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Proteolytic Cleavage Sites of Band 3 Protein in Alkali-Treated Membranes: Fidelity of Hydropathy Prediction for Band 3 Protein[1]

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To assess the fidelity of hydropathy prediction for band 3 protein, we determined the cleavage sites of the protein and the portions of the protein tightly bound to the membrane lipid bilayer by means of *in situ* proteolytic digestion. For the removal of all anticipated hydrophilic connector loops from membranes, we had to denature the band 3 protein molecule *in situ* by alkali treatment. When the alkali-treated membranes were digested with trypsin, chymotrypsin, and pepsin, the majority of the anticipated transmembrane portions remained in the membrane fraction. However, five anticipated transmembrane portions were released into the supernatant fraction. Thus, the first, second, third, sixth and tenth anticipated transmembrane portions, in accordance with the hydropathy prediction, were released into the supernatant with the proteolytic digestion method. This indicates that these anticipated transmembrane portions are not bound with the boundary lipids although the hydrophobicity of these portions is comparable to that of the portions experimentally remaining in the membrane fraction. It is conceivable that the membrane peptide portions of band 3 protein could be classified into at least two categories, *i.e.* one bound to the boundary lipids and the other free from the boundary lipids. Approximately 90% of the transmembrane domain of the band 3 protein are recovered in either the supernatant fraction or the membrane fraction. The fidelity of hydropathy prediction for polytopic membrane proteins and the nature of the membrane embedded peptide portions are discussed.

Key words: band 3 protein, category for transmembrane segments, classification of transmembrane peptide portions, fidelity of hydropathy prediction, proteolytic cleavage sites.

Human erythrocyte band 3 protein is a typical multi-spanning polytopic membrane protein that mediates chloride-bicarbonate exchange across the erythrocyte membrane (*1*). In peripheral capillaries, band 3 protein mediates the exchange of cellular HCO_3^- with Cl^- in plasma. This is known as the "Chloride Shift." As a result of the anion exchange, the intracellular pH of red blood cells is rendered acidic. This acidification is the trigger for the dissociation of O_2 from oxyhemoglobin. Thus, red blood cells can discriminate metabolically active tissues from inactive tissues and supply oxygen preferentially to metabolically active tissues. Accordingly, as long as oxygen is delivered by red blood cells, a minimum but sufficient amount of oxygen is supplied to tissues. As such, oxygen intoxication never occurs (*2*).

Many polytopic integral membrane proteins mediate the transport of ions, solutes, and metabolites as well as proteins themselves across biological membranes. The transport phenomenon plays an important role in cell biology. The clarification of molecular mechanisms related to such transport is clearly a pressing issue. While the structure-function relationships of the transport mechanisms have been discussed, a number of assumptions about structure have been made based on hydropathy predictions. However, the "interpreted" molecular mechanisms have been generalized with the limited amount of experimental evidence. Such "interpretations" have fallen short of contributing to the real understanding of the phenomenon. For comprehending the molecular mechanisms of transport, two-dimensional structural information on membrane-spanning peptide portions and the topology of the functionally important amino acid residues are as important as the three-dimensional high-resolution structure. With this view, we have been investigating the molecular structure of the band 3 protein *in situ* (*3-6*). The complete amino acid sequences of the band 3 family proteins, AE1-AE3, have been deduced from their cDNA sequences (*7-10*). The human erythrocyte band 3 protein, AE1, contains up to 14 putative transmembrane segments and is one of the most investigated polytopic integral membrane pro-

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Abbreviations: H_2DIDS, dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; HPLC, high performance liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TPCK, tosyl-L-phenylalanine chloromethyl ketone.

teins (*11-13*). Even for the band 3 protein, however, only limited structural information is currently available compared with the large body of knowledge derived from kinetic studies. Thus, it is not yet known where the anion binding site is within the band 3 protein or how the conformational changes of the band 3 protein are induced by the binding of the transported anion to this protein.

In this study, we attempt to determine the proteolytic cleavage sites of the band 3 protein and the peptide portions embedded in the membrane lipid bilayer through tight lipid-peptide interactions. We also discuss the fidelity of hydropathy prediction for multi-spanning polytopic membrane proteins, and caution against the use of "interpreted models" without any experimental evidence for elucidating the structure and function relationship of polytopic integral membrane proteins.

EXPERIMENTAL PROCEDURES

Preparation of Leaky Human Erythrocyte Membranes and Denaturation of Band 3 Protein In Situ—Human blood which had been stored for less than 2 weeks was used in this study. Erythrocyte membranes (white ghosts) were prepared as described previously (*5*). Briefly, erythrocytes were pretreated with bovine trypsin to digest the glycophorins. White ghosts were prepared by osmotic lysis and the cytosolic 40-kDa domain of band 3 protein was removed from the white ghosts by trypsin digestion. Although band 3 protein is predicted to have many hydrophilic connector loops, this protein is quite resistant to proteinases (*5*). To remove all anticipated hydrophilic connector loops from membranes, we, therefore, had to denature the band 3 protein molecule *in situ* by treatment with a high concentration of NaOH. The membranes were treated with 100 mM NaOH to remove peripheral membrane proteins and to denature the band 3 protein *in situ*. The denaturation was confirmed by the binding of [^{3}H]H_2DIDS (*5*).

In Situ Proteolytic Digestion of Band 3 Protein—Since the aim of our investigation was elucidation of the molecular mechanism of anion transport, we assessed the validity of hydropathy predictions for band 3 protein by digesting red blood cell membranes by successive proteolytic digestion with trypsin, chymotrypsin, and pepsin under the assumption that peptide portions without lipid-peptide interactions should be released into the supernatant on proteolytic digestion. The alkali-treated membranes were washed and resuspended in 5 mM phosphate buffer (pH 8.0) at a protein concentration of 1.0 mg/ml. The membrane suspension was digested with 25 μg/ml of trypsin at 37°C for 60 min. Supernatants were separated from membranes by centrifugation at 4°C for 30 min at either 27,200 or 130,000$\times g$, and then passed through a 0.45 μm filter. Under both centrifugation conditions, the supernatants were clear with the absence of membranes, as confirmed on SDS-PAGE.

Peptides released into the supernatant were analyzed as described below. The trypsin-treated membranes were washed extensively with 5 mM phosphate buffer (pH 8.0) with or without 1 M KCl, and then resuspended in the same buffer at a protein concentration of 1.0 mg/ml. The trypsin-treated membranes were additionally digested with 25 μg/ml of chymotrypsin at 37°C for 60 min. Peptides released into the supernatant were collected as described above. The trypsin/chymotrypsin-treated membranes were washed extensively with 5 mM phosphate buffer (pH 8.0) with or without 1 M KCl, and then used for the analysis of peptides remaining in the membrane fraction.

The washed trypsin/chymotrypsin-treated membranes were resuspended in 15 mM HCl at a protein concentration of 1.0 mg/ml, followed by overnight digestion with 1.0 mg/ml of pepsin at 37°C. The pepsin-treated membranes were washed extensively with 5 mM phosphate buffer (pH 8.0) with or without 1 M KCl, and then used for the analysis of peptides remaining in the membrane fraction.

Analysis of Peptides Released from Membranes on Proteolysis—Supernatants collected as described above were filtered and analyzed on a HPLC equipped with a reversed phase column (Cosmosil 5C18-AR-300, 4.6$\times$250 mm; Chemco, Osaka), using a linear gradient of 0-100% acetonitrile containing 0.1% trifluoroacetic acid. All peaks were analyzed by a gas-phase sequencer (Applied Biosystem, model 492), and the phenylthiohydantoins were identified with an Applied Biosystems 140C phenylthiohydantoin analyzer on-line system.

Analysis of Peptides Remaining in Membranes after Proteolysis—Washed trypsin/chymotrypsin-treated and pepsin-treated membranes prepared as described above were dissolved in an equal volume of 8 M urea/4% SDS at a protein concentration of 0.3 mg/ml. Peptides in the dissolved membrane solution were separated on a TSKG 3000SW column (21.5$\times$600 mm; TOSOH, Tokyo) equilibrated with 50 mM ammonium bicarbonate containing 2 M urea and 0.1% SDS. Each fraction from the TSKG 3000SW column was collected and applied to a reversed phase column (Cosmosil 5C18-AR-300, 4.6$\times$250 mm; Chemco), using a water-acetonitrile-propanol system containing 0.1% trifluoroacetic acid. While for the first 20 min and at 165 min water containing trifluoroacetic acid was the sole eluent, at other times the percent composition of the eluent mixture was selectively varied between 25 and 155 min. Thus, the gradient composition chosen was as follows: 20 to 25 min, water 100 to 90%, propanol 0 to 10%; 25 to 115 min, water 90 to 0%, acetonitrile 0 to 90%, propanol 10%; 115 to 135 min, acetonitrile 90 to 10%, propanol 10 to 90%; 135 to 145 min, acetonitrile 10%, propanol 90%; 145 to 155 min, water 0 to 90%, acetonitrile 10 to 0%, propanol 90 to 10%; 155 to 165 min, water 90 to 100%. All peaks were analyzed with a gas-phase sequencer (Applied Biosystems, model 492), and the phenylthiohydantoins were identified with an Applied Biosystems 140C phenylthiohydantoin analyzer on-line system.

Analytical Procedures—SDS-PAGE for protein analysis was carried out according to the method of Kawano and Hamasaki (*14*). Protein was determined by the method of Lowry *et al.* (*15*) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Proteolytic Digestion of Alkali-Treated Membranes—The band 3 protein consists of two domains, that is, an amino-terminal 40-kDa domain and a carboxyl-terminal transmembrane 55-kDa domain (*16*). Although the transmembrane domain of band 3 protein is predicted to have many hydrophilic connector loops, the transmembrane 55-kDa domain is quite resistant to proteinases in the

native conformation (*5, 17*). This suggests that the majority of the anticipated hydrophilic connector loops are not loose floppy loops but either assume a compact ordered structure or are embedded in the erythrocyte membrane. To remove such anticipated hydrophilic connector loops from the cell membrane, we denatured the band 3 protein molecule *in situ* with 100 mM NaOH and used the alkali-treated membranes for proteolytic digestion. The alkali-treated membranes were completely precipitated on centrifugation at either 27,200 or 130,000×*g* for 30 min. Essentially similar results were obtained in experiments involving centrifugation at either 27,200 or 130,000×*g*. Thus, there was no cross contamination of peptides released into the supernatant fraction by those remaining in the membrane fraction. The peptides released into the supernatant fraction and remaining in the membrane fraction were analyzed by HPLC.

HPLC Analyses of Peptides Released into the Supernatant Fraction and the Fraction Remaining in the Membrane—Peptides released into the supernatant were analyzed by HPLC (Fig. 1), and all the peaks were sequenced with a gas-phase sequencer. The peaks numbered from 1 to 18 for the trypsin supernatant and from 20 to 43 for the trypsin/chymotrypsin supernatant were due to the band 3 protein molecule. The digested membranes were washed and dissolved in a urea/SDS solution. The peptides in the dissolved membrane solution were also analyzed by HPLC (Fig. 2, a-c), and all the peaks were sequenced with a gas-phase sequencer. The peaks numbered from 1 to 35 and from P1 to P6 were due to the band 3 protein molecule. Peptides recovered in the supernatant fraction and/or the membrane fraction are summarized in Tables I and II, and their quantities are shown in Fig. 3. Among these peptides, the peptides indicated within the boxes in Table I were

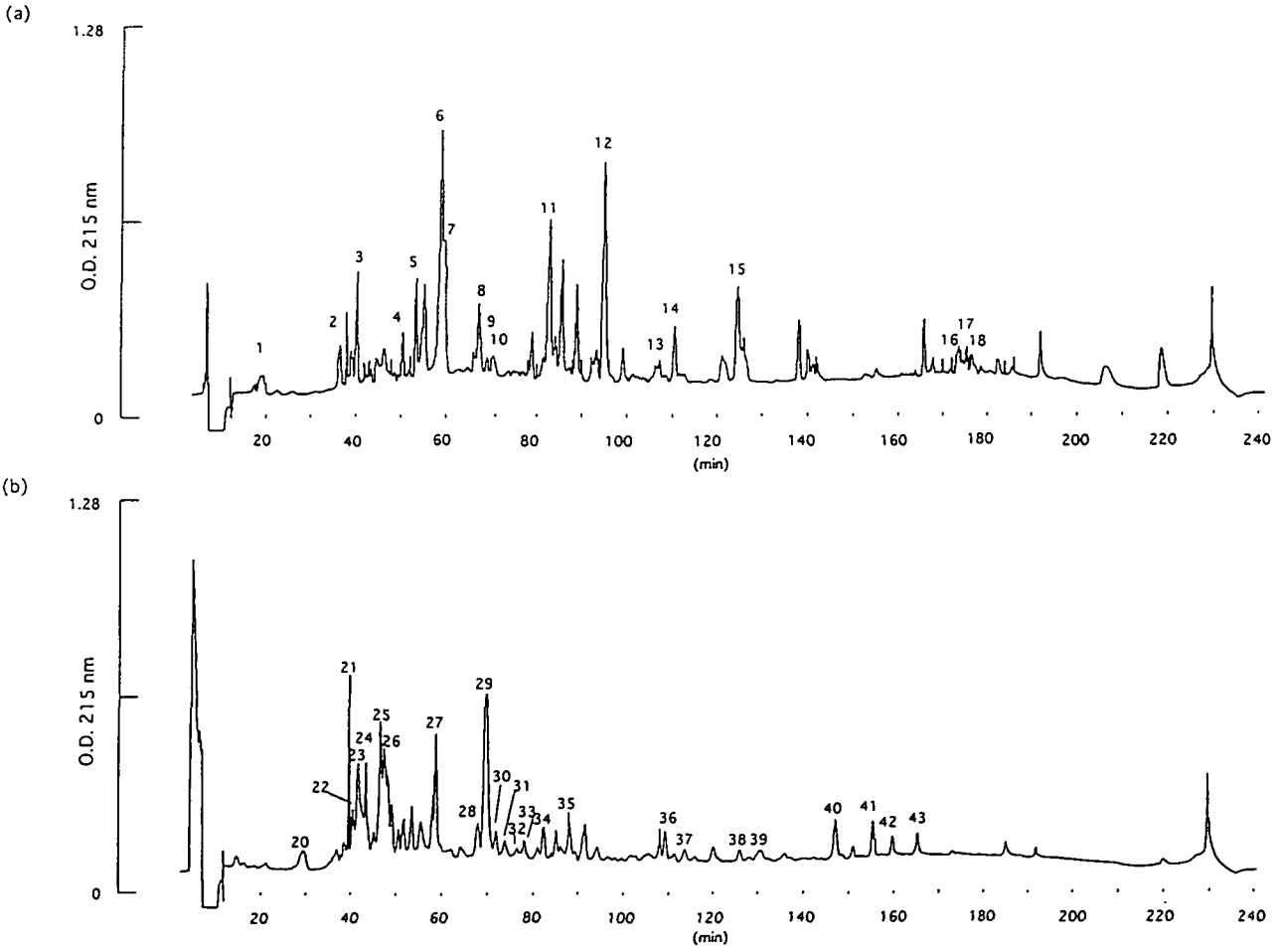

Fig. 1. **HPLC analyses of peptides in the band 3 transmembrane domain released into the supernatant fraction following trypsin treatment (a) and trypsin/chymotrypsin treatment (b).** White ghosts in 5 mM phosphate buffer (pH 8.0) containing 0.14 M NaCl were pretreated with 15 μg/ml of TPCK-trypsin at 4°C for 60 min to remove the cytosolic 40-kDa domain. The membranes were treated with 100 mM NaOH to remove peripheral membrane proteins and to denature the band 3 protein *in situ*. As a result, the band 3 protein was the major component of the remaining membrane proteins. The alkali-stripped membranes were washed and resuspended in 5 mM phosphate buffer (pH 8.0) at a protein concentration of 1.0 mg/ml. (a) The membrane suspension was digested with 25 μg/ml of trypsin at 37°C for 60 min. Peptides released into the supernatant were collected and analyzed as described under "EXPERIMENTAL PROCEDURES." Peptides in the peaks numbered from 1 to 18 were from the band 3 protein molecule. (b) The trypsin-treated membranes were washed extensively and resuspended in 5 mM phosphate buffer (pH 8.0) at a protein concentration of 1.0 mg/ml. The trypsin-treated membranes were additionally digested with 25 μg/ml of chymotrypsin at 37°C for 60 min. Peptides released into the supernatant were collected and analyzed as described under "EXPERIMENTAL PROCEDURES." Peptides in the peaks numbered from 20 to 43 were from the band 3 protein molecule.

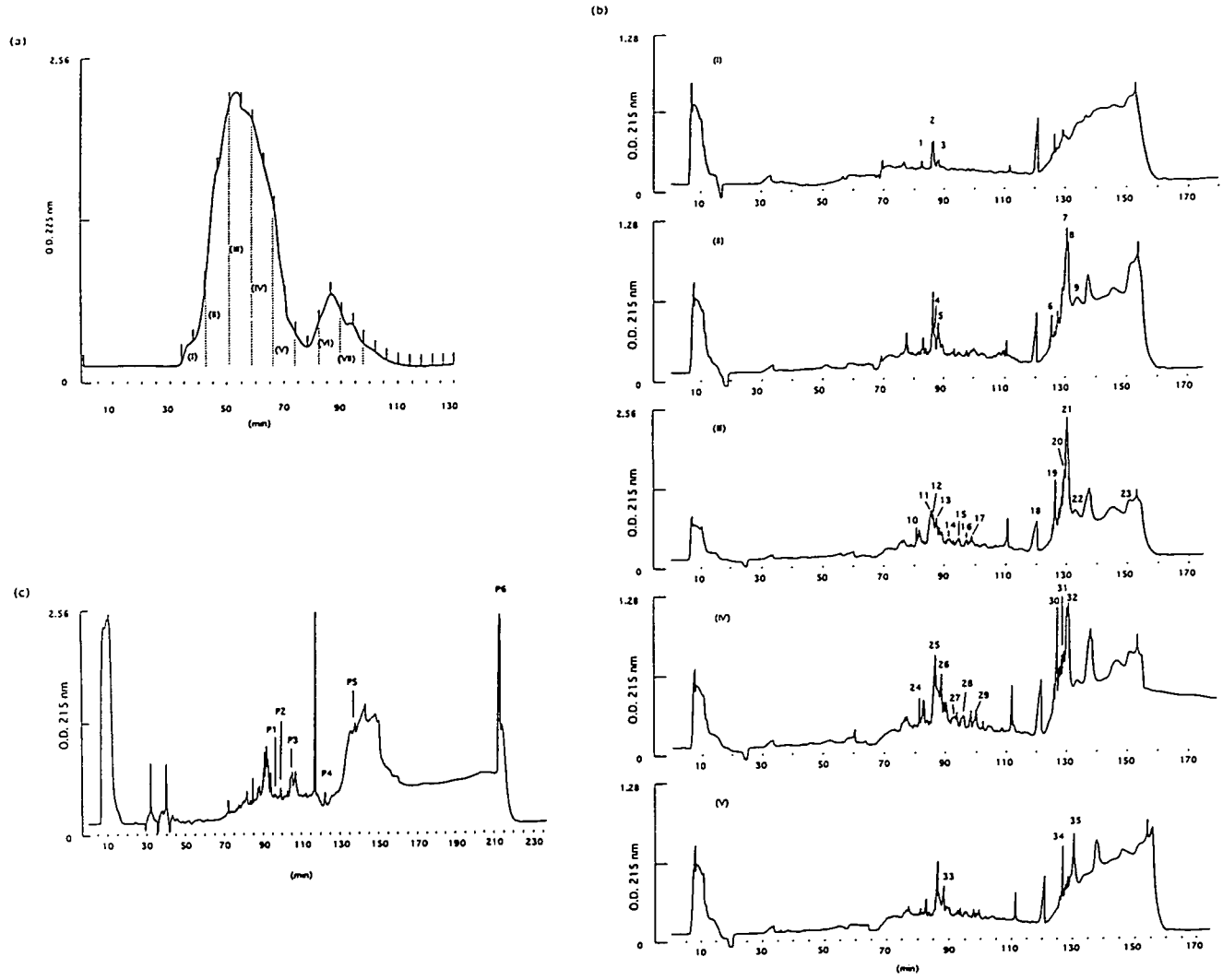

Fig. 2. **HPLC analyses of peptides remaining in the membranes after successive proteinases digestion.** (a) Alkali-stripped membranes were digested with trypsin and chymotrypsin as described under "EXPERIMENTAL PROCEDURES." The treated membranes were washed extensively with 5 mM phosphate buffer (pH 8.0) with or without 1 M KCl, and then dissolved in 8 M urea/4% SDS at a protein concentration of 0.3 mg/ml. Peptides in the dissolved membrane solution were separated on a TSKG 3000SW column (21.5×600 mm; TOSOH, Tokyo) equilibrated with 50 mM ammonium bicarbonate containing 2 M urea and 0.1% SDS. (b) Each fraction, (I)-(VII), in (a) was collected and applied to a reversed phase column (Cosmosil 5C18-AR-300, 4.6×250 mm; Chemco, Osaka), using a water-acetonitrile-propanol system containing 0.1% trifluoroacetic acid. All peaks were analyzed with a gas-phase sequencer as described under "EXPERIMENTAL PROCEDURES." Peptides in the peaks numbered from 1 to 35 were from the band 3 protein molecule. Fractions (VI) and (VII) on TSKG 3000SW separation contained no peptides from the band 3 protein molecule. (c) Trypsin/chymotrypsin-treated membranes were washed and resuspended in 15 mM HCl at a protein concentration of 1.0 mg/ml, followed by 1.0 mg/ml pepsin digestion at 37°C overnight. The pepsin-digested membranes were dissolved in an equal amount of 8 M urea/4% SDS at a protein concentration of 0.3 mg/ml and then applied to a reversed phase column (Cosmosil 5C18-AR-300, 4.6×250 mm; Chemco), using a water-acetonitrile-propanol system containing 0.1% trifluoroacetic acid. All peaks were analyzed with a gas-phase sequencer as described above, and peptides from the band 3 protein molecule were numbered from P1 to P6.

released into the supernatant while also remaining in the membrane fraction (see also Fig. 3).

Anticipated Transmembrane Portions Released into the Supernatant Fraction—The results summarized in Tables I and II were aligned and compared with a hydropathy prediction based on a combination of Kyte-Doolittle plots and Goldman-Engelman-Steitz plots (*18*) (Fig. 4). The proteolytic cleavage sites of band 3 protein in alkali-treated membranes are indicated by arrows in Fig. 4. The data summarized here were obtained not only in this experiment but also in our previous work (*5, 6, 17*). The underlined portions are the transmembrane portions indicated on the hydropathy prediction (*18*). The majority of the anticipated transmembrane portions were precipitated in the membrane fraction and consistent with the prediction, indicating that these portions are tightly bound to the membrane lipid bilayer, even in the denatured band 3 protein conformation. However, the five putative transmembrane portions of Gln-404 to Lys-430, Gly-436 to Gln-457, Leu-459 to Cys-479, Pro-568 to Lys-590, and Trp-723 to Ser-745 were liberated from the alkali-treated membrane on proteolytic digestion. Thus, the first, second,

TABLE I.
(a) Peptides released into trypsin Sup and trypsin/chymotrypsin Sup.

Amino acid sequence	Position in band 3	Peak No.	Reference[a]
GLDLNGGPDDPLQQTGQLFGGLVR	361-384	14, 15	KS-5
DIR	385-387	1	
RYPYYLSDITDAFSP.....	389-?	16	KM-7
YPYYLSDITDAFSPQVLAA....	390-?	17	KM-7
YLSDITDAF	393-401	36	KM-7
YLSDITDAFSPQVLAAVI	393-410	41	KM-7
IYFAALSPAITF	412-423	38	
AALSPAITF	414-423	35	
GGLLGEK	424-430	25, 26	
NQMGVSELLISTAVQGIL	433-450	42	
ALLGAQ	452-457	32	
ALLGAQPL	452-459	31	
ALLGAQPLLVVGF	452-464	39	
ALLGAQPLLVVGFSGPL.....	452-?	43	
SGPLLVFEEAFF	465-476	40	
KTYNYN	551-556	29	
TYNYNVL	552-558	30	
TYNYNVLMVPK	552-562	9	
TYNYNVLMVPKPQGPLPNTA.....	552-?	18	
KNSSY	592-596	20	
NSSYFPGK	593-600	4	
LSVPDGFK	632-639	8	KS-3
VS(N)SSAR	640-646	2	
GWVIHPLGLR	647-656	12	KS-4
IVSKPER	688-694	21, 22	
MVK	696-698	1	
GMP	720-722	30	
LSATTVR	724-730	23	
SVTHANA	731-737	24	
SVTHANALTVMGK	731-743	7	KS-2
ASTPGAAAQIQEVK	744-757	5	
LFKPPKYHPDVPY	812-824	33	
YHPDVPY	818-824	27	KS-1
YHPDVPYVK	818-826	6	
TWR	830-832	3	
VLILT	862-866	37	
RNVELQ	879-884	28	
NVELQ(C)LDADDAK	880-892	10,13	C-2
ATFDEEEGR	893-901	11	C-1
ATFDEEEGRDEY	893-904	34	

TABLE I. Continued.
(b) Peptides remaining in trypsin/chymotrypsin membranes.

Amino acid sequence	Position in band 3	Peak No.	Reference[a]
YLSDITDAFSPQVLAAVIFIYFA....	393-?	3,4,26,33	KM-7
NQMGVSELLISTAVQGILFALLG-AQ.....	433-?	7,18,22, 32,35	
ALLGAQPLL	452-460	14	
SF(C)ETNGLEYIVGRVWIGFW-LIL....	477-?	8,23	
MVPKPQGPLPNTALLSLVLMAG-TFFF	559-584	13	
MVPKPQGPLPNTALLSLVVLMA-GTFF...	559-?	5,11,12,25, 26,33	
PQGPLPNTA.....	563-?	14,27	
RVIGDFGVPISILIMVLVDFFIQDTY	603-628	15,28	KM-8
VIGDFGVPISILIMVLVDFFIQDTY	604-628	17,28	KM-8
VIGDFGVPISILIMVLVDFFIQDTY-TQK	604-631	15,16,29	KM-8
SEFPIWMMFASALPALLVF	657-675	19	
SEFPIWMMFASALPALLVFILIFL	657-680	20	
SEFPIWMMFASALPALLVFILIF-LE...	657-?	6	
SEFPIWMMFASALPALLVFILIFL-ESQIT...	657-?	30,34	
GSGFHLDLLLVVGMGGVAALF	699-719	10	KM-6
GSGFHLDLLLVVGMGGVAALF...	699-?	1,24	KM-6
ASTPGAAAQIQEVKEQRISGLL-VA...	744-?	21,31,35	KM-9
EQRISGLLVAVLVGLSI...	758-?	21	KM-9
DVPY	821-824	2	
STPASLAL.....	852-?	9	

[a]Designated in our previous publications (*5, 17*). The peptides surrounded by boxes were released into the supernatant as well as remaining in the membrane fraction. The letters in parentheses indicate that the amino acid residues could not be determined by sequencing.

TABLE II. **Peptides remaining in pepsin membranes.**

Amino acid sequence	Position in band 3	Peak No.	Reference[a]
YIVGRVWIGFWLILLVVLVVAFE-GSFLVRFISRY...	486-?	P5, P6	
VIGDFGVPISILIMVLVDFFIQDTY	604-628	P3	KM-8
VIGDFGVPISILIMVLVDFFIQDTY-TQK	604-631	P2	KM-8
FGVPISILIMVLVDFFIQDT	608-627	P1	KM-8
SEFPI...	657-?	P4	

[a]Designated in our previous publication (*5*).

third, sixth and tenth anticipated transmembrane portions were released into the supernatant fraction on proteolytic digestion. These data suggest that these portions are components of the embedded portions in the membrane lipid bilayer without tight lipid-peptide interactions, even though the hydrophobicity of these portions is comparable to that of the portions remaining in the membrane fraction, as demonstrated experimentally.

Proteolysis of the connecting loops or high concentration alkali-treatment ought to destabilize the membrane lipid bilayer and induce the liberation of these five anticipated transmembrane peptides from the lipid bilayer in spite of the fact that they were bound with boundary lipids in the membrane lipid bilayer. However, this was not the case, because the remaining nine anticipated transmembrane portions were not released into the supernatant fraction at all (see Tables I and II, and Fig. 3). From the evidence presented here it could be interpreted that the remaining peptides and the peptides released from the alkali-treated membranes are embedded in the native erythrocyte membranes in a different manners, respectively.

Nature of Peptide Portions Embedded in the Membrane Lipid Bilayer in the Native Band 3 Protein Conformation—The transmembrane 55-kDa domain of band 3 protein was resistant to proteolytic digestion, and only two anticipated hydrophilic connector loops, from Thr-552 to Lys-562 and from Thr-629 to Arg-656, were susceptible to proteinases in the native conformation (*5, 19*). The remainder of the anticipated hydrophilic connector loops were resistant to proteinases in the native conformation. However, they were released into the supernatant fraction on proteolytic digestion only when band 3 protein was denatured in alkali-treated membranes, as shown in Table I and Fig. 3, as well as in our previous work (*5, 17*), suggesting that the rest of the anticipated hydrophilic loops are not floppy loops but are embedded in the membrane lipid bilayer or assume

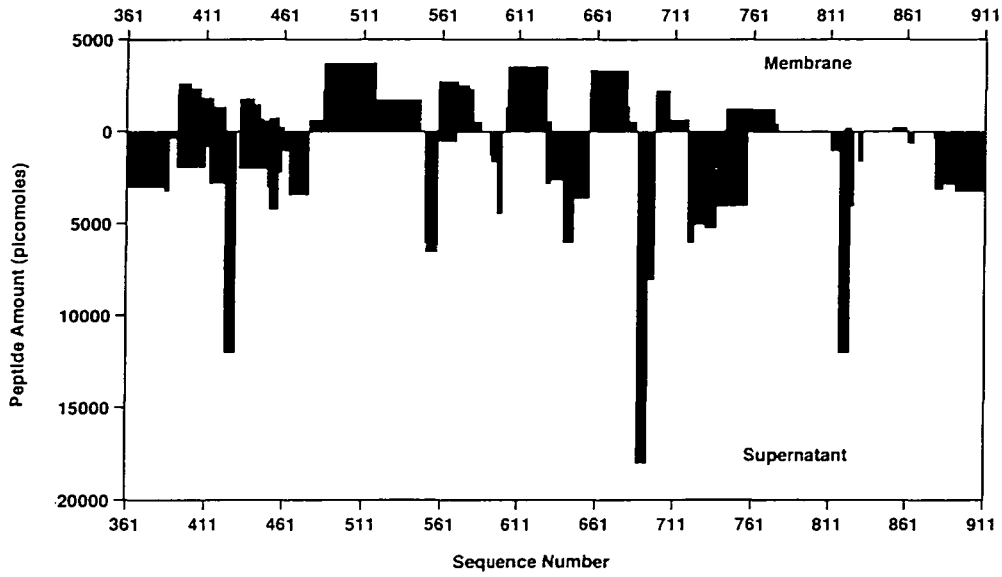

Fig. 3. **Peptides recovered in the supernatant fraction and in the membrane fraction after successive proteinase digestion of alkali-treated membranes.** Alkali-treated membranes (5 mg of protein) were digested as described under "EXPERIMENTAL PROCEDURES." Peptides recovered in the supernatant fraction and in the membrane fraction are expressed in picomoles.

compact ordered structures which are resistant to proteinases.

Evidence obtained in this study suggests that the peptide portions embedded in the membrane lipid bilayer can be classified into at least two categories, one being embedded in the native membrane but exposed to the membrane surface in alkali-treated membranes, and the other portion remaining embedded in the alkali-treated membranes. In other words, the former peptide portions could be parts of the embedded portions in the membrane lipid bilayer without tight lipid-peptide interactions (category 2 in Fig. 5), and the latter portions could be parts of the embedded portions in the membrane lipid bilayer with tight lipid-peptide interactions (category 3 in Fig. 5). Tentative category models are depicted in Fig. 5 (see also Ref. *2*). Only the portions from Thr-552 to Lys-562 and from Thr-629 to Arg-656 belong to category 1 in Fig. 5.

Cluster Formation by Peptide Portions in the Membrane Lipid Bilayer—The unexpected behavior of the five incompatible peptide portions can be ascribed to the peptide-peptide interactions in the membrane lipid bilayer (*20*). Unlike bitopic membrane proteins which have only one transmembrane peptide portion, multi-spanning polytopic membrane proteins can form clusters of transmembrane peptide portions in the membrane lipid bilayer through peptide-peptide interactions within the native molecule conformation. Because band 3 protein is a polytopic membrane protein, some of the transmembrane peptide portions belonging to category 3 could form clusters surrounding other peptide portions within clusters. Owing to the cluster formation, only a restricted hydrophobic surface area of the surrounded peptide portions would be in contact with the lipids in membranes. Such peptide portions could be exposed to the membrane surface when the cluster formation is destroyed in the alkali-treated membranes and subsequently released into the supernatant fraction on proteolytic digestion (see Fig. 5). In the native band 3 protein conformation, it is conceivable that the incompatible peptide portion from Tyr-393 to Tyr-486 in Fig. 4 would be surrounded by a cluster(s) formed by other compatible transmembrane peptide portions, with the corresponding peptide portions being released into the supernatant fraction from alkali-treated membranes on proteolytic digestion upon destabilization of the cluster formation, as schematically depicted as category 2 in Fig. 5. Similar phenomena should be occurring in the sixth and tenth anticipated transmembrane portions, from Pro-568 to Lys-592 and from Trp-723 to Ser-745, in Fig. 4, respectively.

Peptide portions within such clusters in the membrane lipid bilayer are not necessarily hydrophobic if the clusters are formed with amphipathic peptide portions. In other words, hydrophilic peptide portions are not always parts of the hydrophilic connector loops but could be embedded in the membrane lipid bilayer in an energetically stable state. Therefore, peptide portions belonging to category 2 could consist of both hydrophobic and hydrophilic peptide portions. When the clusters in the membrane lipid bilayer were still strong enough as to the alkali-treatment but were partially destabilized, peptide portions in the clusters could be either released into the supernatant fraction or remain within the membrane fraction depending on the denaturation conditions (category 2(b) in Fig. 5).

It is possible that the destabilized peptide portions previously observed in Bacteriorhodopsin (*21*) could be surrounded in such clusters and released into the supernatant fraction on denaturation of the protein during prolonged proteolytic digestion.

Fidelity of Hydropathy Prediction for Band 3 Protein—In this communication, we have assessed hydropathy prediction for band 3 protein using a proteolytic digestion method, and the majority of the anticipated transmembrane peptide portions were found to be consistent with hydropathy prediction based on a combination of Kyte-Doolittle plots and Goldman-Engelman-Steitz plots (*18*). The remaining five anticipated transmembrane peptide portions, however, were released into the supernatant fraction, which was inconsistent with the hydropathy prediction. The incompatibility could be ascribed to the cluster formation due to peptide-peptide interactions of the

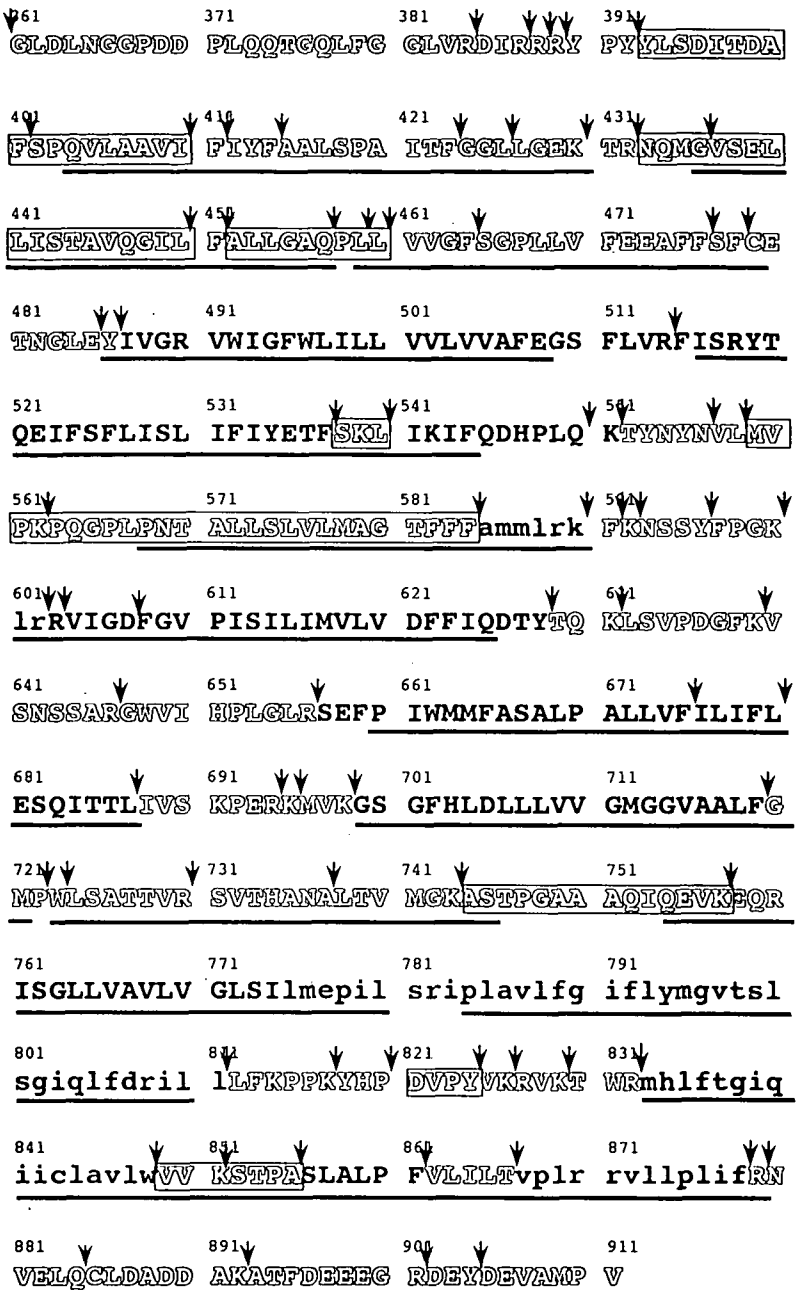

Fig. 4. **Comparison with hydropathy prediction based on a combination of Kyte-Doolittle plots and Goldman-Engelman-Steitz plots** (*18*). The amino acid sequences in bold capitals indicate the portions remaining in the membrane fraction, meaning that the portions belong to category 3 in Fig. 5, and the sequences in outlined capitals indicate the peptides released into the supernatant fraction by proteolytic digestion, meaning that the portions belong to category 1 or 2 in Fig. 5. The outlined capitals surrounded by boxes indicate the portions remaining in the membrane fraction as well as released into the supernatant fraction. The amino acid sequences in small letters indicate the portions which could not been detected in either the supernatant fraction or the membrane fraction. Arrows indicate the *in situ* proteolytic cleavage sites of the band 3 protein in the alkali-treated membrane. The data summarized here were obtained in this and our previous work (*5, 6, 17*). The underlined portions are the anticipated transmembrane portions predicted by Wood (*18*).

multi-spanning band 3 protein. Unlike bitopic membrane proteins, the hydrophilic peptide portions of polytopic membrane proteins would not be always exposed to the membrane surface but could be embedded inside peptide clusters in the membrane lipid bilayer in an energetically stable state. Conversely, hydrophobic peptide portions within clusters could exist in the membrane lipid bilayer shielded from boundary lipids.

Considering the results presented here, we realize that portions including functionally important residues or portions such as Lys-539, Lys-851 (*2, 6*), and the portion from Ala-400 to Ala-408 (*22, 23*) in Fig. 4 belong to category 2 and as such are either released into the supernatant fraction or remain within the membrane fraction. This would make hydropathy prediction for band 3 protein inadequate for understanding the structure and function relationship. As shown by our current results and other publications (*24-29*), conventional methods involving hydropathy analysis for two-dimensional structure predictions for polytopic membrane proteins may be inappropriate, and may give misleading information when used in attempts to understand the molecular mechanisms of polytopic membrane proteins. The gene fusion approach with alkaline phosphatase or prolactin (*30-32*) has been used successfully for the topological mapping of a variety of polytopic membrane proteins. In interpreting the results of fusion experiments, however, we need to take into account the possibility of disturbing the cluster formation on fusing with the reporter proteins.

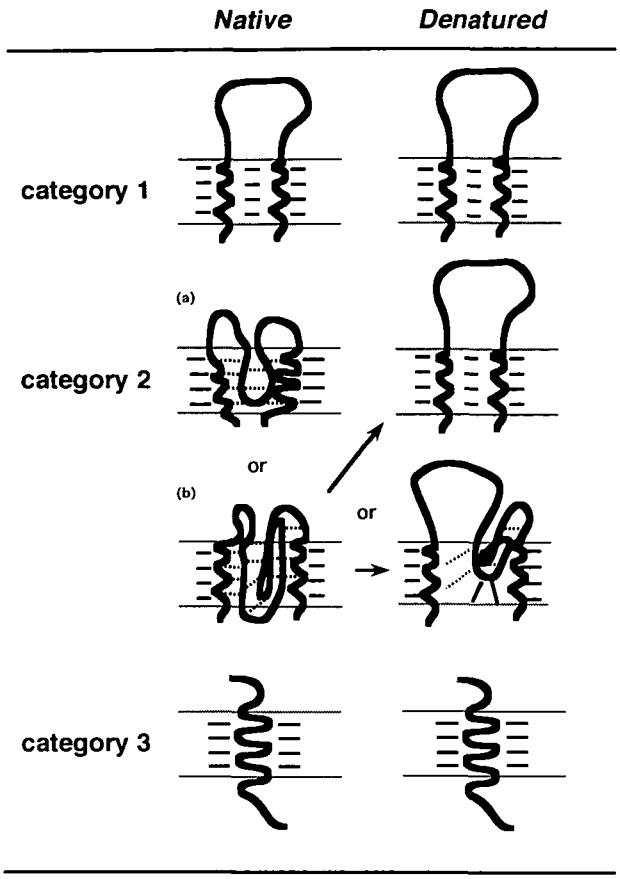

Fig. 5. **Classification of the transmembrane portions into three categories.** Considering the experimental results in the text, we classified the membrane protein portions of band 3 protein into categories 1 to 3. Only portions belonging to category 1 are released into supernatant on proteolytic digestion in the native state. Dotted lines (·····) indicate peptide-peptide interactions, and solid lines (——) indicate lipid-peptide interactions.

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