the aldehyde, which is then reduced to the primary alcohol (Jennings and Anderson, 1987). If borohydride is added after the *N*-acyl shift has taken place, the borohydride reduces the adduct, but the arylsulfonate remains covalently attached to the protein (Jennings and Anderson, 1987).

In our earlier study of the effect of Woodward's reagent K on band 3 function, the transport measurements were carried out with the arylsulfonate still covalently bound to the protein (Jennings and Anderson, 1987). The goal of the present study was to examine the functional consequences of converting the reactive carboxyl groups to alcohols. We therefore tried to minimize the formation of the *N*-acyl derivative by performing the Woodward's reagent K modification at 0°C and adding BH₄ directly to the suspension of cells without washing off the unreacted Wood-

ward's reagent K. We previously showed (Jennings and Anderson, 1987), using liquid chromatography, that in band 3 from cells labeled with Woodward's reagent K and [^3H]BH₄ at neutral pH, most of the radioactivity is recovered as hydroxynorvaline and proline, both of which are derived from glutamate. (The proline is formed from chloronorvaline, which is produced during acid hydrolysis of hydroxynorvaline [Thomas et al., 1983].) There is no detectable labeled homoserine in the modified band 3, which indicates that the main modified residues are glutamates, not aspartates.

Effects on Monovalent Anion Exchange

Fig. 2 shows that treatment of cells with Woodward's reagent K/BH₄ at 0–3°C causes irreversible inhibition of monovalent anion exchange, measured as ^{36}Cl

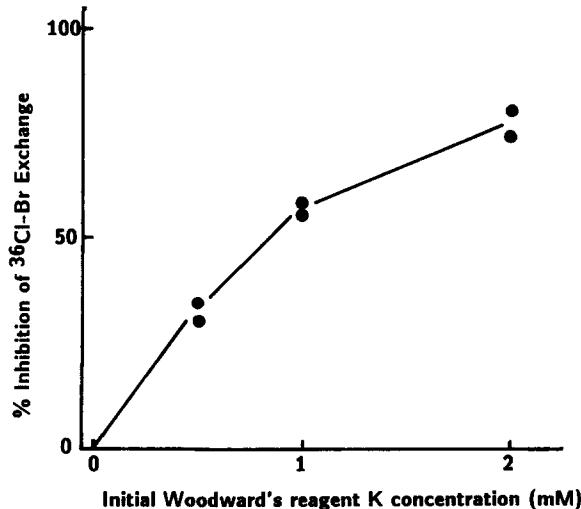


FIGURE 2. Irreversible inhibition of monovalent anion exchange by Woodward's reagent K/BH₄. Cells were treated with the indicated initial concentration of Woodward's reagent K on ice for 10 min, reduced with two additions of 2 mM BH₄ 5 min apart, and then washed and loaded with ^{36}Cl in 150 mM KCl, 10 mM MOPS, pH 7.0. Efflux of ^{36}Cl was measured at 0°C in 150 mM KBr, 10 mM MOPS, pH 7.0. The data shown are from a single experiment with duplicate fluxes (four time points) at each concentration. In two further experiments (not shown), 2 mM Woodward's reagent K/BH₄ gave 76 and 78% inhibition of Cl-Br exchange.

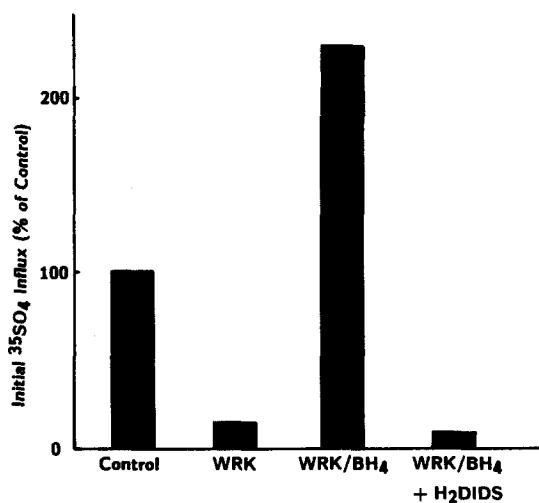
efflux into Br media. Treatment with 2 mM Woodward's reagent K/BH₄ caused 75% irreversible inhibition of chloride-bromide exchange. The 75% inhibition does not represent the maximum that can be produced by the modification; a second treatment with 2 mM Woodward's reagent K/BH₄ increased the inhibition to 87% (one experiment with duplicate fluxes). In all the following studies of sulfate transport, the cells were treated once with 2 mM Woodward's reagent K/BH₄. We assume that this treatment modifies ~75% of the copies of the protein and leaves 25% unmodified (see Discussion).

Sulfate Influx into Chloride-loaded Cells

Woodward's reagent K alone, without BH₄ reduction, inhibits net sulfate influx into chloride-loaded cells. However, reductive cleavage of the Woodward's reagent K-

glutamate adduct (with concomitant conversion of the carboxyl to the alcohol) reverses the inhibition and actually stimulates the influx (Fig. 3). The stimulated transport is inhibited completely by the stilbenedisulfonate derivative H₂DIDS (4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate). In unmodified cells, the net sulfate influx into chloride-loaded cells is very strongly stimulated by lowering the extracellular pH (Milanick and Gunn, 1984). Pretreatment with Woodward's reagent K/BH₄ nearly eliminates this pH dependence (Fig. 4). That is, the transport protein in the modified cells behaves qualitatively as if the proton required for sulfate influx is bound even at pH > 7.

FIGURE 3. Stimulation by Woodward's reagent K/BH₄ of sulfate influx into chloride-containing cells. Control cells received neither Woodward's reagent K nor BH₄; separate controls showed that BH₄ alone has no effect. Cells treated with 2 mM Woodward's reagent K and BH₄ (WRK/BH₄) were processed exactly as described for Fig. 2. Cells treated with Woodward's reagent K alone (WRK) were incubated for 10 min on ice with 2 mM reagent, followed by 30 mM glycine to stop the reaction. The cells were then washed twice in 40 vol of 150 mM KCl/10 mM MOPS, pH 7, and the influx of [³⁵S]SO₄ was measured from a chloride-free MOPS-buffered 100 mM K₂SO₄ medium, pH 7.0, 20°C. When present, H₂DIDS (far right) was added directly to the flux medium at a concentration of 12 μ M. The data shown are average of duplicate flux determinations in a single experiment, representative of two others.



Proton-Sulfate Cotransport in the Modified Cells

Net sulfate influx into chloride-containing red cells at pH below 7 is accompanied by a stoichiometric influx of protons (Jennings, 1976), as originally predicted in the titratable carrier model (Gunn, 1972). The catalytic cycle for net chloride-sulfate exchange is shown in Fig. 5. If the titratable carboxyl group were converted to an alcohol, the transporter would be locked (at neutral pH) into the charge state that is appropriate for sulfate influx. The conversion of this carboxyl to an alcohol would be expected to cause the observed stimulation of sulfate influx at neutral pH. Fig. 6 shows that the proton influx associated with the chloride-sulfate exchange is 75% inhibited by the standard 2 mM Woodward's reagent K/BH₄ treatment. The incomplete inhibition is expected, because, under the conditions used, only ~75% of the copies of band 3 are modified (as indicated by the incomplete inhibition of chloride-bromide exchange; Fig. 2). Therefore, in the same cells that exhibit accelerated (H₂DIDS inhibitable) sulfate influx into chloride-loaded cells, there is negligible

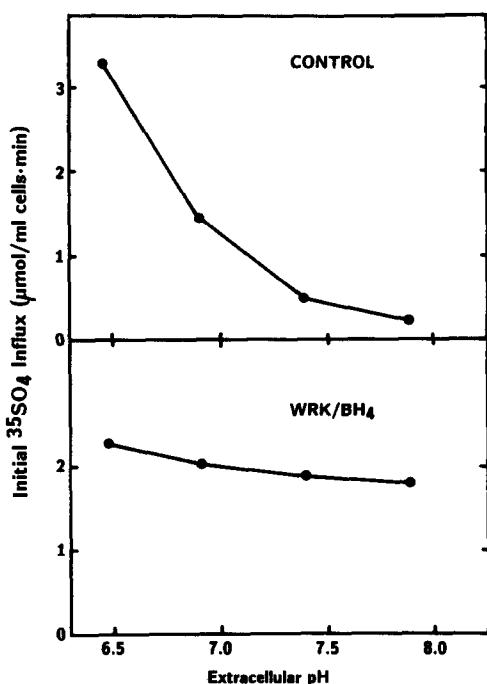


FIGURE 4. Extracellular pH dependence of sulfate influx into chloride-loaded cells. (Top) Control cells were washed in KCl/MOPS, and the initial influx of [^{35}S]SO₄ was measured in a chloride-free 100 mM K₂SO₄ medium buffered with 10 mM MOPS, pH 6.5–7.0, or 20 mM Tris base titrated to the desired pH (7.5–8) with MOPS acid. Temperature, 20°C. (Bottom) Cells were treated with 2 mM Woodward's reagent K/BH₄ as described for Figs. 2 and 3. After two washes in KCl/MOPS, the sulfate influx was measured as for the controls. Each point represents the average of four to six flux measurements. The standard deviations are <10%, except for the control experiments at pH 6.5, in which the SD is 18%. Under the conditions used for the modification of the cells, roughly 25% of the copies of band 3 are unmodified. To illustrate the pH dependence of the flux in the modified protein, without interference from the remaining copies of native protein, 25% of the control flux at each pH has been subtracted.

proton-sulfate cotransport, other than the residual 25% arising from the unmodified band 3. This is consistent with the idea that the carboxyl group that is normally associated with proton-sulfate cotransport has been converted to the untitratable, neutral alcohol.

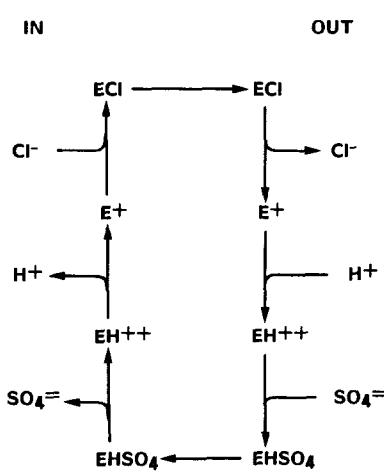


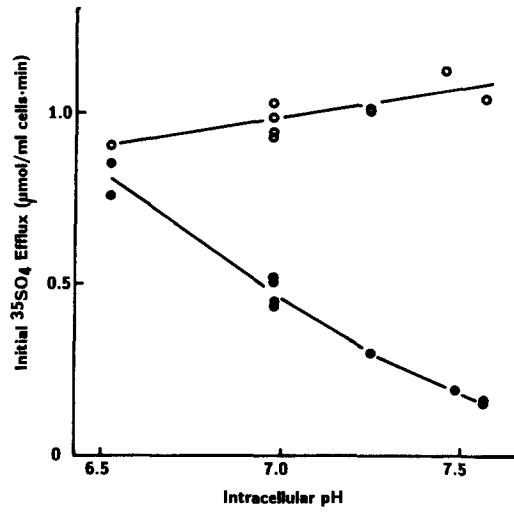
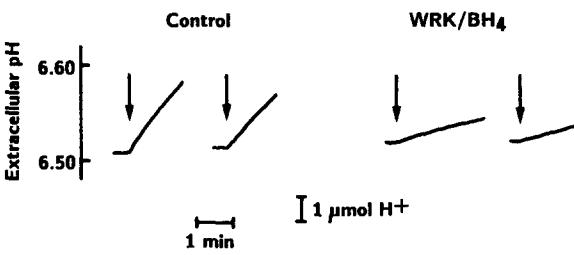
FIGURE 5. Catalytic cycle for the net exchange of chloride for sulfate catalyzed by band 3. E⁺ represents the form of the protein that is normally present at pH 7.4. Lowering the pH causes the formation of EH⁺⁺, which is the form that transports sulfate. The addition of proton and sulfate to E⁺ are in random order (Milanick and Gunn, 1982). After inward translocation, proton and sulfate are released into the intracellular medium and the cycle is completed. The net result of one catalytic cycle is the efflux of one chloride ion and the influx of one sulfate ion and one proton.

FIGURE 6. Inhibition by Woodward's reagent K/BH₄ of the net proton influx associated with chloride-sulfate exchange. Cells were treated with 2 mM Woodward's reagent K/BH₄ exactly as in Figs. 2–4 and then washed in 150 mM KCl, 10 mM MOPS, pH 6.5 (the lower pH was used to facilitate CO₂ removal). Cells were then suspended at 5% hematocrit in a CO₂-free 100 mM K₂SO₄ medium buffered with 10 mM MOPS at pH 6.5. The extracellular pH was monitored continuously with the glass electrode. (*Left*) Two experiments with control cells. The proton influx is 2.8 $\mu\text{mol}/\text{ml cells} \cdot \text{min}$, which is 85% of the net sulfate influx measured at the same temperature and pH. (*Right*) Two experiments with cells that had been treated with Woodward's reagent K/BH₄. The stilbenedisulfonate-sensitive sulfate influx in these cells is the same as in control cells, but the proton influx is only 0.74 $\mu\text{mol}/\text{ml cells min}$ (26% of control). In a separate set of experiments with a different cell preparation, the proton flux in treated cells (three flux measurements) was 31% of the control flux.

Sulfate-Sulfate Exchange in the Modified Cells

To determine whether the Woodward's reagent K/BH₄ modification alters the intracellular pH dependence of sulfate transport, it is preferable to use sulfate-sulfate exchange rather than chloride-sulfate exchange. (Initial sulfate efflux into a

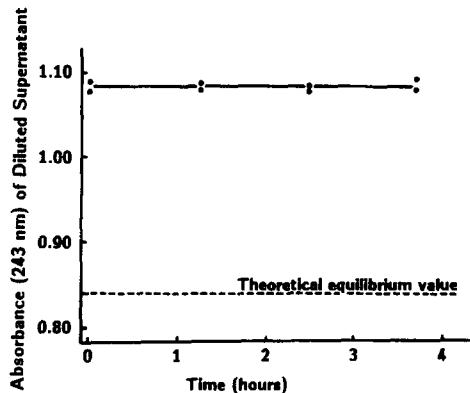
FIGURE 7. Removal by Woodward's reagent K/BH₄ of the intracellular pH dependence of sulfate-sulfate exchange at constant extracellular pH. Cells were treated with (open symbols) or without (filled symbols) 2 mM Woodward's reagent K/BH₄ in KCl/MOPS, pH 7, as in the previous figures and then washed and titrated to an extracellular pH of between 6.3 and 8.0 in an unbuffered 110 mM K₂SO₄ medium. After loading with [³⁵S]SO₄ in the same unbuffered medium, the initial efflux of tracer was measured under nitrogen in 100 mM K₂SO₄, 10 mM MOPS, pH 7.0 (see Materials and Methods). The data represent four different preparations of cells, each of which was split into control and treated aliquots. Two flux measurements were performed at each pH; single data points indicate that the two flux determinations had identical results. The flux unit is defined to correct for the small change in cell volume with pH. Thus, 1 ml of cells is defined as the number of cells ($\sim 1.1 \times 10^{10}$) that have a total volume of 1 ml when they are at Donnan equilibrium with 100 mM K₂SO₄, 10 mM MOPS, pH 7.



chloride medium is complicated by the changing intracellular chloride concentration, which progressively inhibits sulfate efflux. The changing anion concentration is not a problem for sulfate influx into chloride-containing cells, because the catalytic cycle is limited by sulfate influx, and the sulfate entering the cells does not significantly impede further influx for the first 25% of the exchange.)

Fig. 7 illustrates the effect of intracellular pH on the initial tracer sulfate efflux into a medium consisting of 100 mM K_2SO_4 , 10 mM MOPS, pH 7.0. The cells and medium were not at Donnan equilibrium with respect to pH, but neither the intracellular nor the extracellular pH changed by more than 0.1 unit during the efflux measurements (atmospheric CO_2 had been removed by N_2 bubbling). In control cells, lowering the intracellular pH from 7.5 to 6.5 stimulates the tracer efflux by a factor of 5; that is, protonation of an intracellular site, at constant extracellular pH, stimulates sulfate efflux. Treatment of the cells with Woodward's reagent K/BH_4

FIGURE 8. Lack of entry of inactivated Woodward's reagent K into red cells at 0°C. Cells were washed and resuspended gently at 0–2°C to a hematocrit of 28% in 150 mM KCl, 10 mM MOPS, pH 7, which contained 2 mM Woodward's reagent K that had been allowed to hydrolyze (Dunn et al., 1974) at room temperature before chilling. Duplicate samples of suspension were removed at various times, centrifuged, and the absorbance of the supernatant (243 nm) was recorded after diluting 20-fold in KCl/MOPS. A parallel suspension lacked the reagent; the absorbance of the diluted supernatant was constant and was <5% that of the diluted supernatant containing reagent. The absorbance should drop to 0.84 if the reagent were to reach equilibrium with the intracellular water. Two other experiments, with the same result, were carried out over shorter time periods (40 min).



completely changes this intracellular pH dependence. The tracer efflux is nearly independent of intracellular pH over the range examined. The Woodward's reagent K/BH_4 pretreatment causes a sevenfold acceleration of the flux as measured at intracellular pH 7.5; the accelerated flux is >90% inhibited by 12 μM H₂DIDS in the flux medium (data not shown). The large effect of Woodward's reagent K/BH_4 on the intracellular pH dependence of sulfate transport suggests that there is a carboxyl group in the protein that can cross the permeability barrier (see Discussion).

Lack of Permeation by the Reagent at 0°C

In order to interpret the effect of extracellular Woodward's reagent K and BH_4 on the intracellular pH dependence of sulfate transport, it is necessary to investigate as rigorously as possible the permeability properties of the reagent. The ketoketenim-

ine itself is unstable and is hydrolyzed to a compound of the same charge and very similar size (Dunn et al., 1974). We performed some very simple influx measurements with the hydrolyzed compound at pH 7 and 0°C, using the ultraviolet absorbance of the compound as the assay for its disappearance from the extracellular medium. Over times as long as 4 h, there is no detectable loss of the reagent into the cells (Fig. 8). The minimum half-time for penetration of the compound is well over 10 h. We did one similar experiment at 22°C and found that there is detectable uptake, with a half-time of ~25 min. The finite penetration of the compound at higher temperature is of no consequence for the present purposes; all the modifications in this article were performed for 10 min at temperatures below 3°C.

As an independent estimate of the permeation of the reagent, cells were labeled with [³H]BH₄ after a 10-min exposure to Woodward's reagent K. As shown previously, there is no detectable spectrin labeling (Jennings and Anderson, 1987); this lack of labeling of the most massive intracellular membrane protein is evidence for confinement of the label to extracellular sites. We also measured the extent of labeling of hemoglobin in cells so labeled, and compared the labeling with that of hemoglobin labeled in a lysate (same reagent concentrations, same total amount of hemoglobin, same temperature, time, and pH). The labeling of hemoglobin in intact cells is <1% of the labeling of hemoglobin in a lysate.

DISCUSSION

The present results provide the first evidence that there is an amino acid residue in the red cell anion-exchange protein that can sometimes be in contact with extracellular water and at other times be in contact with intracellular water. The reasoning is as follows: Woodward's reagent K, an arylsulfonic acid, cannot cross the permeability barrier during the 10-min exposure at 0°C. The reagent, with BH₄ reduction, converts certain exofacial carboxyl groups to primary alcohols. The modification stimulates sulfate influx into chloride-loaded cells, but it inhibits the proton flux that normally takes place during chloride-sulfate exchange. That is, the modified protein transports sulfate as if the normally titratable carboxyl group were irreversibly protonated, which is exactly what would be expected if it had been converted into an alcohol. The same treatment modifies the intracellular pH dependence of sulfate efflux (at fixed extracellular pH). This indicates that the intracellular titratable group can sometimes be extracellular in order to have been modified by the extracellular nonpenetrating reagent. To our knowledge, this is the first example of the chemical modification of an amino acid residue that can cross the permeability barrier in band 3 or any other transport protein.

It is important to clarify what we mean by permeability barrier and the pH on each side of the barrier. In red cells it is possible to maintain rather large transmembrane pH gradients for times sufficiently long to measure unidirectional ion fluxes (e.g., Milanick and Gunn, 1982, 1984). In the experiment in Fig. 7, the extracellular pH is fixed, and the sulfate tracer efflux varies as a function of the intracellular pH. This implies that there must be a titratable group whose state of protonation is influenced much more strongly by the intracellular pH than by the extracellular pH. The actual pH in the immediate vicinity of this titratable group may not be the same as the bulk intracellular pH, because of local surface charge effects. The titratable

group, however, is nonetheless defined as intracellular because its state of protonation is a function of the intracellular pH. The modification of the cells with Woodward's reagent K/BH₄ causes the sulfate efflux to have the characteristics expected if the intracellular titratable site were permanently protonated. We believe that the carboxyl group is converted to the alcohol when the protein is in the outward-facing conformation during the exposure to Woodward's reagent K/BH₄. Once the conversion to the alcohol has taken place, the modified group can cross the permeability barrier (probably in concert with the "out"-to-"in" conformational transition of the protein-sulfate complex). The resulting inward-facing conformation is in the proper charge state to transport sulfate outward even at neutral intracellular pH, because the former carboxyl group is permanently uncharged.

Lack of Permeation of the Reagent

Our finding that the reagent does not enter the cells under the present labeling conditions is consistent with the known permeability properties of the membrane. The zwitterionic reagent (Fig. 1), with formal charges spaced fairly far apart, is very unlikely to enter the cells in the short times and at the low temperatures used here. The ketoketenimine, which has only a negative charge, could conceivably penetrate the membrane, because some arylsulfonic acids are known to cross the red cell membrane at finite rates. For example, benzenesulfonate has a half-time of penetration of 135 min at 34°C in ox red cells (Aubert and Motaïs, 1975). However, the temperature dependence of red cell anion exchange is rather high (see Passow, 1986), and there is no evidence to our knowledge that any arylsulfonic acid can cross the membrane at a measurable rate at 0°C.

Other Evidence for a Thin-Barrier Model of the Transport Pathway

The idea that a single carboxyl group can (at different times) be in contact with either intracellular or extracellular water is evidence that the transport pathway consists largely of a hydrophilic pathway that is interrupted by a thin barrier. It is useful to enumerate other kinds of evidence in favor of the idea that the red cell anion exchanger contains a thin permeability barrier. Rao et al. (1979) used fluorescence resonance energy transfer to estimate the distance between the stilbenedisulfonate site and cytoplasmic sulfhydryl groups; the distance is only ~40 Å, which indicates that the stilbenedisulfonate site lies in a cleft that extends some distance into the membrane. Macara et al. (1983) subsequently showed that eosinmaleimide, bound at the stilbenedisulfonate site, is quenched by cesium present on the cytoplasmic side of the membrane; this finding is consistent with the existence of a thin barrier between the sites reached by the impermeant intracellular cesium and impermeant extracellular eosinmaleimide. Functional studies of band 3-catalyzed anion conductance indicate that the main mode of the conductance is not the conformational (outward- to inward-facing) transition of the unloaded transporter but rather is the "tunneling" of bound anion through the permeability barrier (Fröhlich et al., 1983; Knauf et al., 1983; Fröhlich, 1984). Falke and Chan (1986) found that some inhibitors of band 3-mediated anion exchange act by slowing the exchange between the transport site and the bulk medium; the most likely explanation of this effect is that

the agents block an access channel. Finally, Milanick and Gunn (1986) measured the inhibitory effect of internal and external hydrogen ion on chloride-chloride exchange; the data can be explained by a model in which a single titratable carboxyl group alternately has access to the intracellular and extracellular solutions. Our chemical modification experiments are in complete agreement with this idea.

*The Transmembrane Effect of Woodward's Reagent K Is Not
the Result of "Recruitment"*

Passow and Zaki (1978) and Grinstein et al. (1979) showed several years ago that nonpenetrating ligands can affect the reactivity of amino acid residues on the opposite side of the membrane. These findings were interpreted (we believe correctly) in terms of the recruitment, by mass action, of transport proteins into predominantly outward-facing or inward-facing forms (see Knauf et al., 1984; Knauf and Mann, 1984). In any functional study of an obligatory exchanger that is believed to obey ping-pong kinetics (Gunn and Fröhlich, 1979), it is important to consider possible effects of recruitment. However, Woodward's reagent K/BH₄ does not appear to have a major effect on the inward- vs. outward-facing distribution of transporters. This was demonstrated by measuring the *trans* acceleration by intracellular chloride of the tracer sulfate influx. The acceleration (at pH 7) is a factor of 2.5 in both control and Woodward's reagent K/BH₄-treated cells. We interpret this acceleration as evidence that 40% (i.e., 1/2.5) of the transporters face outward at Donnan equilibrium in the sulfate medium at pH 7 (see Jennings, 1980). The lack of major effect of Woodward's reagent K/BH₄ on the distribution of transporters indicates that the alteration of the intracellular pH dependence of sulfate efflux cannot be explained by recruitment of transporters.

There are, of course, other possible explanations of the modification of the intracellular pH dependence of sulfate transport. The conversion of an external carboxyl group to an alcohol may have an allosteric effect (see Passow, 1986) on the pK_a of an intracellular titratable group. However, it is very hard to see how removal of an external negative charge can cause an internal carboxyl group to behave as if it were protonated at pH 7.4. Conversion of the extracellular carboxyl to an alcohol would, if anything, be expected to make the protein less negatively charged and hence promote deprotonation of the internal carboxyl.

The same argument applies if the internal titratable group were not a carboxyl. For example, the intracellular pH dependence of sulfate efflux could be controlled by a histidine residue (see below) that lies just inside the permeability barrier. The reactive carboxyl group could lie just outside the barrier, close enough to the intracellular histidine to affect the pK_a of the histidine side chain. Removal of the charge on the carboxyl group should make it more difficult to protonate the histidine and hence shift the intracellular pH dependence of the sulfate efflux to the left, the opposite of the observed result. The above arguments, of course, represent oversimplifications because only simple electrostatics are considered. However, it is difficult to imagine a mechanism by which removal of a single extracellular negative charge could drastically raise the pK_a of an intracellular titratable group. We believe that it is more likely that the extracellular titratable group has periodic access to the intracellular medium.

Comparison with Other Chemical Modification Studies of Band 3

As we have discussed previously (Jennings and Anderson, 1987), it is difficult to compare the studies with Woodward's reagent K with those in other laboratories using carbodiimides (Bjerrum, 1983; Craik and Reithmeier, 1985), because the reagents are oppositely charged and very possibly react with different carboxyl groups. Other than the carbodiimide studies, there are two sets of chemical modification experiments that should be mentioned in the context of the present experiments. First, Passow and co-workers (Lepke and Passow, 1982; Raida and Passow, 1985) have shown that dansylation of red cells causes inverse effects on sulfate and chloride transport that are qualitatively similar to those that we find for Woodward's reagent K/BH₄. The similarity in the effects is intriguing, but, at present, there is no reason to suspect that the same amino acid residues are involved. The conditions that we have used for the modification/labeling experiments cause reasonably selective conversion of carboxyl groups to alcohols, as judged by liquid chromatography of acid hydrolysates of the labeled protein (Jennings and Anderson, 1987). Dansylation can potentially affect several different amino acid residues, and it is not yet clear which residues are responsible for the accelerating effects on sulfate transport (see Passow, 1986). We do not know, therefore, the relationship between the reactive glutamate residue in our experiments and the residue that, when dansylated, causes acceleration of sulfate flux.

There is evidence from the pH dependence of phosphate transport, as well as inhibition of transport with diethylpyrocarbonate, that a histidine residue is of functional importance in band 3 (Chiba et al., 1986; Matsuyama et al., 1986). In cells modified with Woodward's reagent K/BH₄, neither intracellular nor extracellular pH strongly affects sulfate transport in the range 6.5–7.5. If the titration of a histidine residue affects sulfate transport in the modified cells, then the pK_a of the effect must be 6 or below. Our studies do not rule out the presence of a histidine residue associated with the transport pathway, but they do indicate that a carboxyl group is the main determinant of both the intracellular and extracellular pH dependence of sulfate transport in the neutral to slightly acid pH range.

How Many Carboxyl Groups Are Modified?

We previously showed that Woodward's reagent K/[³H]BH₄ labels at least two glutamate residues on band 3 (Jennings and Anderson, 1987). We do not know which of these residues is responsible for the functional effects demonstrated in this article. The labeling of the 35-kD chymotryptic fragment is much more extensive than that of the 60-kD fragment under the conditions used here (data not shown). We have shown elsewhere (Jennings et al., 1988) that the titratable group involved in proton-sulfate cotransport is not removed by papain treatment of intact cells; the most likely location for this residue is the 28-kD COOH-terminal papain fragment. However, we are still far from being able to specify which glutamate residue is associated with proton-sulfate cotransport. We also do not know whether or not all the functional effects of the modification, including the acceleration of Cl conductance (Jennings et al., 1988) are attributable to the modification of a single residue.

Without knowing exactly which residues are modified, it would still be useful to know the stoichiometry of the modification under the standard conditions used in

this article. However, calculation of the stoichiometry of labeling with Woodward's reagent K/[³H]BH₄ is difficult, because we do not know the magnitude of the kinetic isotope effect in the BH₄ reduction. Although much remains unknown about the molecular details of the modification, we emphasize that the main conclusions of this article do not depend on precise knowledge of the number of carboxyl groups modified. Even if more than one carboxyl group is modified, we can still conclude that one of the modified residues is responsible for the intracellular pH dependence of transport. The intracellular residue must have periodic access to the extracellular medium in order to have been modified by the nonpenetrating reagent.

Stoichiometry of Chloride-Sulfate Exchange in the Modified Band 3

In native band 3, the catalytic cycle for chloride-sulfate exchange (Fig. 5) involves one chloride, one proton, and one sulfate. In the modified band 3, sulfate can still be transported inward, but there is no proton cotransport. The catalytic cycle for this transport could be two chloride ions exchanging for one sulfate, or it could be an electrogenic one-for-one exchange in parallel with a conductive chloride efflux. We have attempted to distinguish between these possibilities by measuring the electrical properties of the transport in the modified cells, but thus far the data are inconclusive.

Effect of Uncleaved Arylsulfonate

The sulfate influx into modified cells at pH 7.5 is ~4.5 times larger than the flux into control cells, but it is still much smaller than would be expected if the titratable carboxyl group on 75% of the copies of band 3 were protonated (Milanick and Gunn, 1984). One reason that the flux at neutral pH in the modified cells is not higher is that the BH₄ cleavage of bound arylsulfonic acid is incomplete. As shown in Fig. 1, BH₄ cleaves the arylsulfonate to produce the alcohol only if the active ester has not yet rearranged to the *N*-acyl derivative. Unfortunately, some formation of the *N*-acyl derivative is unavoidable, even at 0°C.

To try to estimate the amount of uncleaved reagent on band 3 after the usual BH₄ reduction, cells were treated with Woodward's reagent K/BH₄, washed, and then reduced further with [³H]BH₄. Results of preliminary experiments of this kind suggest that as many as half the copies of band 3 have uncleaved arylsulfonate bound under our standard conditions of Woodward's reagent K/BH₄ treatment. The presence of the bound reagent on some of the copies of band 3 does not affect any of the conclusions of this article, because those copies of the protein are functionally silent. That is, the bound arylsulfonate causes essentially complete inhibition of sulfate transport, as would be expected from the effects of other negatively charged agents of similar size (Cabantchik et al., 1975; Knauf et al., 1978). If not for the presence of the nonfunctioning copies of the protein, the acceleration of sulfate transport by Woodward's reagent K/BH₄ would be even larger than we have observed.

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