Lysine-691 of the anion exchanger from human erythrocytes is located on its cytoplasmic surface

Hans K. ERICKSON¹ and Jack KYTE

Department of Chemistry, 0506, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0506, U.S.A.

A combination of vectorial modification and site-directed immunochemistry has been used to determine the disposition, with respect to the membrane, of Lys-691 of the anion exchanger from human erythrocytes. Intact erythrocytes and inside-out vesicles were vectorially modified in the same container with pyridoxal phosphate and sodium [³H]borohydride. The modified inside-out vesicles were separated from erythrocytes by differential centrifugation and the vesicles and erythrocyte membranes were treated with alkali and digested with trypsin and thermolysin to liberate the peptides IVSKPER and IVSK{N^ε-[4'-(5'-phospho-[4-³H]pyridoxyl)]}PER. These peptides, containing

the unmodified and modified versions of Lys-691, were retrieved from the digests by site-directed immunochemistry and were identified by HPLC and liquid scintillation spectroscopy. Both the inside-out vesicles and the intact erythrocytes contained the peptide IVSKPER, however, the ³H-label from the phosphopyridoxylated peptide could be detected only in the inside-out vesicles. The incorporation of ³H into Lys-691 of the anion exchanger from inside-out vesicles was at least 30-fold greater than the incorporation into Lys-691 of the anion exchanger from intact erythrocytes. It follows that Lys-691 of the anion exchanger is located on the cytoplasmic surface of the plasma membrane.

INTRODUCTION

The anion exchanger (Band 3 or AE1), a membrane-spanning glycoprotein of human erythrocytes, catalyses the exchange of chloride and bicarbonate across the plasma membrane of the cell. Physiologically, this anion exchange facilitates the transport of carbon dioxide from the peripheral tissues to the lungs. The complete amino acid sequence of human anion exchanger has been determined from the cDNA [1]. The polypeptide that folds to produce the protein is 911 amino acids long and contains two distinct domains. The first 360 amino acids form a hydrophilic, cytoplasmically located domain that binds ankyrin, haemoglobin and glycolytic enzymes [2]. The last 551 amino acids constitute a hydrophobic domain that spans the membrane to form the site through which anions are transported. Mild proteolysis of the native protein with a variety of proteinases removes the cytoplasmic domain and leaves the intact membrane-spanning, C-terminal domain with the ability to transport anions [3].

Plots of hydropathy [4] initially identified 12 potential membrane-spanning hydrophobic segments of at least twenty amino acids in length, designated M1 to M12, that are distributed throughout the membrane-spanning domain. Subsequent experiments [5] have shown that Lys-851, near the centre of the last hydrophobic segment M12, is extracytoplasmic, therefore, this segment must be split into two, M12 and M13. The C-terminus of the protein is located on the cytoplasmic side of the membrane, therefore, the C-terminal domain of the protein spans the membrane an even number of times to form the pathway the ions follow through the protein.

Current topological models of the anion exchanger contain up to 14 membrane-spanning segments but direct experimental evidence exists for only a few of these assignments. One approach to establishing whether or not one of these segments spans the membrane is to determine the disposition, relative to the membrane, of amino acids in the hydrophilic regions before and after that segment. This has been accomplished in several of these

regions by chemical labelling [5–11], proteolytic digestion [12,13] or immunological techniques [14–16]. Natural markers on the protein, such as glycosylation, have also been used to define the disposition of amino acids. The anion exchanger contains one site of glycosylation at Asp-642 [16], located in the hydrophilic region preceding the hydrophobic segment M8. This observation localizes this region to the extracytoplasmic surface of the native protein. The disposition of the hydrophilic region of amino acids following the hydrophobic segment M8 was previously unknown. Therefore although the hydrophobic segment M8, on the basis of its hydropathy, has the potential to form a membrane-spanning segment, no experimental evidence to confirm this possibility has yet been reported. In the present study, the disposition of Lys-691, contained in the hydrophilic region between hydrophobic segments M8 and M9, was shown to be on the cytoplasmic surface of native anion exchanger by a combination of vectorial modification and site-directed immunochemistry. Therefore the hydrophobic segment M8 must span the bilayer.

MATERIALS AND METHODS

Materials

Human blood that had exceeded the expiry date was obtained from the San Diego Blood Bank. The N^ε-fluoren-9-ylmethoxycarbonyl (Fmoc) amino acids, thermolysin, 1-hydroxybenzotriazole hydrate, carboxypeptidase B and *p*-alkoxybenzyl solid phase was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, U.S.A.). Sodium [³H]borohydride (220 mCi·mmol⁻¹) was purchased from NEN Research Products (Boston, MA, U.S.A.). BSA, carboxypeptidase Y, leucine aminopeptidase, trypsin treated with tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl), Sephadex G-25, acetylthiocholine chloride, pyridoxal phosphate, glyceraldehyde 3-phosphate, β-NAD⁺, sodium arsenate, Triton X-100 and soybean trypsin inhibitor were purchased from Sigma Chemical Co. 1,3-Diisopropylcarbodiimide, 1,3-dicyclohexyl-

Abbreviations used: C₁₈, octadecylsilyl silica gel; pyr, N^ε-[4'-(5'-phosphopyridoxyl)]; [³H]pyr, N^ε-[4'-(5'-phospho-[4-³H]pyridoxyl)]; Fmoc, fluoren-9-ylmethoxycarbonyl; a.m.u., atomic mass units.

¹ To whom correspondence should be addressed (e-mail herickso@ucsd.edu).

carbodiimide, *N*-methylpyrrolidinone, ethanedithiol, hydrindantin, dimethylaminopyridine, acetic anhydride and piperidine were purchased from Aldrich Chemical Co. 1-Methyl-2-pyrrolidinone was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Trifluoroacetic acid was purchased from Halocarbon Products Co. (Hackensack, NJ, U.S.A.). Ninhydrin and *N*-[(*m*-maleimidobenzoyl)hydroxy]sulphosuccinimide were purchased from Pierce Chemical Co. Econo-Safe scintillation cocktail was purchased from Research Products International (Mount Prospect, IL, U.S.A.). Affigel 10 and Affigel 102 were from Bio-Rad Laboratories. Freund's adjuvant was purchased from Difco Laboratories (Detroit, MI, U.S.A.).

Synthesis of peptides

The peptides *N*-acetylIVSKPER and IVSCPER were synthesized by solid phase Fmoc methods [11,17]. *N*^z-Fmoc-*N*³-[(4-methoxy-2,3,6-trimethylphenyl)sulphonyl]-L-arginine was attached to the *p*-alkoxybenzyl solid phase with either diisopropylcarbodiimide or dicyclohexylcarbodiimide. The preformed hydroxybenzotriazole esters [17] of each of the following protected amino acids were then added sequentially to complete each synthesis: *N*^z-Fmoc-L-glutamic-*O*^γ-butyl ester, *N*^z-Fmoc-L-proline, *N*^z-Fmoc-*N*^ε-(*tert*-butyloxycarbonyl)-L-lysine or *N*^z-Fmoc-*S*-(triphenylmethyl)-L-cysteine, *N*^z-Fmoc-*O*^β-butyl-L-serine, *N*^z-Fmoc-L-valine and *N*^z-Fmoc-L-isoleucine. Acetylation was performed in 1-methyl-2-pyrrolidinone containing four equivalents of acetic anhydride and two equivalents of *p*-dimethylaminopyridine. The products, *N*-acetylIVSKPER and IVSCPER, were cleaved from the solid phase and all protecting groups were removed by treatment with trifluoroacetic acid/phenol/water/ethanedithiol/thioanisole (86:5:4:3:2, by vol.) for 12 h (IVSCPER) or trifluoroacetic acid/thioanisole (95:5, v/v) for 4 h (*N*-acetylIVSKPER). Trifluoroacetic acid was removed under reduced pressure and the remaining residue was dissolved in 10% (v/v) acetic acid and extracted with diethyl ether. The aqueous phase was freeze-dried and the crude peptides were purified by reverse-phase HPLC. The two purified peptides were hydrolysed in 300 μl of 6 M HCl and their amino acid compositions were determined. Enzymic digestion followed by amino-acid analysis was also used to ensure that the removal of all protecting groups from the peptides was complete. All amino acid compositions were in agreement with the expected values.

Modification of *N*-acetylIVSKPER with pyridoxal phosphate and sodium cyanoborohydride

The peptide *N*-acetylIVSK(pyr)PER, where pyr indicates *N*^ε-[4'-(5'-phosphopyridoxyl)] modification of the lysine, was prepared as described by Thibault [18]. The modified peptide was digested with thermolysin to remove *N*-acetyllysine and the product IVSK(pyr)PER was purified from the digest by HPLC.

Preparation of immunoadsorbent

Rabbit anti-peptide antisera [19] 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 boosting injections with incomplete Freund's adjuvant and complete Freund's adjuvant after 2 weeks and 1 month respectively. The haptenic conjugates were made by coupling the peptide IVSCPER through its cysteine residue to the amino groups on BSA with the bifunctional cross-linker, *N*-[(*m*-maleimido-benzoyl)hydroxy]sulphosuccinimide [20], as described by Ewalt [21]. Acid hydrolysis and subsequent amino acid analysis of the haptenic conjugate revealed that it contained

10 nmol of IVSCPER per nmol of BSA. The synthetic peptide IVSCPER was also used to prepare a peptide affinity column [21] to isolate immunoglobulins capable of recognizing the N-terminal and C-terminal sequences IVS- and -PER. It was found by amino acid analysis that 2 μmol of peptide · ml⁻¹ had been coupled to the solid phase. Antisera from rabbits immunized with the conjugate 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. An immunoadsorbent was prepared from these affinity-purified immunoglobulins as described by Ewalt [21]. The amount of peptide that this immunoadsorbent was capable of binding was determined by passing the peptide IVSKPER (40 nmol) over the column, washing with PBS and eluting the bound IVSKPER 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 IVSKPER was hydrolysed and subjected to amino acid analysis. The immunoadsorbent (3 ml) had a capacity sufficient to bind one nmol of the peptide. The immunoadsorbent was also found to bind the synthetic peptide IVSK(pyr)PER.

Modification of intact erythrocytes and inside-out vesicles with pyridoxal phosphate and sodium [³H]borohydride

Inside-out vesicles were prepared by the method of Steck and Kant [22]. The sidedness of the vesicles was assessed by assaying for acetylcholinesterase [23] and glyceraldehyde-3-phosphate dehydrogenase [22]. The accessibility of acetylcholinesterase was routinely estimated for each preparation of inside-out vesicles, and 37 ± 9% of the acetylcholinesterase was accessible in the absence of detergent. For two preparations of inside-out vesicles, the accessibility of glyceraldehyde-3-phosphate dehydrogenase in the absence of detergent was also determined and was found to be 87 ± 5%. Inside-out vesicles (prepared from 50 ml of blood) in 8 ml of 150 mM NaCl/20 mM sodium phosphate, pH 8.0, were added to 11 ml of washed erythrocytes (from 20 ml of blood) in the same buffer. Pyridoxal phosphate and sodium borate were added to the mixture so that the final concentrations were 12 mM pyridoxal phosphate, 20 mM sodium phosphate and 30 mM sodium borate, pH 8.0, in 20 ml of solution [24,25]. The reaction was allowed to proceed for 15 min at 37 °C. Sodium [³H]borohydride (25 mCi, 220 mCi · mmol⁻¹) in 0.2 ml of 0.01 M NaOH was added to reduce the imine and after 30 min at room temperature the modified inside-out vesicles were separated from the modified erythrocytes by differential centrifugation as described by Kalo [10].

Digestion of membranes and immunoadsorption

The peptides IVSKPER and IVSK(pyr)PER were released from membranes with trypsin and thermolysin by a modification of the method used by Kang et al. [26]. Membranes from either the modified erythrocytes [27] or the modified inside-out vesicles were suspended in 50 vol. of 0.1 M NaOH, on ice, for 30 min. The membranes that had been stripped with alkali were sedimented by centrifugation at 100 000 *g* for 20 min (Beckman, model L7-55 equipped with a Ti 50.2 rotor) and washed once with 5 mM sodium phosphate, pH 8. They were then digested with trypsin (0.4 mg · ml⁻¹) in 0.2 mM CaCl₂/50 mM ammonium bicarbonate, pH 7.8, for 4 h. Soybean trypsin inhibitor (0.8 mg · ml⁻¹) was added and the membranes were digested with thermolysin (0.4 mg · ml⁻¹) for 4 h and sedimented as before. Thermolysin was inhibited with one equivalent of phosphoramidone and 2 mM EDTA and the digests were passed over the immunoadsorbent specific for peptides with the N-terminus IVS-

and the C-terminus -PER. The immunoadsorbent was washed with 45 ml of PBS and the specifically bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5.

Enzymic digestions

Enzymic digestion of peptides with leucine aminopeptidase (0.007 units·ml⁻¹) was performed in 2.5 mM MgCl₂/0.1 M Tris/SO₄, pH 8.6, for 4 h at 37 °C. The digestion of peptides with carboxypeptidase Y (8 units·ml⁻¹) was performed in 0.1 M pyridinium acetate, pH 5.5, for 4 h at 37 °C. Peptides were digested with thermolysin (0.4 mg·ml⁻¹) in 0.2 mM CaCl₂ and 50 mM ammonium bicarbonate, pH 7.8, for 1 h at 37 °C. Modified peptides were digested with carboxypeptidase B (3 units·ml⁻¹) in 100 mM sodium phosphate, pH 3.7, for 1 h at 37 °C.

Analytical methods

Peptides were purified by HPLC using either an SP8100 system equipped with a variable wavelength Spectra 100 UV-vis detector (Spectra-Physics Chromatography) or a system consisting of two M6000A pumps (Waters) with an automatic gradient controller (model 680, Waters), an octadecylsilyl silica gel (C₁₈) column and an absorbance detector (model 44, Waters) fitted with an extended-wavelength module operating at 229 nm. Radioactivity (c.p.m.) was determined after mixing samples with an equal volume of Econo-Safe liquid scintillation cocktail by scintillation spectroscopy (Beckman LS 233). Peptides were hydrolysed in sealed, evacuated glass hydrolysis tubes containing 6 M HCl for 45 min at 160 °C and the hydrolysates were analysed using a modular system composed of an autosampler (SP8875, Spectra-Physics), a ternary pump (SP8800, Spectra-Physics) and a sodium cation-exchange column (Pickering). Amino acids were quantified as their ninhydrin derivatives. Densitometry was performed using a 2202 Ultrosan (LKB, Bromma, Sweden).

RESULTS

Location of Lys-691 in intact anion exchanger

Inside-out vesicles were mixed with intact erythrocytes estimated to contain an equivalent amount of the anion exchanger. The vesicles and the cells were modified in the same container with pyridoxal phosphate and sodium [³H]borohydride. The inside-out vesicles were separated from the erythrocytes by differential centrifugation [10]. Membranes made from the erythrocytes and the inside-out vesicles were each stripped with alkali [26] and digested with trypsin and thermolysin. Peptides containing the N-terminal and C-terminal sequences IVS- and -PER were retrieved from the digests by immunoadsorption and submitted to reverse-phase HPLC on a C₁₈ analytical column.

A peak of absorbance (peak a, Figure 1A) was observed in the sample from intact erythrocytes. This peak had the same retention time as the synthetic standard IVSKPER (16 min). The fraction associated with this peak was found to have the amino acid composition: I_{0.8}V_{1.0}S_{0.9}K_{1.1}P_{1.0}E_{1.2}R_{1.0}G_{0.7}. No peak at 315 nm (Figure 1A) or ³H-label above background levels (Figure 1B) were evident near the retention time (16.5 min) of the standard IVSK(pyr)PER. The chromatogram of the sample from the inside-out vesicles also contained a peak of absorbance (peak a, Figure 1C) at 229 nm with the same retention time as the unmodified peptide IVSKPER (16 min); however, another peak was evident with a retention time coincident with the standard IVSK(pyr)PER (peak b, Figure 1C). This peak had absorbancies at both 229 and 315 nm and, associated with this second peak,

Figure 1 Incorporation of pyridoxal phosphate into Lys-691 of the anion exchanger from inside-out vesicles and intact erythrocytes

Inside-out vesicles (35 mg protein) and intact erythrocytes (11 ml packed cells) were modified vectorially in the same container (20 ml) with 12 mM pyridoxal phosphate and 5.6 mM sodium [³H]borohydride (25 mCi, 220 mCi·mmol⁻¹). The modified inside-out vesicles and modified erythrocytes were then separated and the modified erythrocytes were washed twice with isotonic buffer and open membranes were prepared by the method of Dodge et al. [27]. The membranes and the inside-out vesicles were stripped with alkali and then digested with thermolysin and trypsin. The digests were passed twice over the immunoadsorbent, each time the column was washed with PBS and the specifically bound peptides eluted. The acid eluates were pooled and the specifically adsorbed peptides were separated by reverse-phase HPLC on an analytical C₁₈ column (0.46 cm × 0.25 cm) using 0.05% (v/v) trifluoroacetic acid in water and a linear gradient of 1% (v/v) acetonitrile·min⁻¹. The eluate was monitored for absorbance at 229 nm and 315 nm and 1 ml fractions were collected and a portion (0.5 ml) of each was assayed for ³H-label by scintillation spectroscopy. (A) Chromatogram of the peptide IVSKPER (peak a) isolated from modified erythrocytes, and (B) the associated ³H-label (c.p.m.). (C) Chromatogram of the peptides IVSKPER (peak a) and IVSK(pyr)PER (peak b) isolated from modified inside-out vesicles, and (D) the associated ³H-label (c.p.m.). Absorbance at 229 nm (lower traces in A and C), absorbance at 315 nm (upper traces in A and C) and c.p.m. of ³H-label are presented as a function of elution time (min).

was a peak of radioactivity containing 2620 c.p.m. of ³H-label (Figure 1D).

The results of this experiment suggest that Lys-691 of the anion exchanger in inside-out vesicles was modified with

Figure 2 Digestion of IVSK(^3H)pyr)PER with carboxypeptidase B

Synthetic IVSK(pyr)PER was added to a pool of the remaining fractions containing the radioactive peak (Figure 1D) and the absorbance peak (peak b, Figure 1C) with the same retention time as IVSK(pyr)PER. The mixture was digested for 1 h with carboxypeptidase B (3 units $\cdot \text{ml}^{-1}$) and products of the digest were separated by reverse-phase HPLC using the system described in the legend to Figure 1, except that a linear gradient of 0.5% acetonitrile $\cdot \text{min}^{-1}$ was used. Fractions were collected at 1 min intervals and the radioactivity was measured. (A) Chromatogram of the digested sample showing IVSK(pyr)PE (peak c) and IVSK(pyr) (peak d), and (B) the associated ^3H -label (c.p.m.). A_{315} and c.p.m. of ^3H -label are presented as a function of elution time (min).

impermeant pyridoxal phosphate and sodium borohydride to a much greater extent than Lys-691 of the anion exchanger of intact erythrocytes. This would imply that Lys-691 is cytoplasmically oriented in the native anion exchanger from human erythrocytes. It was possible, however, that the ^3H -label was associated with some species other than the peptide IVSK(^3H)pyr)PER, where ^3H pyr indicates N^6 -[4'-(5'-phospho-[4- ^3H]pyridoxyl)].

In an experiment to demonstrate that the radioactive peak in the sample from the inside-out vesicles arose from the peptide IVSK(^3H)pyr)PER, the synthetic standard IVSK(pyr)PER was digested with carboxypeptidase B and submitted to reverse-phase HPLC run with a shallower gradient [0.5% (v/v) acetonitrile $\cdot \text{min}^{-1}$]. Two peaks of absorbance at 315 nm were observed. The first peak had the same retention time (31 min) as the undigested standard IVSK(pyr)PER and the fraction associated with this peak was found to have the composition $\text{I}_{0.7}\text{V}_{0.9}\text{S}_{1.0}\text{K}_{0.3}\text{P}_{1.2}\text{E}_{1.2}$. The second peak had a longer retention time (33 min) and its amino acid composition was $\text{I}_{0.8}\text{V}_{0.9}\text{S}_{1.0}\text{K}_{0.2}\text{P}_{1.2}$. These results show that carboxypeptidase B had successively removed the arginine and glutamate residues

from the C-terminus of the peptide IVSK(pyr)PER. To determine if the peak of ^3H -label in the sample from the inside-out vesicles was from the peptide IVSK(^3H)pyr)PER, 0.2 nmol of synthetic IVSK(pyr)PER was added to a pool of the remaining portions of fractions 16 and 17 of the sample (a total of 2620 c.p.m.) from the modified inside-out vesicles (Figure 1D). The mixture was digested with carboxypeptidase B and submitted to reverse-phase HPLC under similar conditions to those used to purify the products of the carboxypeptidase digest of the standard IVSK(pyr)PER. The ^3H -label was associated with an absorbance peak at 315 nm (940 c.p.m.; peak c, Figure 2A), which had a retention time similar to that of the synthetic standard IVSK(pyr)PER and with a peak (1160 c.p.m.; peak d, Figure 2B), which had a retention time similar to the synthetic standard IVSK(pyr). These results show that the peak of radioactivity with the same retention time as the standard IVSK(pyr)PER in the sample from the inside-out vesicles was from the peptide IVSK(^3H)pyr)PER.

In a separate experiment, open erythrocyte membranes were labelled with pyridoxal phosphate and non-radioactive sodium cyanoborohydride by the method used to label the standard peptide *N*-acetylIVSKPER. A peak of absorbance at 315 nm with the same retention time (16.5 min) as the standard IVSK(pyr)PER was observed after immunoadsorption of a digest of the alkali-stripped membranes. The fraction associated with the peak was submitted to electrospray MS. Two ions were observed with m/z values of 530.3 atomic mass units (a.m.u.) and 353.9 a.m.u. These are the values expected for the dication and trication of IVSK(pyr)PER (neutral mass of 1058.6 a.m.u.) (results not shown).

The immunoadsorbent used in these experiments had been constructed from immunoglobulins whose affinities for the peptides IVSKPER and IVSK(pyr)PER should have been equivalent [28]. Contrary to this expectation, however, when the digests from the inside-out vesicles were passed over the immunoadsorbent a third time and the acid eluates were submitted to reverse-phase HPLC, the ratio of the ^3H -label (c.p.m.) from the modified peptide eluting at 16.5 min to the area (cm^2) of the peak of absorbance at 229 nm for the unmodified peptide was much lower than the same ratio calculated from the chromatogram obtained from the first two passes (310 c.p.m. $\cdot \text{cm}^{-2}$ compared with 1990 c.p.m. $\cdot \text{cm}^{-2}$). After the fourth passage over the immunoadsorbent, no ^3H -label remained (< 40 c.p.m.) even though the yield of the unmodified peptide IVSKPER was the same. These results indicated that the immunoadsorbent had a greater affinity for the phosphopyridoxylated peptide IVSK(^3H)pyr)PER than for the unmodified peptide IVSKPER.

All of the peptide IVSKPER was removed from the inside-out vesicles and erythrocytes by repeatedly passing the digests over the immunoadsorbent and monitoring the eluates by HPLC and scintillation spectroscopy until it was clear that no radioactivity was present in any of the peaks. The total area of the absorbance peaks associated with the peptide IVSKPER from all the passes of the erythrocyte digests was 10 cm^2 , and no ^3H -label (< 100 total c.p.m. above background) was found near the retention time of the standard peptide IVSK(pyr)PER in any of the fractions. The total area of the absorbance peaks of the peptide IVSKPER from the inside-out vesicles was 7.1 cm^2 and 2960 c.p.m. of ^3H -label was associated with the peptide IVSK(^3H)pyr)PER from passes one to four.

The area of the absorbance peak of the unmodified peptide can be used as an internal standard to correct for the yield of peptide from the various separations and digestions. By dividing the c.p.m. in the radioactive peak of the modified peptide by the area of the absorbance peak of the unmodified peptide, a measure of

Table 1 IVSK([³H]pyr)PER from inside-out vesicles and intact erythrocytes

The radioactive peaks (c.p.m.) representing the peptide IVSK([³H]pyr)PER were determined as described in detail in the legend to Figure 1.

Experiment	³ H-label (c.p.m.)	
	Erythrocytes	Inside-out vesicles
1	< 30	550
2	< 40	860
3	< 40	550
4*	< 70	2620

* The results from these experiments are shown in Figure 1.

the initial specific radioactivity of the modified protein in each sample immediately after the labelling reaction can be obtained. The specific radioactivity of IVSK([³H]pyr)PER obtained from inside-out vesicles (385 c.p.m. · cm⁻²) was > 30-fold greater than the specific radioactivity of the same peptide obtained from erythrocytes (< 10 c.p.m. · cm⁻²), when the two specimens were labelled in the same container. As Lys-691 in the anion exchanger from inside-out vesicles was at least 30 times more accessible to modification by pyridoxal phosphate and sodium borohydride than Lys-691 in the anion exchanger from intact erythrocytes, this residue must lie on the cytoplasmic surface of the plasma membrane. Three additional experiments were carried out identical to the one described above, except that the digests were passed over the immunoadsorbent only twice or three times and were not exhaustively depleted of peptide. The results of all of the experiments (Table 1) are in agreement with the cytoplasmic disposition of Lys-691.

Yield of modified Lys-691 in the anion exchanger from inside-out vesicles

To prevent disruption of the native conformation of membrane-spanning proteins during chemical modification, it has been suggested that the level of incorporation of a general labelling reagent should be low [29]. In the experiments described above, the high specific radioactivity of sodium [³H]borohydride (220 mCi · mmol⁻¹) enabled the identification of the peptide IVSK([³H]pyr)PER, even when the level of incorporation was intentionally limited (< 1%). A disadvantage of this strategy, however, is that the incorporation observed may result from the modification of a reactive residue in a small unrepresentative subset of the total population of the protein. For example, during the preparation of the inside-out vesicles, some of the anion exchanger could have been denatured and Lys-691 exposed, making it more reactive. To examine this possibility, the degree of phosphopyridoxylation at Lys-691 in the anion exchanger from inside-out vesicles was increased systematically. Samples of inside-out vesicles were modified with increasing equimolar amounts of pyridoxal phosphate and sodium borohydride. The vesicles were layered over 15% (w/v) dextran and centrifuged to determine the degree of lysis occurring as a result of the modification. It was found that all samples of the modified vesicles floated, indicating that the vesicles had not been ruptured by high concentrations of pyridoxal phosphate and sodium borohydride. The modified and unmodified IVSKPER peptides were then isolated from the vesicles as before. In these experiments, however, only a portion of each digest was passed over the immunoadsorbent in order not to exceed its capacity and a quantitative yield of both modified and unmodified peptides

Figure 3 Yield of phosphopyridoxylation at Lys-691 of the anion exchanger from inside-out vesicles

Samples (7.2 mg of protein) of inside-out vesicles were modified in 3 ml of 150 mM NaCl/20 mM sodium phosphate with increasing equimolar amounts of pyridoxal phosphate and sodium borohydride. Sealed vesicles were separated by differential centrifugation and IVSKPER and IVSK(pyr)PER were isolated as described in the legend to Figure 1. The yield of the incorporation (percentage of the total modified peptide) is presented as a function of the concentration of pyridoxal phosphate (M). Open and closed circles represent the results of two separate experiments.

would then be obtained. The amounts (nmol) of the two peptides retrieved, IVSKPER and IVSK(pyr)PER, were calculated from the respective absorbance peaks after HPLC (Figure 1) and their absorption coefficients [$0.97 \text{ nmol} \cdot \text{cm}^{-2}$ for IVSKPER at 229 nm and $0.41 \text{ nmol} \cdot \text{cm}^{-2}$ for IVSK(pyr)PER at 315 nm]. The degree of incorporation was calculated by dividing the nmol of IVSK(pyr)PER observed by the total nmol of IVSKPER and IVSK(pyr)PER. It was found that the yield of incorporation increased linearly (Figure 3) with the increase in concentrations of pyridoxal phosphate and sodium borohydride. This result demonstrates that the incorporation observed in the earlier experiments was not into a small, unrepresentative population of anion exchanger.

It is unclear why the incorporation observed at the higher concentrations of reagents levelled out at 37% (Figure 3). About two thirds ($63 \pm 9\%$) of the acetylcholinesterase in the inside-out vesicles was normally inaccessible to its substrates. In the preparation of vesicles used in the two experiments presented in Figure 3, the inaccessibility of acetylcholinesterase to its substrate was 67%. Acetylcholinesterase is a marker enzyme for the extracytoplasmic surface of the erythrocytic plasma membrane [22]. The accessibility of glyceraldehyde-3-phosphate dehydrogenase to its substrates was assessed as a marker of the exposure of the cytoplasmic surface [22]. A higher percentage of the glyceraldehyde 3-phosphate ($87 \pm 5\%$) was accessible in the absence of detergent. Taken together these observations suggest that in the preparations of inside-out vesicles used in these experiments, about two thirds of the vesicles were sealed inside-out, about 10% were sealed right-side-out and about 20% were open. Therefore the cytoplasmic surface of 90% of the anion exchangers should have been accessible for modification, rather than the 40% found.

DISCUSSION

Lys-691 of the human erythrocyte anion exchanger is found in the hydrophilic sequence -ESQITTLIVSKPERKMKVKGSGF-HLD- stretching from Glu-681 to Asp-702. This section of the polypeptide lies between two potential membrane-spanning segments that have been designated M8 and M9 by Kopito and

Lodish [4]. In current topological models, the region between the hydrophobic segments M8 and M9 is given a cytoplasmic location because the hydrophilic region preceding it is extracytoplasmic and the intervening hydrophobic segment M8 has the potential to span the bilayer [30–33]. There is no experimental evidence, however, indicating that the hydrophobic segment M8 does span the bilayer. Lux et al. [33] have explicitly noted that the two hydrophilic regions between the hydrophobic segments M8 and M9 and the hydrophobic segments M11 and M12 respectively, are regions of the protein with poorly defined orientation. Recently, it has been shown [11], by a combination of vectorial modification and site-directed immunochemistry, that Asp-821, between the hydrophobic segments M11 and M12, is located on the cytoplasmic side of the membrane. To examine the orientation with respect to the membrane of the hydrophilic region between the hydrophobic segments M8 and M9 of the anion exchanger, we have now applied vectorial modification and site-directed immunochemistry to Lys-691.

Kang et al. [26] have shown that a number of hydrophilic peptides can be cleaved enzymically from the anion exchanger in erythrocyte membranes that have been stripped with alkali. Previously, the peptide YHPDVPYVK, designated KS1 by Kang et al. [26], was isolated by site-directed immunochemistry from tryptic digests of alkali-stripped membranes of erythrocytes [11]. This earlier success in obtaining the peptide YHPDVPYVK prompted the present search for a peptide that could be released from the hydrophilic region between the hydrophobic segments M8 and M9 of the anion exchanger in these alkali-stripped membranes. It has been shown that digestion of the synthetic peptide *N*-acetylIVSKPER, corresponding to the amino acid sequence between Leu-687 and Arg-694 of the human anion exchanger, with thermolysin produces the peptide IVSKPER (M. Kalo, personal communication). Therefore we attempted to release this short peptide, containing Lys-691, from alkali-stripped erythrocyte membranes by digesting them with a combination of trypsin and thermolysin. This combination proved successful in liberating the short peptide IVSKPER, which was then isolated by site-directed immunochemistry.

To determine the disposition of Lys-691 with respect to the membrane, it was necessary to submit the native protein to vectorial modification both in intact erythrocytes and inside-out vesicles. Pyridoxal phosphate is unable to cross most membranes because of its negative charge, therefore, it has been used extensively in conjunction with sodium borohydride to modify membrane-spanning proteins vectorially [18,24,25,28,34]. Human erythrocyte membranes are unique in that pyridoxal phosphate can act as a substrate for the anion exchanger and slowly traverse the membranes, however, Cabantchik et al. [35] have shown that this transport is negligible when erythrocytes are modified with pyridoxal phosphate for short duration at high pH. The reaction conditions at which transport was inhibited were similar to those used in the present study. Furthermore, the results of the present experiments demonstrate that pyridoxal phosphate was reacting vectorially.

The current study provides new information on the topography of the region of the anion exchanger following the hydrophobic segment M8 and containing Lys-691. The nearest amino acid to the N-terminus of this region that has been assigned a location with respect to the plasma membrane is Asp-642, which is located in the hydrophilic region between the hydrophobic segments M7 and M8. Jay [36] has determined that Asp-642 in the native human anion exchanger is glycosylated. Since this carbohydrate can be cleaved in intact cells with endo- β -galactosidase [37], the disposition with respect to the membrane of Asp-642 must be extracytoplasmic. Because Lys-691 is on the

cytoplasmic surface of the anion exchanger, the hydrophobic segment M8 must traverse the bilayer from its extracytoplasmic to its cytoplasmic side.

To the C-terminal side of Lys-691, the situation is less clear. Jennings et al. [38] have shown that when trypsin is sealed into human erythrocyte right-side-out vesicles, cleavage of some of the anion exchanger occurs at Lys-743, which lies in the hydrophilic region between hydrophobic segments M9 and M10. They assigned this region to the cytoplasmic surface of the membrane even though the yield of the cleavage was extremely low. Recently, Popov et al. [39] have suggested that Lys-743 may be located on the extracytoplasmic side of the membrane. Cell-free translation of several mutants of the anion exchanger, into which novel glycosylation sites had been inserted, was examined in a reticulocyte lysate supplemented with microsomal membranes. The only naturally occurring glycosylation site, Asp-642, was mutated to an aspartic acid residue so that the glycosylation of the mutants could be analysed by shifts in the electrophoretic mobility on polyacrylamide gels. Mutation of Lys-743 to an asparagine residue resulted in the glycosylation of 37% of the protein as judged by the shift in electrophoretic mobility. Other mutants in which amino acids in the same hydrophilic region were mutated to asparagine residues were also glycosylated to a similar extent. These results were taken to indicate that the hydrophilic loop containing Lys-743 was extracytoplasmic, even though the levels of glycosylation were lower in the mutants than that observed at Asp-642 for the wild-type protein (67%). Unfortunately, the abilities of the glycosylated mutants to transport anions could not be addressed in the translation system that was used and it may be the case that the expressed proteins were improperly folded.

Jennings and co-workers [40–42] suggested that Glu-681, immediately following the hydrophobic segment M8 and in the same hydrophilic region as Lys-691, may have access to both the cytoplasmic and extracytoplasmic medium while fulfilling the role of the functional group responsible for the H⁺ transport accompanying the transport of SO₄²⁻. Studies on the transport properties of the mutant murine erythrocyte anion exchanger expressed in oocytes from *Xenopus laevis*, in which Glu-699 (equivalent to human Glu-681) had been mutated, are consistent with this proposal [43–45]. It has been suggested that the positively charged lysine residues at positions 691, 695 and 698 and the arginine at position 694 in the human enzyme would then anchor this portion of the hydrophilic region on the cytoplasmic surface and form part of the inward facing mouth of an anion selective access channel [41]. The present study provides the first direct evidence for the cytoplasmic disposition of this region of the protein that surrounds Lys-691.

We thank Larry Gross for performing the electrospray MS, and Anne Kuller for synthesizing the peptide *N*-acetylIVSKPER. This research was supported by Grant MCB-9406321 from the National Science Foundation to J.K., and a Training Grant DK07233 from the National Institutes of Health to H.K.E.

REFERENCES

- 1 Tanner, M. J. A., Martin, P. G. and High, S. (1988) *Biochem. J.* **256**, 703–712
- 2 Low, P. S. (1986) *Biochim. Biophys. Acta* **864**, 145–167
- 3 Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol.* **103**, 61–223
- 4 Kopito, R. R. and Lodish, H. F. (1985) *Nature (London)* **316**, 234–238
- 5 Okubo, K., Kang, D., Hamasaki, N. and Jennings, M. L. (1994) *J. Biol. Chem.* **269**, 1918–1926
- 6 Tanner, M. J. A., Williams, D. G. and Kyle, D. (1979) *Biochem. J.* **183**, 417–427
- 7 Jennings, M. L. and Nicknisch, J. S. (1984) *Biochemistry* **23**, 6432–6436
- 8 Cobb, C. E. and Beth, A. H. (1990) *Biochemistry* **29**, 8283–8290
- 9 Brock, C. J., Tanner, M. J. A. and Kempf, C. (1983) *Biochem. J.* **213**, 577–586

- 10 Kalo, M. S. (1996) *Biochemistry* **35**, 999–1009
- 11 Erickson, H. K. (1997) *Biochemistry* **36**, 9958–9967
- 12 Mawby, W. J. and Findlay, J. B. C. (1982) *Biochem. J.* **205**, 465–475
- 13 Jennings, M. L. and Adams, M. F. (1981) *Biochemistry* **20**, 7118–7123
- 14 Wainwright, S. D., Tanner, M. J. A., Martin, G. E. M., Yenole, J. E. and Holmes, C. (1989) *Biochem. J.* **258**, 211–220
- 15 Wainwright, S. D., Mawby, W. J. and Tanner, M. J. A. (1990) *Biochem. J.* **272**, 265–268
- 16 Liebermann, D. M. and Reithmeier, R. A. F. (1988) *J. Biol. Chem.* **263**, 10022–10028
- 17 Stewart, J. M. and Young, J. D. (1984) *Solid Phase Peptide Synthesis*, 2nd edn., Pierce Chemical Co., Rockford, IL, U.S.A.
- 18 Thibault, D. (1993) *Biochemistry* **32**, 2813–2821
- 19 Walter, G., Scheidtmann, K. H., Carbone, A., Laudano, A. P. and Doolittle, R. F. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5197–5200
- 20 Liu, F. T., Zinnecker, M., Hamaoka, T. and Katz, D. H. (1979) *Biochemistry* **18**, 690–697
- 21 Ewalt, K. L. (1994) *Biochemistry* **33**, 5077–5088
- 22 Steck, T. L. and Kant, J. A. (1974) *Methods Enzymol.* **173**, 172–181
- 23 Ellman, G. L., Courtney, K. D., Valentino, Jr., A. and Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88–95
- 24 Ohkawa, I. and Webster, R. E. (1981) *J. Biol. Chem.* **56**, 9951–9958
- 25 Dwyer, B. P. (1991) *Biochemistry* **30**, 4105–4112
- 26 Kang, D., Okubo, K., Hamasaki, N., Kuroda, N. and Shiraki, H. (1992) *J. Biol. Chem.* **267**, 19211–19217
- 27 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130
- 28 Kyte, J., Xu, K. and Bayer, R. (1987) *Biochemistry* **26**, 8350–8360
- 29 Bretscher, M. S. (1971) *Nature New Biol.* **231**, 229–232
- 30 Tanner, M. J. A. (1993) *Semin. Hematol.* **30**, 34–57
- 31 Reithmeier, R. A. F. (1993) *Curr. Opin. Struct. Biol.* **3**, 515–523
- 32 Passow, H., Wood, P. G., Lepke, S., Müller, H. and Sovak, M. (1992) *Biophys. J.* **62**, 98–100
- 33 Lux, S. E., John, K. M., Kopito, R. R. and Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9089–9093
- 34 Anderberg, S. J. (1995) *Biochemistry* **34**, 9508–9516
- 35 Cabantchik, Z. I., Balshin, M., Breuer, W. and Rothstein, A. (1975) *J. Biol. Chem.* **250**, 5130–5136
- 36 Jay, D. G. (1986) *Biochemistry* **25**, 554–556
- 37 Mueller, T. J., Li, Y. T. and Morrison, M. (1979) *J. Biol. Chem.* **254**, 8103–8106
- 38 Jennings, M. L., Anderson, M. P. and Monaghan, R. (1986) *J. Biol. Chem.* **261**, 9002–9010
- 39 Popov, M., Tam, L. Y., Jing, L. and Reithmeier, R. A. F. (1997) *J. Biol. Chem.* **272**, 18325–18332
- 40 Jennings, M. L. and Anderson, M. P. (1987) *J. Biol. Chem.* **262**, 1691–1697
- 41 Jennings, M. L. and Smith, J. S. (1992) *J. Biol. Chem.* **267**, 13964–13971
- 42 Jennings, M. L. (1995) *J. Gen. Physiol.* **105**, 21–47
- 43 Müller-Berger, S., Karbach, D., Kang, D., Aranibar, N., Wood, P. G., Rüterjans, H. and Passow, H. (1995) *Biochemistry* **34**, 9325–9332
- 44 Sekler, I., Lo, R. S. and Kopito, R. R. (1995) *J. Biol. Chem.* **270**, 28751–28758
- 45 Chernova, M. N., Jiang, L., Crest, M., Hand, M., Vondorpe, D. H., Strange, K. and Alper, S. L. (1997) *J. Gen. Physiol.* **109**, 345–360