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Massspectrometric Analyses of Transmembrane Proteins in Human Erythrocyte Membrane

Yoshito Abe, Tomohito Chaen, Xiu Ri Jin, Tomohiro Hamasaki and Naotaka Hamasaki*

Department of Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

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It is difficult to understand the functional mechanisms of integral membrane proteins without having protein chemical information on these proteins. Although there have been many attempts to identify functionally important amino acids in membrane proteins, chemically and enzymatically cleaved peptides of integral membrane proteins have been difficult to handle because of their hydrophobic properties. In the present study, we have applied an analytical method to transmembrane proteins combining amino acid sequencing, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, and liquid chromatography with electrospray ionization (LC/ESI) mass spectrometry. We could analyze most (97%) of the tryptic fragments of the transmembrane domains of band 3 as well as other minor membrane proteins. The peptide mapping of the transmembrane domain of band 3 was completed and the peptide mapping information allowed us to identify the fragments containing lysine residues susceptible to 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and to 2,4-dinitrofluorobenzene (DNFB). This method should be applicable to membrane proteins not only in erythrocyte membranes but also in other membranes.

Key words: LC/ESI mass spectrometry, MALDI-TOF mass spectrometry, membrane protein, peptide mapping, transmembrane.

Abbreviations: BSA, bovine serum albumin; CNBr, cyanogen bromide; $C_{12}E_8$, octaethylene glycol monododecyl ether; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenyl; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; LC/ESI, liquid chromatography with electrospray ionization; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PTH, phenylthiohydantoin; RP-HPLC, reverse phase-high performance liquid chromatography; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TFA, trifluoroacetic acid; TM, transmembrane spanning portion; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

Membrane proteins are integrated in the lipid bilayer and play key roles in cell viability and cell metabolism. They function as transporters and signal transmitters between intra- and extra-cellular compartments. Only a limited number of integral membrane proteins have been crystallized and analyzed by X-ray crystallography (*1–4*). Most membrane protein structures have been topologically predicted simply by hydropathy predictions based on their DNA sequences. Recent studies have indicated, however, that those hydropathy predictions are not necessarily and convincingly applicable to multi-spanning membrane proteins (*5–7*). Therefore, it is necessary to collect protein chemical information as well as tertiary structural information on membrane proteins.

In the context of the structure and function relationship, protein chemical information helps us to understand the molecular mechanisms of proteins. To identify modified amino acid residues, proteins are usually fragmented either enzymatically or chemically, and the resulting fragments analyzed. The recent development of mass spectrometric techniques has enabled the rapid and sensitive identification of fragmented peptides at the pmol to fmol level. In the case of membrane proteins, however, especially multi-spanning integral membrane proteins, it is difficult to determine modified residues because of the difficulty in analyzing transmembrane peptides. Since these peptides are buried in the membrane, they are not easily accessible to proteinases. The hydrophobic properties of transmembrane segments result in their aggregation during enzymatically and chemically-mediated cleavage and high performance liquid chromatography (HPLC) separation. In a recent proteome project, the treatment of hydrophobic peptides was problematic for protein identification (*8*, *9*). Therefore, hydrophobic transmembrane peptide treatment methods need to be established.

Band 3 is a typical multi-spanning integral membrane protein in erythrocytes that exchanges chloride and bicarbonate ions between the intra and extracellular side. It comprises two structurally and functionally distinct domains (*10–12*). The 40-kDa NH_2-terminal domain is located in the cytosol and interacts with the cytoskeleton. The COOH-terminal 55-kDa domain is integrated into the membrane lipid bilayer with 14 spanning transmembrane segments assumed by hydropathy predictions

*To whom correspondence should be addressed: Naotaka Hamasaki, Tel: +81-92-642-5748, Fax: +81-92-642-5772, E-mail: hamasaki@cclm.med.kyushu-u.ac.jp

(*13*), and is both necessary and sufficient for anion transport function after proteolytic removal of the cytosolic 40-kDa domain (*14*, *15*). Studies of the function and structure of the 55-kDa transmembrane domain suggest that some arginine, lysine, glutamic acid, and histidine residues play an important role in anion transport (*16–20*). These important residues, namely Lys 539 (*21*), Lys 851 (*21*, *22*), Glu 681 (*20*) and His 834 (*23*) were identified by chemical modification and mutation studies; however, the other residues have not yet been identified, because handling the transmembrane peptides of band 3 is as difficult as in the case of other integral membrane proteins.

In the present study, we have applied a peptide analytical method to erythrocyte membrane proteins and identified all peptides originating from the 55-kDa band 3 transmembrane domains (Gly 361–Val 911) using amino acid sequencing, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, and liquid chromatography with electrospray ionization (LC/ESI) mass spectrometry. The method allowed us to identify the lysines susceptible to chemical modifications in band 3 by various reagents. Further, we identified peptide fragments from other minor erythrocyte membrane proteins using the LC/MS/MS system. These methods are applicable to the study of transmembrane proteins in erythrocyte membranes.

MATERIALS AND METHODS

Materials—3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid were purchased from Aldrich Chemicals (Milwaukee, WI). L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-trypsin (sequence grade) and N-glycosidase F were purchased from Roche Diagnostics (Mannheim, Germany). Trifluoroacetic acid (TFA) and other chemical reagents were purchased from Wako Co., Ltd. (Osaka, Japan).

Preparation of Erythrocyte Membranes for Peptide Analysis—Human blood stored at 4°C in acid/citrate/dextrose solution was obtained from the Fukuoka Red Cross Center. Erythrocytes were stored for less than 2 wk. Erythrocyte membranes (white ghosts) were prepared as described previously (*24*). The membranes (containing 1 mg of protein) were treated with *N*-glycosidase F (2 U) in 200 μl of 20 mM phosphate buffer (pH 7.2) for 24 h at room temperature. To remove the NH_2-teminal 40-kDa domain of band 3, membranes were pretreated with a low concentration of TPCK-trypsin (1 μg/ml) in 5 mM $NaHCO_3$ on ice for 30 min. Peripheral membrane proteins and peptides on erythrocyte membranes were removed with 10 mM NaOH. The membranes were then washed three times with 5 mM $NaHCO_3$.

Preparation of Tryptic Peptide Fragments of Erythrocyte Membrane Proteins—Tryptic peptide fragments for analyses were prepared as follows. The pretreated membranes were solubilized in a total volume of 200 μl of 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$ (octaethylene glycol monododecyl ether). The proteins in the solution (0.6 mg/ml) were digested with 4 μg TPCK-trypsin for 2 h at 37°C. The purified transmembrane domain of band 3 in 0.1% $C_{12}E_8$ solution was prepared according to Casey *et al*. (*25*), and digested with TPCK-trypsin under the same conditions.

Preparation of Modified Membranes with 4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic Acid (SITS) and 2,4-Dinitrofluorobenzene (DNFB)—Membranes were modified with SITS and DNFB according to previous studies (*24*, *26*). In brief, the pretreated membranes were modified with 0.1 mM SITS in 20 mM borate-buffer, pH 9.5 at 4°C for 90 min, and with 1 mM DNFB in 20 mM Tris-HCl buffer, pH 8, at 37°C for 30 min. Some membranes were preincubated with 100 μM 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), pH 8, at 37°C for 30 min before modification. The modified membranes were washed three times with 6 volumes of 5 mM $NaHCO_3$ containing 0.5% bovine serum albumin (BSA) and then washed three times with 5 mM $NaHCO_3$ at 4°C.

CNBr (Cyanogen Bromide) Treatment—The peptides were dissolved in 100 μl of 50% TFA solution, and incubated with 10 μg CNBr for 12 h at room temperature. After CNBr treatment, the peptides were lyophilized and stored at 4°C until analysis.

HPLC Separation of Peptide Fragments—After centrifugation (13,000 × *g* 10 min), the peptides were applied directly to an HPLC system. Samples (each 50 μl; 30 μg) were loaded onto a C_{18} reverse phase–HPLC column (RP-HPLC; cosmosil 4.6 ID × 250 mm), and eluted with a gradient of water (solvent A) and 2:1 (v/v) isopropanol/acetonitrile (solvent B), each containing 0.1% TFA, at a flow rate of 800 μl/min. The mobile phase composition was maintained at 5% B for 5 min, and then raised from 5% to 85% over 60 min and from 85 to 100% over 20 min. The eluted peptides were monitored by their absorbance at 214-nm. The SITS-modified and DNFB-modified peptides were monitored by their fluorescence at 430-nm (excitation, 340-nm) and absorbance at 340-nm, respectively. We collected the peptide fractions using the reference peaks, and the fractions were lyophilized and stored at 4°C until analysis.

Amino Acid Sequencing and Mass Spectrometry—The peptides were analyzed on a gas phase sequencer (Applied Biosystems, model 492, Foster City, CA). The phenylthiohydantions (PTHs) were identified by an Applied Biosystems 140C PTH analyzer on line system. MALDI-TOF mass spectrometric analyses were performed using a Voyager RP spectrometer (Applied Biosystems) with the acceleration voltage set to 20 kV. Data were acquired in the positive linear mode of operation, and the spectra were externally calibrated with calibration kits I and II (Applied Biosystems). The collected peptides were dissolved in 2:1 (v/v) isopropanol/acetonitrile and 0.1% TFA. The peptide solutions (0.5 μl) were mixed with 0.5 μl of matrix solutions (sinapinic acid and α-cyano-4-hydroxycinnamic acid saturated with 0.1% TFA and 50% acetonitrile in aqueous solution, respectively). The theoretical masses of each peptide were calculated using protein prospector on the World Wide Web (http://prospector.ucsf.edu/).

LC/ESI-MS was performed using a combination of HPLC (Waters 600E, Waters) and an LCQ Advantage Ion Trap Mass Spectrometer (Finnigan, San Jose, CA, USA). Each sample (10 μl; 6 μg) was loaded onto a C_{18} RP-HPLC column (2.1 ID × 250 mm;Waters Symmetry 300 C_{18} 5 mm) after centrifugation (13,000 × *g* 10 min). The peptides were separated by HPLC using a gradient of water

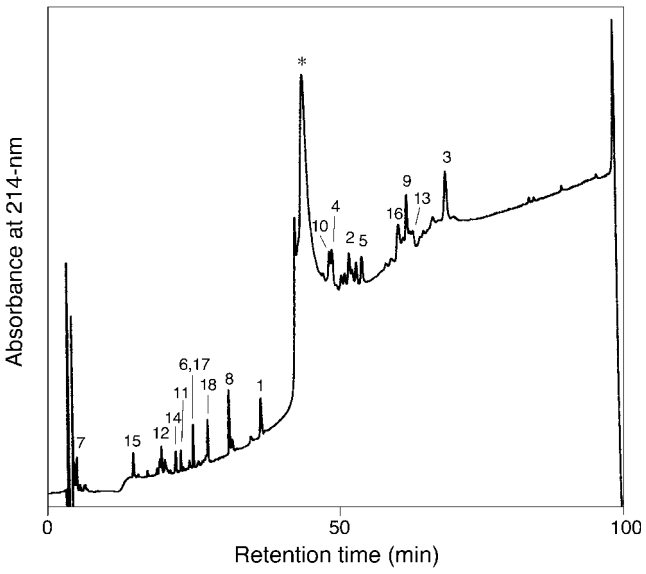

Fig. 1. **HPLC separation profile of tryptic fragments of the purified transmembrane domain of band 3 with detection at 214 nm.** The protein was dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$, and digested with trypsin. The tryptic peptides were separated by HPLC on a reverse phase column (Cosmosil 4.6 ID × 250 mm, Nakarai, Tokyo, Japan) with a water/acetonitrile/isopropanol eluent system containing 0.1% TFA as described under "MATERIALS AND METHODS." The peak fractions from 1 to 18 were analyzed by amino acid sequencing and mass spectrometry and the results of the analyses are summarized in Table 1. The peaks indicated by asterisks contained no peptide fragments detectable by amino acid sequencing and mass spectrometry.

(solvent A) and 2:1 (v/v) isopropanol/acetonitrile (solvent B), each containing 0.025% TFA, at a flow rate of 1 ml/min. The mobile phase composition was maintained at 5% B for 5 min, and then B was increased from 5 to 85% in 60 min and from 85 to 100% in 20 min. The column eluent was split and 20% of the flow (200 μl/min) was directed into the ESI source. Data were acquired and analyzed using LCQ version 2.0 software (Finnigan, San Jose, CA). Instrument parameters were as follows: ESI needle voltage, 5 kV; ESI capillary temp, 260°C; ion energy, 35%; isolation window, 2 amu; scan range 550–2,000 amu. Peptides from the MS/MS results were identified using TURBO SEQUEST software ver. 2.0 (Finnigan, San Jose, CA) (*27*) against a human protein database extracted from the NCBI protein database. Matches to peptides identified by TURBO SEQUEST were filtered according to their cross correlation scores (X_{corr}), normalized difference in correlation scores (ΔC_n), and the tryptic nature of each peptide. The parameters used were conservative and chosen to filter the results to minimize the inclusion of false positives. All accepted results had a ΔC_n of at least 0.35 and X_{corr} of at least 2.0.

Analytical Procedures—Sodium dodecyl sulfate–polyacrylamide gel electrophoresis for protein and peptide analyses was performed according to the methods of Laemmli (*28*) and Kawano and Hamasaki (*29*). Protein concentration was determined by the method of Lowry *et al*. (*30*) using BSA as the standard.

RESULTS

HPLC Separation and Identification of Tryptic Peptides from Band 3—For analysis, the purified transmembrane domain of band 3 was dissolved in 0.1% $C_{12}E_8$ (final concentration) and digested with TPCK-trypsin. After tryptic digestion, the peptides were separated by RP-HPLC using an acetonitrile and isopropanol mixture as the solvent. We collected each peptide fraction as a reference at 214-nm absorbance (Fig. 1). The peptide fractions were analyzed for their N-terminal sequences and molec-

Table 1. **Tryptic peptide fragments of the transmembrane domain of band 3 analyzed by amino acid sequencing, MALDI-TOF mass spectrometry, and LC/ESI mass spectrometry.**

No.	Start	End	Mass calculated [MH+][a]	Mass observed by MALDI-TOF MS[b]	Amino acid sequencer	The most intense mass observed in LC/ESI MS	The mass from LC/ESI MS[c]
1	361	384	2,468.73	2,468.73	GLDLNGG	1,234.9 (+2)[d]	2,468.2
2	388	430	4,757.57	4,758.00	RRYPYYLS	1,586.2 (+3)	4,757.0
3	433	551	1,3504.17	1,3504.00	NQMGVSE	1,350.9 (+10)	13,503.0
4	552	600	5,546.76	5,546.78	TYNYNVL	1,387.1 (+4)	5,545.5
5	604	631	3,173.78	3,173.35	VIGDFGV	1,058.3 (+3)	3,173.0
6	632	639	863.00	861.99	LSVPDGF	862.4 (+1)	863.4
7	640	646	720.76	721.78	VSDSSAR[e]	ND[f]	ND
8	647	656	1,148.40	1,147.53	GWVIHPL	574.6 (+2)	1,148.6
9	657	694	4,354.29	4,354.36	SEFPIWM	1,451.8 (+3)	4,353.3
10	699	730	3,274.91	3,274.83	GSGFHLD	1,092.0 (+3)	3,274.6
11	731	743	1,329.56	1,328.66	SVTHANA	665.0 (+2)	1,329.4
12	744	757	1,371.53	1,370.02	ASTPGAA	686.1 (+2)	1,370.2
13	758	817	6,612.26	6,612.29	EQRISGLL	1,653.5 (+4)	6,610.9
14	818	826	1,118.28	1,118.65	YHPDVPY	559.4 (+2)	1,118.3
15	830	832	462.53	ND	TWR	ND	ND
16	833	879	5,284.75	5,284.76	MHLFTGI	1,761.8 (+3)	5,284.0
17	880	892	1,434.56	1,435.85	NVELQXL	717.5 (+2)	1,434.0
18	893	911	2,203.32	2,202.79	ATFDEEE	1,101.6 (+2)	2,202.1

[a]Calculated from average isotopic masses.[b]Mass spectrometry.[c]Calculated from the masses of multiple charge ions. [d]Most intense ions in the charge state are shown in parentheses. [e]Sugar-linked asparagine 642 changes to aspartate following digestion with N-glycosidase F. [f]Not detected.

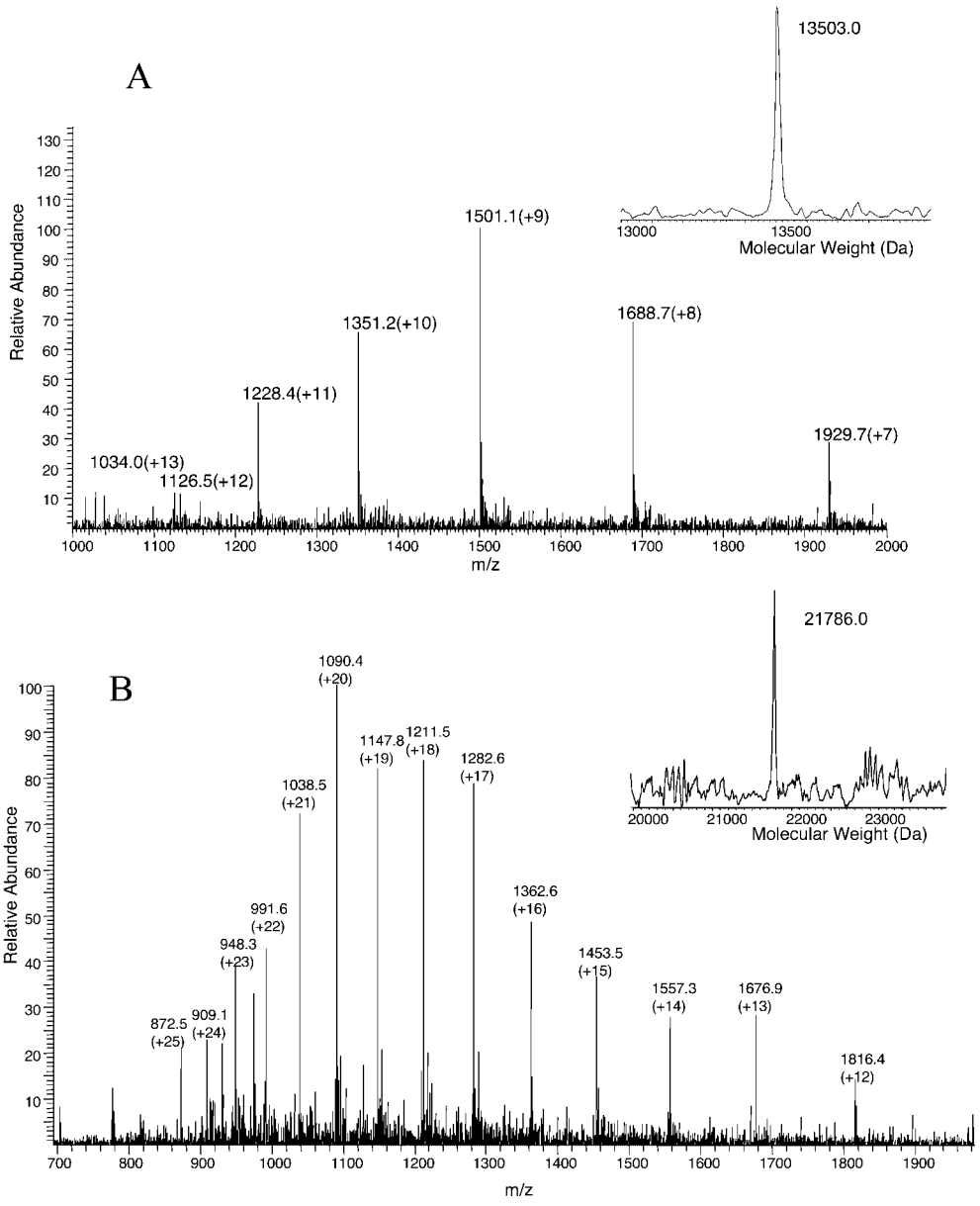

Fig. 2. **The ESI mass spectra of tryptic fragment from intact membranes and SITS modified membranes.** (A) The spectrum is shown for peptide fragment No.3 with ions carrying positive charges from 7 to 13. The insets show the reconstructed mass peaks for the peptide. The molecular mass, 13503, is identical to the predicted mass of tryptic fragment No. 3. (B) The spectrum is shown for the peptide fragment from SITS-modified membranes with ions carrying positive charges from 12 to 25. The inset molecular mass, 21,786, suggests that the peptide comprises Gly 361 to Lys 551 (predicted mass, 21,334) containing one SITS (molecular weight 452) molecule.

ular masses using an amino acid sequencer and MALDI-TOF mass spectrometry (Table 1). We analyzed band 3 fragment peptides by LC/ESI mass spectrometry. Mainly, peptides were determined by detection of the molecular ions derived from multiple charge states. As an example, for fragment no. 3, we could observe the masses from the multiple charged ions, 1,929.7 (+7), 1,688.7 (+8), 1,501.1 (+9), 1,351.2 (+10), 1,228.4 (+11), 1,126.5 (+12), 1,034.0 (+13) (Fig. 2A). Reconstruction of the masses from each ion indicates that the molecular weight of fragment no. 3 is 13,503 (Fig. 2A, inset). The molecular weights of other fragments were also determined (Table 1). The molecular weights determined by LC/ESI mass spectrometry were identical to the masses determined by MALDI-TOF mass spectrometry and the calculated theoretical molecular weights. Further, we could confirm the band 3 peptides using MS/MS, except for large fragments with mass greater than 5,000 kDa. Figure 3 shows the selected ion chromatograms generated by the most intense molecular ion charge state for each tryptic fragment. These peaks were well separated and well shaped, and had little tendency to aggregate. As a result, almost all tryptic peptides in the transmembrane domain were identified (Fig. 4, 97% of the sequence) except for fragments too short to be observed by this analytical method. Thus, we were able to produce a peptide map of the transmembrane domains of band 3.

Peptide Analyses of Transmembrane Proteins in Intact Human Erythrocyte Membrane—To analyze the peptides from band 3 in intact erythrocyte membranes, the membranes were solubilized in 0.1% $C_{12}E_8$ solution and digested with TPCK-trypsin. To simplify the peptide analysis, the N-terminal region (40-kDa) of band 3, *N*-linked suger and peripheral proteins were removed before membrane solubilization. The peptides were separated by RP-HPLC and analyzed by an amino acid sequencing, MALDI-TOF, and LC/ESI mass spectrometry. These fragments originating from band 3 coincided

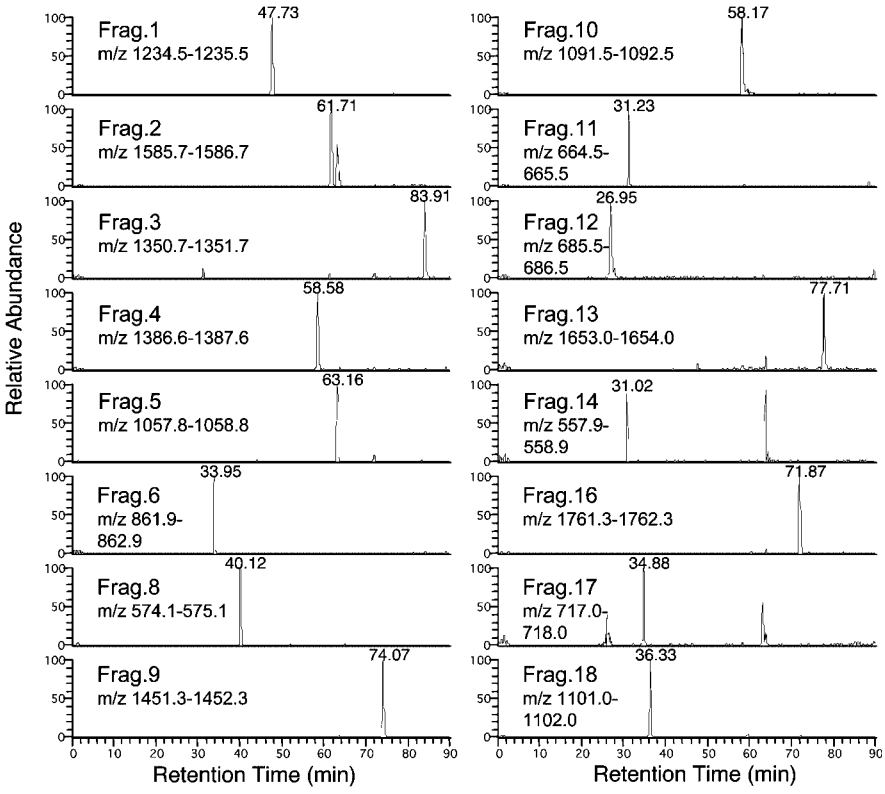

Fig. 3. **Selected ion chromatograms from ESI-MS detection of peaks from the HPLC separation of tryptic fragments of the transmembrane domain of band 3.** The protein was dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$, and digested with trypsin. The tryptic peptides were separated by HPLC using a reversed-phase column (Waters Symmetry 300 C_{18} 5 μm, 2.1 ID × 250 mm; Waters, Milford, MA) with a water/acetonitrile/2-propanol eluant system containing 0.025% trifluoroacetic acid as described under "MATERIALS AND METHODS." Chromatograms were generated by the most intense molecular ion charge state within the mass/charge range of the instrument.

completely with the peptide fragments from the identified purified proteins (data not shown). This shows that the method can be applied to the study of the band 3 protein in intact erythrocyte membranes.

Based on the results of the analysis of intact membranes, we also could analyze peptide fragments from other erythrocyte integral membrane proteins using this analytical method. Mainly, analyses were performed using the TURBO SEQUEST program based on the MS/MS data for each peptide fragment as obtained by LC/ESI mass spectrometry. The identified peptides and analytical data are summarized in Table 2. All data for the peptide fragments had ΔC_n values greater than 0.35 and an X_{corr} values greater than 2, demonstrating good reliability. We found some peptides originating from flotillin, aquaporin1, glucose transporter 1, glycophorins, rhesus antigens, and stomatin, all of which are erythrocyte integral membrane proteins. Even peptides from low quantity membrane proteins, such as glycophorin C, were identified using this analytical method (Fig. 5 and Table 2). These findings indicate that this method is applicable to the rapid and sensitive identification of peptides from erythrocyte membrane proteins containing hydrophobic transmembrane regions.

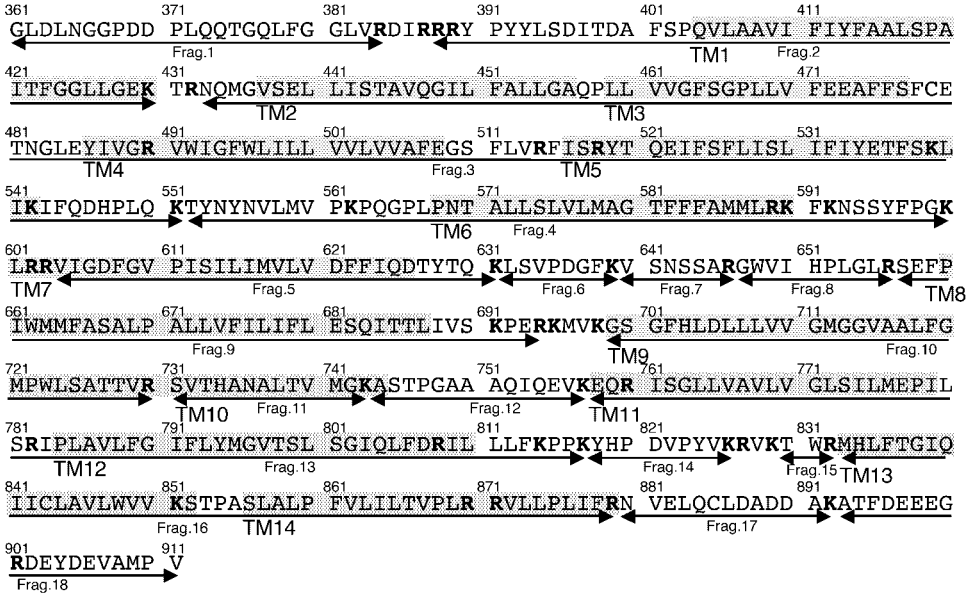

Fig. 4. **Amino acid sequence of the transmembrane domain of human band 3.** The tryptic cleavable lysines and arginines are indicated by bold type. The peptides identified by this analytical method are underlined. TM segments by hydropathic prediction (*13*) are indicated by gray boxes. The peptide fraction numbers are those in Fig. 1 and Table 1.

Table 2. **Tryptic peptide fragments of erythrocyte integral membrane proteins analyzed by LC/MS/MS systems (see text for details).**

Protein	Time (s)	*m/z*	Start	End	Sequence	ΔC_n	X_{corr}
Stomatin	9.26	1,248.40	221	232	VIAAEGEMNASR	0.502	3.268
Stomatin	24.04	1,447.65	219	232	AKVIAAEGEMNASR	0.513	3.095
Glucose transporter	31.90	1,418.60	213	223	FLLINRNEENR	0.373	3.021
Stomatin	32.48	1,931.11	126	144	VQNATLAVANITNADSATR	0.700	6.692
Glucose transporter	32.57	2,216.43	231	249	LRGTADVTHDLQEMKEESR	0.526	4.839
Glucose transporter	33.47	1,143.24	459	468	TFDEIASGFR	0.525	3.973
Glycophorin C	34.11	2,580.64	98	123	GTEFAESADAALQGDPALQDAGDSSR	0.656	5.173
Glucose transporter	34.35	1,947.08	233	249	GTADVTHDLQEMKEESR	0.497	4.287
Stomatin	35.48	1,716.99	236	251	EASMVITESPAALQLR	0.454	3.792
Glucose transporter	35.54	2,499.65	469	492	QGGASQSDKTPEELFHPLGADSQV	0.534	2.927
Aquaporin1	35.56	2,313.40	244	263	VWTSGQVEEYDLDADDINSR	0.608	4.351
Rhesus C/E antigens	36.07	1,248.38	401	409	YFDDQVFWK	0.485	2.944
Stomatin	36.11	2,029.40	233	251	ALKEASMVITESPAALQLR	0.644	5.675
Glycophorin A	36.28	3,309.56	101	131	KSPSDVKPLPSPDTDVPLSSVEIENPETSDQ	0.623	4.236
Glycophorin A	36.56	3,181.39	102	131	SPSDVKPLPSPDTDVPLSSVEIENPETSDQ	0.524	5.418
Flotillin	36.94	1,379.60	52	63	NVVLQTLEGHLR	0.480	2.722
Stomatin	37.82	1,602.87	98	111	TISFDIPPQEILTK	0.378	2.493
Glycophorin C	38.57	3,261.44	98	128	GTEFAESADAALQGDPALQDAGDSSRKEYFI	0.548	4.705
Rhesus C/E antigens	39.60	888.06	410	417	FPHLAVGF	0.564	2.014
Flotillin	40.10	1,468.84	308	321	MALVLEALPQIAAK	0.421	2.956
Stomatin	42.40	3,758.11	159	191	NLSQILSDREEIAHNMQSTLDDATDAWGIKVER	0.570	7.127
Stomatin	42.99	4,227.69	159	195	NLSQILSDREEIAHNMQSTLDDATDAWGIKVERVEIK	0.439	4.217
Glucose transporter	45.03	3,275.88	7	38	KLTGRLMLAVGGAVLGSLQFGYNTGVINAPQK[a]	0.400	3.715
Rhesus C/E antigens	45.33	2,117.41	401	417	YFDDQVFWKFPHLAVGF	0.418	3.274
Stomatin	46.89	1,756.11	78	93	GPGLFFILPCTDSFIK	0.473	2.972
Stomatin	48.45	2,128.58	264	283	NSTIVFPLPIDMLQGIIGAK	0.364	4.000
Stomatin	49.04	3,462.13	252	283	YLQTLTTIAAEKNSTIVFPLPIDMLQGIIGAK	0.403	3.849
Rhesus C/E antigens	49.37	3,098.55	235	263	KNAMFNTYYALAVSVVTAISGSSLAHPQR	0.567	3.500
Rhesus C/E antigens	51.05	2,970.37	236	263	NAMFNTYYALAVSVVTAISGSSLAHPQR	0.470	3.434
Aquaporin1	51.75	3,207.72	163	195	DLGGSAPLAIGLSVALGHLLAIDYTGCGINPAR	0.429	2.563
Rhesus antigen	54.34	2,833.28	239	264	AIVDTYFSLAACVLTAFAFSSLVEHR	0.602	2.823
Glucose transporter	54.36	3,408.93	301	333	AGVQQPVYATIGSGIVNTAFTVVSLFVVERAGR	0.489	3.376
Glucose transporter	57.76	3,124.61	301	330	AGVQQPVYATIGSGIVNTAFTVVSLFVVER	0.417	3.138
Glucose transporter	58.12	4,046.70	265	300	SPAYRQPILIAVVLQLSQQLSGINAVFYYSTSIFEK	0.525	5.275
Rhesus antigen	58.98	4,602.15	195	235	KGHENEESAYYSDLFAMIGTLFLWMFWPSFNSAIAEPGDK	0.570	4.909
Glucose transporter	59.58	2,760.45	93	127	RNSMLMMNLLAFVSAVLMGFSKLGK	0.385	3.302
Rhesus antigen	59.95	4,473.97	196	235	GHENEESAYYSDLFAMIGTLFLWMFWPSFNSAIAEPGDK	0.561	5.169
Glycophorin A	64.66	3,966.76	62	97	VQLAHHFSEPEITLIIFGVMAGVIGTILLISYGIRR	0.628	6.301
Glucose transporter	66.08	4,383.11	52	92	YGESILPTTLTTLWSLSVAIFSVGGMIGSFSVGLFVNRFGR	0.542	4.797
Glucose transporter	66.52	4,022.69	52	89	YGESILPTTLTTLWSLSVAIFSVGGMIGSFSVGLFVNR	0.393	4.265
Glycophorin A	67.65	4,449.43	62	101	VQLAHHFSEPEITLIIFGVMAGVIGTILLISYGIRRLIKK	0.509	4.102
Glycophorin A	70.73	4,321.26	62	100	VQLAHHFSEPEITLIIFGVMAGVIGTILLISYGIRRLIK	0.449	3.194
Glycophorin C	77.23	4,467.54	49	88	METSTPTIMDIVVIAGVIAAVAIVLVSLLFVMLRYMYRHK	0.584	3.066
Glycophorin B	81.86	3,996.12	55	89	FTVPAPVVIILIILCVMAGIIGTILLISYSIRRLIKA	0.441	4.149

Underlined letter indicates the transmembrane region of aquaporin 1 originating from X-ray crystallography (*3*) and other proteins predicted by the SOSUI program (*41*).

Identification of the Lysine Residues Modified by Inhibitors in Band 3—SITS and DNFB, well-known inhibitors of band 3, react covalently with lysine residues in the transmembrane region of band 3. The modified lysines have not yet been identified until now. We identified the lysines susceptible to these reagents by peptide mapping of band 3.

SITS Modified Lysine: The stilbene compounds DIDS and H_2DIDS have two isothiocyanate groups that react with amino groups in proteins. H_2DIDS reacts with Lys 539 and Lys 851 in band 3, and induces an intra-molecular cross-linkage between them (*21*). DIDS reacts with Lys 539, but no cross-link with Lys 851 is formed except under partial denaturing conditions (*24*). The other stilbene compound, SITS, has only one isothiocyanate group. Therefore, we examined which of Lys 539 or Lys 851 is more reactive toward SITS. After SITS modification and trypsin digestion, the membrane was analyzed by LC/ESI mass spectrometry. Although no molecular ions derived from fragments 1, 2, and 3 could be found, the reconstructed mass of 21,786 from the ESI mass spectrum was confirmed (Fig. 2B). To determine the SITS modified fragment, we analyzed SITS-modified membranes using the RP-HPLC system and traced the pep-

tide modified by SITS using the reference peak at a fluorescence of 430-nm (Fig. 6A). Only one peak was detected (Fig. 6A, peak a), and this peak fraction was collected and analyzed by amino acid sequencing and MALDI-TOF mass spectrometry (Table 3). The molecular mass from MALDI-TOF mass spectrometry was identical to the molecular weight identified by the LC/ESI mass. The analytical results indicate that the peptide fragment from Gly 361 to Lys 551 contains one SITS molecule, indicating that SITS modifies only Lys 539.

DNFB Modified Lysines: In the case of DNFB, two susceptible lysines are found in one band 3 molecule (*20*, *26*). Passow named these lysines Lys a and Lys c (*20*, *26*). Previous mutational analysis has suggested that Lys 539 is Lys a (*31*), however, Lys c has not yet been identified. First, to identify the 2,4-dinitrophenyl (DNP)-lysine adduct, we used LC/ESI mass spectrometry. However, the molecular ions derived from fragments 3 and 4 disappeared on the chromatogram, so that no molecular ions from peptides containing DNP-lysine adducts could be

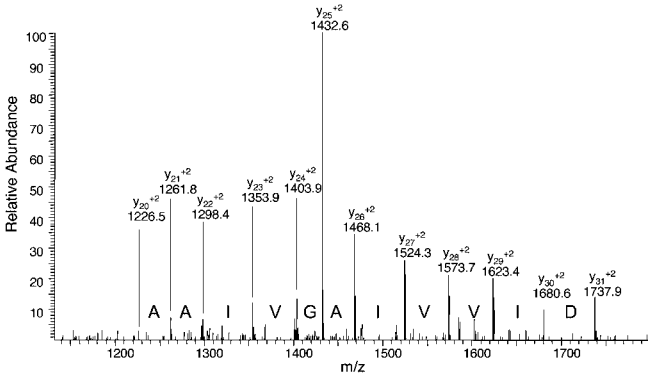

Fig. 5. **Part of the MS/MS product ion spectrum from the dissociation of the double charged molecular ion of the tryptic peptide from Met 49 to Lys 88 in glycophorin C.** The spectrum shows the sequence DIVVIAGVIAA, which corresponds to the sequence from Asp 58 to Ala 67 in glycophorin C.

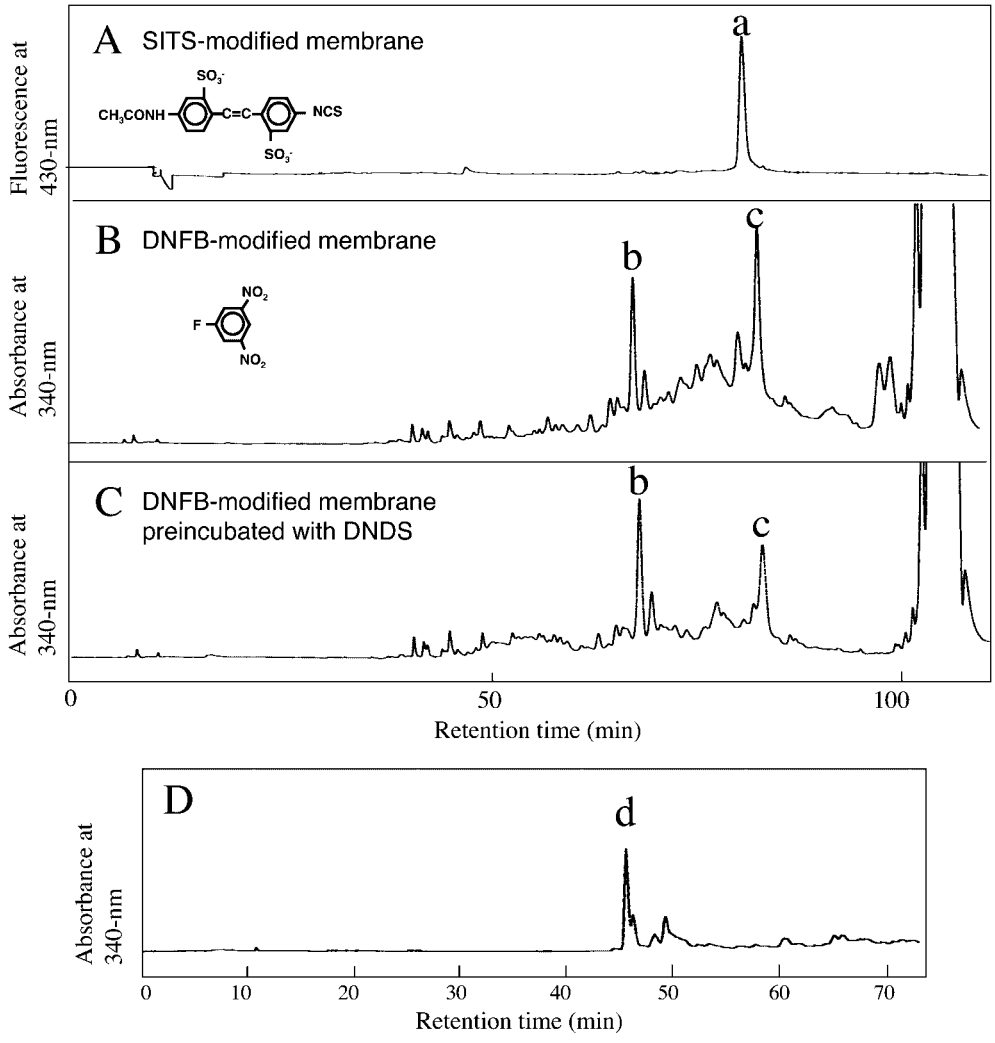

Fig. 6. **HPLC separation profiles of membranes modified by DNFB and SITS.** Membranes were modified with 0.1 mM SITS in 20 mM borate buffer, pH 9.5, at 4°C for 90 min, or with 1 mM DNFB in 20 mM Tris-HCl buffer, pH 8, at 37°C for 30 min. The modified membranes were dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$, and digested with trypsin. The tryptic peptides in the solubilized membranes were separated by HPLC on a reverse phase column (Cosmosil 4.6 ID × 250 mm, Nakarai, Tokyo, Japan) with a water/acetonitrile/isopropanol eluent system containing 0.1% TFA as described under "MATERIALS AND METHODS." SITS-modified peptides were monitored by fluorescence at 430-nm (excitation 340-nm) and DNFB-modified peptides were monitored by absorbance at 340-nm. (A) HPLC separation profiles of tryptic peptide fragments in SITS-modified membranes. (B) HPLC separation profiles of tryptic peptide fragments in DNFB-modified membranes. (C) HPLC separation profiles of tryptic peptide fragments in DNFB-modified membrane preincubated with 100 μM DNDS for 30 min at pH 8 and 37°C. The DNFB modified peptide fraction b in (B) was collected and lyophilized. The peptide was cleaved with 10 μg CNBr in 100 μl of 50% TFA solution for 12 h at room temperature. The CNBr-treated peptides were separated by HPLC on a reverse phase column (Cosmosil 4.6 ID × 250 mm, Nakarai, Tokyo) with a water/acetonitrile/isopropanol eluent system containing 0.1% TFA as described under "MATERIALS AND METHODS." The peptides were monitored by their absorbance at 340 nm. The HPLC separation profile of the CNBr cleavage fragments is shown in D). The peak fractions a, b, c, and d were analyzed by MALDI-TOF mass spectrometry and amino acid sequencing, and the results are summarized in Table 3.

Table 3. **Peptide analyses of peptides from SITS- and DNFB-modified membranes.**

Peak	The observed mass[a]	Amino acid sequencing	The identified peptide fragment
Fig. 6a	21,787.0	GLDLNG	Gly 361 to Lys 551 (predicted mass, 21,334) containing one SITS (molecular weight 452) molecule
Fig. 6b	5,712.54	TYNYNV	Thr 552 to Lys 600 (predicted mass, 5,546.7) containing one DNP (molecular weight 167) molecule
Fig. 6c	13,669.9	NQMGVS	Asn 433 to Lys 551 (predicted mass, 13,504) containing one DNP molecule
Fig. 6d	1,739.24	LRXFKN[b]	Leu 588 to Lys 600 (predicted mass, 1,571.87) containing one DNP molecule and Lys 590 modified with DNFB

[a]Mass of a single charged ion by MALDI-TOF mass spectrometry. [b]The third PTH-amino acid was not detectable.

found. Therefore, we analyzed DNFB-modified membranes using the RP-HPLC system and traced the peptide modified by DNFB using the reference peak at 340-nm absorbance (Fig. 6B). We observed two main peaks at 63 min (Fig. 6B, peak b) and 80 min (Fig. 6B, peak c), and these peak fractions were collected and analyzed (Table 3). Analysis of peak b suggested that a fragment from Thr 552 to Lys 600 contains one DNP molecule. This peptide fragment contains three possible lysines, at positions 562, 590, and 592, that are susceptible to DNFB. To identify the DNFB-susceptible lysine, we carried out CNBr treatment of this fragment. Figure 6D shows the HPLC profile of the CNBr treated fragments. The peak d fraction was collected and analyzed. The peptide molecular weight of was 1739.24 and was estimated to comprise the region from Leu 588 to Lys 600, containing one DNP molecule (Table 3). The N-terminal sequence was LRXFKN and level of the 3rd PTH-lysine was diminished. These results indicate that Lys 590 is modified by DNFB. Peak c comprised the region from Asn 433 to Lys 551 and contained one DNP molecule (Table 3). Preincubation with 100 μM of the non-covalent binding stilbene compound DNDS resulted in a decrease in the intensity of peak c (Fig. 6D). This is consistent with previous findings of competition between DNFB and H_2DIDS for reaction with Lys 539 (*26*). Therefore, we determined that Lys c is Lys 590, further supporting the identity of Lys a as Lys 539.

DISCUSSION

In the present study, we applied a peptide analytical method to tryptic fragments of erythrocyte membrane proteins. Using this method, we could analyze peptides originating from the 55-kDa band 3 transmembrane domain (Gly 361–Val 911) and other erythrocyte integral membrane proteins (see Figs. 1–5, Tables 1 and 2). This method has many advantages for the study of erythrocyte membrane proteins. First, the membrane proteins can be solubilized in mild detergent and digested with protease under mild conditions. Second, peptides can be analyzed rapidly using LC/ESI mass spectrometry. These factors are useful for the determination of the residues modified by unstable chemical reagents. In our recent study, we determined the DEPC-modified histidine that is important for the anion transport mechanism of band 3 (*23*). DEPC histidine adducts are easily hydrolyzed (*32*); however, we could overcome this obstacle by using mild conditions and rapid analysis.

The greatest advantage of this method is its ability to analyze directly peptides and proteins originating from intact erythrocyte membranes. Furthermore, we identified most peptides in the band 3 transmembrane domain (97% of sequence). Therefore, the method allowed us to determine band 3 residues susceptible to certain chemical modifications directly. In previous chemical modification studies, lysine, arginine, glutamic acid and histidine residues have been shown to be essential for transport activity (*16–23*).

SITS and DNFB are lysine specific reaction reagents and anion transport inhibitors. In this study, we determined that lysine 539 is susceptible to SITS and lysines 539 and 590 are susceptible to DNFB using peptide mapping of band 3. SITS is a well-known and well-studied inhibitor of band 3 anion transport, and the predominant modified lysine has been assumed to be Lys 539 base on studies of other stilbene compounds bound to band 3 (*21*, *24*). In the case of DNFB, Lys 539 had been identified as one modification site (*31*); however, here we provide the first evidence that Lys 590 is another DNFB modification site. The inhibition of anion transport depends on SITS and DNFB modification of Lys 539, which is the main reaction site of other lysine-reactive inhibitors, for example, stilbene compounds including DIDS and H2DIDS (*21*, *24*). The sites must not be anion binding sites within the anion exchange center, because the mouse band 3 mutant K558N, which corresponds to K539N of human band 3, has the same specific activity as wild-type band 3 in *Xenopus oocytes* (*18*). Thus, we conclude that Lys 539 is part of the extracellular rim of the anion transport channel (*20*). While the transport activity of band 3 is slightly increased by the DNFB modification of Lys 590 (*26*), Lys 590 is also the site modified by phenylisothiocyanate, which modifies three lysines in the band 3 molecule (*33*). Interestingly, modification by phenylisothiocyanate leads to an inhibition of the anion transport activity of band 3 (*33*). Therefore, Lys 590 plays a role in regulating the transport activity of band 3. Passow *et al*. examined the anion effect of DNFB modification of two lysines (*20*). They found that when Lys 590 is modified by DNFB, the rate of DNFB modification at Lys 539 is reduced in chloride medium but not in sulfate medium. When Lys 590 is modified by DNFB, the rate of DNFB modification at Lys 539 is slightly faster in sulfate medium than in chloride medium. On the other hand, when Lys 590 is free, the rate of DNFB modification at Lys 539 is about 10 fold faster in chloride medium than in sulfate medium. These complex results in different anions media indicate that two lysines undergo allosteric interactions with the anion binding site (*20*). The regulation of anion transport activity and the allosteric interactions with the anion binding site indicate that Lys 590 is located on the same part of the anion transport channel as Lys 539.

Transmembrane segment containing Lys 590 (TM 6) are resistant to proteases in the native conformation;

however, the protease sensitivity increases under alkali conditions (*5*, *34*). These transmembrane peptide portions, classified as "category 2," are shielded from the lipid bilayer by other membrane peptide portions, and peptide-peptide interactions occur (*5*, *6*). These portions are more flexible than typical transmembrane segments that interact with lipids, and must be important for a transporter mechanism induced by conformational changes (*35*). Other chemical anion transport inhibitors that attack Lys 851 (*21*, *22*), Glu 681 (*20*) and His 834 (*23*) are concentrated in category 2 peptide portions (*34*). Especially, His 834 play an essential role in the conformational change that occurs during band 3 mediated anion transport (*23*). As discussed above, Lys 590 has a role in regulating anion transport activity and allosteric interactions with the anion binding site. These findings support our idea that the "category 2" peptide portions, including that containing Lys 590 in TM 6, play an important role in the anion transport mechanism (*5*, *6*, *35*).

An interesting result was obtained in the case of tryptic peptides of the transmembrane domain of band 3 modified by SITS. Trypsin cleavable sites on band 3, Arg 384, Arg 387, Arg 388, Arg 389, Arg 432 and Lys 430, were all susceptible to trypsin when band 3 was not modified by SITS (*5*, *34*). When band 3 was covalently modified with SITS, all these trypsin cleavable sites became resistant and a 22-kDa fragment (from Gly 361 to Lys 551) was recovered from the SITS-modified membranes even though the membranes were extensively digested with trypsin (see Fig. 2B, peak a in Fig. 6A and Table 3). The same 22-kDa fragment peak (peak a in Fig. 6A) was also recovered from SITS-modified membranes when the membranes were digested with trypsin, chymotrypsin or proteinase K (data not shown), indicating that a region of band 3 including the 22-kDa fragment is resistant to proteinases when band 3 is covalently modified by SITS. We have shown that the N-terminal region of the transmembrane domain of band 3 is essential for the anion transport activity (*36*), and that the N-terminal region of the membrane domain (from Gly 361 to Ala 408) interacts with the loop between TM 13 and TM 14 (*34*). These regions are hidden from the cytoplasmic surface when band 3 takes the outward conformation (Takazaki *et al*., unpublished data). It has been shown that DIDS stabilizes the band 3 conformation (*37*) and that stilbene compounds such as DIDS and SITS alter the band 3 conformation from the inward to the outward conformation (*38*, *39*). Previous studies have suggested that band 3 contains subdomains comprising TM 1–5, TM 6–8, and TM 9–12 that interact with each other (*5*, *6*, *34*, *40*). The present results indicate that the 22-kDa fragment region including TM 1–5 (from Ser 401 to Phe 544) forms a subdomain of band 3, and that the subdomain is resistant to proteinases *in situ* when SITS binds covalently to band 3. Although DNFB also binds to the same Lys 539 as SITS, DNFB does not stabilize the 22-kDa fragment region.

In conclusion, we present here an analytical method for use with erythrocyte membrane proteins. The method comprising amino acid sequencing, MALDI-TOF and LC/ESI-MS is useful for the molecular study of band 3 and other minor membrane proteins in erythrocyte membranes. Furthermore we determined the important residues in band 3 for anion transport by identifying the chemically modified sites, and these residues play essential roles in anion transport mechanism. These findings will promote a better understanding of membrane protein structure and function relationships.

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