J. Biochem. 124, 509-518 (1998)

Secondary Structures of Synthetic Peptides Corresponding to the First Membrane-Contact Portion of Normal Band 3 and Its Deletion Mutant (Southeast Asian Ovalocytosis)[1]

Hiroyuki Kuma,*,† Kaori Inoue,* Guohui Fu,† Setsuko Ando,* Sannamu Lee,* Gohsuke Sugihara,* and Naotaka Hamasaki†,[2]

*Department of Chemistry, Faculty of Science, Fukuoka University, Fukuoka 814-0180; and †Department of Clinical Chemistry and Laboratory Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812-8582

Received for publication, March 2, 1998

The conformations of synthetic peptides corresponding to the first membrane-contact portion from Tyr[390] to Lys[430] of band 3 (band 3-1a) and the counterpart portion of South-East Asian ovalocytosis (SAO) band 3 (band 3-1b) in lipid bilayers were examined by means of circular dichroism (CD), Fourier transform infrared (FTIR) spectroscopy as well as a proteolytic digestion method. The CD and FTIR studies showed that band 3-1a and band 3-1b in a membrane lipid bilayer cannot assume an α-helix rich structure but instead assume a β-structure rich conformation. The proteolytic digestion experiments demonstrated that the cleavage sites of Tyr[392] and Phe[423] were common to both the model and erythrocyte membranes. Taken together with our previous work, which indicated that the first membrane-contact portion was the portion embedded in the erythrocyte membrane without tight lipid-peptide interactions [Hamasaki *et al.* (1997) *J. Biochem.* 122, 577-585], we imply herein that the first membrane-contact portion of band 3 by itself can not assume the ordinary α-helix conformation in the membrane lipid bilayers. A proteinase-resistant portion, from Ser[402] to Phe[423], was observed when liposomes containing band 3-1a were digested with proteinase K, while no proteinase-resistant core portion was found in the case of band 3-1b (ΔAla[400]-Ala[408]). This suggests the crucial role of the deleted portion, from Ala[400] to Ala[408], in the interaction of the first membrane-contact portion of band 3 with a membrane lipid bilayer.

Key words: band 3 protein, circular dichroism (CD), Fourier transform infrared (FTIR), liposome, South-East Asian ovalocytosis (SAO) band 3.

Band 3 protein is a typical multi-spanning polytopic membrane protein, and plays a key role in respiration as a metabolic sensor due to the anion exchange activity between Cl^- and HCO_3^- (*1*). Although electron microscopic images of negatively-stained band 3 protein were obtained by two groups (*2, 3*), they were not sufficient to permit discussion of the structure/function relationship. To understand the molecular mechanisms of the anion exchange, therefore, we have been investigating the molecular structure of band 3 protein based on affinity labeling of the active center for anion exchange (*4, 5*), on assessment of the hydropathy prediction to band 3 protein (*6, 7*), and on the dynamic conformational changes of band 3 protein during the anion exchange process (*8*).

Unlike bitopic membrane proteins which have only one transmembrane peptide portion, polytopic membrane proteins have multi-transmembrane peptide portions, and their structures in a membrane lipid bilayer are more complicated than those of bitopic membrane proteins due to the mutual peptide-peptide interactions and/or lipid-peptide interactions in the membrane lipid bilayer (*1, 7*). Actually, five anticipated transmembrane peptide portions

[1] This work was supported in part by Grants-in-Aid for Scientific Research on the Priority Area of "Channel-Transport Correlation" (09257235) and for International Scientific Research (09044319) from the Ministry of Education, Science, Sports and Culture of Japan, and a Grant-in-Aid from the Kyushu University Interdisciplinary Programs in Education and Projects in Research Development.

[2] To whom correspondence should be addressed at: Department of Clinical Chemistry and Laboratory Medicine, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582. Tel: +81-92-642-5748, Fax: +81-92-642-5772, E-mail: hamasaki@cclm.med.kyushu-u.ac.jp

Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-AA-PEG-Resin, Fmoc-amino acid polyethylene glycol/polystyrene resin; Fmoc-Lys(Boc)-PEG, *N*-α-Fmoc-*N*-ε-*t*-butoxycarbonyl-L-lysine-polyethyleneglycol; TBTU, 2-(1*H*-benztriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; DMF, dimethylformamide; DPPC, dipalmitoyl-D,L-phosphatidylcholine; SM, sphingomyelin; PS, L-α-phosphatidylserine; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PTH, phenylthiohydantoin; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; DCM, dichloromethane; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TFE, trifluoroethanol; *HPLC*, high performance liquid chromatography; CD, circular dichroism; FTIR, Fourier transform infrared spectroscopy; FAB, fast atom bombardment; ER, endoplasmic reticulum.

were released from the alkaline-pretreated membrane on trypsin/chymotrypsin digestion, indicating that these portions are differently embedded in the membrane lipid bilayer compared to the rest of the transmembrane peptide portions, which remained in the alkaline-pretreated membrane due to peptide-lipid interactions with the boundary lipids (7). Thus, the peptide portions embedded in a membrane lipid bilayer can be classified into at least two categories, one embedded in the membrane lipid bilayer without tight lipid-peptide interactions (category 2 in Ref. 7), and the other portions embedded in the membrane lipid bilayer with tight lipid-peptide interactions (category 3 in Ref. 7).

The results obtained with the proteolytic digestion method (7) suggested that the peptide portion from Tyr^{393} to Leu^{460} comprises an assembly of peptide portions belonging to category 2 and includes a cluster(s) with peptide-peptide interactions in the erythrocyte membrane. However, in accordance with a hydropathy prediction based on a combination of Kyte-Doolittle plots and Goldman-Engelman-Steitz plots (9), a similar portion from Gln^{404} to Cys^{479} had been anticipated to be the first, second or third transmembrane portion, which should be embedded in a membrane lipid bilayer with tight lipid-peptide interactions. To elucidate the structure of the first membrane-contact portion in lipid bilayers, in this study, using circular dichroism (CD) and Fourier transform infrared (FTIR) spectra in combination with a proteolytic digestion method (7), we examine the conformations of synthetic peptide corresponding to the peptide portion from Tyr^{390} to Lys^{430} of band 3 protein in model phospholipid membranes. We also examine the counterpart peptide portion of South-East Asian ovalocytosis (SAO) band 3 protein, because the deletion from Ala^{400} to Ala^{408} of the mutant band 3 occurs in the peptide region from Tyr^{390} to Lys^{430} (10, 11).

EXPERIMENTAL PROCEDURES

Peptide Synthesis of Band 3-1a, Band 3-1b, and Transmembrane α-Helix Model Peptide P24—Peptides were synthesized with a Milligen 9050 automatic solid phase synthesizer with the standard Fmoc chemistry as described previously (12). Fmoc-AA-PEG-Resin (1 g, 0.2 mmol) was employed as the COOH-terminal polymer support. Fmoc-amino acids were coupled using four equivalents of TBTU. The unreacted N-terminal amino group at each coupling step was acetylated using acetic anhydride, and Fmoc groups were deprotected with 20% piperidine in DMF. The peptides were cleaved from the resin by incubation with a solution of trifluoroacetic acid:*m*-cresol:1,2-ethanedithiol:thioanisole [53:1.3:3.6:7.0 (v/v)] for 3 h at room temperature. The trifluoroacetic acid was evaporated off and the peptides were precipitated by the addition of diethyl ether. After the crude peptides had been immersed in a small amount of HCOOH and then dissolved in 30% acetic acid, the solution was passed through Sephadex G-25 (15×1,200 mm; Pharmacia Biotech, Uppsala, Sweden) with 30% acetic acid, and then purified by means of HPLC with Cosmosil 5C8-AR-300 (10×250 mm; Nacalai Tesque, Kyoto) using a gradient system of water-acetonitrile containing 0.1% trifluoroacetic acid. The band 3-1a peptide was purified by gel-filtration HPLC (Sepharose 12; Pharmacia Biotech) with 50% acetonitrile-water containing 0.1% trifluoroacetic acid.

Amino acid analysis was performed with a JASCO system fluorescent amino acid analyzer (JASCO, Tokyo) after hydrolysis in 5.7 M HCl in a sealed tube at 110°C for 48 h. The analytical data for band 3-1a (i) and band 3-1b (ii) were as follows: (i) Asp, 1.8 (2); Thr, 1.8 (2); Ser, 2.8 (3); Glu, 1.9 (2); Pro, 2.6 (3); Gly, 3.6 (3); Ala, 6.0 (6); Val, 2.4 (2); Leu, 4.7 (5); Ile, 4.3 (4); Phe, 3.7 (4); Lys, 1.4 (1) and (ii) Asp, 1.7 (2); Thr, 1.8 (2); Ser, 2.2 (2); Glu, 0.9 (1); Pro, 1.4 (2); Gly, 3.2 (3); Ala 3.0 (3); Val, 0.9 (1); Leu, 3.8 (4); Ile, 3.5 (4); Phe, 2.6 (3); Lys, 1.4 (1). The molecular weights were determined from FAB-mass spectra obtained with a JEOL JMX-HX110 (JEOL, Tokyo); base peak, 4,445.3; calculated for $C_{216}H_{315}O_{58}N_{43}\cdot H^+$, 4,444.164 for band 3-1a; and base peak, 3,559.5; calculated for $C_{174}H_{261}O_{47}N_{33}\cdot H^+$, 3559.133 for band 3-1b. The sequences of the peptides were also confirmed with a gas-phase sequencer (Applied Biosystems, model 492, California, USA), and the phenylthiohydantoins (PTH-derivatives) were identified with an Applied Biosystems 140C PTH-derivative analyzer on-line system. A transmembrane α-helix model peptide, P24 (Ac-Lys_2-Gly-Leu_{24}-Lys_2-Ala-NH_2), was also synthesized by Fmoc-chemistry according to Davis *et al.* (13).

CD Measurement of Peptides in TFE or Liposomes—CD spectra were recorded with a JASCO J-600 spectropolarimeter (JASCO) using a quartz cell of 1-mm path length, and the data were expressed in terms of the molar ellipticity. Peptide concentrations were determined from the UV-absorbance of Tyr ($\varepsilon = 1{,}340$ at 275 nm). Spectra in TFE or liposomes were measured with peptide concentrations of 100 and 10 μM, respectively. Micelle solutions (3 mM) were prepared by dissolving a desired amount of LPC or LPC:LPG (3:1) in 5 mM TES buffer, followed by solubilization by the addition of the peptides (30 μM).

Phospholipid liposomes were prepared and characterized as described previously (14, 15). In brief, phospholipid (egg yolk PC:egg yolk PE:bovine erythrocytes SM:bovine brain PS = 12:12:10:5.2) (20 mg, ~25 μmol) and peptides (2.5 μmol) were codissolved in chloroform (1 ml) and then dried by spraying of nitrogen in a conical glass tube. The dried peptide-lipid film was hydrated in 2 ml of 5 mM TES buffer or HEPES buffer (pH 7.4) with repeated vigorous vortex mixing at 20°C for 30 min. The suspension was sonicated at 0°C for 30 min with 10 min intervals using a probe-type sonicator (Kaijo Denki ultrasonic disrupter model TA-4280, Tokyo), centrifuged and then diluted to 25 ml with the same buffer, in which the lipid concentration was ~1 mM. The small unilamellar vesicles obtained (16) were used for the CD measurements. Spectra were analyzed as a linear combination of spectra for poly (L-lysine) (MW, 15,000-30,000) adopting the three well-known conformations depending on the solvent conditions; α-helix, β-structure, and random coil (17). All measurements were carried out at 25°C. CD data were expressed as mean residue ellipticity. To minimize scattering due to liposomes, each CD spectrum for liposomes was subtracted from that for a peptide obtained in the presence of liposomes.

FTIR Measurement of Peptides in Lipid Bilayers—Before the samples were prepared, the trifluoroacetate counter ions of the peptides which originated from the trifluoroacetic acid used during the HPLC purification were

removed by lyophilization twice from 10 mM HCl. The spectral samples for FTIR in a lipid dispersion were prepared by codissolving lipid (6×10^{-6} mol) and a peptide (2×10^{-7} mol) in chloroform at a lipid:peptide ratio of 30:1. After drying the samples under N_2 gas and vacuum, they were hydrated with a small amount of saline (150 mM NaCl, pH 7.4) [lipid:saline = 4:6 (w/v)] at 0°C and then stood at 20°C for 30 min. The cooling (0°C) and warming (20°C) were repeated again to sufficiently incorporate the peptides into the lipid according to the method of Rizzo *et al.* (*18*). Samples were placed in a microcell fitted with CaF_2 and a 15-μm Teflon spacer. Spectra were obtained with a Bio Rad FTS-165 (Bio-Rad, California, USA) and a Hewlett Packerd Vectra VL2 computer (Hewlett Packard, California, USA) for data acquisition and analysis. Each spectrum was the average of 250 scans and was recorded at ambient temperature (21-23°C) with 2-cm^{-1} resolution. Reference spectra of lipid dispersions prepared by completely the same procedure were recorded in the same microcells and under identical instrument conditions as the sample spectra. Difference spectra were obtained by digitally subtracting the lipid dispersion spectra from the corresponding sample spectra. The subtracting factor was varied until the second derivative of the peptide spectrum between 1,800 and 1,700 cm^{-1} was featureless (*19*). The second-derivative spectrum was obtained by twice application of the first-derivative function with nine-point smoothing using the Savitzky-Golay smoothing procedure (*20, 21*). Then the spectra were deconvoluted to obtain estimates of the numbers of component bands and their frequency ($\gamma = 4.25$, and smoothing factor = 0.3), followed by a curve-fitting procedure to obtain estimates of the bandwidth and band area as described by Fabian *et al.* (*19*). After smoothing on the deconvolution, the spectra were offset to place the minimum absorbance value at zero. Only the 1,700-1,620 cm^{-1} range was used for secondary structure analysis.

Proteolytic Digestion Experiments on Peptides in Liposomes—Liposomes containing band 3-1a and band 3-1b ([lipid]:[peptide] = 10:1) were prepared as described above. Peptides incorporated into the lipid bilayer were digested with chymotrypsin or proteinase K [peptide: proteinase = 50:1 (mol/mol)] at 37°C for 1 h. The peptide portions released into the supernatant from the lipid bilayer on proteolytic digestion and those remaining in the lipid bilayer were separated by using a Millipore Ultrafree filter (Millipore, Massachusetts, USA). Peptide portions in the supernatant or the lipid bilayer fraction were analyzed as described previously (*6, 7*).

Analysis of Peptides Released into the Supernatant Fraction or Remaining in the Lipid Bilayer Fraction—The peptide portions released into the supernatant fraction were analyzed by HPLC on a reversed phase column (Cosmosil 5C18-AR-300; 4.6×250 mm, Nacalai Tesque), using a linear gradient of 0-100% acetonitrile containing 0.1% trifluoacetic acid. The peptide portions remaining in the lipid bilayer fraction were dissolved in an equal volume of 8 M urea/4% SDS and then applied to a reversed phase column (Cosmosil 5C18-AR-300; 4.6×250 mm, Nacalai Tesque), using a water-acetonitrile-propanol system containing 0.1% trifluoroacetic acid (*7*). All peaks were analyzed with a gas-phase sequencer as described above.

Materials—Fmoc-Lys(Boc)-PEG and Fmoc-amino acids were from Millipore. DMF, DCM, piperidine, acetic anhydride, diisopropylethylamine, 1-hydroxybenzotriazole, and TBTU were purchased from Nacalai Tesque. Egg yolk PC, egg yolk PE, bovine erythrocytes SM, bovine brain PS, and reagents for amino acid sequencing were obtained from Sigma (Missouri, USA) and Applied Biosystems (California, USA), respectively. Other chemicals were of analytical grade.

RESULTS

CD Spectral Study—Figure 1 shows CD spectra of band 3 peptides (band 3-1a and band 3-1b) in TFE (Fig. 1a) and in lipid bilayers of liposomes with a phospholipid composition similar to that of human erythrocyte membranes (Fig. 1b). The interpretation of CD spectra for membrane lipid bilayers is complicated due to differential light scattering and absorption flattening that can lead to nonlinear attenuation of the CD bands at strong absorption peaks (*22, 23*). We, therefore, measured CD spectra of band 3 peptides not only in liposomes (Fig. 1b) but also in micelle solutions (Fig. 1c) to overcome such complications. The CD spectrum of a transmembrane α-helix model peptide, P24 (*13, 24-26*),

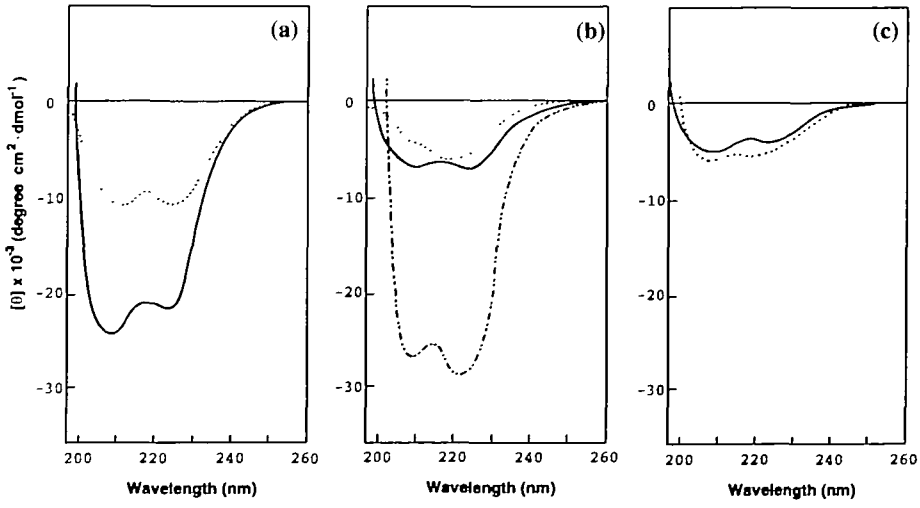

Fig. 1. **CD spectra of band 3-1a, band 3-1b, and/or P24 in TFE (a), lipid bilayers of liposomes (b), and micelle solutions (c).** (a and b) Spectra of band 3-1a, band 3-1b, and a transmembrane α-helix model peptide, P24, are indicated by a solid line (——), a dotted line (·····), and a dashed line (– · · –), respectively. The concentrations of the peptides were 100 μM in TFE and 10 μM in lipid bilayers of liposomes with a phospholipid composition similar to that of human erythrocyte membranes (PC:PE:SM:PS = 12:12:10:5.2). (c) CD spectra of band 3-1a peptide (30 μM) in micelle solutions (3 mM) of LPC and LPC:LPG (3:1) are indicated by a solid line (——), and a dotted line (·····), respectively.

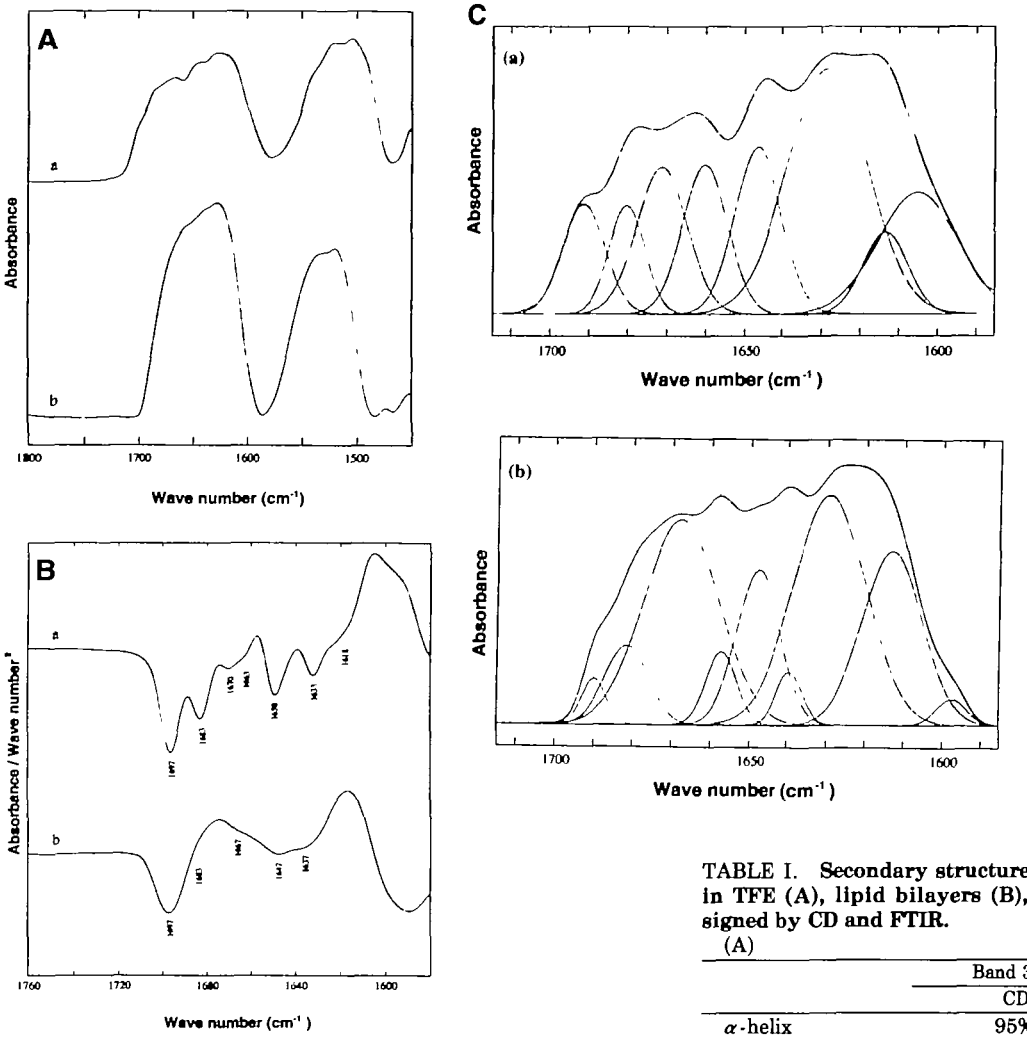

Fig. 2. **(A) FTIR difference spectra of band 3-1a (a) and band 3-1b (b) in lipid bilayers.** Difference spectra were obtained by digitally subtracting the lipid dispersion spectrum from the corresponding sample spectrum. The subtracting factor was varied until the second derivative of the peptide spectrum between 1,800 and 1,700 cm^{-1} was featureless using Bio-Rad software according to Fabian *et al.* (*19*). **(B) Second-derivative infrared spectra of band 3-1a (a) and band 3-1b (b) in lipid bilayers in the amide I region. (C) Deconvoluted infrared spectra and curve fitting analyses of amide I of band 3-1a (a) and band 3-1b (b) in lipid bilayers.** Deconvoluted infrared spectra and curve fitting analyses were performed according to Fabian *et al.* (*19*) as described under "EXPERIMENTAL PROCEDURES."

TABLE I. **Secondary structure contents of synthetic peptides in TFE (A), lipid bilayers (B), and micelle solutions (C) assigned by CD and FTIR.**

(A)

	Band 3-1a	Band 3-1b
	CD	CD
α-helix	95%	30%
β-structure	5%	30%
Random	0%	40%

(B)

	Band 3-1a		Band 3-1b	
	CD	FTIR	CD	FTIR
α-helix	15%	13%	0%	5%
β-structure	60%	71%	65%	81%
Random (and/or others)	25%	16%	35%	14%

(C)

	Band 3-1a	
	CD	
	LPC	LPC/LPG (3:1)
α-helix	28%	15%
β-structure	42%	40%
Random	30%	45%

was also obtained in liposomes as a reference (Fig. 1b).

TFE, an α-helix promoting solvent, is considered to reflect a hydrophil-lipophil dynamic membrane environment and has been used to study the conformational requirements of biological reaction membranes (*27*). In TFE, band 3-1a have a double minimum spectrum at 208-222 nm that is characteristic of the α-helical structure. The helical content was 95% (Table I). Band 3-1b also have a double minimum spectrum but with much lower intensity as compared with that in the case of band 3-1a; 30% α-helix, 30% β-sheet, and 40% random structure (Table I). The helical content of band 3-1a was much greater than that of band 3-1b, indicating that band 3-1a exhibits inherent helicity in a hydrophobic membrane-like milieu, which is lacked by band 3-1b.

CD spectra of band 3-1a, band 3-1b, and P24 in liposomes with a phospholipid composition similar to that of human erythrocyte membranes are shown in Fig. 1b. The sample solutions after sonication were slightly turbid. Red shifts (~1-2 nm) of CD spectra in turbid samples have often been observed in biological systems due to multiple scattering in turbid suspensions (*28, 29*). The transmembrane α-helix model peptide, P24, completely had the α-helical structure in the lipid bilayers with a double minimum spectrum at 208-222 nm (Fig. 1b). The band 3-1a spectrum was also a double minimum spectrum at 209-224 nm. However, the double minimum band of the band 3-1a spectrum was not simply assigned to the α-helical structure because of the low molar eclipticity. This spectrum was analyzed as a mixture of 15% α-helix, 60% β-sheet, and 25% random structure according to the method of Fukushima *et al.* (*17*) (Table I). On the other hand, band 3-1b exhibited a minimum at 220 nm (Fig. 1b), indicating that the conformation of band 3-1b in liposomes is attributable to 65%

β-sheet and 35% random structure (Table I). To make sure that the low intensity of the spectrum was not due to turbidity, we measured CD spectra of band 3-1a in micelle solutions of LPC and LPC/LPG (3:1) (Fig. 1c). The spectra in micelle solutions of LPC and LPC/LPG (3:1) were analyzed also as a mixture of 15-28% α-helix, 40-42% β-sheet, and 30-45% random structure (Table I).

FTIR Study—FTIR is an established method for studying the secondary structures of polypeptides and proteins in aqueous solution, and has recently been applied for studyings their conformational analysis in the presence of lipid bilayers, extensively (*19, 21, 25, 30, 31*). Thus, to obtain further information on the structures of the band 3 peptides, we measured FTIR spectra using a 15-μm thin cell to compensate for the strong absorption of water in the amide I region, and prepared specimens for FTIR with a lipid: water ratio of 4:3 (w/v), because phospholipids are able to form a lipid bilayer under these conditions (*32*). Peptides were dispersed in fully hydrated lipid bilayers consisting of erythrocyte membrane lipid components by repeated cooling (0°C) and warming (20°C). The spectra of the lipid bilayers with or without the peptides were recorded between 3,500 and 1,000 cm^{-1}. Difinite differences were observed in the regions of the amide I and amide II bands. The different absorption spectra for the peptides and the reference lipid bilayers of band 3-1a and band 3-1b in the region from 1,800 to 1,450 nm^{-1} are shown in Fig. 2A. In order to avoid artificial bands, and in correct band positions for amide I and amide II, the subtraction factor for the absorption spectrum was changed until the second derivative of the peptide spectrum between 1,800 and 1,700 cm^{-1} became featureless (Fig. 2B) before being resolved by Fourier deconvolution of the spectra (*19*). The deconvoluted spectrum was analyzed by curve fitting between 1,715 and 1,585 cm^{-1} (Fig. 2C). The quantitative contribution of each band to the total amide I contour was given at 6 peaks for the spectra of band 3-1a and 7 peaks for band 3-1b. The peak parameters and their assigned structures are listed in Table II. When the component bands at 1,691, 1,680, and 1,628 cm^{-1} were assigned to β-sheet structure, the β-structure of band 3-1a represented 58% of the total area of amide I (Table II). The components at 1,660 and 1,646 cm^{-1} assigned to α-helical structure and random structure (and/or 3^{10}-helix), respectively, were estimated to represent 13 and 16%. It is noteworthy that turn structure (13%) at 1,671 cm^{-1} was observed. The component around 1,670 cm^{-1}, which is assigned as turn structure (1,671 cm^{-1}, 13%), drastically increased in the band 3-1b spectrum and instead the α-helix component was decreased. The results of FTIR were fairly consistent with those of CD analysis when peaks assigned as β-structure and turn in FTIR were

TABLE II. **Curve-fitting analysis of FTIR spectra of band 3-1a and band 3-1b.**

Band 3-1a		Band 3-1b		
Band position	Band area	Band position	Band area	Band assignment*
(cm^{-1})	(%)	(cm^{-1})	(%)	
1,691	10	1,690	2	Turn/β-sheet
1,680	8	1,681	7	Turn/β-sheet
1,671	13	1,667	33	Turn
1,660	13	1,657	5	α-helix
1,646	16	1,647	14	Random and/or 3^{10}-helix, turn (β-sheet)
		1,639	3	β-sheet
1,628	40	1,628	36	β-sheet

*The assignments are based on recent literature (*19, 21, 25, 44*).

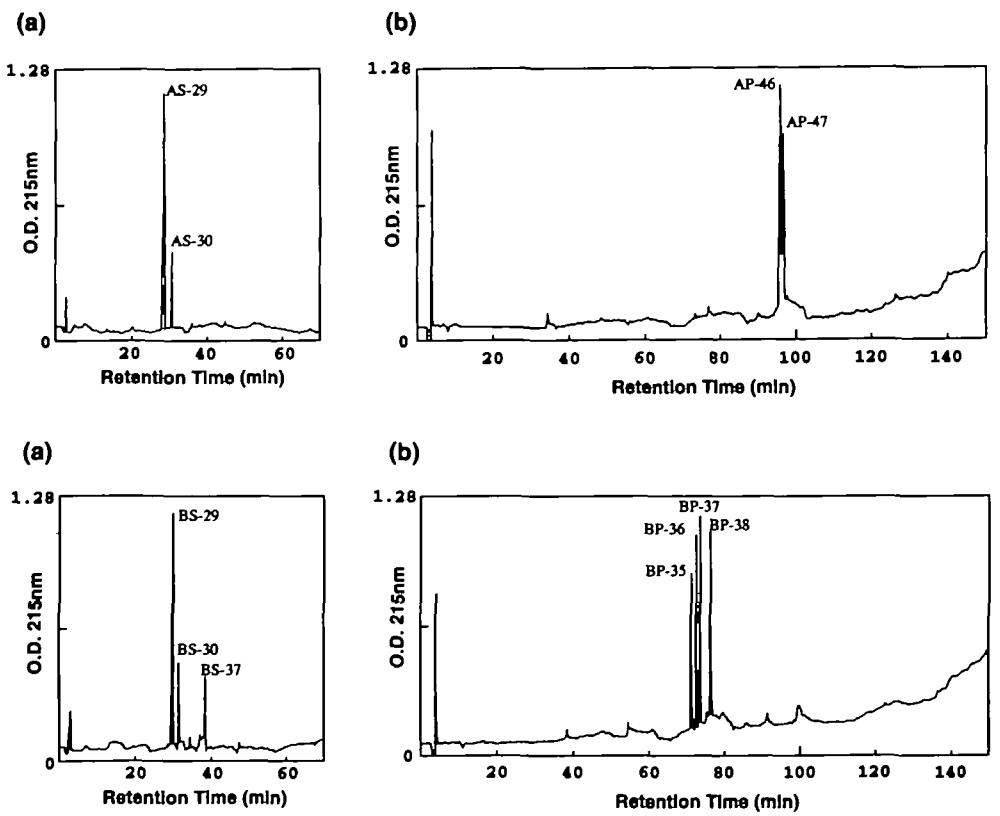

Fig. 3. **HPLC analyses of peptides released into the supernatant fraction (a) and remaining in the lipid bilayer fraction (b).** Liposomes containing band 3-1a and band 3-1b ([lipid]:[peptide] = 10:1) were prepared as described under "EXPERIMENTAL PROCEDURES." Peptides incorporated into the lipid bilayer were digested with chymotrypsin [peptide:proteinase = 50:1 (mol/mol)] at 37°C for 1 h as described under "EXPERIMENTAL PROCEDURES." Peptide portions released into the supernatant from the lipid bilayer on proteolytic digestion and those remaining in the lipid bilayer were separated by using a filter of Millipore Ultrafree size (Millipore, Massachusetts, USA). Peptide portions in the supernatant or the lipid bilayer fraction were analyzed as described previously (5-7). Peptides in the peaks numbered AS-29, 30, and AP-46, 47, and BS-29, 30, 37, or BP-35-38 were of band 3-1a and band 3-1b, respectively.

TABLE III. **Peptides released into the supernatant fraction and remaining in the membrane lipid bilayers.**
(A) After chymotryptic digestion.

Fraction	Peak No.	Amino acid sequence	Amount (pmol)	Position in band 3 protein
	Band 3-1a:	300 YPYYLSDITDAFSPQVLAAVIFIYFAALSPAITFGGLLGEK 430		
Supernatant	AS-29	YPY	2,000	390-392
		YPYY		390-393
	AS-30	GGLLGEK	2,000	424-430
Membrane	AP-46	YLSDITDAFSPQVLAAVIFIYFAALSPAITF	1,500	393-423
	AP-47	LSDITDAFSPQVLAAVIFIYFAALSPAITF	1,500	394-423
	Band 3-1b:	390 YPYYLSDITD 399 — 409 VIFIYFAALSPAITFGGLLGEK 430		
Supernatant	BS-29	YPY	1,000	390-392
		YPYY		390-393
	BS-30	GGLLGEK	1,000	Δ424-Δ430
	BS-37	IYF	800	Δ412-Δ414
Membrane	BP-35	SPAITF	1,000	Δ418-Δ423
	BP-36	AALSPAITF	2,000	Δ415-Δ423
	BP-37	LSDITD — VIF	1,200	394-Δ411
	BP-38	YLSDITD — VIF	600	393-Δ411

(B) After proteinase K digestion.

Fraction	Peak No.	Amino acid sequence	Amount (pmol)	Position in band 3 protein
	Band 3-1a:	390 YPYYLSDITDAFSPQVLAAVIFIYFAALSPAITFGGLLGEK 430		
Supernatant	KAS-25	YLSD	4,000	393-396
	KAS-29	YPY	5,000	390-392
	KAS-36	DITDAF	1,200	396-401
Membrane	KAP-17	DITDAF	2,000	396-401
	KAP-76	DAFSPQVLAAVIFIYFAALSPAITF	2,000	399-423
	KAP-78	DITDAFSPQVLAAVIFIYFAALSPAITF	1,000	396-423
	Band 3-1b:	390 YPYYLSDITD 400 — Δ410 VIFIYFAALSPAITFGGLLGEK Δ430		
Supernatant	KBS-23	LSPA	3,000	Δ417-Δ420
	KBS-25	SPAIT	3,000	Δ418-Δ422
	KBS-26	AAL	1,500	Δ412-Δ414
	KBS-29	YPY	6,000	390-392
	KBS-37	SPAITF	3,000	Δ418-Δ423
Membrane	None			

taken as β-structure (Table I).

Proteolytic Digestion of Liposomes—The modes of interaction of band 3-1a and band 3-1b with the model phospholipid membranes were examined by means of a proteolytic digestion method, that is, the cleavage sites of band 3-1a and band 3-1b in a model membrane lipid bilayer were compared with those of the corresponding portion of band 3 protein in the erythrocyte membrane as a reflection of the mode of interaction between the peptides (or the protein) and the membrane lipid bilayer. Peptides in liposomes were digested with chymotrypsin or proteinase K and then separated as described under "EXPERIMENTAL PROCEDURES." Figure 3 shows the HPLC profiles of peptides released into the supernatant fraction and remaining in the lipid bilayer fraction on chymotrypsin digestion. All peaks were analyzed with a gas-phase sequencer. As shown in Table IIIA, portions of band 3-1a, YPY (390-392), YPYY (390-393), and GGLLGEK (424-430), were released from liposomes into the supernatant fraction on chymotrypsin digestion, while the portion from Tyr^{393} (or Leu^{394}) to Phe^{423} of band 3-1a was proteinase-resistant and remained in the lipid bilayer fraction after digestion of the liposomes with chymotrypsin.

When band 3-1b in the liposomes was digested with chymotrypsin, a new peptide, IYF (*Δ412-Δ414*), was released into the supernatant fraction in addition to the peptides, YPY (390-392), YPYY (390-393) and GGLLGEK (*Δ424-Δ430*), while the portions, YLSDITDVIF (393-*Δ411*), LSDITDVIF (394-*Δ411*), SPAITF (*Δ418-Δ423*), and AALSPAITF (*Δ415-Δ423*), remained in the membrane fraction (Table IIIA). Thus, three more cleavage sites, $Phe^{\Delta411}$, $Phe^{\Delta414}$ and $Leu^{\Delta417}$, appeared in band 3-1b, indicating that the mode of interaction of band 3-1b with the lipid bilayer is different from that of band 3-1a with the membrane lipid bilayer.

A proteinase-resistant portion, from Ser^{402} to Phe^{423}, was observed when liposomes containing band 3-1a were digested with proteinase K and the resistant portion remained in the membrane lipid bilayer fraction (Table IIIB), indicating that this portion is embedded in the membrane lipid bilayer. No proteinase-resistant core portion, however, was observed in the membrane lipid bilayer fraction when

liposomes containing band 3-1b were digested with proteinase K (Table IIIB). This suggests the crucial roles of the peptide portion from Ala^{400} to Ala^{408} in the interaction of the peptide from Ser^{402} to Phe^{423} with the lipid bilayer.

Surprisingly, an almost equal amount of a hydrophilic peptide, DITDAF (396-401), remained in the membrane lipid bilayer fraction when compared to the amount released into the supernatant fraction (Table IIIB). This could not be explained simply by methodological problems such as incomplete washing, because no other peptide portions YPY, YPYY, GGLLGEK, IYF, LSPA, AAL, and SPAITF, were distributed at all in either of the fractions (Table III). A similar phenomenon was observed in the alkali-treated erythrocyte membrane, that is, the portion from Tyr^{393} to Ile^{410} which contains the portion, DITDAF (396-401), was distributed in both the supernatant fraction and the membrane fraction when the treated membranes were digested with trypsin and chymotrypsin (7). The reason for the amphipathic distribution of DITDAF (396-401) remains to be determined.

On digesting liposomes containing peptides with proteinases, the membrane-bound peptides containing the carboxy-terminal or amino-terminal of band 3-1a and band 3-1b as well as the undigested full size band 3-1a and band 3-1b were not detected (Fig. 3 and Table III). Liposomes prepared as described under "EXPERIMENTAL PROCEDURES" were small unilamellar vesicles (~300 Å diameter), as shown in previously (*16*), and the intravesicular space was shielded sufficiently from the proteinases (*33*). Therefore, the lack of detection of peptides containing the carboxy-terminal or amino-terminal region indicates that the peptides are embedded in the liposome membranes due to both termini facing the extravesicular surface. In such small unilamellar liposomes, phospholipids are unevenly distributed between the inner and outer monolayers of the membrane lipid bilayer as a result of different phospholipid head sizes, with phospholipids having larger head groups tending to be located in the outer monolayers. Thus, the nonuniform distribution of peptides may be a reflection of the heterogeneous distribution of phospholipids. It should be mentioned that the heterogeneous distribution of phospholipids did not affect the peptide conformation because the contents of α-helix and β-structure of the peptides in the liposomes, as assessed by CD, were almost identical to those in the homogenous fully hydrated lipid bilayers examined by FTIR (Table I). Under the experimental conditions, the phospholipids are homogeneously distributed in the hydrated lipid bilayers.

DISCUSSION

When the fidelity of hydropathy prediction for band 3 protein in the erythrocyte membrane was examined by means of a proteolytic digestion method, the portion from Tyr^{393} to Tyr^{486} behaved uniquely. Namely, the whole peptide portion was liberated from the membrane lipid bilayers on proteolysis (*7*), although the portion would be expected to be hydrophobic enough to contain three putative transmembrane peptide portions on hydropathy prediction. To characterize such a unique region, from Tyr^{393} to Tyr^{486}, in the erythrocyte membrane, we examined the modes of interaction with model lipid bilayers using the peptide portion from Tyr^{390} to Lys^{430} (band 3-1a).

The secondary structure of band 3-1a in model lipid bilayers was unexpectedly confirmed to be a β-structure rich conformation by the CD and FTIR studies, while the structure in TFE solution was α-helical as the helical content was approximately 90% (Figs. 1 and 2, Table I). The CD spectrum of band 3-1a in model lipid bilayers indicated that the peptide structure could be assigned as 15% α-helix, 60% β-structure, and 25% random, while the FTIR study suggested 13% α-helix, 51% β-structure, 16% random (or/and 3^{10} helical structure, β-structure), and 13% turn (Table II). The spectrum of band 3-1b in model lipid bilayers was also estimated to be β-structure rich: 65% β-structure and 35% random by CD, and 5% α-helix, 48% β-structure, 14% random, and 33% turn by FTIR. The contents of the various secondary structures in both peptides estimated by CD and FTIR spectroscopy are generally in agreement with each other. The general limitations of the quantitative determination of the secondary structures of proteins through CD and FTIR studies have been discussed in detail by Fabian *et al.* (*19*), such as the uncertainties regarding the evaluation of β-sheet (and turn) contents by CD and underestimation of random structure by FTIR. Moreover, the assignment of the peaks in the amide I region with different secondary and tertiary structures by FTIR is complicated by the fact that secondary structures depend on the peptide sequence, the type of sample preparation, and other environmental factors. Even considering all of these uncertainties, however, we could conclude that band 3-1a and band 3-1b did not assume an α-helix rich structure, but instead assumed a β-structure rich conformation in the model lipid bilayers.

The solution structures of the peptide portions from Val^{405} to Gly^{424} and Gly^{436} to Ala^{456} have been studied using NMR by Gargaro *et al.* (*34*), who found that the peptide portions adopted a predominantly α-helical structure in a TFE solution that is coincident with our CD data in TFE, as shown in Fig. 1 and Table I. However, this evidence could not simply mean that the peptide portions assume an α-helix rich structure in the membrane lipid bilayer, because the α-helix rich structure in a TFE solution changed to a β-structure rich conformation in the model lipid bilayer used in this investigation.

The proteolytic cleavage sites of the peptides in the model lipid bilayer and the erythrocyte membrane were compared. When the liposomes were digested with

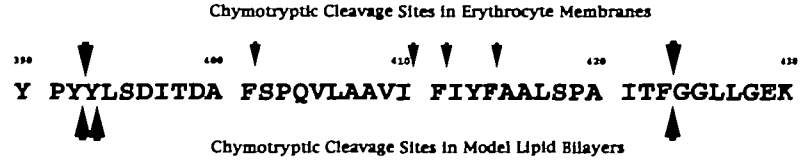

Fig. 4. **Chymotryptic cleavage sites of band 3-1a in liposomes and the corresponding peptide portion of band 3 protein in erythrocyte membranes.** Comparison of the cleavage sites in erythrocyte membranes (top) and in liposomes (bottom). The major and minor cleavage sites are indicated by large and small arrows, respectively. The data summarized here were obtained in this study and from a previous paper (*7*).

chymotrypsin as described under "EXPERIMENTAL PROCEDURES," three peptide fragments YPY (390-392), YPYY (390-393), and GGLLGEK (424-430), were released into the supernatant fraction, while the peptide portion from Tyr^{393} (or Leu^{394}) to Phe^{423} remained in the membrane fraction. As summarized in Fig. 4, the chymotryptic cleavage sites were the carboxyl sites of Tyr^{392}, Tyr^{393}, and Phe^{423} in the liposomes, and the carboxyl sites of Tyr^{392} and Phe^{423} as major cleavage sites and Phe^{401}, Ile^{410}, Phe^{411}, Phe^{414} as minor cleavage sites in the erythrocyte membrane, respectively. The common major chymotryptic cleavage sites in liposomes and the erythrocyte membrane were Tyr^{392} and Phe^{423}, although many more theoretical chymotryptic cleavage sites exist between Tyr^{392} and Phe^{423}. The additional cleavage sites, Phe^{401}, Ile^{410}, Phe^{411}, and Phe^{414}, observed in the erythrocyte membrane are supposed to be cleaved in the supernatant fraction after which portion was liberated from the membrane lipid bilayer, because peptides digested at the additional site of Phe^{401}, Ile^{410}, Phe^{411}, and Phe^{414} were recovered only from the supernatant fraction (7). The additional cleavage site of Tyr^{393} in liposomes may reflect a subtle difference between the model and erythrocyte lipid bilayers.

The chymotrypsin-resistant peptide portion from Tyr^{393} (or Leu^{394}) to Phe^{423} in the model lipid bilayer preferentially remained in the membrane lipid bilayer fraction. The corresponding portion of band 3 protein in the erythrocyte membrane, however, was distributed to both the supernatant fraction and the membrane fraction on digestion with chymotrypsin (7). The different modes of interactions with the two membrane lipid bilayers could be explained as follows. As we proposed previously (*1, 7*), peptide portions embedded in a membrane lipid bilayer could be classified into at least two categories (categories 2 and 3 in Ref. 7), that is, one is the portions embedded in the membrane lipid bilayer when band 3 protein assumes the native conformation, but exposed on the membrane surface when band 3 protein takes on the denatured conformation, and the other is the portions remaining embedded in the membrane lipid bilayer under the native and denatured conditions. In other words, the former peptide portions could be parts of the portion embedded in the membrane lipid bilayer without tight lipid-peptide interactions, and the latter portions could be parts of the portion embedded in the membrane lipid bilayer with tight lipid-peptide interactions. Unlike bitopic membrane proteins which have only one transmembrane peptide portion, a multi-spanning polytopic membrane protein, band 3 protein, could form clusters consisting of multi-membrane peptide portions in the membrane lipid bilayer through peptide-peptide interactions within the native molecule conformation. Owing to the cluster formation, only a restricted hydrophobic surface area of the peptide portion from Tyr^{390} to Lys^{430} would be in contacted with lipids in erythrocyte membranes and, therefore, liberated from the membranes on proteolytic digestion depending on the denaturation conditions. The synthetic peptide from Tyr^{390} to Lys^{430} in the model lipid bilayer could mimic bitopic membrane proteins which have only one hydrophobic peptide portion. Thus, contrary to the whole band 3 protein molecule in the erythrocyte membrane, the hydrophobic synthetic peptide in the model lipid bilayer would be in direct contact with boundary lipids and form tight lipid-peptide interactions, because the cluster formation can not occur with just the peptide portion from Tyr^{390} to Lys^{430}. Accordingly, the chymotrypsin-resistant portion from Tyr^{393} (Tyr^{394}) to Phe^{423} preferentially remained in the membrane lipid bilayer.

Band 3-1b also takes on a mainly β-structure in a model lipid bilayer, as shown by the CD and FTIR studies (Table II), but is fully digested by proteinase K, contrary to band 3-1a (Table IIIB), suggesting that the deleted portion, from Ala^{400} to Ala^{408}, is important for formation of the proteinase-resistant core portion in the membrane lipid bilayer. This result could be correlated to the abnormal conformation of the SAO band 3 protein molecule.

Isolated β-strands can not exist stably as transmembrane segments in a lipid bilayer due to unfavorable interactions of the peptide backbone with the nonpolar phase of the lipid bilayer, that is, the peptide portion from Tyr^{390} to Lys^{430} may stay on the lipid surface after taking on a β-structure. We could not obtain direct evidence of the incorporation of the band 3 peptides into the membrane lipid bilayers using Trp fluorescence, because no Trp residue is present in the peptides. The following evidence, however, strongly suggests that the peptides are incorporated into lipid bilayers through a hydrophobic interactions: (i) the band 3-1a peptide was solubilized completely in micelle solutions of LPC and LPC/LPG (3:1); (ii) the CD spectra of band 3-1a in micelles and liposomes were almost the same, despite the slightly different locations of the double minimum bands (Fig. 1, b and c); and (iii) the common major chymotryptic cleavage sites in liposomes and the erythrocyte membrane were Tyr^{392} and Phe^{423}. Recent extensive studies by Deber and co-workers involving *de novo*-designed model peptides with systematically varied helical propensity or segmental hydrophobicity have shown that in membrane mimetic environments of both vesicles (liposomes) and micelle solutions of LPC and LPC/LPG (3:1) the peptides interact with the lipid in parallel manners (*35, 36*). These findings indicate that the peptides were incorporated into lipid bilayers a taking on a β-structure.

The FTIR study showed that band 3-1a has after considerable contents of antiparallel β-structure and β-turn in a model lipid bilayer (Table II). Although such conformations of antiparallel β-structure and β-turn would reflect a reaction of the peptide due to the instability in the lipid bilayer, at least we could say that the peptide portion from Tyr^{390} to Lys^{430} has an inherent tendency to form a β-structure rich conformation in the model lipid bilayer. Taken together with the common proteolytic cleavage sites of band 3 protein in the erythrocyte membrane and of the synthetic peptide in the model lipid bilayer, we could safely assume that the peptide portion from Tyr^{390} to Lys^{430} in the membrane lipid bilayer does not take on the ordinary α-helix transmembrane conformation but instead assumes the β-structure rich conformation, as observed for the synthetic peptide in the model lipid bilayer.

Recent X-ray crystallographic analyses of integral membrane proteins have shown that their hydrophobic sequences span the lipid bilayers predominantly as not only α-helix but also β-structure (*37, 38*). Hucho *et al.* (*39*) also revealed through FTIR experiments that the secondary structure of the four-spanning transmembrane segments in the nicotinic acetylcholine receptor was composed of a mixture of α-helix and β-structure. Our present results

show that band 3-1a has considerable contents of antiparallel β-structure and β-turn in the model lipid bilayer. The chymotrypsin-resistant membrane bound region from Leu^{394} to Phe^{423} consists of 30 amino acid residues, the chain length of which is often observed as a hairpin-like structure (β-loop) formed into membranes by antiparallel β-structure bent by β-turn (*40-44*). Aggeli *et al.* (*45*) have also reported that a 27-residue peptide, having a sequence corresponding to that of the transmembrane segment of the Isk protein with slow voltage-gated potassium channel activity, which is also incorporated into synthetic phospholipid membranes after taking on a β-structure conformation. The observations cited above could also support our theory that the conformation of the peptide portion from Tyr^{390} to Lys^{430} in the erythrocyte membrane is not necessarily the ordinary α-helix conformation but the β-structure rich conformation. The coexisting effect of the following peptide portion, from Thr^{431} to Gln^{457}, on the conformation of the peptide portion in the model lipid bilayer is now under investigation.

REFERENCES

1. Hamasaki, N. and Okubo, K. (1996) Band 3 protein: Physiology, function and structure. *Cell. Mol. Biol.* **42**, 1025-1039
2. Dolder, M., Walz, T., Hefti, A., and Engel, A. (1993) Human erythrocyte band 3. Solubilization and reconstitution into two-dimensional crystals. *J. Mol. Biol.* **231**, 119-132
3. Wang, D.N., Sarabia, V.E., Reithmeier, R.A.F., and Kuhlbrandt, W. (1994) Three-dimensional map of the dimeric membrane domain of the human erythrocyte anion exchanger, band 3. *EMBO J.* **13**, 3230-3235
4. Kawano, Y., Okubo, K., Tokunaga, F., Miyata, T., Iwanaga, S., and Hamasaki, N. (1988) Localization of the pyridoxal phosphate binding site at the COOH-terminal region of erythrocyte band 3 protein. *J. Biol. Chem.* **263**, 8232-8238
5. Okubo, K., Kang, D., Hamasaki, N., and Jennings, M.L. (1994) Red blood cell band 3. Lysine 539 and lysine 851 react with the same H2DIDS (4,4′-disothiocyanodihydrostilbene-2,2′-disulfonic acid). *J. Biol. Chem.* **269**, 1918-1926
6. Kang, D., Okubo, K., Hamasaki, N., Kuroda, N., and Shiraki, H. (1992) A structural study of the membrane domain of band 3 by tryptic digestion. Conformational change of band 3 *in situ* induced by alkali treatment. *J. Biol. Chem.* **267**, 19211-19217
7. Hamasaki, N., Okubo, K., Kuma, H., Kang, D., and Yae, Y. (1997) Proteolytic cleavage sites of band 3 protein in alkali-treated membranes: Fidelity of hydropathy prediction for band 3 protein. *J. Biochem.* **122**, 577-585
8. Izuhara, K., Okubo, K., and Hamasaki, N. (1989) Conformational change of band 3 protein induced by diethyl pyrocarbonate modification in human erythrocyte ghosts. *Biochemistry* **28**, 4725-4728
9. Wood, P.G. (1992) The anion exchange proteins: homology and secondary structure. *Prog. Cell Res.* **2**, 325-352
10. Jarolim, P., Palek, J., Amato, D., Hassan, K., Sapak, P., Nurse, G.T., Rubin, H.L., Zhai, S., Sahr, K.E., and Liu, S.C. (1991) Deletion in erythrocyte band 3 gene in malaria-resistant Southeast Asian ovalocytosis. *Proc. Natl. Acad. Sci. USA* **88**, 11022-11026
11. Schofield, A.E., Reardon, D.M., and Tanner, M.J.A. (1992) Defective anion transport activity of the abnormal band 3 in hereditary ovalocytic red blood cells. *Nature* **355**, 836-838
12. Kiyota, T., Lee, S., and Sugihara, G. (1996) Design and synthesis of amphiphilic alpha-helical model peptides with systematically varied hydrophobic-hydrophilic balance and their interaction with lipid- and bio-membranes. *Biochemistry* **35**, 13196-13204
13. Davis, J.H., Clare, D.M., Hodges, R.S., and Bloom, M. (1983) Interaction of a synthetic amphiphilic polypeptide and lipids in a bilayer structure. *Biochemistry* **22**, 5298-5305
14. Agawa, Y., Lee, S., Ono, S., Aoyagi, H., Ohno, M., Tanigichi, T., Anzai, K., and Kirino, Y. (1991) Interaction with phospholipid bilayers, ion channel formation, and antimicrobial activity of basic amphipathic alpha-helical model peptides of various chain lengths. *J. Biol. Chem.* **266**, 20218-20222
15. Iwata, T., Lee, S., Oishi, O., Aoyagi, H., Ohno, M., Anzai, K., Kirino, Y., and Sugihara, G. (1994) Design and synthesis of amphipathic 3(10)-helical peptides and their interactions with phospholipid bilayers and ion channel formation. *J. Biol. Chem.* **269**, 4928-4933
16. Suenaga, M., Lee, S., Park, N.G., Aoyagi, H., Kato, T., Umeda, A., and Amako, K. (1989) Basic amphipathic helical peptides induce destabilization and fusion of acidic and neutral liposomes. *Biochim. Biophys. Acta* **981**, 143-150
17. Fukushima, K., Muraoka, Y., Inoue, T., and Shimozawa, R. (1988) Conformational change of poly(L-lysine) induced by lipid vesicles of dilauroylphosphatidic acid. *Biophys. Chem.* **30**, 237-244
18. Rizzo, V., Stankowski, S., and Schwarz, G. (1987) Alamethicin incorporation in lipid bilayers: a thermodynamic study. *Biochemistry* **26**, 2751-2759
19. Fabian, H., Naumann, D., Misselwitz, R., Ristau, O., Gerlach, D., and Welfle, H. (1992) Secondary structure of streptokinase in aqueous solution: a Fourier transform infrared spectroscopic study. *Biochemistry* **31**, 6532-6538
20. Savitzky, A. and Golay, M.J.E. (1964) Smoothing and differentation of data by simplified least squares procedures. *Anal. Chem.* **36**, 1627-1639
21. Chung, L.A. and Thompson, T.E. (1996) Design of membrane-inserting peptides: spectroscopic characterization with and without lipid bilayers. *Biochemistry* **35**, 11343-11354
22. Park, K., Perczel, A., and Fasman, G.D. (1992) Differentiation between transmembrane helices and peripheral helices by the deconvolution of circular dichroism spectra of membrane proteins. *Protein Sci.* **1**, 1032-1049
23. Smith, S.O., Jonas, R., Braiman, M., and Bormann, B.J. (1994) Structure and orientation of the transmembrane domain of glycophorin A in lipid bilayers. *Biochemistry* **33**, 6334-6341
24. Zhang, Y.-P., Lewis, R.N.A.H., Hodges, R.S., and McElhaney, R.N. (1992) Interaction of a peptide model of a hydrophobic transmembrane alpha-helical segment of a membrane protein with phosphatidylcholine bilayers: differential scanning calorimetric and FTIR spectroscopic studies. *Biochemistry* **31**, 11579-11588
25. Zhang, Y.-P., Lewis, R.N.A.H., Henry, G.D., Sykes, B.D., Hodges, R.S., and McElhaney, R.N. (1995) Peptide models of helical hydrophobic transmembrane segments of membrane proteins. 1. Studies of the conformation, intrabilayer orientation, and amide hydrogen exchangeability of Ac-K2-(LA)12-K2-amide. *Biochemistry* **34**, 2348-2361
26. Zhang, Y.-P., Lewis, R.N.A.H., Hodges, R.S., and McElhaney, R.N. (1995) Peptide models of helical hydrophobic transmembrane segments of membrane proteins. 2. Differential scanning calorimetric and FTIR spectroscopic studies of the interaction of Ac-K2-(LA)12-K2-amide with phosphatidylcholine bilayers. *Biochemistry* **34**, 2362-2371
27. Abergel, C., Loret, E., and Claverie, J.M. (1989) Conformational analysis of T immunogenic peptides by circular dichroism spectroscopy. *Eur. J. Immunol.* **19**, 1969-1972
28. Gordon, D.J. and Holzwarth, G. (1971) Optical activity of membrane suspensions: calculation of artifacts by Mie scattering theory. *Proc. Natl. Acad. Sci. USA* **68**, 2365-2369
29. Rosenheck, K. and Schneider, A.S. (1973) Circular dichroism of chromaffin granule proteins in situ: analysis of turbidity effects and protein conformation. *Proc. Natl. Acad. Sci. USA* **70**, 3458-3462
30. Méthot, N., McCarthy, M.P., and Baenziger, J.E. (1994) Secondary structure of the nicotinic acetylcholine receptor: implications for structural models of a ligand-gated ion channel. *Biochemistry* **33**, 7709-7717
31. Parker, F.S. (1983) *Applications of Infrared, Rraman, and Resonance Ranman Spectroscopy in Biochemistry*, Plenum Press,

New York
32. Kodama, M., Kuwabara, M., and Seki, S. (1982) Successive phase-transition phenomena and phase diagram of the phosphatidylcholine-water system as revealed by differential scanning calorimetry. *Biochim. Biophys. Acta* **689**, 567-570
33. Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, K. (1995) Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore. *Biochemistry* **34**, 6521-6526
34. Gargaro, A.R., Bloomberg, G.B., Dempsey, C.E., Murray, M., and Tanner, M.J.A. (1994) The solution structures of the first and second transmembrane-spanning segments of band 3. *Eur. J. Biochem.* **221**, 445-454
35. Li, S.-C. and Deber, C.M. (1993) Peptide environment specifies conformation. Helicity of hydrophobic segments compared in aqueous, organic, and membrane environments. *J. Biol. Chem.* **268**, 22975-22978
36. Liu, L.-P., Li, S.-C., Goto, N.K., and Deber, C.M. (1996) Threshold hydrophobicity dictates helical conformations of peptides in membrane environments. *Biopolymers* **39**, 465-470
37. Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985) Structure of the protein subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3 Å resolution. *Nature* **318**, 618-621
38. Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J.E. (1996) Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* **274**, 1859-1866
39. Hucho, F., Gorne-Tschelnokow, U., and Strecker, A. (1994) Beta-structure in the membrane-spanning part of the nicotinic acetylcholine receptor (or how helical are transmembrane helices?). *Trends Biochem. Sci.* **19**, 383-389
40. Bennett, J.A. and Dingledine, R. (1995) Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant membrane loop. *Neuron* **14**, 373-384
41. Philipson, L.H. and Steiner, D.F. (1995) Pas de deux or more: the sulfonylurea receptor and K^+ channels. *Science* **268**, 372-373
42. Yellen, G., Jurman, M.E., Abramson, T., and MacKinnon, R. (1991) Mutations affecting internal TEA blockade identify the probable pore-forming region of a K^+ channel. *Science* **251**, 939-942
43. Hartmann, H.A., Kirsch, G.E., Drewe, J.A., Taglialatela, M., Joho, R.H., and Brown, A.M. (1991) Exchange of conduction pathways between two related K^+ channels. *Science* **251**, 942-944
44. Shinozaki, K., Anzai, K., Kirino, Y., Lee, S., and Aoyagi, H. (1994) Ion channel activity of a synthetic peptide with a primary structure corresponding to the presumed pore-forming region of the voltage dependent potassium channel. *Biochem. Biophys. Res. Commun.* **198**, 445-450
45. Aggeli, A., Boden, N., Cheng, Ling, Y., Findlay, J.B., Knowles, P.F., Kovatchev, P., and Turnbull, P.J. (1996) Peptides modeled on the transmembrane region of the slow voltage-gated IsK potassium channel: structural characterization of peptide assemblies in the beta-strand conformation. *Biochemistry* **35**, 16213-16221