Topological Disposition of Tyrosine 486 in Anion Exchanger from Human Erythrocytes†

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ABSTRACT: The location with respect to the plasma membrane of tyrosine 486 in the native anion exchanger of human erythrocytes has been determined by site-directed immunochemistry. Intact erythrocytes and inside-out vesicles were [^{125}I]radioiodinated by lactoperoxidase in the same vessel. After the erythrocytes and inside-out vesicles had been separated by differential centrifugation, the modified polypeptide of the anion exchanger was isolated from each sample and digested with the proteinase from *Staphylococcus aureus* strain V8 and trypsin to generate the peptide YIVGR. An immunoadsorbent that was specific for the carboxy-terminal sequence −IVGR was used to purify the peptide YIVGR, which contains tyrosine 486 of the anion exchanger, from the products of the digestion. The [^{125}I]radioiodinated peptides isolated by the immunoadsorbent were submitted to high-pressure liquid chromatography, and their respective mobilities were compared to those of synthetic peptides that had been iodinated at tyrosine. By applying this technique, the peptide containing tyrosine 486 was unambiguously identified, and the incorporation of [^{125}I]iodine into this residue in anion exchanger could be monitored. When inside-out vesicles and intact cells were [^{125}I]radioiodinated in the same suspension, tyrosine 486 was modified to at least a 6-fold greater specific radioactivity in the inside-out vesicles than it was in the intact cells. This amino acid, therefore, was assigned to the cytoplasmic surface of native anion exchanger. It follows that the polypeptide of anion exchanger spans the membrane three times before it reaches the extracellular region surrounding glutamine 550.

Erythrocytic anion exchanger (band 3) is an abundant glycoprotein embedded in the plasma membrane of erythrocytes. It is best known for its ability to catalyze the coupled transport of bicarbonate and chloride across the membrane of erythrocytes, an ability that provides for the efficient clearance of carbon dioxide from peripheral tissues (Passow, 1986). Erythrocytic anion exchanger is a member of a family of membrane-embedded proteins facilitating anion transport in a wide variety of cells (Kopito, 1990; Alper, 1991).

Human erythrocytic anion exchanger, composed of a polypeptide that is 911 amino acids in length (Tanner et al., 1988), is divided into two distinct domains. The first 400 amino acids constitute an amino-terminal, cytoplasmic domain that can be cleaved from the membrane with proteinases (Steck et al., 1976; Appell & Low, 1981; Mawby & Findlay, 1982). The remaining amino acids form a carboxy-terminal, membrane-embedded domain, and this portion of the polypeptide spans the lipid bilayer several times to produce the channel for anion transport (Kopito & Lodish, 1985). Among the different members of the family of anion exchangers, the amino-terminal domain varies in its length and composition, but the membrane-embedded domain is highly conserved over the entire family (Kopito, 1990; Alper, 1991). Distributed evenly over the membrane-embedded domain of anion exchangers from erythrocytes, there are thirteen hydrophobic segments of about twenty amino acids in length, designated as segments M1−M13, if segment M12 is split into segments M12 and M13 (Kopito & Lodish, 1985). It has been proposed that some or all these hydrophobic segments span the membrane as α-helices and form the channel through which the anions are transported.

In order to ascertain how the anion exchanger accomplishes the transport of anions across the plasma membrane of erythrocytes, studies have been performed to define the segments of the amino acid sequence that pass through the membrane to form the channel for anions. Several of the hydrophilic segments in the sequence of human erythrocytic anion exchanger have been assigned to one side of the lipid bilayer or the other by various chemical, enzymatic, and immunological strategies, and models for the topology of anion exchanger have been proposed (Wang et al., 1994; Tanner, 1993; Reithmeier, 1993; Passow et al., 1992; Passow, 1986). Despite the assignment of amino acids in the sequence of human erythrocytic anion exchanger to one side of the membrane or the other, the structure of the protein and its mechanism of transporting anions are still poorly understood.

In the studies described in this report, the topological disposition of the hydrophilic region between segments M3 and M4 was investigated by evaluating the accessibility of tyrosine 486 to radioiodination by lactoperoxidase in intact erythrocytes and inside-out vesicles. Previous results have placed the hydrophilic segment containing this tyrosine on the extracytoplasmic side of the bilayer (Wang et al., 1994; Tanner, 1993; Reithmeier, 1993). This inference, however, was based on profiles of hydropathy (Kopito & Lodish, 1985) and results that assigned cysteine 479 (Solomon et al., 1983) and tyrosine 486 (Tanner et al., 1979) as extracellular either

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on the basis of negative observations or on an inference as to the identity of a modified amino acid by the electrophoretic mobility of a large, labeled fragment generated from cleavage of the protein. The results presented here show that tyrosine 486 is exposed on the cytoplasmic surface of human erythrocytic anion exchanger.

EXPERIMENTAL PROCEDURES

Materials. Outdated human blood was obtained from the San Diego Blood Bank. The N^{α}-(fluorenylmethyloxycarbonyl) amino acids (N^{α}-FMOC amino acids)[1] and the *p*-alkoxybenzyl alcohol solid phase were obtained from either Bachem, Inc. or Calbiochem-Novabiochem Corp. Other chemicals purchased from Calbiochem-Novabiochem Corp. included sodium dodecyl sulfate (SDS) and carboxypeptidase Y. Sodium dodecyl sulfate was recrystallized from 95% ethanol (Burgess, 1969). Sodium [^{125}I]iodide (2−25 mCi), carrier and reducing agent free, was purchased from Amersham Life Science or NEN Research Products, Du Pont Co. Amberlite XAD4, bovine serum albumin (BSA), leucine aminopeptidase, phenylmethanesulfonyl fluoride, subtilisin, trypsin that had been treated with *N*-(*p*-tolylsulfonyl)-L-phenylalanyl chloromethyl ketone, and lactoperoxidase from bovine milk [$A_{412}(A_{280})^{-1} = 0.82$] were purchased from Sigma Chemical Co. The proteinase from *Staphylococcus aureus* strain V8 was purchased from Worthington Biochemical Corp. Affigel 10 and Biogel A1.5m were purchased from Bio-Rad Laboratories. Dextran T-70 was purchased from Pharmacia Biotech. Ninhydrin was purchased from Pierce Chemical Co. Trifluoroacetic acid (TFA) was purchased from Halocarbon Products Corp. Freund's adjuvant was purchased from Difco Laboratories. BioSafe II was purchased from Research Products International Corp. Diisopropylcarbodiimide, hydrindantin, 1-hydroxybenzotriazole, *N*-methylpyrrolidinone, and piperidine were purchased from Aldrich Chemical Co.

Synthesis of Peptides. The peptides, KIVGR and LEYIVGR, were synthesized according to the fluorenylmethoxycarbonyl method (Stewart & Young, 1984). The first four cycles, which were identical for the two peptides, were performed in the same vessel. At this point, the solid phase containing the unfinished peptide was split, and synthesis of the two different peptides was completed separately. The first protected amino acid, N^{α}-FMOC-N^{δ}-[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]-L-arginine, was attached to *p*-alkoxybenzyl alcohol solid phase with dicyclohexylcarbodiimide. The following protected L-amino acids were added in order before the resin was split: N^{α}-FMOC-glycine, N^{α}-FMOC-L-valine, and N^{α}-FMOC-L-isoleucine. To complete the synthesis of KIVGR, N^{α}-FMOC-N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine was added. Synthesis of LEYIVGR was completed by adding, in order, N^{α}-FMOC-*O*-butyl-L-tyrosine, N^{α}-FMOC-L-glutamic acid O^{γ}-butyl ester, and N^{α}-FMOC-L-leucine. The peptides were cleaved from the resin in 95:5 TFA:phenol, and the TFA was removed under reduced pressure. The resulting residue was dissolved in water and extracted with diethyl ether. The aqueous phase was lyophilized, and each crude peptide was purified by preparative high-pressure liquid chromatography (HPLC) on an octadecylsilane (C_{18}) column (2.2 cm × 25 cm). To ensure that all of the protecting groups had been removed and that no isopeptide linkages were present, the structures of the purified peptides were verified by amino acid analysis following both acid hydrolysis and exopeptidase digestion with carboxypeptidase Y and leucine aminopeptidase. For KIVGR, the amino acid composition was found to be $K_{1.0}I_{0.6}V_{1.0}G_{1.2}R_{1.2}$ upon acid hydrolysis. Leucine aminopeptidase and carboxypeptidase Y digestions gave the following amino acid compositions: $K_{1.0}I_{1.0}V_{0.9}G_{0.4}R_{0.4}$ and $R_{1.0}G_{1.0}V_{0.9}I_{<0.1}K_{<0.1}$, respectively.

To produce standards with which radioactive peptides isolated from enzymatic digests of radioiodinated anion exchanger could be identified, the peptide LEYIVGR was synthesized as described above. After acid hydrolysis and amino acid analysis, the composition of the purified peptide was $L_{1.1}E_{1.0}Y_{0.9}I_{0.5}V_{0.7}G_{1.0}R_{1.0}$. Further characterization by digestion with carboxypeptidase Y and leucine aminopeptidase gave the following respective amino acid compositions: $R_{1.0}G_{1.1}V_{1.1}I_{1.1}Y_{1.0}E_{0.2}L_{0.2}$ and $L_{1.0}E_{1.0}Y_{1.0}I_{0.9}V_{0.9}G_{0.2}R_{0.2}$. The synthetic peptide LEYIVGR was digested with the proteinase from *S. aureus* strain V8 to generate the unmodified, chromatographic standard, YIVGR. The purified standard ran as a single peak on reverse-phase HPLC with a retention time of 21 min and gave an amino acid composition of $Y_{1.0}I_{0.7}V_{0.9}G_{1.1}R_{1.0}$ after acid hydrolysis.

Preparation of an Immunoadsorbent Specific for −IVGR. Antipeptide immunoglobulins specific for the carboxy-terminal sequence −IVGR were produced from a conjugate of the peptide KIVGR and BSA. Following the method of Kagen and Glick (1979), the purified peptide (15 μmol) was conjugated through its lysyl side chain and its amino terminus to BSA (20 mg in 3 mL) by glutaraldehyde (7 mM). To block any unreacted glutaraldehyde, glycine (80 mM) was added after 30 min. Excess peptide and reagents were removed by dialysis against phosphate-buffered saline: 150 mM NaCl, 0.1 mM ethylenediaminetetraacetate, 20 mM sodium phosphate, pH 7.4 (PBS). By amino acid analysis, it was determined that 18 nmol of peptide had been covalently attached to each nanomole of the carrier. This conjugate was injected subcutaneously and intramuscularly into white New Zealand rabbits initially as a 1:1 suspension of conjugate (0.75 mg mL^{-1}) and Freund's complete adjuvant. Later injections were performed with a similar suspension made with Freund's incomplete adjuvant.

To purify immunoglobulins that recognize the carboxy-terminal sequence −IVGR from the antisera, an affinity adsorbent that had KIVGR attached through its lysyl side chain and amino terminus was constructed. Purified KIVGR (13 mg) was dissolved in 5 mL of 100 mM sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate (NaHEPES), pH 7.5, and 3.5 mL of washed succinylated agarose activated with *N*-hydroxysuccinimide (Affigel 10) was added. The slurry was shaken overnight at 4 °C, and the modified agarose was washed with 100mM pyridinium acetate, pH 5.5. A small portion of the final slurry was treated with carboxypeptidase Y overnight at 37 °C, and amino acid analysis of the products established the nominal capacity to be 50 nmol of epitope (mL of packed agarose)$^{-1}$.

[1] Abbreviations: FMOC, 9-fluorenylmethyloxycarbonyl; SDS, sodium dodecyl sulfate; 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; NaHEPES, sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Affigel 10, succinylated agarose activated with *N*-hydroxysuccinimide; iodo, monoiodinated; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

Fresh rabbit antiserum was passed over this affinity adsorbent to purify the specific immunoglobulins for the construction of an immunoadsorbent that would selectively isolate peptides with the carboxy-terminal sequence −IVGR. After the affinity adsorbent had been washed with PBS, specifically bound immunoglobulins were eluted with 0.1 M sodium phosphate, pH 2.5, and these immunoglobulins were stored as a precipitate after mixing with an equal volume of saturated ammonium sulfate. The precipitated immunoglobulins were concentrated by pelleting them into a thick paste and dialyzing this small volume into PBS. The redissolved immunoglobulins (4 mg in 0.7 mL) were added to 0.4 mL of washed beads of Affigel 10 in 100 mM NaHEPES, pH 7.5, and the slurry was gently stirred overnight at 4 °C. The capacity of this immunoadsorbent was determined by passing a solution containing an excess of synthetic YIVGR over it, washing the column with PBS, and eluting the bound peptide with 0.1 M sodium phosphate, pH 2.5 in 1 mL fractions. The acid eluates were submitted to HPLC, and the isolated peaks of YIVGR were pooled. Amino acid analysis of this pool after acid hydrolysis showed that the immunoadsorbent could bind 6 nmol of a peptide with a carboxy-terminal sequence −IVGR.

Iodination of Peptides. To produce the peptide YIVGR monoiodinated on the phenolic group of tyrosine, the synthetic peptide LEYIVGR was modified with iodide, lactoperoxidase, and hydrogen peroxide (Morrison & Bayse, 1970; Morrison, 1980). Purification of the products of this reaction on reverse-phase HPLC revealed two iodinated peptides, a mono- and a diiodotyrosyl derivative, each with a longer retention time (31 and 34 min, respectively) than the unmodified version (26 min). Digestion of the mono- and diiodinated peptides with leucine aminopeptidase gave the compositions $L_{1.0}E_{1.0}Y_{0.0}I_{0.9}V_{0.9}G_{0.7}R_{0.6}$ and $L_{1.0}E_{1.0}Y_{0.0}I_{0.8}V_{0.8}G_{0.7}R_{0.7}$, respectively. Tyrosine was absent from the amino acid analyses of these digests, and mono- and diiodotyrosine (at mole fractions of 0.7 and 0.3, respectively) appeared at longer, characteristic retention times on the chromatograms. Tyrosine displays a shift in the maximum of its ultraviolet absorbance when it is iodinated (Sun & Dunford, 1993), and the mono- and diiodotyrosyl peptides also showed similar shifts in maximum absorbance to 285 and 290 nm, respectively, from the maximum absorbance 278 nm of the unmodified peptide. These observations indicated that the tyrosine in the peptide LEYIVGR had become a mixture of mono- and diiodotyrosine upon treatment with lactoperoxidase.

Monoiodinated LEYIVGR was digested with the proteinase from *S. aureus* strain V8 to produce the chromatographic standard, Y(iodo)IVGR, which ran as a single peak on HPLC (26 min). The amino acid composition of the purified, modified peptide following acid hydrolysis was $Y_{0.8}I_{0.8}V_{1.0}G_{1.3}R_{1.0}$. Unmodified tyrosine appeared in these hydrolysates because the iodination reverts in the strong acid. Because only a very small percentage of the tyrosines in anion exchanger would be modified in the experiments to be described, it was thought to be unlikely that lactoperoxidase would iodinate the same tyrosines twice; therefore, only a monoiodinated standard was produced for use as an internal standard.

Iodination of Denatured Anion Exchanger. Denatured anion exchanger (30 nmol), after dialysis into 150 mM NaCl, 25 mM sodium phosphate, pH 7.5, was enzymatically radioiodinated with Na[^{125}I]I (2 mCi), lactoperoxidase, and hydrogen peroxide (Morrison & Bayse, 1970; Morrison, 1980). After the last addition of hydrogen peroxide, the iodinated polypeptide was dialyzed into 10 mM NaI, 100 mM ammonium bicarbonate, pH 8.2, to remove excess [^{125}I]-iodine by exchange and prepare the polypeptide for proteolytic digestion.

Iodination of Intact Erythrocytes and Inside-Out Vesicles. The procedure used to iodinate intact erythrocytes and inside-out vesicles was modified from Phillips and Morrison (1971) and Boxer et al. (1974). Ghosts of erythrocytes were prepared by an adaptation of the procedure of Steck et al. (1970). Inside-out vesicles were prepared from ghosts of erythrocytes (Steck et al., 1970; Fairbanks et al., 1971). The sidedness of the inside-out vesicles was confirmed by the accessibility of sialic acid (Steck et al., 1970; Warren, 1959). Inside-out vesicles (prepared from 80 mL of blood) that had been dialyzed into 150 mM NaCl, 25 mM phosphate, pH 7.5, were mixed with washed human erythrocytes (from 30 mL of blood) in a total volume of 40 mL of 150 mM NaCl, 25 mM phosphate, pH 7.5. To the suspension were added lactoperoxidase and KI to final concentrations of 0.6 and 1.0 μM, respectively, and 15 mCi of Na^{125}I. The iodination of the sample was performed by adding of 0.1 M hydrogen peroxide (40 μL) once every 2 min for 40 min. After the iodination was completed, the mixture was layered on a cushion of 15% dextran T-70, 150 mM NaCl, 25 mM sodium phosphate, pH 7.5, and centrifuged in a SW 27 rotor (Beckman Corp.) at 15 000 rpm for 1 h. The mixture was separated into a pellet of erythrocytes and a floating band of inside-out vesicles, and each was collected separately. From this point, the [^{125}I]radioiodinated erythrocytes and inside-out vesicles were treated as separate samples. After washing the [^{125}I]radioiodinated erythrocytes twice in 150 mM NaCl, 25 mM sodium phosphate, pH 7.5, ghosts were prepared as described earlier. The [^{125}I]radioiodinated inside-out vesicles were also washed by diluting them with 30 volumes of the same buffer and sedimenting the suspension in a Ti 45 rotor (Beckman Corp.) at 20 000 rpm for 30 min. Each sample was then dissolved by adding SDS, and the denatured protein free from phospholipid was prepared from both.

Preparation of Anion Exchanger Free of Phospholipid. Ghosts of erythrocytes or inside-out vesicles were dissolved by adding at least 5 mg of SDS for each mg of protein and then applied to a gel filtration column (2.6 cm × 90 cm) of Biogel A1.5m that had been equilibrated with 0.1% SDS, 40 mM tris(hydroxymethyl)aminomethane (Tris) sulfate, pH 8 (Kyte, 1972). Fractions from the column were examined for the polypeptide of anion exchanger by gel electrophoresis in 0.1% SDS on gels cast from 10% acrylamide (Laemmli, 1970), and those fractions containing the polypeptide were pooled and lyophilized. After the sample had been dissolved in a minimum volume of water and made 8 M in urea, the detergent was removed according to the method developed by Weber and Kuter (1971) as modified by Nicholas (1984). Finally, the sample was dialyzed into either 0.1 M ammonium bicarbonate, pH 8.2, in preparation for proteolytic digestion or 150 mM NaCl, 25 mM sodium phosphate, pH 7.5, so that the polypeptide could be iodinated.

Isolation of Peptides by Immunoadsorption and High-Pressure Liquid Chromatography. After the [^{125}I]radioiodinated polypeptides (30 nmol in 8 mL) had been treated

first with the proteinase from *S. aureus* strain V8 followed by trypsin, the digests were passed over the immunoadsorbent. The immunoadsorbent was washed with 40 mL of PBS, and the bound peptides were eluted with 0.1 M phosphate, pH 2.5, in fractions of 2 mL. The acid eluates were submitted to reverse-phase HPLC on an analytical column (Vydac, 0.46 cm × 25 cm) packed with C_{18} reverse-phase medium. The column was run at 1 mL min^{-1} in 0.05% TFA in water and developed with a linear gradient of 1% min^{-1}, from 0 to 40 min, with 0.2% TFA in neat acetonitrile (Mahoney & Hermodson, 1980). The effluent from the column was continuously monitored for absorbance at 229 nm and collected in fractions of 1 mL. The HPLC system included a Spectra-Physics Chromatograph SP8100 with a variable-wavelength Spectra-Physics Spectra 100 UV−vis detector. [^{125}I]Iodine was assessed by dissolving samples in BioSafe II liquid scintillation cocktail and submitting them to liquid scintillation counting on a Beckman LS-1701 scintillation counter.

Enzymatic Digestions and Amino Acid Analysis. [^{125}I]-Radioiodinated polypeptides of anion exchanger stripped of dodecyl sulfate and dialyzed into 0.1 M ammonium bicarbonate, pH 8.2, were digested first with the proteinase from *S. aureus* strain V8 (50−60 μg mL^{-1}) for 6 h at 37 °C followed by digestion with trypsin (50−60 μg mL^{-1}), after adding $CaCl_2$ to 1 mM, overnight at 37 °C. Trypsin was inhibited by adding 1 mg of phenylmethanesulfonyl fluoride. Peptides were digested with subtilisin (50 μg mL^{-1}) or the proteinase from *S. aureus* strain V8 (50 μg mL^{-1}) in 0.1 M ammonium bicarbonate, pH 8.2, for 2 h at 37 °C. Digestions with leucine aminopeptidase (10 μg mL^{-1}) were performed in 2.5 mM $MgCl_2$, 0.1 M Tris sulfate, pH 8.6 for 4 h at 37 °C. Digestions with carboxypeptidase Y (120 μg mL^{-1}) were performed in 0.1 M pyridinium acetate, pH 5.5 for 4 h at 37 °C. Hydrolysis of peptides in acid was performed by heating them in 6 M HCl at 155 °C for 40 min. Amino acid analysis following acid hydrolysis or digestion with leucine aminopeptidase or carboxypeptidase Y was performed on a modular system including a Spectra-Physics autosampler SP8875, a Spectra-Physics HPLC pump SP8800, and a Pickering sodium cation-exchange analytical column with postcolumn derivatization by ninhydrin. Protein concentrations were determined by the method of Lowry et al. (1951) with BSA as the standard.

RESULTS

Isolation of Y(iodo)IVGR from Denatured Anion Exchanger Iodinated by Lactoperoxidase. The amino acid sequence −FFSFCETNGLEYIVGRVWI− comprises positions 475−493 in the complete sequence of human erythrocytic anion exchanger. The following studies focus on the radioiodination of tyrosine 486 within the native protein and digestion of the resulting radioiodinated form of the polypeptide of anion exchanger with the proteinase from *S. aureus* strain V8 and trypsin to produce the peptide Y([^{125}I]iodo)-IVGR, where the abbreviation iodo indicates that the tyrosine is monoiodinated. Initially, the denatured polypeptide of anion exchanger was isolated from membranes of human erythrocytes by gel filtration after the membranes had been dissolved in a solution of SDS (Kyte, 1972). Fractions containing the polypeptide were pooled, and the dodecyl sulfate was removed (Weber & Kuter, 1971; Nicholas, 1984). The denatured anion exchanger was radioiodinated with [^{125}I]-iodide and lactoperoxidase by adding small aliquots of hydrogen peroxide to the mixture, and the sample was then digested with the proteinase from *S. aureus* strain V8 followed by trypsin. The digests were passed over an immunoadsorbent specific for the carboxy-terminal sequence −IVGR and eluted with a low-pH phosphate buffer. The acid eluates were submitted to HPLC, and half of each fraction was submitted to liquid scintillation (Figure 1). In the chromatogram (Figure 1B) of the peptides obtained from the labeled denatured protein, there was a peak of absorbance (peak a) eluting at the same retention time (21 min) as the synthetic standard, YIVGR (Figure 1A). Throughout the description of these experiments, the area of absorbance for this peak will be used as an internal standard to account for differences in yield between samples. The chromatogram of the peptides from the labeled protein also showed a smaller peak of absorbance (peak b) at 26 min that had the same retention time as the modified standard, Y(iodo)IVGR (Figure 1A). Only one major peak of radioactivity (Figure 1C) was obtained from the digest of the labeled, denatured protein, and it had the same chromatographic mobility as the iodinated standard, Y(iodo)IVGR (peak b).

To verify that the peak of radioactivity indeed represented the peptide Y([^{125}I]iodo)IVGR, a small amount of synthetic Y(iodo)IVGR was added to half of the fraction containing peak b (Figure 1B), and the mixture was digested with subtilisin. Synthetic Y(iodo)IVGR was added to the digest because the products of its digestion could be easily followed by absorbance at 229 nm, and they could be used as standards for radioactive products. After the products of the digestion with subtilisin were separated on HPLC, half of each fraction was assayed for radioactivity. If the radioactive iodine had been attached to tyrosine and the product was the peptide Y([^{125}I]iodo)IVGR, then after digestion, the new radioactive peak isolated from modified protein and the new peak of absorbance from the synthetic standard should have coincided during separation by HPLC. The chromatograms show a peak of absorbance (Figure 1D) and a peak of radioactivity (Figure 1E) that coelute at 31 min (peak c). All the smaller peaks of absorbance observed (Figure 1D) were shown to be fragments of subtilisin generated as the enzyme digested itself because they were present in the control sample to which no peptides had been added. In a separate experiment, synthetic YIVGR was digested with subtilisin, and the product migrating at the longer retention time (5 min later) on HPLC was identified as YIV by amino acid analysis, which gave a composition of $Y_{1.1}I_{0.9}V_{1.0}$ after acid hydrolysis. Under the conditions of this digestion with subtilisin, essentially all of the Y([^{125}I]iodo)IVGR isolated from the denatured protein was converted to the product, Y([^{125}I]iodo)-IV. The results of the digestion with subtilisin confirmed that the major radioactive peak (peak b) represents Y([^{125}I]-iodo)IVGR.

Isolation of Y(iodo)IVGR from Native Human Anion Exchanger Iodinated with Lactoperoxidase in Inside-Out Vesicles and Intact Erythrocytes. Inside-out vesicles that had been prepared from human erythrocytes were mixed with an amount of intact human erythrocytes estimated to contain an equivalent amount of anion exchanger. The mixture was radioiodinated with [^{125}I]iodide and lactoperoxidase by adding small aliquots of hydrogen peroxide to the mixture. Under the conditions purposely chosen for the labeling, only a small percentage of the tyrosines present in the protein

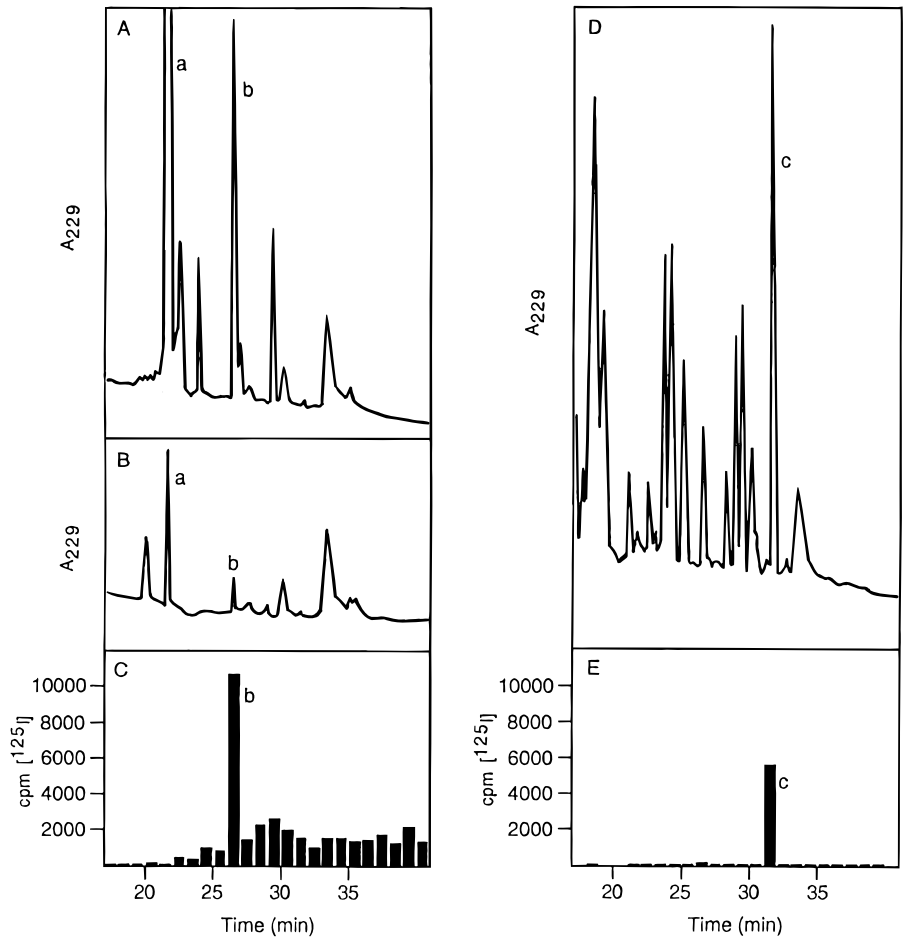

FIGURE 1: Incorporation of [^{125}I]iodine into tyrosine 486 of denatured human anion exchanger. Isolated and denatured polypeptides (about 30 nmol) of anion exchanger were [^{125}I]radioiodinated and then digested first with the proteinase from *S. aureus* strain V8 (50 μg mL^{-1}) followed by trypsin (50 μg mL^{-1}). The digests were passed over the immunoadsorbent specific for the carboxy-terminal sequence −IVGR (6 nmol capacity). The immunoadsorbent was washed with 40 mL of PBS, and bound peptides were eluted with 0.1 M phosphate, pH 2.5, and submitted to C_{18} reverse-phase HPLC. The column (0.46 cm × 25 cm) for HPLC was run at 1 mL min^{-1} in 0.05% TFA in water and developed with a linear gradient of 1% min^{-1}, from 0 to 40 min, with 0.02% TFA in neat acetonitrile. The effluent from the column was continuously monitored for absorbance at 229 nm (A_{229}) and collected in fractions of 1 min. Half of each of these fractions was submitted to liquid scintillation (cpm [^{125}I]). The other half of the one fraction from the chromatogram that contained Y([^{125}I]iodo)IVGR from the labeled protein (peak b) was mixed with synthetic Y(iodo)IVGR and subtilisin (50 μg mL^{-1}). After 2 h, the products of the digestion were submitted to C_{18} reverse-phase HPLC under the same conditions described previously, and each of the fractions from this chromatogram was submitted to liquid scintillation. (A) Chromatogram of a mixture of synthetic peptides, YIVGR (peak a) and YIVGR monoiodinated at its tyrosine (peak b). (B) Chromatogram of peptides isolated by immunoadsorption from [^{125}I]radioiodinated denatured anion exchanger. (C) Radioactivity (cpm [^{125}I]) in fractions from the chromatogram in panel B. (D) Chromatogram of the products of digestion by subtilisin. Peak c contains the fragment Y(iodo)IV. (E) Radioactivity (cpm [^{125}I]) in fractions from the chromatogram in panel D.

were iodinated by the lactoperoxidase. The modified erythrocytes and the modified inside-out vesicles were then separated by differential centrifugation, and after this point the erythrocytes and inside-out vesicles were treated separately so that the extent of radioiodination at tyrosine 486 in the two samples could be compared. From the inside-out vesicles and the erythrocytes, the polypeptides of anion exchanger were purified by gel filtration. Even at this stage, the confinement of the iodination to the extracytoplasmic surface of the intact erythrocytes was readily apparent because the specific radioactivity of the polypeptide of anion exchanger from the inside-out vesicles (43 000 cpm A_{280}^{-1} mL^{-1}) was 20-fold greater than that from intact erythrocytes (2400 cpm A_{280}^{-1} mL^{-1}). Following digestion of the polypeptide with the proteinase from *S. aureus* strain V8 and trypsin, each sample was submitted to immunoadsorption and reverse-phase HPLC. Half of each fraction from the HPLC was assayed for radioactive iodine. The peptides isolated from the inside-out vesicles (Figure 2B) and the intact erythrocytes (Figure 2D) were detected as peaks of absorbance in the two separate chromatograms; the two designated peaks (peaks a) migrated at the same location as synthetic YIVGR (peak a, Figure 2A). These peptides represent in the final samples the majority of the anion exchanger present in the initial samples because only a small fraction (<0.01) of the tyrosines in the protein had been iodinated. These peaks act as internal standards for the yield of the peptides through the various steps between the initial mixtures of cells and vesicles and the final digests. They accounted for all of the peptide YIVGR produced in each digestion because reapplication to the immunoadsorbent of the flow through from the first immunoadsorption produced no further peak of absorbance with this chromatographic mobility. A distinct peak of absorbance with the chromatographic mobility of the iodinated standard peptide Y(iodo)-IVGR at 26 min (peak b, Figure 2A) was not observed in the acid eluates of either sample (Figure 2B,D). Nor was a distinct peak of radioactivity representing Y([^{125}I]iodo)IVGR detected in either sample (Figure 2C,E) after HPLC and scintillation counting; instead, both chromatograms displayed

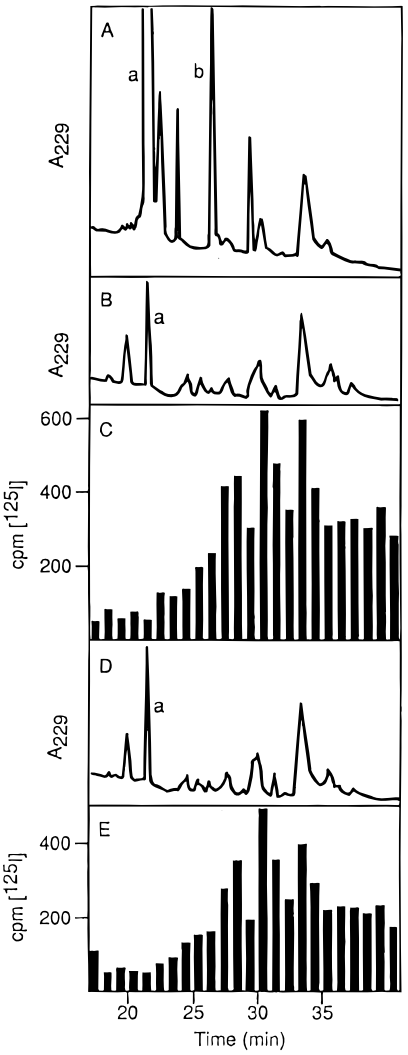

FIGURE 2: Incorporation of [^{125}I]iodine into tyrosine 486 of human anion exchanger in inside-out vesicles and intact erythrocytes. Isolated polypeptides (about 30 nmol of anion exchanger in each sample) of anion exchanger that had been radioiodinated either in inside-out vesicles or intact erythrocytes were digested with the proteinase from *S. aureus* strain V8 (60 μg mL^{-1}) followed by trypsin (60 μg mL^{-1}), and the digests were passed over an immunoadsorbent that recognizes the carboxy-terminal sequence −IVGR (6 nmol capacity). The immunoadsorbent was washed with 40 mL of PBS, the bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5, and the eluates were submitted to C_{18} reverse-phase HPLC. The column (0.46 cm × 25 cm) was run at 1 mL min^{-1} in 0.5% TFA in water and developed with a linear gradient 1% min^{-1}, from 0 to 40 min, with 0.2% TFA in neat acetonitrile. The effluent from the column was monitored continuously for absorbance at 229 nm (A_{229}) and collected in fractions of 1 min. Half of each of these fractions was submitted to liquid scintillation (cpm of [^{125}I]). (A) Chromatogram of a mixture of the synthetic peptides, YIVGR (peak a) and YIVGR monoiodinated at its tyrosine (peak b). (B) Chromatogram of peptides isolated from anion exchanger [^{125}I]radioiodinated in inside-out vesicles. (C) Radioactivity (cpm of [^{125}I]) in fractions from chromatogram in panel B. (D) Chromatogram of peptides isolated from anion exchanger [^{125}I]-radioiodinated in intact erythrocytes. (E) Radioactivity (cpm of [^{125}I]) in fractions from chromatogram in panel D.

a broad pattern of radioactivity. It was possible, however, that some or all of the radioactivity detected at 26 min, the same retention time as the synthetic standard (peak b), represented the peptide Y([^{125}I]iodo)IVGR.

Digestion with subtilisin was used to determine what portion of the radioactivity eluting in the same place as the synthetic standard (peak b) represented the modified peptide Y([^{125}I]iodo)IVGR. This experiment was similar to the one performed on denatured anion exchanger (Figure 1D,E). The other halves of the two fractions containing radioactivity that had migrated at 25 and 26 min were pooled; those from the erythrocytes were pooled separately from those from the inside-out vesicles. Each of the two resulting samples was mixed with a small amount of synthetic Y(iodo)IVGR and digested with subtilisin. After the products of digestion were separated on HPLC, each fraction was assayed for radioactivity. If a portion of the radioactivity that had migrated at 25 and 26 min represented the modified peptide Y([^{125}I]iodo)-IVGR, then, after digestion, a distinct peak of radioactivity that coincided with the peak of absorbance from the internal standard, corresponding to the iodinated fragment Y(iodo)-IV (peak c in Figure 1E), should have appeared. In the sample from the inside-out vesicles, a distinct radioactive peak (peak c in Figure 3B) coeluted with the peak of absorbance (Figure 3A) at 31 min from the fragment Y(iodo)-IV. Even though there had been an almost equivalent peak of unmodified YIVGR in the sample from intact erythrocytes (peak a in Figure 2B), no distinct peak of radioactivity (marked c in Figure 3D) coeluted with the modified fragment Y(iodo)IV (peak c in Figure 3C). The product, Y(iodo)IV, of the digestion of the internal standard can be used in turn as an internal standard to account for any differences between the two samples in the overall yield from the digestion with subtilisin of the fragment Y([^{125}I]iodo)IVGR. In both cases, a significant amount of radioactivity resisted digestion with subtilisin and continued to migrate at its original position; these peaks of radioactive iodine are of undetermined origin. They do not represent undigested Y([^{125}I]iodo)IVGR because, upon digestion with subtilisin, essentially all (>95%) of the added synthetic Y(iodo)IVGR was digested to the product, Y(iodo)IV. This was established from comparison of the experimental chromatograms (Figure 3A,C) with control digests to which no peptides had been added.

Determination of the Location of Tyrosine 486 in Native Human Anion Exchanger of Erythrocytes. By quantifying the amount of the radiolabeled target peptide Y([^{125}I]iodo)-IV (Figure 3) and the amount of the unmodifed peptide YIVGR (Figure 2) on the appropriate chromatograms, the relative incorporation of [^{125}I]iodine into tyrosine 486 for the original iodination of the native protein with lactoperoxidase could be calculated. Because both YIVGR and Y([^{125}I]iodo)IVGR were generated by the same cleavages of the polypeptide of anion exchanger with the proteinase from *S. aureus* strain V8 and trypsin and because the digestion of synthetic Y(iodo)IVGR to Y(iodo)IV was essentially complete with subtilisin (Figure 1E), it was assumed that the yield of Y([^{125}I]iodo)IVGR was directly proportional to the yield of Y([^{125}I]iodo)IV in all samples. The differences in specific radioactivity of the peptides between protein iodinated in intact cells and protein iodinated in inside-out vesicles were then used to estimate the relative accessibility of tyrosine 486 and thus determine on which side of the membrane this amino acid is located. The yield of incorporation of [^{125}I]iodine into Y([^{125}I]iodo)IV from the inside-out vesicles relative to that from the erythrocytes was calculated by dividing the specific radioactivity of the peptide YIVGR isolated from inside-out vesicles by the specific radioactivity of the peptide YIVGR isolated from intact erythrocytes. This calculation was assumed to provide an indirect but accurate measurement of the relative incorpora-

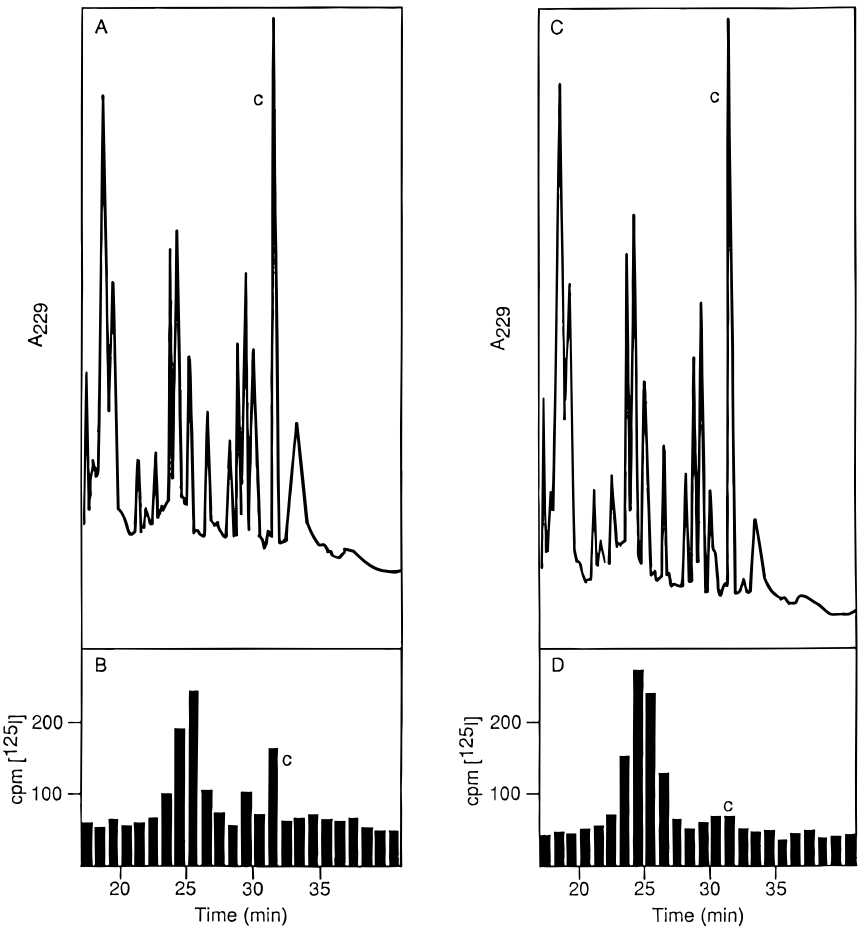

FIGURE 3: Digestion of Y([^{125}I]iodo)IVGR with subtilisin. The other halves of the two fractions containing peptides that migrated at 25 and 26 min (peak b) in the chromatograms shown in Figure 2B,D were pooled to give two different samples; these samples contained Y([^{125}I]-iodo)IVGR from either erythrocytes or inside-out vesicles. The two different pools were each evaporated, redissolved in 0.1 M ammonium bicarbonate, pH 8.2, and mixed with synthetic Y(iodo)IVGR (about 1 nmol) and subtilisin (50 μg mL^{-1}). The products of the digestion were submitted to C_{18} reverse-phase HPLC under the same conditions as described in Figure 2, and each fraction was submitted to liquid scintillation (cpm of [^{125}I]). (A) Chromatogram of a mixture of the synthetic peptide Y(iodo)IVGR and pooled radioactive fractions containing peptides from inside-out vesicles that were digested with subtilisin. (B) Radioactivity (cpm of [^{125}I]) in fractions from chromatogram in panel A. (C) Chromatogram of a mixture of synthetic peptide Y(iodo)IVGR and pooled radioactive fractions containing peptides from erythrocytes that were digested with subtilisin. (D) Radioactivity (cpm of [^{125}I]) in fractions from chromatogram in panel C. Peak c represents the fragment Y(iodo)IV.

tion of [^{125}I]iodine into tyrosine 486 in the native anion exchanger during the initial reaction. In the different preparations, the specific radioactivities have been defined as the counts per minute in peak c, representing Y([^{125}I]iodo)-IV, divided by the area of the absorbance for the peptide YIVGR (peak a), representing the anion exchanger unmodified at tyrosine 486 and hence almost the entire amount. For example, in the experiment shown in Figure 3, the specific radioactivity of the peptide YIVGR isolated from inside-out vesicles was 5.6 cpm $(\text{area}_{229})^{-1}$ and that from intact erythrocytes was $\leq$0.4 cpm $(\text{area}_{229})^{-1}$. Basing the specific radioactivities on the areas of the unmodified peptides corrects for the different amounts of anion exchanger in the erythrocytes and the vesicles and for the different yields of the final fragment in the two samples during labeling, digestion, immunoadsorption, and chromatography. The relative incorporation of [^{125}I]iodine into tyrosine 486 in the inside-out vesicles was $\geq$17 in this experiment.

Two other experiments (Table 1) were performed that were identical except for one difference: in these other experiments, the acid eluates from the immunoadsorbent were neutralized, passed over the immunoadsorbent again, eluted with acid, and then submitted to HPLC. The second pass over the immunoadsorbent was an attempt to reduce nonspecific radioactivity further, so that the peptide Y([^{125}I]iodo)-IVGR, instead of its fragment, could be used to assess the

Table 1: Relative Specific Radioactivities of Y([^{125}I]iodo)IV from Inside-Out Vesicles and Intact Erythrocytes[a]

	inside-out vesicles		erythrocytes		[(cpm IOV[d])(area RBC[e])]/
expt	cpm[b]	area[c]	cpm	area	[(cpm RBC)(area IOV)]
1[f]	97	34	$\leq$8	48	$\geq$17
2[g]	84	15	$\leq$7	10	$\geq$8
3[g]	195	64	22	40	6

[a] The counts per minute of Y([^{125}I]iodo)IV (peaks c, Figure 3) and the areas of the peaks of absorbance at 229 nm associated with the unmodified peptide YIVGR (peaks a, Figure 2) were determined from experiments similar to those described in the legends to Figures 2 and 3. [b] These are the counts per minute in the peptide Y([^{125}I]iodo)IV (peak c, Figure 3) above background. The background was assumed to be the average of the counts per minute in fractions to both sides of peak c. [c] Areas of the peaks of absorbance (peaks a, Figure 2) are given in millimeters^2 of chart paper. [d] Inside-out vesicles. [e] Erythrocytes. [f] This is the experiment displayed in Figures 2 and 3. [g] For these two experiments, the digests were passed over the immunoadsorbent and eluted with low pH, and these eluates were neutralized, passed over the immunoadsorbent a second time, eluted again with low pH, and then submitted to HPLC.

relative incorporation. Although this improved the chromatogram somewhat, the digestion with subtilisin was still required to produce an unambiguous peak of radioactivity. When inside-out vesicles and intact cells were [^{125}I]radioiodinated in the same suspension, tyrosine 486 was modified to at least a 6-fold greater specific radioactivity in the inside-out vesicles than it was in the intact erythrocytes (Table 1). In some of the experiments very little radioiodination, if any, could be detected at this tyrosine in the sample from intact cells. This lack of incorporation further indicated how tightly sealed the cells were to the iodinating agent. Because tyrosine 486 was accessible to the iodinating agent when the protein was labeled in inside-out vesicles but not when it was labeled in intact erythrocytes, even though the intact erythrocytes were present in the same vessel, it must be exposed on the cytoplasmic surface of anion exchanger.

DISCUSSION

The most recent models of anion exchanger have placed the hydrophilic segment containing tyrosine 486 on the extracytoplasmic side of the bilayer (Wang et al., 1994; Tanner, 1993; Reithmeier, 1993; Passow et al., 1992). This assignment was based on profiles of hydropathy (Kopito & Lodish, 1985) and on results that assigned cysteine 479 (Solomon et al., 1983) and tyrosine 486 (Tanner et al., 1979) as extracellular. The reagent, *p*-chloromercuribenzenesulfonate, has been reported to react with cysteine 479 in intact erythrocytes and block the binding of stilbene disulfonates, which are inhibitors of anion transport used to define the site recognizing the anions (Lepke et al., 1976; Shami et al., 1978), after the other five cysteines had been protected with *N*-ethylmaleimide (Solomon et al., 1983). On the basis of this inhibition of binding, cysteine 479 was tentatively assigned to the extracytoplasmic surface of the membrane. An intracellular site of iodination (Williams et al., 1979) has been identified on a hydrophobic fragment about 157 amino acids in length that begins around isoleucine 397, the amino terminus of the membrane-spanning domain (Lepke & Passow, 1976; Grinstein et al., 1978; Brock & Tanner, 1986) and ends at the major extracellular chymotryptic site at tyrosine 553 (Cabantchik & Rothstein, 1974; Steck et al., 1978; Markowitz & Marchesi, 1981). An additional site of iodination has also been located on the same fragment, and the residue modified in this reaction can be labeled only from the extracellular face of the membrane (Tanner et al., 1979). Although tyrosine 486 is found within this 157 amino acid fragment (Tanner et al., 1988), it could not be definitively assigned as one or the other of these sites of iodination because this fragment, which is long enough to span the membrane more than once, contains five tyrosines in its sequence. Because the [^{125}I]radioiodinated fragment of the protein was at the time identified only by its electrophoretic mobility, no information about the location of the sites of the iodination within the fragment was obtained. The location of this hydrophilic segment, therefore, has remained in doubt mainly because the modification of specific amino acids in the protein has not been adequately defined whenever probes were applied vectorially.

In the present studies, site-directed immunochemistry was used to show that tyrosine 486 lies on the cytoplasmic surface of native anion exchanger. This technique was originally developed for evaluating the accessibility of specific amino acids in the sequence of a large protein (Kyte et al., 1987), and it has been applied successfully to topological studies of Na^+/K^+-transporting ATPase (Kyte et al., 1987; Thibault, 1993; Anderberg, 1995) and acetylcholine receptor (Dwyer, 1988, 1991; Ewalt, 1994).

Essential to this experimental strategy, when it is applied to studying the topology of anion exchanger, is a reagent that is specific for the functional group of only one particular type of amino acid and that cannot pass through the barrier provided by the plasma membranes of the erythrocytes. Lactoperoxidase, a high molecular weight protein obtained from bovine milk, has often been used to catalyze the iodination of tyrosine residues on anion exchanger (Morisson & Bayse, 1970; Morrison, 1980), even though the exact identity of the iodinating species generated when lactoperoxidase, iodide, and hydrogen peroxide are mixed together is unclear (Morrison & Schonbaum, 1976; Sun & Dunford, 1993; Huwiler et al., 1985; Huber et al., 1989). The conditions that have been established before and those that were used here, however, have been shown in both intact erythrocytes and sealed vesicles prepared from rough microsomes of rat liver to iodinate only the proteins on the exposed, exterior surface of the respective membranes while neither the intracellular hemoglobin nor the soluble components inside the microsomes were significantly labeled (Phillips & Morrison, 1970; Hubbard & Cohn, 1972; Kreibich et al., 1974). The control against the possibility that the signal produced by the radioiodination might result from nonenzymatic, membrane permeable iodination is provided by simultaneous modification of the cells and the vesicles in the same vessel. In my experiments, the confinement of the iodination to the exterior surface of the intact erythrocytes was readily apparent during isolation of the polypeptides of anion exchanger by gel filtration since the specific radioactivity of the polypeptide from inside-out vesicles was 20-fold greater than that from intact erythrocytes. In addition, the plasma membranes of the erythrocytes were not extensively damaged since very little or no hemoglobin leaked from them during the iodination. From these considerations, it was concluded that the iodinating agent produced by lactoperoxidase, although its properties are still uncertain, could not effectively label the interior surface of anion exchanger either in intact erythrocytes or in the inside-out vesicles and could be considered impermeant under these conditions.

To determine on which side of the membrane tyrosine 486 is located, intact erythrocytes and inside-out vesicles prepared from the erythrocytes were [^{125}I]radioiodinated with lactoperoxidase in the same reaction vessel and later separated by differential centrifugation. The modified protein from each population was analyzed independently by cleaving and purifying the modified target peptide Y([^{125}I]iodo)IVGR from the polypeptide of anion exchanger and quantifying the relative amounts of [^{125}I]iodine that had been incorporated into the tyrosine during the labeling of the native protein. In contrast to the results obtained from the labeling experiment with the denatured protein, the samples from both the intact cells and the inside-out vesicles revealed a broad pattern of radioactive peaks in the chromatograms of the peptides eluted from the immunoadsorbent and submitted to HPLC (Figure 2). From these chromatograms alone, it was not possible to determine what portion of the radioactivity coeluting with the synthetic Y(iodo)IVGR represented incorporation of [^{125}I]-iodine into the tyrosine of the target peptide. In the

experiments with the radioiodinated denatured protein, a clean peak of radioactivity representing Y([^{125}I]iodo)IVGR was isolated in high yield from the proteolytic cleavage. This result indicates that, with the native protein, it was not problems in the digestion or the isolation of Y([^{125}I]iodo)-IVGR by immunoadsorption but a difficulty in labeling tyrosine 486 in the native protein that caused the low yield of Y([^{125}I]iodo)IVGR. The problem of the high background of radioactivity concealing the small signal from the [^{125}I]-iodine on Y(iodo)IVGR could have arisen in the following way. During the labeling of the native protein, tyrosine 486 must compete with a large number of other potential sites for iodination in the reaction vessel; and, if this tyrosine is significantly less reactive than a large portion of these other sites, as the low yield of radioactivity suggests, then the small signal from the incorporation of [^{125}I]iodine into this tyrosine would be obscured by a higher degree of incorporation of [^{125}I]iodine into other sites, which would appear as a high background of radioactivity in the chromatogram. In previous experiments using site-directed immunochemistry (Dwyer, 1988, 1991; Thibault, 1993; Ewalt, 1994; Anderberg, 1995), purification of the radiolabeled target peptide from the native protein by immunoadsorption was sufficient to observe a clean peak of radioactivity on HPLC. In the present experiments, however, contaminating peptides, which were present in small amounts but possessed a high degree of labeling with [^{125}I]iodine, were able to conceal the signal associated with Y([^{125}I]iodo)IVGR. To determine what portion of the radioactivity represented incorporation at tyrosine 486, the radioactivity coeluting with the synthetic Y(iodo)IVGR was mixed with synthetic Y(iodo)IVGR and digested with subtilisin (Figure 3). Upon resubmission to HPLC, the products of this cleavage appeared as a broad peak of radioactivity resistant to proteolysis and, only in the sample purified from inside-out vesicles, as a peak of radioactivity migrating at a later time and coeluting with Y(iodo)IV, the proteolytic product of the modified standard. The peak of radioactivity appearing at the longer retention time is the entire signal associated with incorporation of [^{125}I]-iodine in the tyrosine of YIVGR because the digestion with subtilisin was essentially complete and because this portion of the radioactivity moved to the characteristic retention time of Y(iodo)IV.

Within the region containing the hydrophobic segments M1−M5 in the sequence of human erythrocytic anion exchanger, three hydrophilic segments have been assigned to one side of the lipid bilayer or the other. That the hydrophilic segment to the amino-terminal side of segment M1 is cytoplasmic has been established from the intracellular sites of chymotryptic or tryptic digestion at tyrosine 359 or lysine 360, respectively (Mawby & Findlay, 1982). Lysine 430 between segments M1 and M2 can be either reductively methylated with impermeant sodium [^{3}H]borohydride (Jennings & Nicknish, 1984) or labeled with impermeant eosin-5-maleimide (Cobb & Beth, 1990) in intact erythrocytes and has been assigned to the extracytoplasmic surface. Because the amino terminus of the membrane-embedded domain is on the cytoplasmic side of the native protein and lysine 430 is on the extracytoplasmic side, segment M1 must cross the lipid bilayer. To the carboxy-terminal side of segment M5, the hydrophilic segment surrounding glutamine 564 in the protein is quite susceptible to a variety of proteinases from the external surface of intact erythrocytes (Jennings & Adams, 1981; Jennings et al., 1984; Cabantchik & Rothstein, 1974; Steck et al., 1978; Markowitz & Marchesi, 1981; Brock et al. 1983) and has, therefore, been assigned to the extracytoplasmic surface of the protein.

The present assignment of tyrosine 486 of human anion exchanger as cytoplasmic provides new topological information about the disposition of the polypeptide as it is embedded in the lipid bilayer between segments M1 and M5. On the amino-terminal side of tyrosine 486 between it and extracellular lysine 430 (Jennings & Nicknish, 1984; Cobb & Beth, 1990) lie segments M2 and M3 in a long, hydrophobic stretch of forty-four amino acids between methionine 435 and phenylalanine 478 (Figure 4). In the middle of this stretch are glutamine 457 and proline 458, hardly a hydrophilic segment. If it is assumed that twenty amino acids folded into an α-helix are necessary to cross the lipid bilayer once, this entire stretch of the polypeptide must contain only one segment that traverses the membrane because it is too short to span the membrane three times. In fact, the central twenty amino acids in this stretch, from alanine 452 to phenylalanine 471 have an average hydropathy (1.9) that is significantly greater than that of either segment M2 (1.6) or segment M3 (1.5) and could form the single membrane-spanning segment in this region (Kyte & Doolittle, 1982). In addition, further support for the revised assignment of the second membrane-spanning segment of anion exchanger to these positions comes from alignment of the amino acid sequences of different members from the family of anion exchangers (Figure 4). Comparison of segments M2 and M3 for the different anion exchangers reveals considerable evolutionary variation in the sequences of segment M2 but extensive conservation in the sequences of segment M3. The carboxy-terminal portion of segment M3, however, has polar amino acids at three of the last seven positions within its consensus sequence, and one of these three positions shows a high degree of variation. I have assumed that those stretches of amino acids that are hydrophobic enough to reside in the membrane and that display the highest degree of sequence identity among the different members of the family of anion exchangers are the segments that actually do span the lipid bilayer. On the basis of these criteria, the revised second membrane-spanning segment is proposed to contain the amino acids stretching from alanine 452 to phenylalanine 471.

There is a similar situation to the carboxy-terminal side of tyrosine 486. Segments M4 and M5 lie between the extracellular site (surrounding glutamine 564) on the protein that is susceptible to various proteinases in intact cells (Jennings & Adams, 1981; Jennings et al., 1984; Cabantchik & Rothstein, 1974; Steck et al., 1978; Markowitz & Marchesi, 1981; Brock et al. 1983) and this intracellular tyrosine. This part of the polypeptide is fifty-four amino acids in length and should cross the membrane only once because, again, it seems too short to span it three times. This long segment is of particular interest because it contains lysines 539 and 542. Although each remained a candidate for a time, lysine 539 was recently identified as the site (Bartel et al., 1989a,b; Okubo et al., 1994) that can react covalently with isothiocyanate groups on derivatives of stilbenedisulfonates but only from the outside of the erythrocyte when the transport site is facing outward (Kaplan et al., 1976; Barzilay & Cabantchik, 1979). Because stilbenedisulfonates are potent inhibitors of anion transport (Lepke

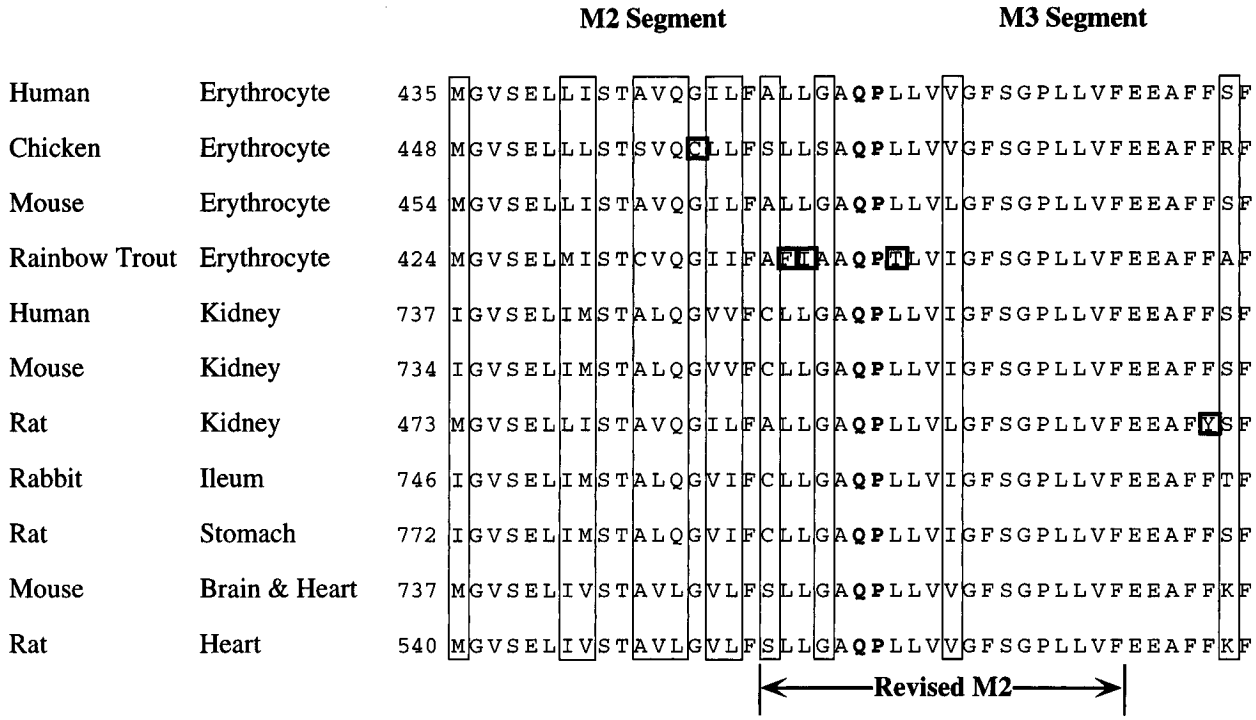

FIGURE 4: Alignment of the amino acid sequences for the M2 segment and the M3 segment in different members of the family of anion exchangers. The sequences of anion exchangers from erythrocytes of humans (Tanner et al., 1988), chickens (Kim et al., 1988), mice (Kopito & Lodish, 1985), and rainbow trout (Huebner et al., 1992); anion exchangers from kidney of humans (Gehrig et al., 1992), mice (Alper et al., 1988), and rats (Kudrycki & Shull, 1989); anion exchangers from gastrointestinal tract of rabbits (Chow et al., 1992) and rats (Kudrycki et al., 1990); anion exchangers from heart of mice (Kopito et al., 1989) and rats (Linn et al., 1992); and anion exchanger from brain of mice (Kopito et al., 1989) were aligned over their respective M2 and M3 segments. The short, intervening hydrophilic segment, −QP−, proposed to divide the displayed sequences into the two potential membrane-spanning segments is in boldface type. The number of the position of the first amino acid for each M2 segment is designated to the left of its respective sequence. Boxed regions indicate positions at which there is substantial variation in the identity of the amino acid in its aligned position among the different members of the family of anion exchangers. A single amino acid enclosed in a bold square indicates a variation in the identity of the amino acid at that position for only one member of the family. At the bottom of the figure, the revised M2 segment is demarcated by the two arrowheads.

& Passow, 1976; Shami et al., 1978), it is thought that this lysine is in the vicinity of the channel responsible for the transport of anions. Neither of these lysines, however, is itself directly involved in the function of the protein because site-directed mutation of either one or both of them has little if any effect on anion transport (Garcia & Lodish, 1989; Bartel et al., 1989b; Wood et al., 1992). Therefore, the intuition which was prevalent before these site-directed mutations were performed, that these lysines formed part of the channel for the anions and consequently were buried in the membrane, is probably incorrect. In fact, segment M5 with an average hydropathy of 1.5 over its twenty residues may not even be a membrane-spanning segment (Kyte & Doolittle, 1982). At this time, segment M4 with an average hydropathy of 2.5 over its twenty residues would appear to be the most likely candidate to traverse the lipid bilayer because it is so much more hydrophobic. This assignment would leave segment M5 lying on the extracellular face of native human anion exchanger. Lysine 539 could be positioned at the rim of the channel for anions where it could react with an isothiocyanate group of derivatives of stilbene-disulfonates.

Of importance to understanding anion transport through the plasma membrane of erythrocytes is a knowledge of the topology of the folded polypeptide as it is embedded in the lipid bilayer. By using site-directed immunochemistry, the location of tyrosine 486 was assigned to the cytoplasmic surface of native anion exchanger. Other segments of the amino acid sequence in the amino-terminal portion of the membrane-embedded domain have been previously assigned to one side of the bilayer or the other by various experimental strategies involving modification and cleavage of the protein. From all of these results, it can be concluded that the polypeptide, starting at its cytoplasmic point of entry, spans the membrane three times, instead of the five times initially predicted by plots of hydropathy, before it reaches the extracellular region around glutamine 564.

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