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Modification of glycophorin A during oxidation of erythrocyte membrane

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Human erythrocyte ghosts were oxidized with *tert*-butyl hydroperoxide and subsequently treated with tritiated borohydride to label the membrane proteins modified during the membrane oxidation. From the ghosts, oxidized-and-tritiated glycophorin A was isolated and characterized. No intermolecular cross-links were observed as analyzed by sodium dodecylsulfate gel electrophoresis. But, the number of lysine residues was significantly reduced and susceptibility to proteinases such as trypsin, chymotrypsin and pronase was lower than that of control glycophorin A. Trypsinization of the oxidized-and-tritiated glycophorin A gave insoluble and soluble trypsin fragments. After dansylation, N-terminal amino acids of the trypsin-fragments were determined. Dansyl amino acids from the insoluble trypsin fragments were not identical with those from control insoluble counterparts in the membrane-spanning region of glycophorin A molecule. Fractionation by gel filtration of dansyl-soluble trypsin fragments, and the N-terminal amino acid analysis of the fractionated peptides indicated that the peptides derived from the glycosylated region located in the outside of the membrane matrix were identical with those from control soluble counterparts. The results suggest that the glycosylated outside region of glycophorin A was modified only slightly but the hydrophobic membrane-spanning region was extensively modified during membrane oxidation, most likely by oxidized lipids.

Introduction

A number of evidence have demonstrated that lipid oxidation in cell membrane causes modification and damage of membrane proteins and thereby could impair the functions of membrane and cells [1–3]. The modifications of membrane proteins that occur during lipid oxidation include cross-linking, and fluorescence formation [4–8]. We have previously demonstrated that erythrocyte membrane treated with an organic hydroperoxide incorporated radioactivity on reduction with tritiated borohydride [9]. Tritium was incorporated into the membrane proteins as well as lipids, which indicated that the tritium incorporation is an another index for membrane protein modification during lipid oxidation. By this technique, erythrocyte membrane proteins of bands 1, 2, 3, 4.1, 4.2, 5, 6, and PAS-1 (glycophorin A) were found to be modified during lipid oxidation [9]. However, for further understanding of the membrane protein damage, characterization of the modified proteins is necessary.

Glycophorin A is a transmembrane glycoprotein of erythrocyte membrane, and its primary structure has been fully elucidated [10–13]. To investigate how erythrocyte membrane proteins are damaged during membrane oxidation, we have isolated glycophorin A from oxidized-and-tritiated ghosts, and characterized the molecule with respect to intermolecular cross-links, amino acid composition, proteolytic susceptibility, and the sites of tryptic fragmentation.

Materials and Methods

Materials

tert-Butyl hydroperoxide (*t*-BuOOH) was purchased from Nakarai Chemicals, Ltd., Kyoto. Dansyl (Dns) chloride was obtained from Wako Pure Chemical Industries, Ltd., Osaka. *N*-Acetylglucosamine was obtained from Tokyo Kasei Kogyo Company, Tokyo.

Abbreviations: *t*-BuOOH, *tert*-butyl hydroperoxide; Dns, dansyl; PMSF, phenylmethylsulfonyl fluoride; TPCK, L-1-tosylamide-2-phenylethylchloromethylketone; SDS, sodium dodecylsulfate.

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Wheat germ agglutinin-agarose gel was obtained from Seikagaku Kogyo Company, Tokyo. Fluorescamine, dansyl (Dns) amino acid standards, phenylmethylsulfonyl fluoride (PMSF), L-1-tosylamide-2-phenylethylchloromethylketone (TPCK)-trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1) were purchased from Sigma Chemical Company, St. Louis. Pronase (Actinase E) was obtained from Kakenseiyaku Company, Tokyo. NaB^3H_4 (solid 10 Ci/mmol) from Amersham, U.K., was dissolved in 0.01 M NaOH, and stored frozen at −80 °C under nitrogen gas. Hemoglobin (CO-liganded form) was purified from the human erythrocyte lysate by CM-Sephadex C-50 ion exchange column chromatography as described elsewhere [14]. All other chemicals were reagent grade products of Wako Pure Chemical Industries, Ltd.

Analyses

Fluorescence intensity was measured on a Hitachi 650-60 fluorescence spectrometer. Amino acid analysis of glycophorin A was carried out by use of a Hitachi amino acid analyzer L-8500. Dry sample of glycophorin A (1 mg) was dissolved in 1 ml of 6 M HCl and heated at 110 °C for 24 h for analysis of most amino acids, and 25 μg of the sample was dissolved in 1 ml of performic acid and kept at 0 °C for 18 h for methionine analysis. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was performed in the discontinuous buffer system of Laemmli [15] with a 4% stacking and 7.5% separating gel. Protein bands were detected by Coomassie blue and periodic acid Schiff's reagent (PAS) staining and by fluorography [9]. The amount of proteins in erythrocyte ghosts was determined by the method of Lowry et al. [16]. The amount of glycophorin A was determined by sialic acid [17] on the basis of its content in glycophorin A: 18.6% [11]. Dns amino acids were analyzed by high performance liquid chromatography (HPLC) [18–20] with modifications. HPLC of Dns amino acids was performed on a Hitachi 655 liquid chromatograph equipped with a column (4.6 mm i.d. × 25 cm) of YMC A-303 ODS (Yamamura Chemical Laboratories, Ltd., Kyoto). The chromatograph was operated with methanol/10 mM Tris-HCl (pH 6.5), 1 : 1, 6 : 4 and 7 : 3, at a flow rate of 0.5 ml/min, and the peaks were detected by a Shimadzu RF 530 fluorescence spectromonitor.

Human erythrocyte ghosts

Human erythrocyte ghosts were isolated as described [9]. Hemoglobin content of the ghosts was 1–3% of the total proteins as determined by the pyridine hemochromogen method [21].

Oxidized-and-tritiated ghosts

Oxidized-and-tritiated ghosts were prepared according to the method described previously [9]. Two volumes of a suspension of ghosts (5 mg protein/ml) in 0.1 M phosphate buffer (pH 7.0) was mixed with 1 vol. of 10 μM hemoglobin, an oxidizing catalyst, in 0.1 M phosphate buffer (pH 7.0), and the mixture was kept at 4 °C for 30 min. To this was added one volume of 4 mM *t*-BuOOH in 0.1 M phosphate buffer (pH 7.0), and the mixture was incubated at 37 °C for 1 h. Ghosts were washed four times in 5 mM phosphate buffer (pH 7.0) by centrifugation at 11 000 × *g* for 20 min, and resuspended in 0.1 M phosphate buffer (pH 7.0) at 5 mg protein/ml. The suspension of ghosts was mixed with an equal vol of 0.2 mM NaB^3H_4 (2 mCi/ml) in 0.01 M NaOH, and the mixture was allowed to stand at room temperature for 90 min. The ghost suspension was dialyzed extensively against 10 mM phosphate buffer (pH 7.0) containing 0.1 mM PMSF to obtain oxidized-and-tritiated ghosts. Radioactivity incorporated into oxidized-and-tritiated ghosts was 6460 cpm/μg protein. Tritium incorporation into control ghosts not treated with *t*-BuOOH but similarly processed was only less than 540 cpm/μg protein.

Oxidized-and-tritiated glycophorin A

Oxidized-and-tritiated glycophorin A was isolated by use of lithium diiodosalicylate extraction according to the method of Marchesi and Andrews [22]. A suspension of oxidized-and-tritiated ghosts (83 mg protein) was mixed with a suspension of the oxidized ghosts treated with nonradioactive $NaBH_4$ (400 mg protein) to obtain sufficient amounts of the ghosts for isolation of glycophorin A. From the mixture, about 13 mg of oxidized-and-tritiated sialoglycoproteins (180 cpm/μg protein) were obtained. The sialoglycoprotein preparations were mostly composed of glycophorin A, and other minor sialoglycoproteins [23] were not detected as analyzed by SDS-polyacrylamide gel electrophoresis and PAS staining. Accordingly, the glycophorin A preparations were used without further purification. Control glycophorin A was similarly prepared from control ghosts treated with nonradioactive $NaBH_4$.

Proteolysis of oxidized-and-tritiated glycophorin A

Susceptibility of oxidized-and-tritiated glycophorin A to enzymatic proteolysis was assessed by measurement of N-terminal amino residues of liberated peptides using fluorescamine [24]. Thus, control and oxidized-and-tritiated glycophorin A (50 μg/ml) in 25 mM borate-HCl buffer (pH 8.0) was mixed with 100 μl of TPCK-trypsin (1.0 μg/ml), α-chymotrypsin (10 μg/ml) or pronase (10 μg/ml) in the same buffer. The mixture was incubated at 37 °C for indicated periods. The reaction was stopped by addition of 0.1 mM PMSF for the trypsin and the chymotrypsin digestions and by heating at 100 °C for 3 min for the pronase digestion. To the mixture were added 1.3 ml of 50 mM phosphate buffer

(pH 8.0) and 0.5 ml of fluorescamine (3 mg/10 ml) in dioxane, and the mixture was shaken well. Fluorescence intensity at 390 (excitation) and 475 (emission) nm of the mixture was measured. Blank fluorescence intensity was obtained from the fluorescamine-treatment of the mixture of the sample and the enzymes which had been inactivated by PMSF or heating.

Dansyl trypsin fragments of oxidized-and-tritiated glycophorin A

Trypsin-fragments of oxidized-and-tritiated glycophorin A were prepared according to the methods previously reported [12,13] with slight modifications. Thus, 13 mg of oxidized-and-tritiated glycophorin A (2 380 000 cpm) was dissolved in 1.2 ml of 50 mM Tris-HCl buffer (pH 8.5), and to this solution 130 μl of TPCK-trypsin (3.3 mg/ml) in 50 mM Tris-HCl buffer (pH 8.5)/1 mM $CaCl_2$ was added. After 24-h incubation at 37 °C, 2 μl of 0.2 M PMSF solution in ethanol was added, and the mixture was incubated at 37 °C for 1 h in order to inactivate trypsin. The solution, which was still clear, was adjusted to pH 3.5 by addition of 3.5 ml of 5 M acetate buffer (pH 3.5). The trypsin fragments precipitated at this pH were separated by centrifugation at 40 000 rpm for 30 min, washed with 5 mM acetate buffer (pH 3.5) several times, and lyophilized. The radioactivity in the precipitate was 489 800 cpm. The supernatant containing soluble trypsin fragments was decanted and lyophilized.

Dansylation of oxidized-and-tritiated glycophorin A, the insoluble and the soluble fractions of its trypsin fragments was performed according to the method previously reported [25]. Whole glycophorin A molecule (200 μg) or the insoluble trypsin fragments (1/70 the amount from 13 mg glycophorin A) were mixed with 50 μl of 1% SDS. After the mixture was heated at 100 °C for 5 min, 100 μl of *N*-ethylmorpholine and 150 μl of a solution of dansyl chloride (25 mg/ml) in acetone were added. The reaction mixture was kept at 37 °C for 24 h, then evaporated to dryness and resuspended in 1 ml of water. Insoluble material was removed by centrifugation at 14 000 rpm for 30 min. In order to remove dansic acid (Dns-OH), the solution was passed through a column (0.9 × 20 cm) of Sephadex G-15 equilibrated with 0.1 M ammonium acetate (pH 6.8). The first eluting fluorescent peak fraction was lyophilized to obtain dansyl oxidized-and-tritiated glycophorin A and a dansyl-insoluble fraction of the trypsin-fragments (Dns-ox-1).

For dansylation of the soluble trypsin-fragments, the fragments were dissolved in 8 ml of 0.2 M sodium bicarbonate and 8 ml of a solution of dansyl chloride (5.4 mg/ml) in acetone. The mixture was incubated at 37 °C for 24 h. Acetone was removed by evaporation and Dns-OH was removed by gel filtration through a column of Sephadex G-15. The eluate was lyophilized to obtain dansyl-soluble trypsin fragments (242 000 cpm).

Dansyl oxidized-and-tritiated glycophorin A and the dansyl-soluble trypsin fragments were subjected to gel filtration on a Sephadex G-150 SF column (1.3 × 145 cm) equilibrated with 0.1 M ammonium acetate (pH 6.8). Fluorescence at 365 (excitation) and 525 (emission) nm due to the dansyl group of each fraction was measured, and fluorescence intensity was expressed against that of 0.1 μM quinine sulfate in 0.05 M sulfuric acid. Radioactivity of the eluate was also monitored. The recovery of the radioactivity was 94.6%. Fluorescent peak fractions of the dansyl soluble trypsin-fragments, Dns-ox-2 and Dns-ox-3 (see Fig. 4D), were lyophilized for subsequent analysis.

Control glycophorin A was similarly trypsinized, and insoluble and soluble fractions of trypsin-fragments were obtained. The insoluble fraction was dansylated to obtain Dns-1, and the soluble fraction was dansylated and separated by the gel filtration to afford fractions, Dns-2, Dns-3 and Dns-4 (see Fig. 4B).

Determination of N-terminal amino acid residues of dansyl trypsin fragments of oxidized-and-tritiated glycophorin A

Dansyl-soluble and -insoluble trypsin-fragments from oxidized-and-tritiated or control glycophorin A were dissolved in 0.5 ml of 6 M HCl and the solution was heated at 105 °C for 18 h in a sealed tube [25]. The mixture was evaporated to dryness to remove hydrochloric acid, dissolved in 0.5 ml of methyl alcohol, and then subjected to HPLC analysis by use of a YMC A-303 ODS column. The chromatograph was operated by elution with methyl alcohol/10 mM Tris-HCl (pH 6.5) (1 : 1 (solvent A), 6 : 4 (solvent B) and 7 : 3 (solvent C)) at a flow rate of 0.5 ml/min. The peaks were detected by fluorescence at 340 (excitation) and 530 (emission) nm. Dansyl amino acids were identified by comparison of their retention times with that of the dansyl amino acid standard and by cochromatograph with the standard. Retention time of each dansyl amino acid standard was: Dns-OH (dansic acid) 9.5, Dns-Asp 9.5, Dns-Glu 9.8, Dns-Asn 11.0, Dns-Gln 11.5, Dns-Ser 13.0, Dns-Gly 14.5, Dns-Thr 15.0, Dns-Ala 16.0, Dns-Pro 23.0, ε-Dns-Lys 23.5, Dns-Val 29.0, Dns-Met 30.5, Dns-Trp 43.0, Dns-Ileu 49.0, Dns-Leu 52.0, Dns-Phe 61.0 (solvent A); di-Dns-Lys 47.5 (solvent B); di-Dns-Lys 13.0 and di-Dns-Tyr 27.5 min (solvent C).

Results

Human erythrocyte ghosts were treated with 1 mM *tert*-butyl hydroperoxide at pH 7 and 37 °C for 1 h in the presence of catalytic amount of hemoglobin. Under the reaction conditions, the membrane lipids were extensively oxidized as assessed by increases in thiobarbituric acid-reactive substances and fluorescence in the

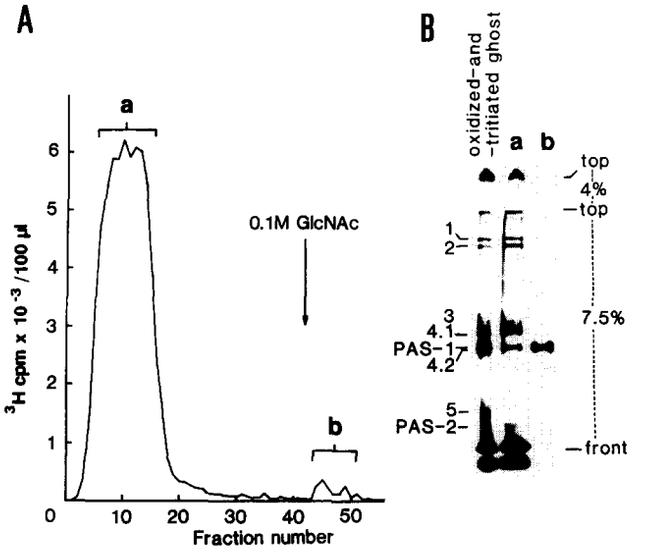

Fig. 1. Separation of oxidized-and-tritiated glycophorin A by affinity chromatography on wheat germ agglutinin-agarose beads. (A) Oxidized-and-tritiated glycophorin A was separated according to the method of Kahane et al. [26]. A suspension of oxidized-and-tritiated ghosts (200 µl, 120 µg protein, 640000 cpm) was solubilized with an equal vol of 2% SDS and 7.6 ml of 0.25 M NaCl, 15 mM phosphate buffer (pH 7.0), 0.025% NaN_3. The mixture was passed through a column (0.6×5 cm) of wheat germ agglutinin-agarose beads at room temperature. The column was washed with the buffer containing 0.05% SDS, and the bound material was eluted with 0.1 M *N*-acetyl-D-glucosamine in the buffer containing 0.05% SDS. Fraction size was 850 µl. Two radioactive peak fractions 'a' (520000 cpm) and 'b' (28000 cpm) were obtained. (B) Oxidized-and-tritiated ghosts (60000 cpm), fraction 'a' (60000 cpm) and fraction 'b' (5000 cpm) were subjected to SDS-polyacrylamide gel electrophoresis and the protein bands were detected by fluorography. The numerals indicate the position of the protein bands from control ghosts [33] simultaneously developed and stained by Coomassie blue and PAS reagent.

membrane [9]. To label the membrane proteins modified during membrane oxidation, oxidized ghosts were then treated with tritiated borohydride. The tritium radioactivity incorporated into the oxidized ghosts (6460 cpm/µg protein) was more than 10-fold that incorporated into unoxidized ghosts.

To confirm the tritium incorporation into glycophorin A molecules, glycophorin A was separated from the oxidized-and-tritiated ghosts by use of a wheat germ agglutinin-agarose column that specifically binds to the sugar moieties of glycophorin A [26] (Fig. 1A). Most of the radioactivity was eluted without being absorbed to the column (fraction 'a') and about 4.4% of the radioactivity was eluted with 0.1 M *N*-acetylglucosamine (fraction 'b'). Whole oxidized-and-tritiated ghosts, and fractions 'a' and 'b' eluted from the column were subjected to SDS-polyacrylamide gel electrophoresis, and the radioactive protein bands were detected by fluorography (Fig. 1B). While whole oxidized-and-tritiated ghosts and fraction 'a' revealed many radioactive protein bands corresponding to native protein bands 1 (spectrin), 2 (spectrin), 3, 4.1, 4.2, 5 (actin), PAS-1 (glycophorin A) and PAS-2; and intermolecularly cross-linked protein bands at the top of the stacking and the separating gels, fraction 'b' revealed a single radioactive protein band corresponding to PAS-1 band (glycophorin A). The results indicate that glycophorin A in oxidized-and-tritiated ghosts must have been modified but not cross-linked during oxidation of ghosts.

Oxidized-and-tritiated glycophorin A was isolated by use of lithium diiodsalicylate extraction method [22] for subsequent analysis. For comparison, control glycophorin A was similarly prepared from unoxidized ghosts after treatment with nonradioactive borohydride. SDS-polyacrylamide gel electrophoresis of oxidized-and-tritiated and control glycophorin A preparations revealed a single PAS-1 band. Radioactivity incorporated into the isolated glycophorin A was 180 cpm/µg protein for the preparation isolated from the 1:5 mixture of labeled and unlabeled ghosts. Comparison of amino acid composition of oxidized-and-tritiated glycophorin A with that of control glycophorin A indicated that the number of lysine residues of oxidized-and-tritiated glycophorin A (3.9 per 100 amino acids) was 66% of that of control glycophorin A (5.9 per 100 amino acids). Thus, it is evident that glycophorin A underwent amino acid damage during the membrane oxidation.

Susceptibility of oxidized-and-tritiated glycophorin A to digestion by trypsin, chymotrypsin and pronase was investigated. The number of peptides liberated from oxidized-and-tritiated glycophorin A on digestion with the proteinases was lower, as estimated by the increases in the number of free amino groups, than that from control glycophorin A throughout the incubation periods in trypsin, chymotrypsin and pronase digests (Fig. 2). Thus, susceptibility of glycophorin A to proteolysis by trypsin, chymotrypsin and pronase was reduced after the oxidative modification.

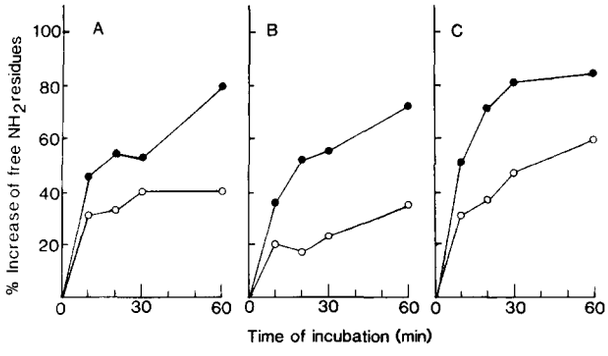

Fig. 2. Susceptibility of oxidized-and-tritiated glycophorin A to trypsin (A), chymotrypsin (B) and pronase (C). Oxidized-and-tritiated glycophorin A (○) and control glycophorin A (●) were treated with TPCK-trypsin, α-chymotrypsin and pronase at pH 8.0 and 37°C. % Increase of amino groups liberated was estimated by use of fluorescamine (see Materials and Methods). Incubation of the enzyme alone showed no significant increases in free amino levels.

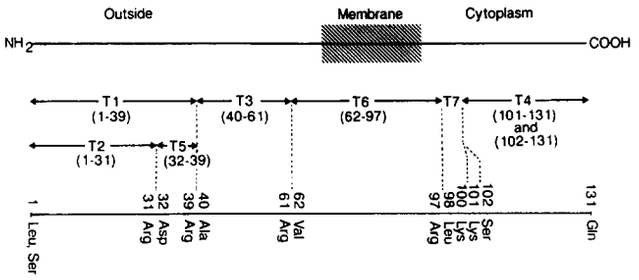

Fig. 3. Alignment of trypsin fragments of glycophorin A, illustrated based on the information from Refs. 11–13.

It is known that native glycophorin A is cleaved into several well-identified peptides, designated T1–T7 fragments, by trypsin [11–13]. As illustrated in Fig. 3, fragments T1 (1–39), T2 (1–31), T3 (40–61), and T5 (32–39) consist in the N-terminus-containing and glycosylated region at the outside of the membrane. Fragment T6 (62–97) is a hydrophobic and membrane-spanning peptide, and fragment T4 (101–131 and 102–131) is the C-terminus-containing peptide located in the inside of the membrane. Fragment T7 is a tri- (98–100) or tetra- (98–101) peptide which has not been isolated. To investigate whether oxidative modification of glycophorin A affects its tryptic fragmentation, oxidized-and-tritiated glycophorin A was subjected to trypsin digestion. As well as control glycophorin A, oxidized-and-tritiated glycophorin A produced insoluble hydrophobic peptides. About 20% of tritium radioactivity was recovered in the insoluble peptides from oxidized-and-tritiated glycophorin A, indicating that peptide modification took place in this region during membrane oxidation. The insoluble peptide fractions from control and oxidized-and-tritiated glycophorin A were dansylated and designated Dns-1 and Dns-ox-1, respectively. Soluble fractions of trypsin-fragments from control and oxidized-and-tritiated glycophorin A were dansylated and subjected to gel filtration on a Sephadex G-150 column (Fig. 4). Gel filtration of dansyl-soluble fragments from control glycophorin A (Fig. 4B) indicated that the glycoprotein was completely digested, and peptide fractions Dns-2, Dns-3, Dns-4, and lower-molecular-weight peptides were produced. Judging from the elution position of Dns-2, Dns-3 and Dns-4, these fractions appeared to correspond to trypsin fragments T1 + T2, T3 and T4 of native glycophorin A [12,13], respectively. Gel filtration of dansyl-soluble fragments from oxidized-and-tritiated glycophorin A (Fig. 4D) revealed a peak fraction at the position of untrypsinized glycophorin A (Fig. 4C), a peak fraction Dns-ox-2 corresponding to Dns-2, a peak fraction Dns-ox-3 corresponding to Dns-3, and a peak fraction with lower molecular weight peptides. The peak corresponding to Dns-4 that may be T4 fragment was missing. The incorporated tritium radioactivity, and therefore the sites of oxidative modification, distributed throughout the fractions.

HPLC analysis of dansyl amino acids liberated by acid hydrolysis of the trypsin-fragments from control (Fig. 5) and oxidized-and-tritiated glycophorin A (Fig. 6) was performed to identify the N-terminal amino acid of each fragment. Dansyl amino acids in Dns-1 were ε-Dns-Lys and Dns-Val (Fig. 5A), those in Dns-2 were Dns-Ser, ε-Dns-Lys and Dns-Leu (Fig. 5B), that in Dns-3 was Dns-Ala (Fig. 5C), and those in Dns-4 were Dns-Ser, Dns-Ala, ε-Dns-Lys and di-Dns-Lys (Fig. 5D). The results indicate that Dns-1, Dns-2, Dns-3 and Dns-4 correspond, respectively, to trypsin fragments T6 + T7

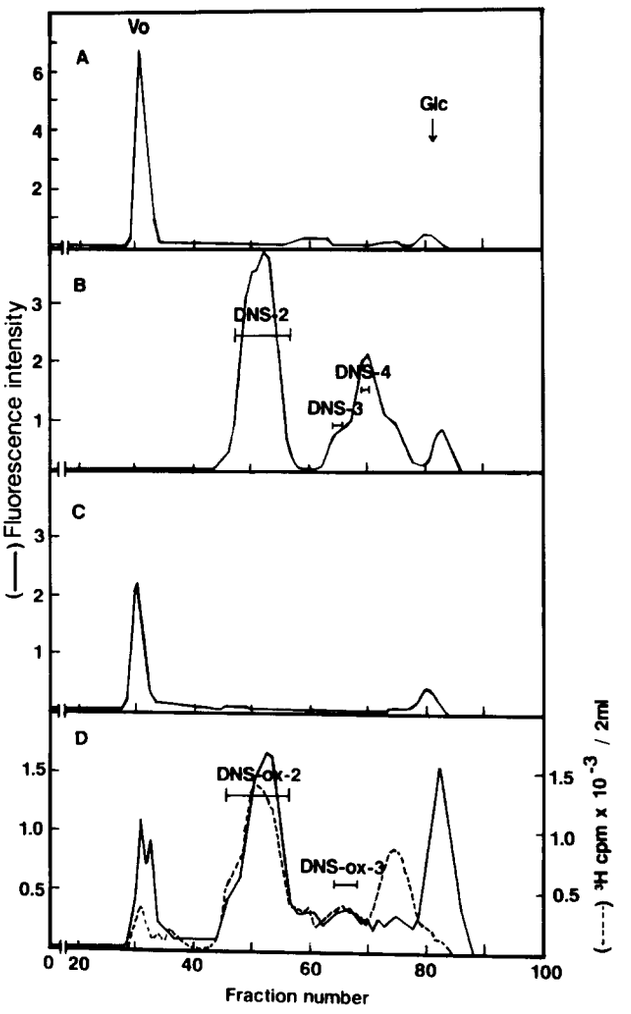

Fig. 4. Gel filtration of dansyl-soluble trypsin fragments from oxidized-and-tritiated glycophorin A and control glycophorin A. (A) Dansyl control glycophorin A; (B) dansyl-soluble trypsin fragments of control glycophorin A; (C) dansyl oxidized-and-tritiated glycophorin A; and (D) dansyl-soluble trypsin fragments of oxidized-and-tritiated glycophorin A (242000 cpm). Gel filtration was performed on a column (1.3×145 cm) of Sephadex G-150 SF equilibrated with 0.1 M ammonium acetate (pH 6.8), and fluorescence of the dansyl group was measured at 365 (excitation) and 525 (emission) nm. Fraction size was 2.0 ml. V_0, void volume estimated by Blue dextran. Glc, elution position of standard D-glucose.

(62–100 or/and 62–101, N-terminal amino acid: Val), a mixture of T1 (1–39, N-terminal amino acid: Ser and Leu) and T2 (1–31, N-terminal amino acids: Ser and Leu), T3 (40–61, N-terminal amino acid: Ala) and a mixture of T3 (40–61, N-terminal amino acid: Ala) and T4 (101–131, and 102–131, N-terminal amino acids: Lys and Ser) [12,13] (see Fig. 3). Dansyl amino acids in Dns-ox-1 were quite different from those of Dns-1 (Fig. 6A), and any of the dansyl amino acids detected did not correspond to the dansyl amino acid standards. This indicates that the N-terminal amino acids of DNS-ox-1 were oxidatively modified amino acids. Trypsin must have cleaved the glycoprotein at the modified amino acid residues in this membrane-spanning hydrophobic region to afford the insoluble segment with unidentified N-terminal amino acids. Dansyl amino acids in Dns-ox-2 were Dns-Ser, ε-Dns-Lys and Dns-Leu, indicating that the fraction corresponded to Dns-2 and thus a

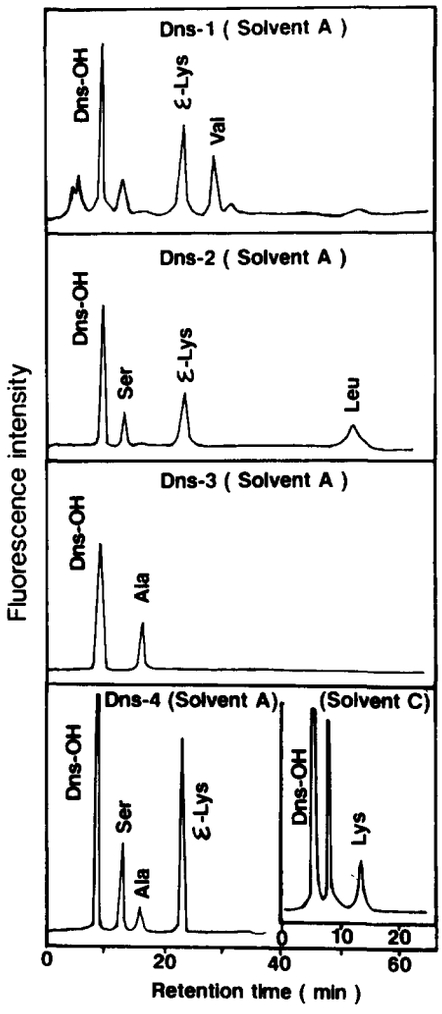

Fig. 5. N-Terminal analysis of dansyl trypsin fragments of Dns-1, Dns-2, Dns-3 and Dns-4 from control glycophorin A. The dansyl trypsin-fragments were hydrolyzed in 6 M HCl at 105°C for 18 h, and the resultant dansyl amino acids were separated and identified by HPLC as described in Materials and Methods.

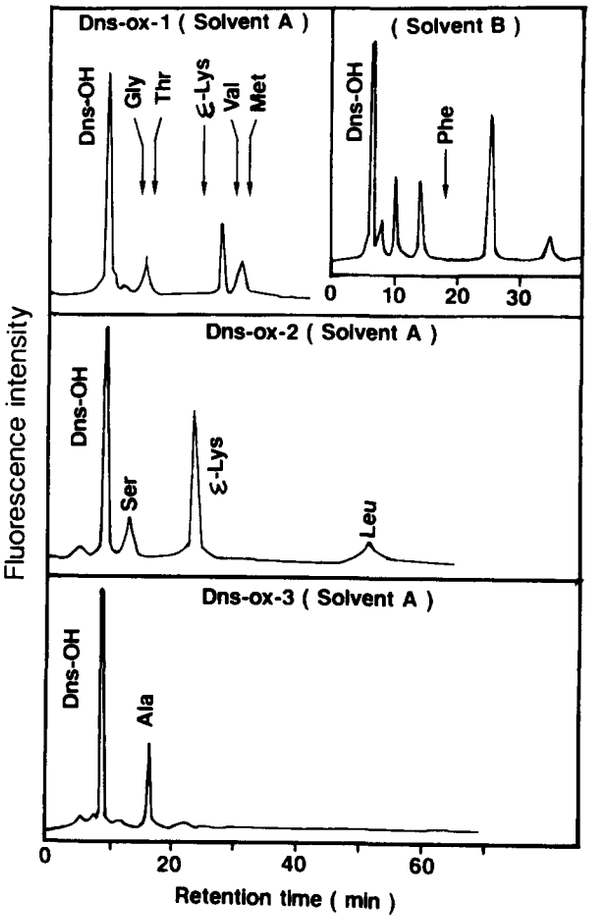

Fig. 6. N-Terminal analysis of dansyl trypsin fragments of Dns-ox-1, Dns-ox-2 and Dns-ox-3 from oxidized-and-tritiated glycophorin A. The dansyl trypsin fragments were hydrolyzed as described in the legend to Fig. 5, and the resultant dansyl amino acids were separated and identified by HPLC as described in Materials and Methods.

mixture of trypsin-fragments T1 and T2 (Fig. 6B). The dansyl amino acid in Dns-ox-3 was Dns-Ala, indicating that the fraction corresponded to Dns-3 and thus T3 trypsin-fragment (Fig. 6C). These results of the dansyl amino acid analysis indicate that the glycosylated outside region of oxidized-and-tritiated glycophorin A was cleaved by trypsin at the same positions as control glycophorin A, but the membrane-spanning hydrophobic region was cleaved differently. Thus, it is likely that the membrane-spanning region of glycophorin A was extensively modified, while the glycosylated outside region was not.

Discussion

In the present study, we have isolated glycophorin A from the ghosts oxidized with *tert*-butylhydroperoxide and characterized its oxidative damage. The membrane proteins could be modified either by direct radical attack of the oxidizing agent or by induced oxidized lipids. Our previous work [9] has demonstrated that

borohydride-reducible functions are formed on the membrane proteins, and their formation is closely correlated with membrane lipid oxidation, which suggests that such protein modification is mainly caused by oxidized lipids. Hence, association of tritium radioactivity with isolated glycophorin A suggests that oxidative modification took place on the glycoprotein molecule during membrane lipid oxidation induced by *tert*-butyl hydroperoxide. Although it is not known what structures are involved in the incorporation of tritium, most probable structures of the borohydride-reducible functions on the proteins are Schiff bases formed by the reaction of aldehyde species generated by degradation of lipid hydroperoxides with amino groups of the proteins [9]. The observed decrease in the number of lysine residues of the oxidized-and-tritiated glycophorin A may be due to the reaction of ε-amino groups of the lysine residues with such aldehyde species. Another candidate for the structure of the borohydride-reducible functions is carbonyl groups generated in oxidatively modified glycophorin A molecules as has recently been suggested for oxidatively damaged proteins in solutions [27].

Glycophorin A was not cross-linked in the ghosts during membrane oxidation by *tert*-butyl hydroperoxide, while most of other membrane proteins were cross-linked extensively [9]. The difference may be due to the difference in protein–protein interaction around the lipid matrix or to the intrinsic properties of the proteins. Formation of cross-links may require highly ordered protein net-work [28] in the membrane.

The oxidative damage of proteins may be reflected by alterations in their biochemical and biological properties. For example, functionally active proteins like enzymes and receptors loose their activities by membrane lipid oxidation [29,30]. Since enzymatic proteolysis depends on primary, secondary, and tertiary structures of substrate proteins, enzymatic proteolysis of the membrane proteins may be affected by oxidative modification. Glycophorin A from oxidized ghosts was less susceptible to various proteinases than that from unoxidized control ghosts as judged by the rate of the release of free amino groups. Recently, Davies [31] proposed that oxidatively damaged proteins become susceptible to proteolysis due to denaturation and an increased hydrophobicity of the proteins but become less susceptible when the proteins are cross-linked. Although glycophorin A was not cross-linked in the membrane during oxidation, it is quite likely that extensive modification of glycophorin A turned the molecule less susceptible to the proteolysis.

The more important finding in this study is that oxidized-and-tritiated glycophorin A liberated, on trypsinization, hydrophobic and membrane-spanning segments with unusual N-terminal amino acids, while soluble other trypsin fragments contained the same N-terminal amino acids as those in control trypsin fragments. This suggests that the membrane-spanning region was preferentially modified during lipid oxidation because of closer proximity to the lipid matrix. In agreement with this result, rough estimation of the incorporated ^{3}H radioactivity per amino acid in the membrane-spanning region marked higher radioactivity than that in the glycosylated outside region. It is likely that various lipid oxidation products including peroxy radicals and aldehyde species [32] reacted preferentially with adjacent hydrophobic and membrane-spanning region of the glycoprotein molecule.

The present study is, to our knowledge, the first example for the isolation and characterization of a particular membrane protein which underwent oxidative modifications in the membrane. It is conceivable that similar oxidative damage also takes place in other erythrocyte membrane proteins during lipid oxidation. Further experimentation directly demonstrating the membrane protein modification by oxidized lipids (e.g., presence of lipid-protein adducts) is necessary to substantiate the role of lipid oxidation in the membrane protein damage suggested here.

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