Identification of Oxidized Methionine Sites in Erythrocyte Membrane Protein by Liquid Chromatography/Electrospray Ionization Mass Spectrometry Peptide Mapping†

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ABSTRACT: In this study, we used peptide mapping combined with liquid chromatography/electrospray ionization mass spectrometry (LC/ESI MS) to examine the methionine oxidation of band 3 of erythrocyte membrane protein. Initially, we identified the methionine sites oxidized by chloramine T (*N*-chloro-*p*-toluenesulfoamide), a hydrophilic reagent. There were three oxidized methionines (Met 559, Met 741, and Met 909) in band 3, and these methionines were located in a hydrophilic region determined by previous topological studies of band 3. In addition, we found that $C_{12}E_8$, a polyoxyethylene detergent, leads to the oxidation of methionines in a transmembrane segment in band 3, and this oxidation occurs in a $C_{12}E_8$ preincubation time-dependent manner. In a previous study, it was found that peroxides accumulate in a polyoxyethylene detergent. Thus, our method enabled the direct and quantitative detection of protein damage due to detergent peroxides. Furthermore, we examined methionine oxidation in the presence of 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) or diethyl pyrocarbonate (DEPC), which induced either an outward or an inward conformation in band 3, respectively. Our results indicated that the location of Met 741 was associated with the band 3 conformation induced by band 3-mediated anion transport. In conclusion, we found that methionine oxidation can be applied to examine membrane protein structures as follows: (1) for topological studies of membrane proteins, (2) for assessing the quality of proteins in detergent solubilization studies, and (3) for the detection of conformational changes in membrane proteins.

There is still little known about the structural and functional relationships of integral membrane proteins such as transporters. This lack of information is partly due to the difficulty of analyzing transmembrane segments according to the standard methods used for the examination of soluble proteins. Since these peptides are buried in membranes, they are not easily accessible to proteases or chemical modification reagents. Furthermore, the hydrophobic properties of transmembrane segments result in their aggregation during enzymatically and chemically mediated cleavage and high-performance liquid chromatography (HPLC) separation. To overcome these problems, we recently developed and established a method of analysis of transmembrane peptides obtained from erythrocyte membrane protein (*1*). Band 3 protein is an erythrocyte membrane protein and it functions as an inorganic anion transporter. The peptides of the transmembrane domain of band 3 were completely analyzed, and the results of the peptide analysis enabled us to identify chemical modification sites that play functional roles in band 3-mediated anion transport (*1*−*3*).

Methionine has a hydrophobic, aliphatic side chain. Unlike other aliphatic amino acids, methionine contains one sulfur that is easily oxidized by oxidants, a reaction by which methionine is converted to methionine sulfoxide and methionine sulfoxide is convert to methionine sulfone by further oxidation (Figure 1, top panel). Numerous previous studies have indicated that information regarding such oxidized methionines could be useful for characterizing protein structures (e.g., for analyses of modifications of active sites in enzymes, protein−protein interactions, and conformational changes in proteins) (*4*−*7*). Methionines frequently appear in transmembrane regions due to their hydrophobic properties. We therefore considered the possibility that methionine oxidation might be useful for the examination of membrane protein structures and functions.

In this study, we applied methionine oxidation to examine erythrocyte membrane protein band 3. We then determined the oxidized methionine sites using peptide mapping combined with a liquid chromatography/electrospray ionization mass spectrometry (LC/ESI MS)[1] technique. The present approach was found to be applicable for the detection of conformational changes in band 3.

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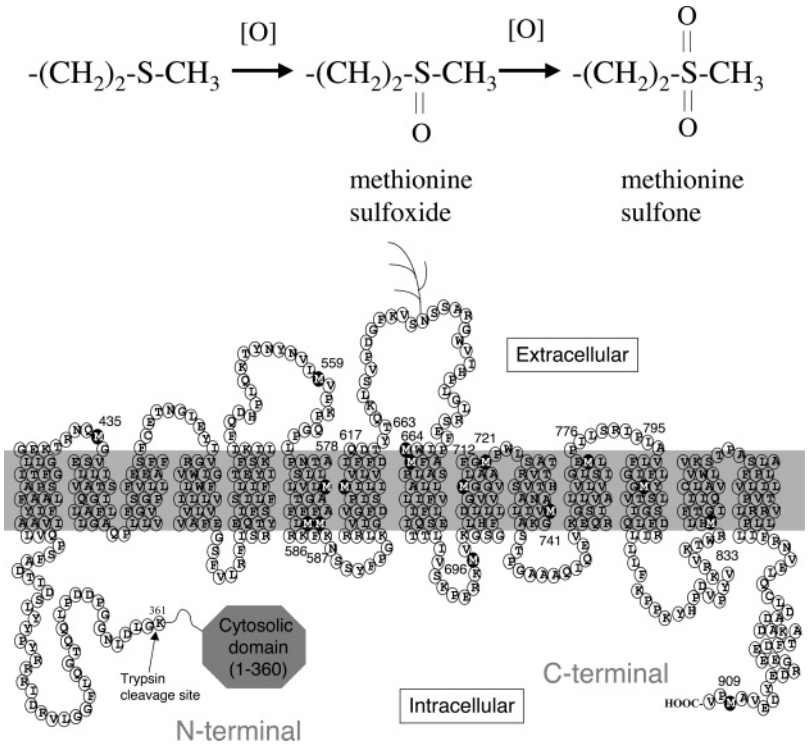

FIGURE 1: (Top panel) Diagram of methionine oxidation. Methionine is converted to methionine sulfoxide by oxidation, and methionine sulfoxide is converted to methionine sulfone by further oxidation. By the oxidation to methionine sulfoxide and methionine sulfone, the mass of methionine was increased 16 and 32 Da, respectively. (Bottom panel) Location of the methionine residues in band 3 in the membrane domain, as based on a 14-span model of band 3 topology. A 14-span model of band 3 folding indicates the location of the methionine sites (indicated by black circles). To remove the 40-kDa NH_2-terminal domain of band 3, the membranes were pretreated with a low concentration of TPCK-trypsin. The tryptic cleavage site (Lys 360) is indicated in the model.

EXPERIMENTAL PROCEDURES

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 Membrane 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 weeks. Erythrocyte membranes (white ghosts) were prepared as described previously (*8*). To remove N-linked sugar, the membrane (1 mg of protein contained in the membrane) was treated with N-glycosidase F (2 units) in 200 μL of 20 mM phosphate buffer (pH 7.2) for 24 h at room temperature. To remove the 40-kDa NH_2-terminal domain of band 3, the 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 the erythrocyte membrane were removed with 10 mM NaOH. The membranes were then washed three times with 5 mM $NaHCO_3$.

Oxidation of Erythrocyte Membrane Proteins by Chloramine T. The pretreated membranes were modified with 0.001, 0.01, 0.1, and 1 mM chloramine T (*N*-chloro-*p*-toluenesulfoamide) in 0.1 M Tris-HCl buffer at pH 8 and 4 °C. Some membranes were preincubated with 1 mM 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) or 0.5 mM diethypyrocarbonate (DEPC) at pH 8 and 37 °C for 30 min before oxidation. The membranes were washed five times with 6 volumes of 0.1 M Tris-HCl buffer at 4 °C.

Preparation of Tryptic Peptide Fragments from Erythrocyte Membrane Proteins. The tryptic peptide fragments used for analysis were prepared as follows. The membranes were solubilized with a total volume of 200 μL of 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$ [octa(ethylene glycol) monododecyl ether]. $C_{12}E_8$ was freshly prepared and was degassed with argon gas. The solubilized proteins in the solution (0.6 mg/mL) were digested with 4 μg of TPCK-trypsin for 2 h at 37 °C. For the analysis of the time dependence of $C_{12}E_8$ storage, some $C_{12}E_8$ was prepared at a concentration of 1% (v/v) in an aqueous solution and the solution was preincubated for 1 or 2 weeks at 37 °C.

Mass Spectrometry. LC/ESI-MS was performed by a combination of HPLC (Waters 600E, Waters) and an LCQ Advantage ion trap mass spectrometer (Finnigan, San Jose, CA). Each sample (10 μL; 6 μg) was loaded onto a C_{18} RP-HPLC column (2.1 i.d. × 250 mm; Waters Symmetry 300 C_{18}, 5 mm) after centrifugation (13000*g* × 10 min). The peptides were separated by HPLC by use of a gradient of the aqueous solution (solvent A) and 2:1 (v/v) 2-propanol/

[1] Abbreviations: BSA, bovine serum albumin; $C_{12}E_8$, octa(ethylene glycol) monododecyl ether; chloramine T, *N*-chloro-*p*-toluenesulfoamide; DEPC, diethyl pyrocarbonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; HPG, hydroxyphenylglyoxal; LC/ESI MS, liquid chromatography/electrospray ionization mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; SAO, southeast Asia ovalocytosis; TFA, trifluoroacetic acid; TM, transmembrane spanning portion; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

Table 1: Molecular Ions from Band 3 Tryptic Peptide Fragments Containing Methionine Analyzed by LC/ESI MS

no.[a]	peptide start−end[b]	methionines	oxidation no.	peptide mass[c] (Da)	most intense molecular ion detected in ESI/MS
M1	433−551	435	0	13 504.17	1350.9 (+10)[d]
			1	13 520.17	nd[e]
M2	552−600	559, 578, 586, 587	0	5546.76	1387.1 (+4)
			1	5562.76	1391.1 (+4)
			2	5578.76	1395.1 (+4)
			3	5594.76	1399.1 (+4)
			4	5610.76	1403.1 (+4)
M3	604−631	617	0	3173.78	1058.3 (+3)
			1	3189.78	1063.6 (+3)
M4	657−694	663, 664	0	4354.29	1451.8 (+3)
			1	4370.29	1457.1 (+3)
			2	4386.29	1462.4 (+3)
M5	696−698	696	0	377.22	nd
			1	393.22	nd
M6	699−730	712, 721	0	3274.91	1092.0 (+3)
			1	3290.91	1097.3 (+3)
			2	3306.91	1102.6 (+3)
M7	731−741	741	0	1329.56	665.0 (+2)
			1	1345.56	673.0 (+2)
M8	758−817	776, 795	0	6612.26	1653.5 (+3)
			1	6628.26	1657.5 (+3)
			2	6644.26	1661.5 (+3)
M9	833−879	833	0	5284.75	1761.8 (+3)
			1	5300.75	1767.1 (+3)
M10	893−911	909	0	2203.32	1101.6 (+2)
			1	2219.32	1109.6 (+2)

[a] Number of peptides containing methionines. [b] Start−end refers to amino acid positions of the identified peptide. [c] Calculated from amino acid seqence. [d] The charge state of the most intense ion is presented in parentheses. [e] Not detected.

acetonitrile (solvent B), each of which contained 0.025% TFA and was handled at a flow rate of 1 mL/min. The mobile phase composition was maintained at 5% B for 5 min and then increased from 5% to 85% in 60 min and from 85% to 100% in 20 min. The column eluent from the HPLC separation was split, and 20% of the flow (200 μL/min) was directed into the ESI source. Data were acquired and analyzed with LCQ version 2.0 software (Finnigan, San Jose, CA). The instrument parameters were as follows: ESI needle voltage, 5 kV; ESI capillary temperature, 260 °C; ion energy, 35%; isolation window, 2 amu; scan range, 550−2000 amu.

Analytical Procedures. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) for protein and peptide analyses was performed according to the methods of Laemmli (*9*) and Kawano and Hamasaki (*10*). Protein concentrations were determined by the method of Lowry et al. (*11*) with BSA as the standard.

RESULTS

Methionine Oxidation in Band 3. In this study, we used band 3 as a model of a membrane protein. Band 3 consists of 911 amino acids and the 40-kDa N-terminal domain is involved in attachments to cytoskeleton proteins; this domain also plays a role in the maintenance of the shape of erythrocytes. The 55-kDa C-terminal domain penetrates the membrane 12∼14 times via a transmembrane segment portion (TM), and the C-terminal domain carries out erythrocyte anion transport (*12*, *13*). The 14 TM prediction model (*14*) is shown in Figure 1, bottom panel. We also show 16 methionines in the C-terminal domain in Figure 1.

First, we oxidized the erythrocyte membranes (white ghosts) by chloramine T, which is commonly used as an oxidant of methionines located in hydrophilic environments. To simplify the peptide analysis, the N-terminal region (40-kDa) of band 3, N-linked sugar, and all peripheral proteins were removed before membrane oxidation. We suspended the white ghosts in 0.1 M Tris-HCl buffer (pH 8), and the ghost suspension was incubated with or without 1 mM chloramine T in 0.1 M Tris-HCl buffer (pH 8) at 4 °C for 2 h. The oxidized membranes were washed in 0.1 M Tris-HCl buffer (pH 8) and were solubilized in 0.1% $C_{12}E_8$ solution. After centrifugation, the supernatant was digested with TPCK-trypsin. The tryptic peptide mixture was analyzed by LC/ESI MS in combined with RP-HPLC. When a single methionine in the peptide was oxidized by chloramine T, it was converted to methionine sulfoxide, and then the molecular weight of the peptide increased by 16 Da (Figure 1, top panel). In mass spectrometry, we could not detect the peaks originated from the methionine sulfones, where single methionine is oxidized by double oxygen (Figure 1, top panel). At alkaline pH (pH 8.0), methionines were mildly oxidized by chloramine T, and formation of methionine sulfones was suppressed (*15*).

With the use of LC/ESI MS, we were able to identify individual oxidized peptides derived from band 3 as new peptides with an increased molecular mass of 16 Da, divided by the charge state. For example, for M2 peptide (the peptide mass is 5546.76) we could observe the masses from the multiple charged ions, 1849.6 (+3), 1387.4 (+4), and 1110.4 (+5) (data not shown). The ratio of peak areas originating from each multiple charged ion was almost the same under LC/ESI MS measurements. And the charge state of the most intense ion of the M2 peptide from Thr 552 to Lys 600 was +4. Thus, in the case of M2 peptide, we used the peak area of the +4 charge ion for calculation of oxidation. Furthermore, the patterns of charge state derived from oxidized and nonoxidized peptides were not so different. The peptides

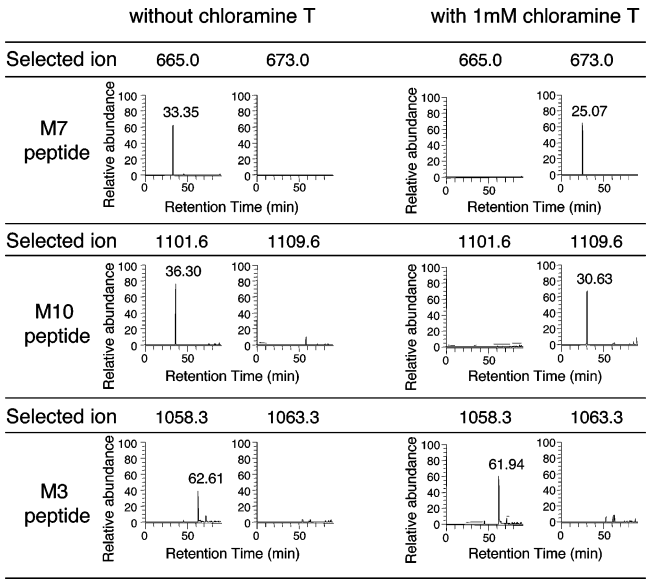

FIGURE 2: Selected ion chromatograms from ESI-MS detection of peaks eluted from HPLC separation of tryptic peptide fragments. Membranes were treated with 1 mM chloramine T at pH 8.0 and 4 °C for 2 h. The treated membranes were dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% $C_{12}E_8$ and the proteins were digested by trypsin for 2 h; the digests were analyzed by LC/ESI MS in combination with a C_{18} RP-HPLC column (2.1 i.d. × 250 mm; Waters Symmetry 300 C_{18}, 5 mm). The peptides were separated by HPLC with a gradient of the aqueous solution (solvent A) and 2:1 (v/v) 2-propanol/acetonitrile (solvent B), each of which contained 0.025% TFA and was handled at a flow rate of 0.2 mL/min. The mobile phase composition was maintained at 5% B for 5 min and then increased from 5% to 85% in 60 min and from 85% to 100% in 20 min. The chromatograms of the oxidized (chromatographs at left, under conditions without chloramine T) and nonoxidized (chromatographs at right, under conditions with chloramine T) M3, M7, and M10 peptide fragments were generated by use of the most intense molecular ion charge state within the mass/charge range of the instrument.

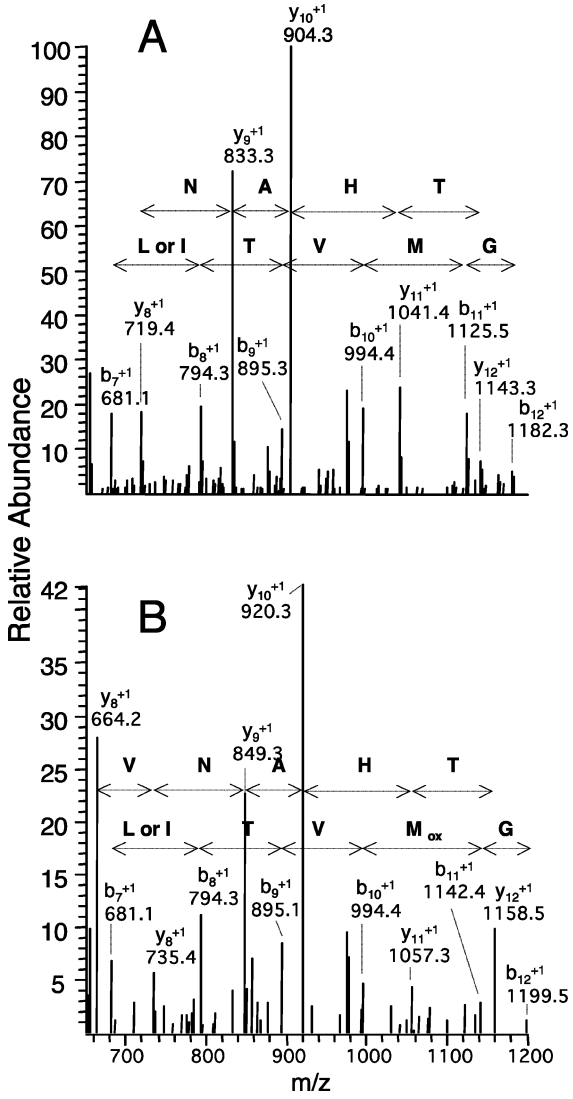

FIGURE 3: MS/MS product ion spectra from the dissociation of a single charged molecular ion of (A) oxidized and (B) nonoxidized M7 tryptic peptide, SVTHANALTV**M**GK, which contains Met 741. MS/MS ions of b and y series are indicated in the spectra. Double-headed arrows indicate the difference of MS/MS ions of b or y series, and one-letter codes for amino acids, estimated from the differences in ion masses, are indicated above the arrows. Comparison of the productive ions of Met 741 in the oxidized and nonoxidized peptide fragments revealed that Met 741 was oxidized only in the oxidized peptide fragment.

containing methionine and methionine sulfoxide could be ionized in a similar manner. The M2 peptide contains four methionines (Met 559, Met 578, Met 586, and Met 587). With the oxidation of one methionine, the original peptide mass was increased by +16 Da; thus, the apparent molecular mass of the peptide was increased by +4. As shown in Table 1, molecular ions at positions 1391.1, 1395.1, 1399.1, and 1403.1 were detected, the molecular masses of which were increased by 4, 8, 12, and 16, respectively, from that of the nonoxidized M2 peptide. Therefore, the detection of these ions indicated single, double, triple, and quadruple oxidation occurred. The molecular ions of the ESI/MS-detected oxidized peptides are summarized in Table 1.

Figure 2 shows the selected ion chromatograms of the M7 (from Ser 731 to Lys 743), M10 (from Ala 893 to Val 911), and M3 (from Val 604 to Lys 631) peptides containing Met 741, Met 909, and Met 617, respectively. As an example of an identification of oxidation, the selected ion chromatogram of the most intense molecular ion of the nonoxidized M7 peptide showed a value of 665.0, and that of the oxidized M7 peptide was 673.0 (Table 1). As shown in Figure 2, treatment with chloramine T gave only the selected ion peak of the oxidized M7 peptide, indicating that Met 741 was oxidized by 1 mM chloramine T. Met 909 in the M10 peptide was also oxidized under the same conditions. In contrast, Met 617 was not oxidized by 1 mM chloramine T (Figure 2). Furthermore, we also detected oxidized methionines using LC/MS/MS. Figure 3 shows the MS/MS analysis of oxidized and nonoxidized M7 peptides. Comparison of the y series and the b series MS/MS ions revealed that the molecular weight of Met 741 had increased by 16 Da. It is clear that only Met 741 was oxidized in the M7 peptide. The oxidation ratios of peptides containing methionines are shown in Figure 4. The ratios of oxidation were estimated from the peak areas of the molecular ions of oxidized and nonoxidized peptides. For example, the peak areas of the nonoxidized and oxidized M2 peptides were 1.22×10^8 and 0.60×10^8, respectively, by 2 h treatment with 0.1 mM chloramine T at 4 °C. Therefore, M2 peptides were 33% oxidized by 2 h treatment with 0.1 mM chloramine T at 4 °C. The analyses revealed that M2, M7, and M10 were completely oxidized by 2 h treatment with 1 mM chloramine T at 4 °C. M7 and M10 each contain a single methionine, Met 741 and Met 909,

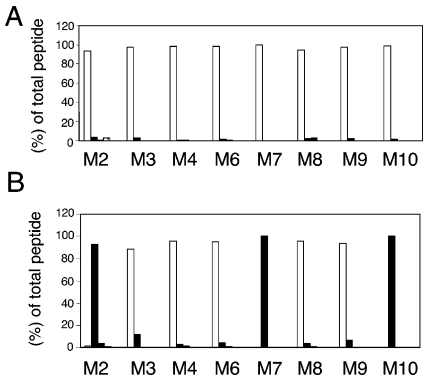

FIGURE 4: Methionine oxidation in band 3. The membranes were treated (A) with or (B) without 1 mM chloramine T at pH 8.0 and at 4 °C for 2 h. The treated membranes were dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% $C_{12}E_8$ and the proteins were digested by trypsin for 2 h; the digests were then analyzed by LC/ESI MS. The bars represent the ratio of oxidized and unoxidized peptides in each sample as follows: white bars indicate unoxidized peptides; black bars, singly oxidized peptides; gray bars, doubly oxidized peptides; and hatched bars, triply oxidized peptide. The oxidation was calculated on the basis of the peak area between oxidized and nonoxidized peptide ion chromatograms.

respectively. Although M2 contains four methionines, we confirmed by MS/MS that only Met 559 was oxidized in the M10 peptide (data not shown). Therefore, Met 559, Met 741, and Met 909 were found to exist in a chloramine T-accessible environment, and other nonoxidized methionines were found to be present in a chloramine T-inaccessible environment.

The chloramine T concentration dependence and the time course of oxidation among the oxidized methionines are shown in Figure 5. The oxidation of Met 909 was completed within a treatment of 100 μM chloramine T or 1 h, respectively. This methionine was found to be very sensitive to oxidation, as compared to the two other methionines. Met 909 is the third amino acid from the C-terminus. Therefore, Met 909 was located in a more hydrophilic environment in the erythrocyte membrane than were the other methionines. The oxidation of the other methionines was not significantly influenced by a longer incubation time or by an excess amount of chloramine T (e.g., see the M3 peptide containing Met 617 in Figure 5B).

Oxidization by $C_{12}E_8$. During the LC/ESI MS analysis, we noticed that methionine oxidation occasionally occurred in a reagent-inaccessible environment. To investigate the source of this type of oxidation, we rechecked each step of our experiment; for example, we looked for membrane deterioration during blood storage, remaining oxidants after washing, and insufficient degassing oxygen in the HPLC system. After performing various assessments, we found that this type of methionine oxidation was dependent on the duration of storage of $C_{12}E_8$. Thus, we prepared $C_{12}E_8$ at a 1% (v/v) concentration in an aqueous solution, and the $C_{12}E_8$ solution was preincubated for 16 days at 37 °C. The white ghosts were solubilized by the preincubated $C_{12}E_8$. After centrifugation, the supernatant was digested with TPCK-trypsin. The tryptic peptide mixture was analyzed by LC/ESI MS in combined with RP-HPLC. Figure 6 shows the methionine oxidation state of each peptide. Comparison of the results obtained by chloramine T oxidation revealed that the transmembrane methionines located in chloramine T-inaccessible regions were oxidized when preincubated $C_{12}E_8$ was used (Figure 6A). With increases in preincubation time, methionine oxidation increases of up to 60% were observed for a period of 2 weeks (Figure 6B).

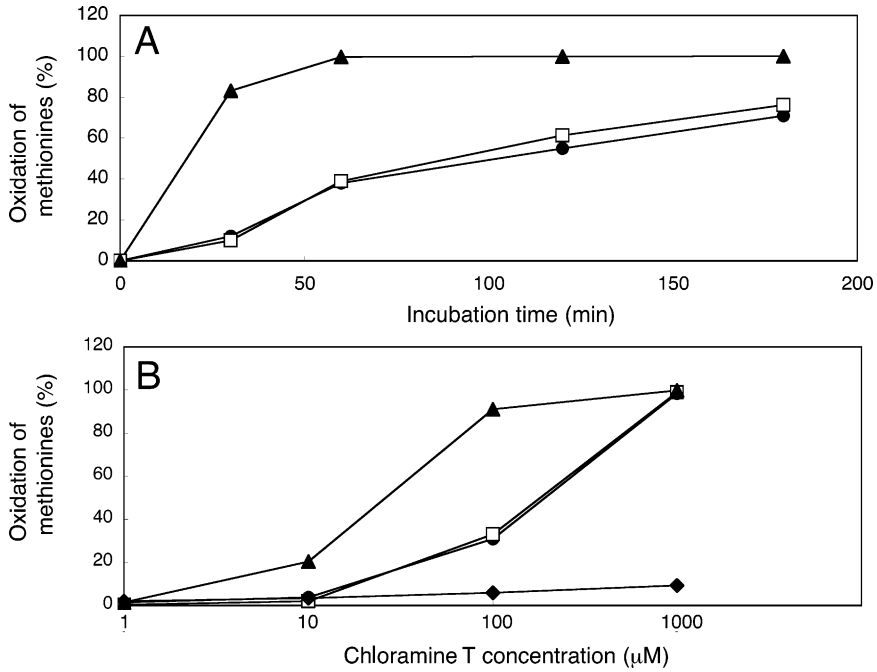

FIGURE 5: Time course and concentration dependence of methionine oxidation by chloramine T. (A) Time course: the membranes were incubated with 100 μM chloramine T in 100 mM Tris-HCl buffer (pH 8) and at 4 °C for each time period noted, until 3 h. (B) Concentration dependence: the membranes were incubated with 0.001, 0.01, 0.1 and 1 mM chloramine T in 100 mM Tris-HCl buffer (pH 8) and at 4 °C for 1 h. Each treated membrane was dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% $C_{12}E8$ and digested by trypsin for 2 h, and the results were analyzed by LC/ESI MS. The oxidation of each methionine was calculated on the basis of the peak area between oxidized and nonoxidized peptide ion chromatograms: (▲) M10; (□) M2; (●) M7; (◆) M3.

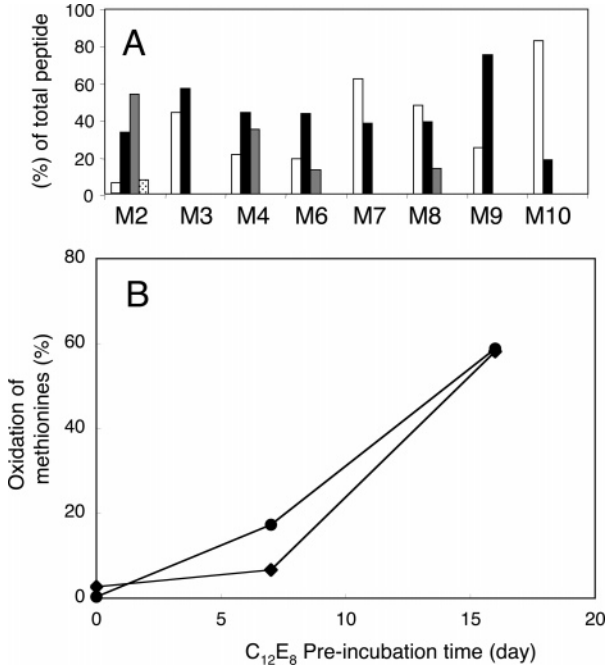

FIGURE 6: Methionine oxidation by preincubated $C_{12}E_8$. (A) We prepared $C_{12}E_8$ at a 1% (v/v) concentration in an aqueous solution, and the $C_{12}E_8$ solution was preincubated at 37 °C for 16 days. The membranes were dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% $C_{12}E_8$, preincubated for 16 days; the proteins were digested by trypsin for 2 h; and the results were analyzed by LC/ESI MS. The bars represent the ratio of oxidized and unoxidized peptides in each sample as follows: white bars indicate unoxidized peptides; black bars, singly oxidized peptides; gray bars, doubly oxidized peptides; and hatched bars, triply oxidized peptide. The oxidation was calculated on the basis of the peak area between oxidized and nonoxidized peptide ion chromatograms. (B) Preincubation time dependence of methionine oxidation by $C_{12}E_8$. We prepared $C_{12}E_8$ at a 1% (v/v) concentration in an aqueous solution, and the $C_{12}E_8$ solution was preincubated at 37 °C for 0, 7, or 16 days. The membranes were dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing each 0.1% preincubated $C_{12}E_8$ and the proteins were digested by trypsin for 2 h; the results were analyzed by LC/ESI MS. Each symbol stands for the oxidation ratios of the M3 peptide (◆) and the M7 peptide (●). The oxidation of each methionine was calculated on the basis of the peak area between oxidized and nonoxidized peptide ion chromatograms.

Methionine Oxidation in the Presence of DNDS or DEPC. The band 3 kinetic studies revealed that the anion exchange is mediated in a one-by-one, electroneutral manner in both directions, as could be accounted for by the "ping pong" model (*16*). This model indicates that the transport is regulated by a single conformational change in band 3 between the outward and inward faces. This conformational change leads to the transfer of a single substrate anion across the membrane, and the rates of association and dissociation of the substrate are much faster than the rate of the conformational change leading to the translocation of the bound anion (*17*). Stilbene compounds such as DIDS, DNDS, and H_2DIDS are known to inhibit anion transport activity and these compounds bind preferentially to band 3 and are immobilized into an outward-facing conformation (*18*−*20*). DEPC modification also inhibits anion transport across the erythrocyte membrane. However, DEPC modification also leads to decreases in H_2DIDS modification of the membrane, and here, DEPC modification of the erythrocyte membrane was reduced in the presence of DNDS in a concentration-dependent manner. Previous results have indicated that DEPC modification leads to an inward-facing conformation (*2*).

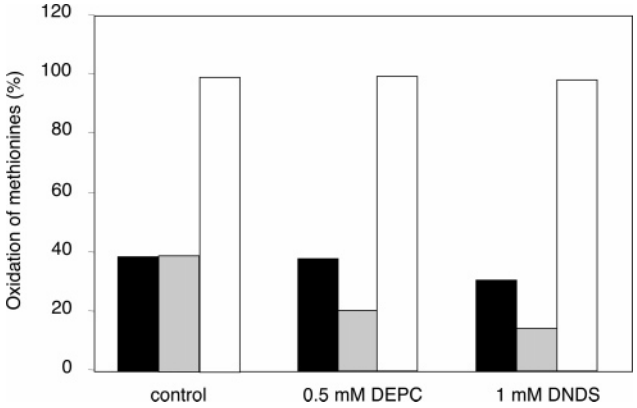

FIGURE 7: Influence of DNDS and DEPC on Met oxidation. The membranes were preincubated without or with 0.5 mM DEPC or 1 mM DNDS at 4 °C for 30 min, and then the samples were treated with 1 mM chloramine T at pH 8.0 and 4 °C for 30 min. The membranes were dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% $C_{12}E_8$ and the proteins were digested by trypsin for 2 h; the results were analyzed by LC/ESI MS. The oxidation of each methionine was calculated on the basis of the peak area between oxidized and nonoxidized peptide ion chromatograms. Black, gray, and white bars represent the oxidation of M2, M7, and M10 peptides in the absence (control) or presence of DEPC or DNDS, respectively.

Thus, we examined methionine oxidation in the presence of DNDS or DEPC. The membranes were pretreated with 1 mM DNDS or 0.5 mM DEPC for 30 min at 4 °C. These concentrations were sufficient to immobilize the outward- and inward-facing conformations, respectively (*2*, *21*). Following pretreatment, oxidation was carried out in Tris buffer at pH 8.0 containing 1 mM chloramine T for 30 min at 4 °C. The oxidation of methionines Met 559, 741, and 909 is shown in Figure 6, as estimated by LC/ESI MS. The oxidation of Met 559 and Met 909 was not influenced by the presence of DEPC or DNDS. On the other hand, the oxidation of Met 741 was reduced in the presence of both reagents. These results indicate that the conformational changes induced by DEPC and DNDS affected the accessibility of Met741 to chloramine T.

DISCUSSION

In the present study, we determined the oxidized methionine sites in membrane proteins using an adaptation of the LC/ESI MS technique. Chloramine T, a hydrophilic oxidation reagent, oxidized three methionines (Met 559, Met 741, and Met 909) in band 3. Band 3 has a single site that is susceptible to extracellular chymotrypsin cleavage, and this site is located between TM5 and TM6. Met 559 is located in such a chymotrypsin-cleavable loop (*22*). Met 909 is located at the terminal end of the C-terminus in band 3, which consists of 911 amino acids. Therefore, these two methionines were present in hydrophilic reagent-accessible regions. Met 741 was located in the transmembrane region (TM10) predicted by the use of a hydropathy model (Figure 1, bottom panel). However, the neighboring residue, Lys 743, has been shown to be cleavable by trypsin in an inside-out vesicle (*23*). Therefore, we were able to confirm that Met 741 was also located in a hydrophilic reagent-accessible region. In contrast, the other methionines were not oxidized. The

erythrocyte bitopic membrane protein glycophorin A also has one methionine in the TM region. The TM segment peptide was detected by the LC/ESI MS system (*1*). The methionine in the TM segment of glycophorin A was not oxidized (data not shown). It is likely that the nonoxidized methionines are buried in the erythrocyte membrane. In addition, oxidation time was not so long (within 2 h) that side reactions by oxidants to membrane lipids or proteins were suppressed. Thus, the intact structure of membrane protein surrounded by lipids might be maintained in the reaction. We therefore concluded that methionine oxidation and LC/ESI MS peptide mapping were useful as a tool for the analysis of the topology of intact membrane proteins. The recent development of mass spectrometric techniques has enabled the rapid and sensitive identification of fragmented peptides at the pico- to femtomole level, even when such peptides have hydrophobic features. Therefore, the present method has powerful advantages for examining the structure of membrane proteins.

We also found that the methionines were oxidized in $C_{12}E_8$ solution. This type of methionine oxidation was found to depend on the duration of $C_{12}E_8$ storage. Furthermore, these oxidations were increased by $C_{12}E_8$ preincubation under solar light (our unpublished result). Previous reports have indicated that peroxides accumulate in distorted polyoxyethylene detergents (*24*−*26*). The polyoxyethylene detergent was used as a general detergent for membrane solubilization, for example, for the purification and crystallization of the membrane protein. Thus, the oxidization of membrane proteins by detergents can lead to a reduction in the quality of purified proteins. In fact, in the case of purification of the acetylcholine receptor, the peroxide yielded by the polyoxyethylene detergent Triton X-100 was modified by membrane protein activity (*27*). In this study, we directly determined the methionines oxidized by $C_{12}E_8$ and we calculated this type of oxidation in a quantitative manner. Therefore, the present method was found to be useful for the examination of the quality of purified membrane proteins. Surprisingly, the oxidation of these methionines occurred primarily in hydrophobic transmembrane segments (Figure 6A). The hydrophobic methionine is converted to the hydrophilic methionine sulfoxide by methionine oxidation (*28*). Therefore, the oxidation of a peptide might be disrupted by a TM−TM assembly stabilized by hydrophobic interactions in a membrane protein, whereby destabilization of membrane protein is induced by membrane protein inactivation. Oxidation occurred in 60% of the methionines for a period of up to 2 weeks (Figure 6B). Protein damage is a very troublesome problem in studies involving protein purification and crystallization by use of polyoxyethylene detergents. Here we suggest that, by using fresh $C_{12}E_8$ and fully degassing the sample in argon gas, oxidation can be prevented.

Stilbene compounds are known to inhibit band 3-mediated anion transport activity, and they bind preferentially to and fix to the outward-facing conformation (*19*−*21*). DEPC modification leads to decreases in H_2DIDS modification of the membrane, and DEPC modification of the erythrocyte membrane was reduced in the presence of DNDS in a concentration-dependent manner (*2*, *21*). The results indicate that DEPC modification of His 834 fixes the molecule in the inward-facing conformation. In addition modification with an arginine-specific reagent, hydroxyphenylglyoxal (HPG), has been shown to lead to the inhibition of anion transport (*3*). As with DEPC modification, HPG modification leads to decreases in DIDS modification of the membrane, and HPG modification of the erythrocyte membrane was found to be decreased in the presence of DNDS. The peptide analysis showed that Lys 539 and Lys 851 were susceptible to modification by H_2DIDS and that His 834 was modified only by DEPC (*2*, *20*). HPG modification occurred specifically at Arg 901 (*3*). Therefore, in a previous report, we suggested that the conformational change in band 3 exerted an effect on the reagent accessibility of these amino acids. In this study, we also revealed that Met 741 was among the residues affected by these conformational changes. Met 741 is in the TM10 segment. Kanki et al. (*29*) suggested that the TM10 segment folds after the TM11 and TM12 segments are incorporated into the membrane. After alkali treatment and protease digestion, the peptides of TM10 were released into the solution from the membrane (*22*, *30*, *31*). Moreover, depending on the expression system and the salt concentration, the location of Lys 743 in the TM10 region can change (*23*). These results indicate that the TM10 region has a flexible character in the band 3 structure. On the other hand, in the presence of either DEPC or DNDS, the oxidation of Met 741 was suppressed. Thus, Met 741 is buried in the membrane when in either the outward- or the inward-facing conformation. In addition, the protease sensitivity of the TM10 region in band 3 was found to be reduced in a SAO mutant membrane in which the band 3 region possessed nine amino acid deletions (residues Ala 400−Ala 408) at the cytoplasmic boundary of the first transmembrane span (TM1) of the normal band 3; in this deletion mutant, band 3 anion transport activity disappeared completely (*22*). The present results, taken together with those of previous studies, suggest that the location of Met 741, and/or the degree to which it is buried in the membrane, is affected by the conformation of band 3, as induced by band 3-mediated anion transport.

Integral membrane protein structures are difficult to investigate due to the difficulty of manipulating hydrophobic membrane segments. A combination of methionine oxidation and peptide mapping has been shown to be useful for the investigation of membrane protein structures, as well as for assessments of the function and quality of purified proteins. Furthermore, mass spectrometry provides a sensitive method for determining the molecular weight of intact proteins. Low levels of recombinant membrane protein expression may be sufficient for such analyses. The present analysis may also be of use in combination with mutation analyses. It should be emphasized that our method is applicable for the examination of the structure of polytopic membrane proteins.

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