Articles

Cytoplasmic Disposition of Aspartate 821 in Anion Exchanger from Human Erythrocytes†

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ABSTRACT: The location with respect to the plasma membrane of aspartate 821 in erythrocytic anion exchanger has been determined by labeling inside-out vesicles and intact erythrocytes with impermeant reagents and following the outcome by site-directed immunochemistry. Intact erythrocytes and inside-out vesicles in the same container were vectorially modified with 1-ethyl-3-[3-(trimethylammonio)propyl]-carbodiimide and [^{35}S]sulfanilic acid. The inside-out vesicles were separated from the erythrocytes by differential centrifugation, and both the vesicles and membranes made from the erythrocytes were stripped with alkali and digested with trypsin to liberate from each sample the peptide YHPDVPYVK containing aspartate 821. The tryptic digests were passed over an immunoadsorbent specific for peptides with the amino-and carboxy-terminal sequences YHPD− and −PYVK. Specifically bound peptides were eluted with acid, and the eluates were pooled and submitted to high-pressure liquid chromatography. A peak of absorbance at 229 nm corresponding to the peptide YHPDVPYVK was present in chromatograms of samples from both the inside-out vesicles and the intact erythrocytes. Another peak that displayed absorbance at 229 and 250 nm, corresponding to the peptide YHP(*p*-[^{35}S]sulfo-β-aspartanilide)VPYVK, was observed in the chromatogram of the sample from the inside-out vesicles but not in the chromatogram of the sample from the erythrocytes. This peak had associated with it a large number of counts per minute of [^{35}S]sulfur, whereas no counts per minute of [^{35}S]sulfur above background were detected on the chromatogram of the sample from the erythrocytes. The incorporation of [^{35}S]sulfanilic acid into aspartate 821 of anion exchanger in inside-out vesicles was at least 10-fold greater than the incorporation of [^{35}S]sulfanilic acid into aspartate 821 of anion exchanger in erythrocytes when the two preparations were labeled in the same solution. These results demonstrate that aspartate 821, found between two hydrophobic segments in the sequence of anion exchanger, is located on the cytoplasmic surface of this membrane-spanning protein.

Human anion exchanger (band 3) catalyzes the coupled transport of various anions across the plasma membrane of erythrocytes. Primarily, chloride is exchanged for bicarbonate, thus facilitating the removal of carbon dioxide from peripheral tissue (*1*). The polypeptide that folds to produce the protein is 911 amino acids long. The native protein can be fragmented into two separable domains: an amino-terminal, cytoplasmic domain 360−370 amino acids in length that functions to bind ankyrin and other cytosolic and cytoskeletal proteins (*2*) and a highly conserved carboxy-terminal, membrane-spanning domain 550 amino acids in length that has been shown to mediate ion transport by itself (*1*). The complete amino acid sequence for anion exchanger has been determined from the cDNAs of human (*3*), murine (*4*), chicken (*5*), and trout (*6*) erythrocytic anion exchangers.

The membrane-spanning domain of the protein contains 13 hydrophobic segments that can be designated M1−M13, if the M12 segment of Kopito and Lodish (*4*) is split into M12 and M13. To understand the mechanism by which anions are transported across the plasma membrane, the assignment of the topography of the folded polypeptide chain in the native enzyme with respect to the plane of the bilayer will be necessary. A variety of chemical, immunological, and enzymatic techniques have been used to assign the hydrophilic segments connecting the 13 hydrophobic segments to one side of the membrane or the other.

A monoclonal immunoglobulin (BRIC 132), thought to be specific for the amino acid sequence −FKPPKYHPD-VPY− between phenylalanine 813 and tyrosine 824, was bound by the plasma membranes only after erythrocytes had been lysed (*7*, *8*). This observation led to the conclusion that this amino acid sequence is on the cytoplasmic surface of the protein. Unfortunately, the apparent binding of the immunoglobulin was significantly weaker than that of other monoclonal immunoglobulins used in the same experiment; therefore, it is not clear whether the signal arose only because access was afforded to the cytoplasmic face of anion exchanger or for some other reason. It is possible that the signal was from the binding of the immunoglobulin to a small population of anion exchanger that had denatured during lysis or from nonspecific binding to other proteins on the membranes. Such ambiguity has plagued other experiments

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in which peptide-specific monoclonal immunoglobulins have been used to examine the topography of membrane-spanning proteins in their native state, and a number of these observations have led to incorrect conclusions (*9*). In fact, a different set of immunoglobulins that recognize an extracytoplasmic antigenic site on senescent erythrocytes is supposed to associate with an epitope within the amino acid sequence −LFKPPKYHPDVPYVKR− between leucine 812 and arginine 827 of human anion exchanger (*10*). Therefore, there are two contradictory conclusions, both based on binding of immunoglobulins to erythrocytic plasma membranes, that have been drawn about the location of this region of anion exchanger. In the present study, the labeling of intact erythrocytes and inside-out vesicles with an impermeant reagent followed by site-directed immunochemistry was used to determine the disposition of aspartate 821 in the region of the folded polypeptide found between hydrophobic segments M11 and M12 of human anion exchanger.

EXPERIMENTAL PROCEDURES

Materials. Human blood that had recently expired was obtained from the San Diego Blood Bank. The N^{α}-fluorenylmethyloxycarbonyl (N^{α}-FMOC)[1] amino acids, N^{α}-*tert*-butoxycarbonyl (*t*-BOC) amino acids, 1-hydroxybenzotriazole hydrate, and *p*-alkoxybenzyl solid phase were purchased from either Bachem or Calbiochem- Novabiochem. [^{35}S]Sulfuric acid (10 mCi mL^{-1}) was purchased from NEN Research Products. Bovine serum albumin (BSA), carboxypeptidase Y, leucine aminopeptidase, proteinase XIV (pronase), trypsin [treated with *N*-(*p*-tolylsulfonyl)-L-phenylalanyl chloromethyl ketone), Sephadex G-25, iodoacetic acid, acetylthiocholine chloride, and soybean trypsin inhibitor were purchased from Sigma Chemical Co. Iodoacetic acid was recrystallized from petroleum ether. Prolidase was purchased from U.S. Biochemical Corp. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (ED carbodiimide), 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide iodide (ET carbodiimide), 1,3-diisopropylcarbodiimide, *N*-methylpyrrolidinone, sulfanilic acid, ethanedithiol, hydrindantin, and piperidine were purchased from Aldrich Chemical Co. Trifluoroacetic acid (TFA) was purchased from Halocarbon Products. Ninhydrin and *N*-[(*m*-maleimidobenzoyl)hydroxy]sulfosuccinimide (sulfo-MBS) were both purchased from Pierce Chemical Co. Biosafe II scintillation cocktail was purchased from Research Products International. Affi-Gel 10 and Affi-Gel 102 were purchased from Bio-Rad Laboratories. Freund's adjuvant was purchased from Difco Laboratories.

Synthesis of Peptides. The peptides YHPDC and PPKYHPDVPYVKRV were synthesized by solid-phase fluorenylmethyloxycarbonyl methods (*11*). N^{α}-(Fluorenylmethyloxycarbonyl)-*S*-(triphenylmethyl)-L-cysteine or N^{α}-FMOC-L-valine was attached to *p*-alkoxybenzyl solid phase with diisopropylcarbodiimide. The preformed hydroxybenzotriazole esters of each of the following protected amino acids were then added sequentially to complete each synthesis: N^{α}-FMOC-N^{δ}-[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]-L-arginine, N^{α}-FMOC-N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine, N^{α}-FMOC-*S*-(triphenylmethyl)-L-cysteine, N^{α}-FMOC-L-aspartic acid O^{β}-butyl ester, N^{α}-FMOC-L-proline, N^{α}-FMOC-N^{im}-(triphenylmethyl)-L-histidine, N^{α}-FMOC-*O*-butyl-L-tyrosine. The products, YHPDC and PPKYHPDVPYVK, were cleaved from the solid phase, and all protecting groups were removed by treatment with 90:5:5 trifluoroacetic acid/ethanedithiol/thioanisole for 4 h (YHPDC) or 86:5:4:3:2 trifluoroacetic acid/phenol/water/ethanedithiol/thioanisole for 9 h (PPKYHPKVPYVKRV). Trifluoracetic acid was removed under reduced pressure, and the remaining residue was dissolved in 10% acetic acid and extracted with diethyl ether. The aqueous phase was lyophilized, and the crude peptides were purified by reverse-phase high-pressure liquid chromatography (HPLC). The peptide YHPDCPYVK was synthesized by solid-phase *tert*-butyloxycarbonyl methods. N^{α}-(*tert*-Butyloxycarbonyl)-N^{ϵ}-(benzoxycarbonyl)-L-lysine was coupled to chloromethylpolystyrene−divinylbenzene solid phase, and the *t*-BOC-protected amino acids N^{α}-*t*-BOC-L-valine, N^{α}-*t*-BOC-*O*-benzyl-L-tyrosine, N^{α}-*t*-BOC-L-proline, N^{α}-*t*-BOC-L-cysteine, N^{α}-*t*-BOC-O^{β}-benzyl-L-aspartate, and N^{α}-*t*-BOC-N^{im}-dinitrophenyl-L-histidine were then sequentially coupled (*11*). The product, YHPDCPYVK, was cleaved from the solid phase by bubbling anhydrous hydrobromic acid through the suspension in 95:2.5:2.5 TFA/anisole/phenol, and it was purified by HPLC. All three peptides were hydrolyzed, and their amino acid compositions were determined. Total enzymatic digestion followed by amino acid analysis was also used to ensure that the removal of all protecting groups from the peptides was complete. Cysteine content was verified quantitatively by reacting the peptides with iodoacetic acid (*12*, *13*) prior to acid hydrolysis or enzymatic digestion and amino acid analysis. All amino acid compositions were in agreement with the expected values.

Synthesis of [^{35}S]Sulfanilic acid. [^{35}S]Sulfanilic acid was synthesized from 1 μmol of [^{35}S]sulfuric acid (5.0 mCi) and 1 μmol of aniline (*14*). Yields ranged from 200 to 300 nmol. A portion of each sample was submitted to liquid scintillation to determine the specific radioactivity.

Modification of PPKYHPDVPYVKRV with 1-Ethyl-3-[3-(trimethylammonio)propyl]carbodiimide and Sulfanilic Acid. A sample of freshly dissolved ET carbodiimide in ice-cold water was added to a 1 mM solution of pure PPKYHPDVPYVKRV in 0.5 mM sulfanilic acid, 0.2 mM $CaCl_2$, and 50 mM sodium phosphate, pH 4.75, such that the final concentration of ET carbodiimide was 50 mM. The reaction was allowed to proceed at room temperature for 2 h, and then an equal volume of 1 M HCL was added to quench it. The pH of the solution was adjusted to 8 with 1 M NaOH, and trypsin (0.16 mg mL^{-1}) was added to remove the amino-terminal tripeptide and the carboxy-terminal dipeptide. The modified peptide YHP(*p*-sulfo-β-aspartanilide)VPYVK was purified by reverse-phase HPLC.

Preparation of Immunoadsorbent. Rabbit antipeptide antisera were elicited by an initial subcutaneous injection of a 1:1 suspension of a haptenic conjugate (300 μg) and Freund's complete adjuvant, followed by subsequent boosting injections with incomplete Freund's adjuvant and complete Freund's adjuvant after 2 weeks and 1 month, respectively.

[1] Abbreviations: ED carbodiimide, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride; ET carbodiimide, 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide iodide; *t*-BOC, *tert*-butoxycarbonyl; FMOC, 9-fluorenylmethyloxycarbonyl; BSA, bovine serum albumin; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; C-18, octadecylsilyl silica gel; PBS, 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4; Affi-Gel 10, succinylated agarose activated with *N*-hydroxysuccinimide; EDTA, ethylenediaminetetraacetic acid.

The haptenic conjugates were made by coupling either YHPDC or YHPDCPYVK through the respective cysteine to the amino groups on BSA with the bifunctional cross-linker sulfo-MBS (*15*) as described by Ewalt (*9*). Acid hydrolysis and subsequent amino acid analysis of the conjugates revealed that there were 18 nmol of YHPDC (nmol of BSA)$^{-1}$ and 16 nmol of YHPDCPYVK (nmol of BSA)$^{-1}$. An equimolar mixture of the two haptenic conjugates was injected into the rabbits.

The synthetic peptide YHPDCPYVK was used to prepare a peptide affinity column (*9*) to isolate immunoglobulins capable of recognizing the amino- and carboxy-terminal sequences YHPD− and −PYVK. It was found by amino acid analysis that 10 μmol mL^{-1} of peptide had been coupled to the solid phase. Antisera from rabbits immunized with the mixture of the two conjugates were passed over the peptide affinity column (0.5 cm × 3 cm). The column was washed with PBS, and bound immunoglobulins were eluted with 0.1 M sodium phosphate, pH 2.5. The immunoglobulins (10 mg) were pooled, dialyzed against PBS, concentrated to 1.5 mL, and coupled to an equal volume of succinylated agarose that had been activated with *N*-hydroxysuccinimide (Affi-Gel 10) overnight at 4 °C. The slurry was packed in a column (1 cm × 1 cm) and washed with PBS. The amount of peptide that the immunoadsorbent was capable of binding was determined by passing the peptide YHPDVPYVK (20 nmol) over the column, washing with PBS, and eluting the bound YHPKVPYVK with acid. The acid fractions were submitted to reverse-phase HPLC, and the fraction containing the peak of absorbance corresponding to that of the peptide YHPDVPYVK was hydrolyzed and subjected to amino acid analysis. The immunoadsorbent had a capacity sufficient to bind 15 nmol of the peptide. The immunoadsorbent was also found to bind the synthetic peptide YHP(*p*-sulfo-β-aspartanilide)VPYVK.

Modification of Membranes with Sulfanilic Acid and 1-Ethyl-3-[3-(dimethylamino)propyl)carbodiimide. Membranes of erythrocytes rinsed free of hemoglobin were prepared according to Dodge (*16*) by lysis of cells in 5 mM sodium phosphate, pH 8.0. The chemical modification of membranes prepared from 160 mL of blood was performed in 60 mM sulfanilic acid and 100 mM sodium phosphate, pH 6.5. Solid ED carbodiimide (32 mM) was added to the mixture and the reaction was quenched after 15 min by adding 5 M sodium acetate, pH 5, to a final concentration of 100 mM. Modified membranes were sedimented by centrifugation in a Ti 45 rotor at 30 000 rpm for 20 min at 4 °C and washed three times with 5 mM sodium phosphate, pH 8.0.

Modification of Intact Erythrocytes and Inside-Out Vesicles with [^{35}S]Sulfanilic Acid and 1-Ethyl-3-[3-(trimethylammonio)propyl]carbodiimide. The method of Steck et al. (*17*) was used to prepare inside-out vesicles. The sidedness of the vesicles were determined by the ability of acetylcholinesterase to cleave acetylthiocholine (*18*). Inside-out vesicles (prepared from 26 mL of blood) in 3 mL of 150 mM NaCl and 20 mM sodium phosphate, pH 7.4, were added to washed erythrocytes (from 10 mL of blood) in 150 mM NaCl and 5 mM sodium phosphate, pH 6.5. Sulfanilic acid and NaCl was added to the mixture such that the final concentrations in the 14 mL solution were 10 mM sulfanilic acid, 150 mM NaCl, and 55 mM sodium phosphate, pH 6.5. 1-Ethyl-3-[3-(trimethylammonio)propyl]carbodiimide was added to 15 mM, and the reaction was allowed to proceed 10 min before it was quenched it with 240 μL of 5 M sodium acetate, pH 5.0, and then neutralized it to pH 7 with 6.2 M NaOH. The mixture was then layered on a cushion of 15% dextran T-70, 150 mM NaCl, and 50 mM sodium phosphate, pH 6.5, and centrifuged in a SW 28 rotor at 15 000 rpm for 30 min to separate the inside-out vesicles from the intact erythrocytes (*19*). At this point, the incorporation of [^{35}S]-sulfanilic acid into aspartate 821 of anion exchanger could be independently assessed for the intact erythrocytes and the inside-out vesicles that had been modified in the same vessel.

Digestion of Membranes and Immunoadsorption. The modified and unmodified versions of the peptide YHPD-VPYVK were released from membranes with trypsin by a modification of the method used by Kang et al. (*20*). Briefly, labeled erythrocytic membranes were suspended in 50 volumes of 0.1 M NaOH on ice for 30 min to remove peripheral membrane proteins. The membranes that had been stripped with alkali were sedimented in a Ti 45 rotor at 30 000 rpm for 20 min and washed with 5 mM sodium phosphate, pH 8. The stripped membranes were digested with trypsin (0.16 mg mL^{-1}) in 0.2 mM $CaCl_2$ and 50 mM sodium phosphate, pH 8.0, for 1 h and sedimented at 30 000 rpm for 20 min in a Ti 45 rotor. Tryptic digests were brought to pH 7 with 1 M HCl and passed over the immunoadsorbent. The immunoadsorbent was washed with 40 mL of PBS, and those peptides that were specifically bound were eluted with 0.1 M sodium phosphate, pH 2.5. The procedure was repeated until it was clear that the digests were depleted of all of the peptides recognized by the immunoadsorbent.

Enzymatic Digestions. Total enzymatic digestions of peptides with a mixture of leucine aminopeptidase (0.007 unit mL^{-1}), carboxypeptidase Y (8 units mL^{-1}), prolidase (8 units mL^{-1}), and pronase (0.003 mg mL^{-1}) were performed in 2.5 mM $MgCl_2$ and 0.1 M Tris sulfate, pH 8.6, for 9 h at 37 °C. Peptides were digested with trypsin (0.16 mg mL^{-1}) in 0.2 mM $CaCl_2$ and 50 mM sodium phosphate, pH 8.0, for 1 h at 37 °C. Modified peptides were digested with pronase (0.33 mg mL^{-1}) in 100 mM sodium phosphate, pH 7.5, for 2 h at 37 °C.

Analytical Methods. Peptides were purified on either a Spectra-Physics Chromatography SP8100 HPLC with a variable-wavelength Spectra-Physics Spectra 100 UV−vis detector or an HPLC system consisting of two Waters M6000A pumps, a Waters 680 automatic gradient controller, and a Waters 44 absorbance detector fitted with an extended-wavelength module operating at 229 nm. Counts per minute of radioactivity were determined by mixing samples with an equal volume of Biosafe II liquid scintillation cocktail before counting with a Beckman LS 233 scintillation counter. Hydrolyses of peptides were performed in evacuated glass hydrolysis tubes containing 6 M HCl for 55 min at 160 °C. The resulting hydrolysates were analyzed using a modular system composed of a Spectra-Physics autosampler SP8875, Spectra-Physics ternary pump SP8800, and a Pickering sodium cation-exchange column. Amino acids were quantified as their ninhydrin derivatives. Densitrometry was performed using a LKB Bromma 2202 Ultroscan.

RESULTS

Modification of Synthetic PPKYHPDVPYVKRV. The peptide PPKYHPDVPYVKRV, representing the amino acid

sequence from proline 815 to valine 828 of anion exchanger, was synthesized to verify conditions of digestion and to produce the standards YHPDVPYVK and YHP(*p*-sulfo-β-aspartanilide)VPYVK corresponding to the liberated peptides expected upon digestion of unmodified and modified anion exchanger, respectively, with trypsin. Digestion of PPKYHPDVPYVKRV with trypsin generated a peptide that eluted at a shorter retention time (25 min) than that of the parent (27 min) when it was submitted to reverse-phase HPLC. Hydrolysis and amino acid analysis of the product of the digestion gave the composition $Y_{2.1}H_{1.1}P_{1.8}D_{1.1}V_{2.1}K_{0.9}$. The peptide YHP(*p*-sulfo-β-aspartanilide)VPYVK was made by the carbodiimide-mediated sulfanilation of PPKYHPDVPYVKRV followed by digestion with trypsin. The product of this reaction eluted at a longer retention time (27 min) than that of YHPDVPYVK (25 min) and also absorbed strongly at 250 nm (the wavelength at which sulfanilic acid absorbs maximally). The amino acid composition of this modified peptide following acid hydrolysis was $Y_{1.9}H_{1.0}P_{2.1}D_{1.0}V_{2.0}K_{0.9}$. The amide resulting from the modification is acid-labile, which would explain why aspartate is present stoichiometrically in the amino acid analysis. The amino acid composition of the peptide following total enzymatic digestion, however, was $Y_{2.3}H_{1.6}P_{1.6}D_{0.1}V_{1.6}K_{1.0}$. That only aspartate was missing from the digest is evidence that it alone had been modified. A small amount of the sample was subjected to electrospray mass spectrometry, and the observed *m/z* of the ion was 636.8, which agrees with the expected mass (1274.4 amu) for dicationic YHP(*p*-sulfo-β-aspartanilide)VPYVK.

Isolation of YHPDVPYVK and YHP(p-sulfo-β-aspartanilide)VPYVK from Membranes of Erythrocytes. Erythrocytic membranes from 160 mL of blood were labeled in a final volume of 50 mL with 60 mM sulfanilic acid and 32 mM ED carbodiimide. The modified and unmodified versions of the peptide YHPDVPYVK were then digested from membranes with trypsin by a modification of the method used by Kang et al. (*20*). The clear supernatant from the digestion was collected and neutralized before it was passed three times over an immunoadsorbent made from polyclonal immunoglobulins specific for the amino- and carboxy-terminal sequences YHPD− and −PYVK. After the immunoadsorbent was rinsed each time, the specifically bound peptides were eluted with 100 mM sodium phosphate, pH 2.5. The acid eluates were pooled and submitted to HPLC using a reverse-phase C-18 column, and the absorbance was monitored at 250 nm for sulfanilate and 229 nm for peptide. Two peaks of absorbance at 229 nm were detected, the first of which had the same retention time (25 min) as the standard peptide YHPDVPYVK. The amino composition of this peak following acid hydrolysis was $Y_{1.7}H_{1.0}D_{1.2}V_{2.0}K_{1.1}$. The second peak eluted with the same retention time (27 min) as the standard peptide YHP(*p*-sulfo-β-aspartanilide)-VPYVK. The ratio of the areas of the peaks of absorbance at 229 and 250 nm for the modified peptide isolated from the membranes was found to be the same as that for the modified synthetic standard. From this experiment, it could be concluded that aspartate 821 of native anion exchanger could be labeled by ED carbodiimide and sulfanilic acid and that both the modified and the unmodified peptide could be isolated by the immunoadsorbent.

Location of Aspartate 821 in Intact Anion Exchanger. Inside-out vesicles prepared from erythrocytes (*17*) were mixed with an amount of packed red blood cells estimated to contain an equivalent amount of anion exchanger. These intact erythrocytes and inside-out vesicles were labeled together at final concentrations of 10 mM [^{35}S]sulfanilic acid (10 500 cpm nmol^{-1}) and 15 mM ET carbodiimide for 10 min. The inside-out vesicles were then separated from the erythrocytes so that the incorporation of radioactivity resulting from the modification of aspartate 821 of anion exchanger from each of these populations could be independently assessed. Membranes were prepared from the labeled erythrocytes. These membranes and the inside-out vesicles were stripped with alkali, and both samples were digested with trypsin (*20*). Tryptic peptides were isolated by immunoadsorption, and the eluted peptides were submitted to reverse-phase HPLC (Figure 1). Fractions (1 mL) were collected, and half of each fraction was submitted to liquid scintillation. Two peaks of absorbance at 229 nm were observed. The first peak (peak a, Figure 1A) had the same retention time (26 min) as the standard peptide YHPDVPYVK. From amino acid analysis of this fraction following acid hydrolysis, the amount of peptide in the entire peak of absorbance was calculated to be 4.9 nmol with the composition $Y_{1.9}H_{1.1}P_{2.0}D_{1.1}V_{1.9}K_{1.0}$. The second peak (peak b, Figure 1B) displayed absorbance at 229 and 250 nm and had the same retention time (27 min) as the synthetic standard YHP(*p*-sulfo-β-aspartanilide)VPYVK. This second peak also had 830 cpm of [^{35}S]sulfur associated with it. In the sample obtained from the modified intact erythrocytes, a peak of absorbance at 229 nm (peak a, Figure 1C) was observed that had a retention time (26 min) the same as the unlabeled standard. The amino acid composition following acid hydrolysis was $Y_{1.8}H_{1.1}P_{2.1}D_{1.1}V_{2.0}K_{1.0}$, and the amount of peptide was calculated to be 6.4 nmol. No peak of absorbance, however, was observed on the chromatogram with the same retention time as the modified standard, and the counts per minute of radioactivity in the fraction collected at 27 min were indistinguishable from background. From this experiment, it appeared that aspartate 821 of anion exchanger could be modified only when the sulfanilic acid and the impermeant ET carbodiimide had access to the cytoplasmic face of the protein, but the possibility remained that the counts of radioactivity and the peak of absorbance at 27 min were associated with some species other than YHP(*p*-[^{35}S]sulfo-β-aspartanilide)VPYVK.

To demonstrate that the peak of radioactivity (peak b, Figure 1B) was associated with the labeled peptide YHP(*p*-[^{35}S]sulfo-β-aspartanilide)VPYVK, experiments were conducted with modified synthetic standard to find a proteinase that would cleave the labeled peptide and generate a smaller peptide that would retain the original label and have a unique retention time when resubmitted to reverse-phase HPLC. If the peak of radioactivity (Figure 1) is associated with YHP(*p*-[^{35}S]sulfo-β-aspartanilide)VPYVK, then digestion of the fraction eluting at 27 min should cause the original counts per minute of radioactivity to move upon rechromatography from 27 min to the new position. The labeled standard YHP(*p*-[S]sulfo-β-aspartanilide)VPYVK proved to be unexpectedly resistant to proteolysis; however, when it was digested with pronase, a mixture of endo- and exoproteases, three peaks of absorbance at 250 nm with retention times of 21, 22, and 24 min were observed upon rechromatography on the same system. The amino acid compositions following acid hydrolysis of these fractions were $Y_{0.9}H_{1.0}P_{1.9}D_{1.1}V_{1.1}$,

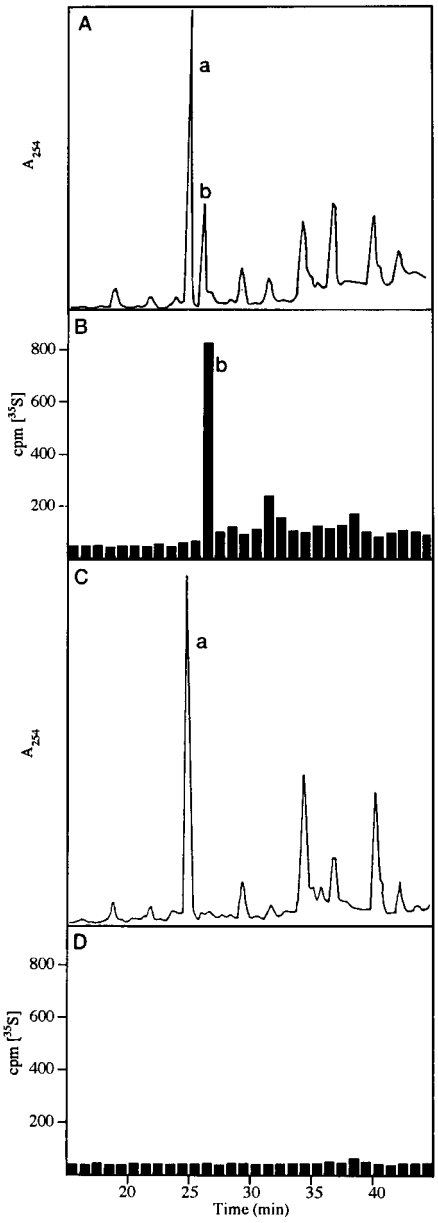

FIGURE 1: Incorporation of [^{35}S]sulfanilic acid into aspartate 821 of anion exchanger in inside-out vesicles and intact erythrocytes. Inside-out vesicles (11 mg from 26 mL of blood) and erythrocytes (10 mL of packed cells) were modified with 10 mM [^{35}S]sulfanilic acid (10 500 cpm nmol^{-1}) and 15 mM ET carbodiimide in the same container. The inside-out vesicles were separated from the erythrocytes by differential centrifugation (*19*). Tryptic digests of alkali-stripped membranes from each sample were neutralized and passed twice over the immunoadsorbent specific for the peptide with the amino- and carboxy-terminal sequences YHPD− and −PYVK (15 nmol capacity). After each pass, the column was washed with phosphate-buffered saline to remove unbound peptides, and specifically bound peptides were eluted with 100 mM sodium phosphate, pH 2.5. Acid eluants from the two passes were pooled, and the volume of the pool was reduced to 2 mL. The sample was submitted to reverse-phase HPLC on an analytical C-18 column (0.046 cm × 0.25 cm) run in 0.05% TFA in water and developed with a linear gradient of 1% CH_3CN min^{-1}. The effluent from the column was monitored for absorbance at 229 and 254 nm and 1 mL fractions were collected. Half of each of these fractions was assayed for [^{35}S]-sulfur by liquid scintillation. (A) Chromatogram of the peptides isolated from modified inside-out vesicles and (B) associated counts per minute of [^{35}S]sulfur. Absorbance at 254 nm (A_{254}) or counts per minute are presented as a function of elution time (minutes). (C) Chromatogram of the peptides isolated from modified erythrocytes and (D) associated counts per minute of [^{35}S]sulfur from the same chromatogram.

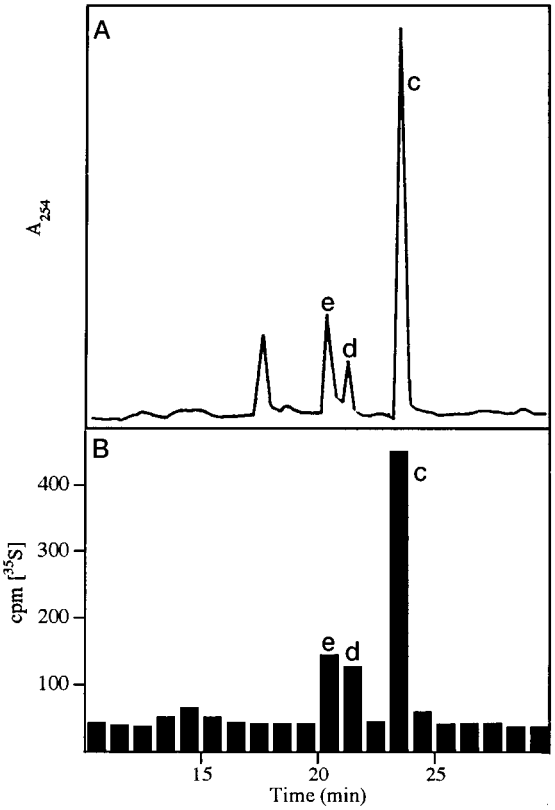

FIGURE 2: Digestion of YHP(*p*-[^{35}S]sulfo-*β*-aspartanilide)VPYVK with pronase. Half of the fraction containing the peak of radioactivity (peak b, Figure 1B) and the peak of absorbance (peak b, Figure 1A) with the same retention time as YHP(*p*-sulfo-*β*-aspartanilide)-VPYVK was evaporated to dryness, and synthetic YHP(*p*-sulfo-*β*-aspartanilide)VPYVK (8 nmol) was added. The sample was digested with pronase (0.33 mg mL^{-1}) in 100 mM sodium phosphate, pH 7.5, for 2 h at 37 °C. Products of the digestion were separated by HPLC under the same conditions as those described in Figure 1. Fractions were collected every minute and submitted to liquid scintillation. (A) Chromatogram of the sample after digestion and (B) associated counts per minute of [^{35}S]sulfur from the same chromatogram. Absorbance at 254 nm (A_{254}) or counts per minute are presented as a function of elution time (min).

$D_{1.2}V_{1.0}P_{1.1}Y_{0.7}K_{0.3}$, and $H_{1.0}P_{1.9}D_{1.1}V_{1.1}$, respectively. The lysine in the amino acid analysis of the second peptide is thought to originate from contaminating peptide derived from self-digested pronase that has a retention time overlapping that of the peptide (*p*-sulfo-*β*-aspartanilide)VPY. It could be concluded that pronase will cut YHP(*p*-sulfo-*β*-aspartanilide)VPYVK into YHP(*p*-sulfo-*β*-aspartanilide)VP, (*p*-sulfo-*β*-aspartanilide)VPY, and YHP(*p*-sulfo-*β*-aspartanilide)V.

A small amount (8 nmol) of standard labeled peptide was added to the remaining half of fraction 27 obtained from the labeled inside-out vesicles (Figure 1). Pronase was added, and the resulting digest was submitted to reverse-phase HPLC (Figure 2). It was found that 150, 130, and 450 cpm were associated with the peptides HP(*p*-sulfo-*β*-aspartanilide)VP (peak c, Figure 2), (*p*-sulfo-*β*-aspartanilide)-VPY (peak d, Figure 2) and YHP(*p*-sulfo-*β*-aspartanilide)V (peak e, Figure 2), respectively. These results show that the peak of radioactivity eluting at the same retention time as the standard YHP(*p*-sulfo-*β*-aspartanilide)VPYVK is the modified peptide YDP(*p*-[^{35}S]sulfo-*β*-aspartanilide)VPYVK. In a separate labeling experiment using unradioactive sulfanilic acid, the peak of absorbance at 229 and 250 nm eluting at 27 min from the sample of labeled inside-out

Table 1: Relative Specific Radioactivities of YHP(*p*-[^{35}S]Sulfo-*β*-aspartanilide)VPY VK from Inside-Out Vesicles and Intact Erythrocytes[a]

	inside-out vesicles		erythrocytes		[(cpm IOV[b])/(nmol IOV)]
expt	cpm	nmol	cpm	nmol	[(cpm RBC[c])/(nmol RBC)]
1	1847	7.7	<107	6.0	>13
2[d]	827	4.9	<48	6.4	>20
3[e]	875	10.8	<50	6.2	>10

[a] The counts per minute of YHPD(*p*-[^{35}S]sulfo-*β*-aspartanilide)VPYVK were determined by liquid scintillation and the nanomoles of YHPD-VPYVK were determined by quantitative amino acid analysis in experiments similar to those described in Figure 1. [b] Inside-out vesicles. [c] Erythrocytes. [d] The results from these experiments are displayed in Figures 1 and 2. [e] The inside-out vesicles and erythrocytes in this experiment were labeled in separate containers.

vesicles was analyzed by electrospray mass spectrometry. The *m/z* of the observed ion was 636.8, which agrees with the expected mass of 1274.4 for dicationic YHP(*p*-sulfo-*β*-aspartanilide)VPYVK.

The specific radioactivity incorporated into aspartate 821 following the modification of anion exchanger was estimated by dividing the counts per minute of radioactivity associated with the fraction eluting upon HPLC at the same position as YHP(*p*-sulfo-*β*-aspartanilide)VPYVK (27 min) by the nanomoles of unmodified YHPDVPYVK on the same chromatogram. The assumptions behind this procedure are that the overall yield of modified and unmodified peptide upon digestion, immunoadsorption, and chromatography were the same and that only a small fraction (< 2%) of the anion exchanger in the inside-out vesicles was modified at aspartate 821 in these experiments. In the experiment just described (Figure 1), the specific radioactivity of YHP(*p*-[^{35}S]sulfo-*β*-aspartanilide)VPYVK from inside-out vesicles and erythrocytes was 170 cpm nmol $^{-1}$ and less than 8 cpm nmol $^{-1}$, respectively. The ratio of the specific radioactivities of the peptide YHP(*p*-[^{35}S]sulfo-*β*-aspartanilide)VPYVK that were obtained from the labeling of inside-out vesicles and intact erythrocytes has been used to assign the orientation of aspartate 821 of anion exchanger in intact erythrocytes. The incorporation of [^{35}S]sulfanilic acid into aspartate 821 of anion exchanger from inside-out vesicles was more than 20-fold greater than the incorporation of [^{35}S]sulfanilic acid into aspartate 821 from erythrocytes when the two preparations were labeled in the same solution. The results of two additional experiments were similar (Table 1) to the results of the experiment just described. Because aspartate 821 was significantly more accessible to modification by the impermeant reagents in inside-out vesicles than in intact erythrocytes, it must be on the cytoplasmic surface of anion exchanger.

Levels of Incorporation of Sulfanilic Acid into Aspartate 821. In the experiments just described, only 1−3% of the anion exchanger was labeled at aspartate 821 during the reaction. The incorporation was purposely kept at a low level to avoid changes in the structure of the protein that might result from the chemical modification of multiple carboxylates in each molecule (*21*). Such a strategy, however, raises the possibility that the incorporation of [^{35}S]sulfanilic acid could result from the labeling of only a small population of anion exchanger that had been denatured in some manner during the preparation of the inside-out vesicles rather than from labeling of aspartate 821 in properly folded, native

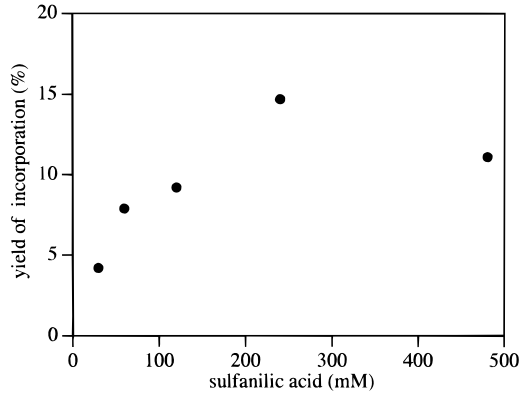

FIGURE 3: Yield of the modification of anion exchanger from inside-out vesicles modified at aspartate 821. Samples of inside-out vesicles (4.8 mg) were modified in 3 mL of 45 mM ET carbodiimide, 150 mM NaCl, and 55 mM sodium phosphate, pH 6.5, containing increasing concentrations of *p*-sulfanilic acid. Reactions were quenched after 10 min, and the vesicles were separated by differential centrifugation. The peptides YHPKVPYVK and YHP(*p*-sulfo-*β*-aspartanilide)VPYVK were isolated as described in Figure 1. The yield of incorporation (percent) was calculated from the ratios of the peaks of absorbance (as seen in Figure 1A) corresponding to the peptides YHPKVPYVK and YHP(*p*-sulfo-*β*-aspartanilide)VPYVK and is presented as a function of the concentration of sulfanilic acid (millimolar).

anion exchanger. To examine this possibility, an experiment was performed to examine the effects of increasing the level of labeling of aspartate 821. Samples of inside-out vesicles were labeled in solutions containing several different concentrations of sulfanilic acid. After the reaction had been quenched, each sample was layered over 15% dextran and centrifuged. Ruptured vesicles do not float on a dextran cushion; therefore, the differential centrifugation was used to determine if any damage had been sustained by the vesicles from the high concentrations of sulfanilic acid. In all of the samples, the majority of the vesicles floated; however, some lysis was evident as the concentration of sulfanilic acid was increased. The floating bands of inside-out vesicles were collected and processed in the usual manner. The areas of the peaks of absorbance at 254 nm (as in Figure 1A) of the peptides YHPDVPYVK and YHP(*p*-sulfo-*β*-aspartanilide)VPYVK were calculated from the high-pressure liquid chromatograms. The extinction coefficients of the two peptides, YHP(*p*-sulfo-*β*-aspartanilide)-VPYVK (0.016 nmol cm^{-2}) and YHPDVPYVK (0.24 nmol cm^{-2}), were determined in a separate experiment. The percentages of incorporation could then be calculated from the ratios of the absorbances (Figure 3). Increasing the concentration of sulfanilic acid resulted in an increase in its incorporation into aspartate 821 that reached a maximum at 240 mM. An additional experiment was performed in which separate 40 mM additions of ET carbodiimide were added to samples of inside-out vesicles in a solution of 150 mM sulfanilic acid every 15 min. The percent incorporation in samples quenched after 5, 15, 30, 45, and 60 min at final concentrations of 40, 80, 120, 160, and 200 mM ET carbodiimide were 11%, 10%, 18%, 23%, and 24%, respectively. The fractions eluting at 27 min from these experiments were pooled, dried down, and rechromatographed on the same HPLC system to be sure that there was no contamination from the peptide YHPDVPYVK. The HPLC chromatogram displayed a single sharp peak at 27 min. The fraction associated with this peak of absorbance was dried

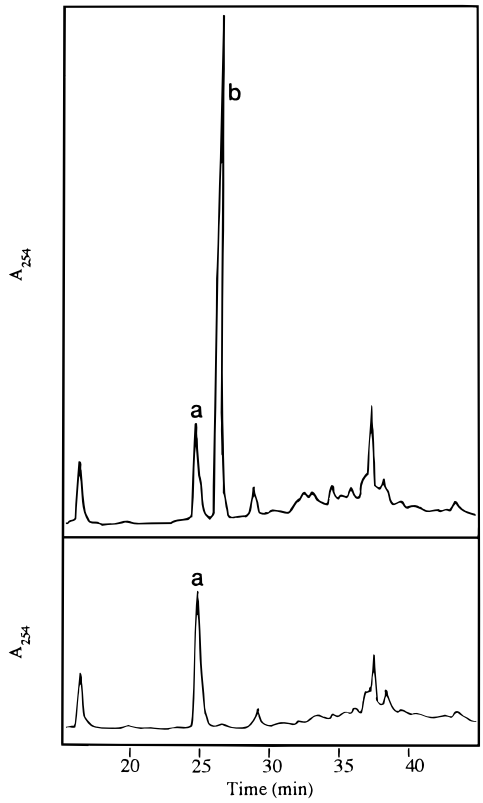

FIGURE 4: High-pressure liquid chromatogram of peptides isolated from modified inside-out vesicles (top panel) and erythrocytes (lower panel) by immunoadsorption. Inside-out vesicles (6.9 mg) and erythrocytes (7 mL of packed cells) containing a similar amount of anion exchanger were labeled in separate vessels containing 150 mM sulfanilic acid, 150 mM NaCl, and 55 mM sodium phosphate, pH 6.5. Three separate additions of ET carbodiimide, each 40 mM, were added every 15 min and the reaction was quenched after 1 h. Peptides were produced by digestion, isolated by immunoadsorption, and separated by chromatography as described in Figure 1.

down and submitted to amino acid analysis following acid hydrolysis. The amino acid composition was $Y_{2.2}H_{1.1}P_{2.4}D_{1.1}V_{1.6}K_{0.6}$.

Inside-out vesicles and erythrocytes were then modified under conditions that produced the highest level of incorporation. The peptides YHPDVPYVK and YHP(*p*-sulfo-β-aspartanilide)VPYVK were isolated by immunoadsorption and chromatographed as usual (Figure 4). Even at these high levels of incorporation of sulfanilic acid into aspartate 821 of anion exchanger in inside-out vesicles (compare Figures 1A and 4A), there was no incorporation of the sulfanilic acid into aspartate 821 of anion exchanger in the erythrocytes.

DISCUSSION

In the region between the potential membrane-spanning segments M11 and M12 of human erythrocytic anion exchanger, there is the amino acid sequence −DRILLLFK-PPKYHPDVPYVKRVKTWR−, from positions 807 to 832 in its overall sequence. Recent topological models of anion exchanger have placed this hydrophilic region both on the interior (*22*−*25*) and on the exterior (*10*) of the cell. In part, the assignment to the interior of the cell was based on the observation that the monoclonal immunoglobulin BRIC 132, believed to be specific for the sequence between amino acid 813 and 824, bound anion exchanger only after disruption of intact erythrocytes (*7*, *8*). Experiments, however, in which the binding of immunoglobulins to a native protein have been used to determine the topography of a membrane-spanning protein have often been misleading. For example, experiments following the binding of immunoglobulins performed in two separate laboratories (*26*, *27*) had indicated that lysine β165 of acetylcholine receptor was exposed to the cytoplasm. It has since been shown that lysine β165 is located on the extracytoplasmic surface of acetylcholine receptor (*9*). The signal that was believed to have resulted from the binding of immunoglobulins to native acetylcholine receptor may have actually represented binding to protein that had been denatured by the treatments used to disrupt the membranes. This is a significant concern if the signal is very weak, as in the binding experiments with the immunoglobulin BRIC 132.

Another set of experiments with a different set of immunoglobulins led to the conclusion that the segment between aspartate 807 and arginine 832 was on the exterior of the cell. Kay et al. (*10*) found that two synthetic peptides corresponding to amino acids 812−827 and 538−554 of human anion exchanger inhibited the binding to intact erythrocytes of immunoglobulins recognizing senescent cells. It was concluded, therefore, that these two regions of anion exchanger were extracytoplasmic and lay in close proximity to each other to form an epitope exposed in senescent erythrocytes. At the time, there was independent support for this conclusion because it was thought that an inhibitor of anion exchange, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid, covalently modified one lysine from each of these two sequences. If this had been the case, the two sequences would have to be extracytoplasmic and close together. It has since been shown, however, that while 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid does modify lysine 539 from the first sequence, the second modified lysine is at position 851 (*28*). The results of the present study demonstrate that the sequence between amino acids 812 and 827 is located on the interior of the cell and should not form part of an extracellular epitope on anion exchanger from senescent erythrocytes.

Indirect evidence for a cytoplasmic location of this hydrophilic loop containing aspartate 821 has also been presented (*29*). Experiments using radioiodination with lactoperoxidase followed by proteolytic dissection of the modified anion exchanger have shown that tyrosine 628 is accessible to iodination from the extracytoplasmic surface of the protein (*30*). Earlier experiments (*31*) were then reinterpreted as indicating that the tyrosines carboxy-terminal to tyrosine 628 were either intracellular or inaccessible to iodination from either surface. Tyrosine 784 is not modified under any circumstances. The argument was, therefore, that if iodination occurred at any other position, it had to be from the cytoplasmic surface and that if iodination was actually confined only to tyrosine, it must result from iodination of one or more of the tyrosines at positions 818, 824, and 905. An inference about the disposition of the region between M11 and M12 was then drawn from the electrophoretic behavior of a peptide observed on a peptide map of a large proteolytic fragment of anion exchanger that had been iodinated by lactoperoxidase in open erythrocytic membranes. This peptide contained [^{125}I]iodine, and its electrophoretic mobility suggested that it contained several basic amino acids. Since the amino acids surrounding the tyrosines in the hydrophilic loop between M11 and M12 are basic, as opposed to the acidic amino acids surrounding tyrosine 905, this tryptic peptide was thought to have originated from this

loop (*29*). It must be stressed, however, that neither a direct identification of this peptide nor an assignment of either tyrosine 818 or tyrosine 824 as the labeled amino acid in this peptide has ever been made. A similar argument was used to conclude that tyrosine 486 was on the extracytoplasmic surface of anion exchanger (*32*). Recently, it has been shown that that conclusion was incorrect. A combination of vectorial modification and site-directed immmunochemistry was used to examine directly the radioiodination of tyrosine 486 catalyzed by lactoperoxidase. A short peptide that contained tyrosine 486 was isolated from [^{125}I] radioiodinated inside-out vesicles and intact erythrocytes, and it was found that tyrosine 486 was modified to at least a 6-fold greater specific radioactivity in inside-out vesicles than it was in intact cells (*19*).

Vectorial modification requires either sealed right-side-out or sealed inside-out preparations or both. In the present experiments both intact erythrocytes and inside-out vesicles were used. The inside-out vesicles were prepared by the method of Steck et al. (*17*), and the proportion of inside-out vesicles in the preparation, as determined by the acetylcholinesterase assay (*18*), ranged from 50% to 80%. It could not be determined whether the other vesicles in the preparation were leaky inside-out vesicles or resealed right-side-out vesicles; regardless, most of the vesicles allowed access of the reagents to the cytoplasmic face of the membrane. Since access is afforded only to the extracytoplasmic face of anion exchanger in the intact erythrocytes, the inside-out vesicles and intact erythrocytes were used together in the experiments to assess the disposition of aspartate 821. The advantage of using the inside-out vesicles in the experiments, as opposed to just membranes of erythrocytes, was that they could be labeled in the same container with intact erythrocytes and easily separated from them by differential centrifugation.

To examine the fate of vectorial modification at aspartate 821, an impermeant reagent was required. Amidation promoted by a carbodiimide was chosen to modify the carboxylate of aspartate 821. Water-soluble carbodiimides, frequently used to label aspartates and glutamates in proteins at acid pH (*33*−*36*), have also been used to label anion exchanger at neutral pH (*37*−*39*). The original goal of the present experiments was to activate aspartate 821 with the membrane-permeant ED carbodiimide to generate the *O*-acyl urea that could then be displaced with an impermeant amine to form a stable amide. This approach has been used to label cytochrome *b*5 vectorially with ED carbodiimide and taurine (*40*). Three criteria for the selection of the amine used in this study were that it react under physiological conditions, that it can be detected in small amounts, and that it not cross the bilayer. Sulfanilic acid was chosen because it fulfilled at least two of these criteria. It reacted rapidly with aspartates in model peptides at pH 6.5, it has a detectable chromophore, and its radioanalogue, [^{35}S]sulfanilic acid, is easily synthesized (*14*). The unexpected reactivity of sulfanilic acid in a reaction mediated by a carbodiimide, even though it is a poorly basic aromatic amine, may possibly result from the fact that the pK_a of 3.26 (*41*) for the amino group favors its complete deprotonation at the pH used in these experiments.

The diazonium salt of sulfanilic acid is impermeant (*42*), which suggests that sulfanilic acid might be impermeant as well. The diazonium salt, however, has a fixed positive charge whereas the corresponding amino group of sulfanilic acid is neutral. Some negatively charged compounds that are impermeant to most membranes will penetrate the membrane of erythrocytes as substrates of anion exchanger. Examples include *N*-*[*(2-aminoethyl)sulfonyl]-7-nitrobenz-2-oxa-1,3-diazole (*43*) and pyridoxal phosphate (*44*). Because it was not clear whether or not sulfanilic acid would cross the bilayer, I chose to use an impermeant carbodiimide. Wieth et al. (*37*) used the quaternary amine 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide as an impermeant reagent that takes advantage of the cation-tight membrane of erythrocytes, and Craik and Reithmeier (*45*) have subsequently demonstrated that ET carbodiimide is impermeant. They showed that when intact erythrocytes were labeled with [^{3}H]ET carbodiimide, no cross-linking of spectrin occurred; however, when erythrocytes that had been opened with detergent were labeled with [^{3}H]ET carbodiimide, cross-linking of spectrin was significant. Since ET carbodiimide does not appear to cross the membrane of human erythrocytes, it is ideally suited to be used with either a permeant or impermeant amine for the vectorial modification of aspartates and glutamates in membrane-spanning proteins.

A major advantage of the strategy used in this study is the ability to isolate a specific peptide from a complicated digest by site-directed immunochemistry. This approach has been successfully used to define the location of selected amino acids in erythrocytic anion exchanger (*19*), Na^+/K^+-transporting ATPase (*46*−*48*), and acetylcholine receptor (*15*, *49*, *50*). Kang et al. (*20*) found that the peptide YHPDVPYVK could be cleaved from membranes of erythrocytes by digestion with trypsin, but only after the membranes had been treated with 0.1 M NaOH. The tryptic peptide was isolated in low yield after multiple chromatographic steps. It could not be released from membranes that had been pretreated with 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate, which stabilizes anion exchanger from conformational changes, and concentrations of 0.01 M NaOH were not high enough to enable release of the peptide even though at this concentration all of the peripheral membrane proteins had been extracted. It follows that the treatment with alkali causes a change in the structure of anion exchanger that produces the susceptibility to tryptic digestion within the hydrophilic loop between M11 and M12. In the present study, site-directed immunochemistry enabled the peptide YHPDVPYVK to be rapidly isolated after a short digestion of alkali-stripped membranes with trypsin.

Ideally, the immunoadsorbent used to isolate peptides from a digest of the modified protein has the same affinity for labeled and unlabeled peptides (*46*). The immunoadsorbent used in these experiments, however, was capable of binding peptides containing the amino- or carboxy-terminal sequences YHPD− or −PYVK, respectively. Since the epitope included the aspartic acid, the affinity of the immunoadsorbent for the peptide YHPDVPYVK was greater than that for the labeled peptide YHP(*p*-sulfo-*β*-aspartanilide)VPYVK, complicating attempts to determine the yield of the incorporation of sulfanilic acid or [^{35}S]sulfanilic acid into aspartate 821. To overcome this problem, the tryptic digests were completely depleted of both the modified and unmodified peptides by repeatedly passing them over the immunoadsorbent until all the peptides had been absorbed as judged by the peaks of absorbance and the counts per minute associated with the peptides YHPDVPYVK and YHP(*p*-[^{35}S]-sulfo-*β*-aspartanilide)VPYVK, respectively, upon subsequent

chromatography.

To avoid disrupting the native conformation of a protein, it has been suggested that general chemical modification should be carried out at conditions where the yield of the incorporation of the label at each site is low (*51*). A disadvantage is that a low yield of incorporation could arise from the modification of a small unrepresentitive population of the protein. The most straightforward way to address this concern is to increase the yield of the incorporation. Care must be taken, however, that the conditions chosen to effect a higher yield of incorporation do not increase the yield by disrupting the native conformation of the protein. In the present experiments, the extent of this problem was assessed in part by monitoring the yield of the incorporation as a function of time. If a dramatic increase in the yield had occurred only after a certain level of incorporation had been attained, it might have been that denaturation of the protein had commenced, leading to the increased reactivity of aspartate 821 toward the reagents. The yield of incorporation, however, increased continuously as the concentration of sulfanilic acid increased to a maximum of 15%, and additional experiments revealed conditions that gradually and continuously boosted the yield of incorporation to 24%. As an additional assessment of the effects of high levels of incorporation, the inside-out vesicles were submitted to differential centrifugation after they were modified to determine whether or not the labeling had disrupted the membranes. Little disruption of the membranes of either the intact erythrocytes or the inside-out vesicles was observed. These results suggest that even when low yields of incorporation were deliberately chosen, as in the experiments using [^{35}S]sulfanilic acid (Figure 1), the observed labeling reflected the behavior of aspartate 821 in properly folded native anion exchanger. It may have been difficult to obtain yields of incorporation higher than 25% because the *O*-acyl urea formed in the carbodiimide reaction can undergo one or more side reactions (*52*) such as intramolecular rearrangement to form a stable *N*-acyl urea or the nucleophilic addition of the primary amine of a lysine residue from anion exchanger. In addition, the optimal pH for the usual carbodiimide reaction with an external amine is much lower than the pH at which these experiments were conducted (*33*, *53*).

Recent experiments have helped to define the topography of anion exchanger near the carboxy-terminus. In discussing these results, the numbering of the hydrophobic segments first proposed by Kopito and Lodish (*4*) will be used. Their long carboxy-terminal segment M12, however, must now be split into two segments, M12 and M13, because lysine 851, in the middle of their segment M12, has recently been shown to reside on the extracytoplasmic side of the membrane. Intact erythrocytes were labeled with the impermeant inhibitor 4,4′-diisothiocyanotodihydrostilbene-2,2′-disulfonic acid, and two small lysine-containing peptides that had been cross-linked by the inhibitor were isolated from a proteolytic digest of the modified protein (*28*). The two lysines cross-linked to the inhibitor were identified as the lysines at positions 539 and 851. Lysine 851, therefore, is extracytoplasmic.

Human anion exchanger is postranslationally modified by fatty acylation that results from a thioester bond (*54*). Okubo et al. (*55*) have localized the site of acylation to cysteine 843 near the center of the hydrophobic segment M12. The thioester bond is not destroyed either by treatment with mercaptoethanol or by digestion with cyanogen bromide (*55*) , results suggesting that it lies protected within the bilayer (*23*).

Jennings et al. (*56*) have provided experiments that have defined the disposition with respect to the membrane of the hydrophilic loop between the potential membrane-spanning segments M9 and M10. Trypsin was sealed in right-side-out vesicles of human erythrocytes to promote the exclusive digestion of the cytoplasmic surface of anion exchanger. A carboxy-terminal fragment was produced during this digestion, and it was isolated by the monoclonal immunoglobulin IV F12. By sequencing the fragment, it was determined that trypsin had cut the protein from its cytoplasmic surface at lysine 743. The hydrophilic loop between M9 and M10, therefore, is located on the cytoplasmic side of the membrane.

The present assignment of aspartate 821, found between the hydrophobic segments M11 and M12 in anion exchanger, to the cytoplasmic surface of the plasma membrane can be added to the results of these earlier experiments to obtain a more complete picture of the situation. Because aspartate 821 is on the cytoplasmic side of the membrane and lysine 851, found between the hydrophobic segments M12 and M13, is extracytoplasmic, the intervening hydrophobic segment M12, containing cysteine 843 that is fatty acylated, spans the bilayer. The carboxy terminus of the protein, which follows segment M13, is cytoplasmically situated (*7*, *8*, *57*), so M13 must also span the membrane. The nearest hydrophilic segment on the amino-terminal side of aspartate 821 should be extracytoplasmic, if segment M11 spans the bilayer. Unfortunately, the location of that hydrophilic segment, which is between hydrophobic segments M10 and M11, is not yet known; however, the assignment by Jennings et al. (*56*) of the hydrophilic segment between M9 and M10, containing lysine 743, to the cytoplasmic side of the bilayer is consistent with an extracytoplasmic location of the hydrophilic segment between hydrophobic segments M10 and M11 if both M10 and M11 span the bilayer.

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