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Characterization of membrane-bound serine protease related to degradation of oxidatively damaged erythrocyte membrane proteins

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Abstract

It has been shown that erythrocyte membrane proteins become susceptible to degradation by membrane-bound serine protease activity after oxidative modification of the membranes (M. Beppu, M. Inoue, T. Ishikawa, K. Kikugawa, Biochim. Biophys. Acta 1196 (1994) 81–87). The aim of the present study was to clarify the presence of the serine protease in oxidized erythrocyte membranes and to characterize the selectivity of the enzyme to oxidized proteins. Human erythrocytes were oxidized in vitro with xanthine/xanthine oxidase/Fe(III) and oxidized membranes isolated. Proteolytic activity of the membranes toward spectrin obtained from oxidized membranes and bovine serum albumin oxidized with H_2O_2/horseradish peroxidase was increased by membrane oxidation, and the degradability of the substrates was increased by substrate oxidation. The proteolytic activity was inhibited by the serine protease inhibitor diisopropyl fluorophosphate (DFP). The 72 kDa and 80 kDa proteins in the membranes were labeled by [^{3}H]DFP when detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and subsequent fluorography. The 72 kDa protein was found to be a serine enzyme, acetylcholine esterase. The 80 kDa protein appeared to be responsible for the degradation of oxidatively damaged proteins. The 80 kDa protein was loosely bound to membranes and readily solubilized into a 0.1% NP-40 detergent solution. The presence of the same 80 kDa protease in intact erythrocyte cytosol was suggested. The increased serine protease activity in oxidized membranes can result from the increased adherence of the cytosolic 80 kDa serine protease to the membranes due to oxidation. 0005-2736/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Oxidative stress; Membrane protein degradation; Membrane-bound serine protease; Oxidized protein; Erythrocyte

1. Introduction

The presence of ATP-independent, and therefore ubiquitin-independent proteases in erythrocyte and reticulocyte extracts [1–8], mitochondria [9,10] and *Escherichia coli* extracts [5,11–13] that are specific to oxidatively damaged intracellular proteins has been demonstrated. A multicatalytic proteolytic complex composed of several subunits has been shown to be responsible for the degradation of the oxidized intracellular proteins in erythrocytes and reticulocytes [6–8]. These proteases have been referred to as the secondary antioxidant defense system of the cells [14].

It has been shown in our previous study that erythrocyte membrane proteins become susceptible

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to degradation by membrane-bound serine protease activity after oxidative modification of the membranes [15]. This suggested the presence of secondary antioxidant defense mechanisms in erythrocyte membranes for the removal of the oxidatively damaged membrane proteins by proteases. The protease in erythrocyte membranes responsible for degradation of oxidatively damaged proteins appears to be different from the multicatalytic proteases previously demonstrated [6–8] because the effect of various protease inhibitors on protein degradation was different between the two systems [15]. The aim of the present study is to clarify the presence of the serine protease in oxidized erythrocyte membranes and to characterize the specificity of the protease to oxidized proteins. It was found that an 80 kDa serine protease is present in erythrocyte membrane that preferentially degrades oxidized proteins. This enzyme may originate in erythrocyte cytosol and become adherent to membranes when the cells are oxidized.

2. Materials and methods

2.1. Materials

Xanthine (X), xanthine oxidase (XO) (EC 1.1.3.22) (from buttermilk, grade III, diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), Nonidet P-40 (NP-40), bovine serum albumin (BSA) (fatty acid free) and horseradish peroxidase (HRP) (EC 1.11.1.7) were obtained from Sigma (St. Louis, MO, USA). XO was treated as follows [15]. A solution of XO (50 U/2.4 ml) was diluted at an enzyme concentration of 2 U/ml with a buffer composed of 25 mM potassium dihydrogen phosphate, 125 mM choline chloride, 0.01 mM ethylenediaminetetraacetic acid (EDTA) and 2.5 mM sodium azide (pH 7.4). To 1 ml of the solution 5 μl of 0.2 M DFP solution in ethanol and 2 μl of 0.2 M PMSF solution in ethanol were added, and the mixture was allowed to stand at 4°C for 12 h to inactivate the contaminating proteases [16]. After the mixture was dialyzed against the same buffer, it was stored at −80°C until use.

^{125}I-Bolton-Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionate; 2335 Ci/mmol), [1,3-^{3}H]DFP ([^{3}H]DFP; 6.0 Ci/mmol), EN^{3}HANCE and Aquasol-2 for ^{3}H measurement were obtained from NEN Research Products (Boston, MA, USA).

Human venous blood withdrawn from a healthy donor using acid-citrate-dextrose as an anticoagulant was stored at 4°C for a couple of days. Blood was centrifuged (320×*g*, 10 min) at 4°C to remove plasma and buffy coats. Erythrocyte pellet was washed four times by centrifugation (320×*g*, 10 min) at 4°C with ice-cold Ca^{2+}−, Mg^{2+}-free Dulbecco's phosphate-buffered saline (pH 7.4) (DPBS(−)). By this procedure, erythrocyte preparations with only about 1/18 000 leukocyte contamination as determined by crystal violet staining were obtained.

2.2. Analysis

Proteins in the membrane preparations were determined by the method of Lowry et al. [17] using BSA as a reference standard. Hemoglobin content in erythrocyte cytosol was determined by absorbance at 523 nm and its molar extinction coefficient of 7880 [16]. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli [18] in a discontinuous buffer system with a 7.5 or 10% separating gel and a 4% stacking gel under reducing conditions. The samples containing 25 μg protein were loaded per lane unless otherwise specified. Protein bands after SDS-PAGE were stained by Coomassie brilliant blue R-250. For fluorography of [^{3}H]DFP-labeled proteins, the gel of SDS-PAGE was rinsed in 10% acetic acid for 1–2 h to fix proteins in the gel. After removal of acetic acid, the gel was shaken in EN^{3}HANCE for 1 h. After removal of EN^{3}HANCE, the gel was shaken in ice-cold water for 1 h. After drying the gel, the radioactivity of the gel was visualized using a Kodak XAR-5 X-ray film with the aid of a lightning plus (Dupont de Nemours, Wilmington, DE, USA) at −80°C.

^{3}H- and ^{125}I-radioactivities were counted using an Aloka LSC 3500 liquid scintillation counter (Tokyo, Japan) and an Aloka ARC 2000 autowell γ-ray counter (Tokyo, Japan), respectively.

2.3. Oxidation of erythrocytes

Oxidation of erythrocytes was performed as described previously [15]. To 100 ml of 40% erythrocyte

suspension in DPBS(−), 100 ml of 2 mM X in Dulbecco's phosphate-buffered saline (pH 7.4) (DPBS(+)), 1.0 ml of 2 U/ml XO solution and 2.0 ml of 10 mM $FeCl_3$ solution in 40 mM HCl were added. The DPBS(+) has sufficient buffer capacity to maintain the pH of the suspension. The suspension was incubated at 37°C for 3 h under aerobic conditions. Oxidized erythrocytes were recovered by centrifugation at $320\times g$ at 4°C for 10 min and washed twice with DPBS(+) by centrifugation. Unoxidized control erythrocytes were obtained by the same incubation conditions without the oxidants.

2.4. Preparation of erythrocyte membranes and cytosol

Erythrocyte pellet was lysed in 40 vols. of 5 mM sodium phosphate buffer (pH 8.0). The lysate was centrifuged at $9600\times g$ for 20 min to obtain erythrocyte membrane pellet. The membrane pellet was washed several times with the buffer by centrifugation until the supernatant became colorless. The membrane pellets from oxidized and unoxidized control erythrocytes were resuspended in DPBS(+) containing 0.1 mM α-tocopherol, and the membrane pellet from intact erythrocytes was resuspended in DPBS(−). All the operations were performed at 4°C. The membrane suspension was stored at −80°C until use.

Erythrocyte cytosol was prepared according to the method previously reported [5]. A preparation of intact erythrocyte pellet was lysed in 1.5 vols. of 1 mM dithiothreitol for 1 h. The mixture was centrifuged at $10\,000\times g$ for 20 min to obtain the supernatant, which was then centrifuged at $18\,500\times g$ for 60 min. The supernatant was dialyzed against 10 mM Tris-HCl buffer (pH 7.8) containing 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 8 mM KCl and 10% glycerol to obtain erythrocyte cytosol. The cytosol preparation was stored at −80°C until use.

2.5. Preparation of oxidized and unoxidized spectrin and ^{125}I-radiolabeling

Oxidized or unoxidized spectrin from oxidized or unoxidized erythrocytes was prepared by the method previously described [19]. Erythrocyte membrane was prepared from 400 ml of oxidized or unoxidized erythrocytes according to the method of Dodge et al. [20]. The membrane preparation was suspended in 1000 ml solution (pH 7.5) containing 1 mM EDTA, 5 mM 2-mercaptoethanol and 0.03 mM PMSF, and spectrin was extracted by standing the solution for 18 h. The suspension was centrifuged at $100\,000\times g$ for 1 h to obtain spectrin extract. To the extract sodium azide at 0.02% was added, and the extract was condensed using an Amicon membrane filter (PM 30) and equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 20 mM sodium chloride, 130 mM potassium chloride, 0.1 mM EDTA, 0.5 mM 2-mercaptoethanol and 0.03 mM PMSF. The solution was passed through a column (2.5×90 cm) of Sepharose CL-4B. A 280 nm absorbing fraction corresponding to spectrin dimer was collected and condensed to obtain spectrin preparation. The preparation was dialyzed against 10 mM sodium phosphate buffer (pH 8.0).

^{125}I-Labeling of spectrin was carried out by the method of Bolton and Hunter [21]. A 150 μl solution of 100 μg spectrin and 250 μCi ^{125}I-Bolton-Hunter reagent were mixed, and the mixture was kept at 4°C overnight under stirring. To stop the reaction 50 μl of 50 mM phosphate buffer (pH 7.5) containing 10 mg/ml glycine were added, and the mixture stirred at 4°C for 1 h. The mixture was applied to a column of Sephadex G-50 and the column eluted with DPBS(−) containing 0.02% sodium azide to obtain ^{125}I-oxidized or unoxidized spectrin. Specific activity of ^{125}I-oxidized and unoxidized spectrin was 1.2×10^5 and 2.26×10^5 cpm/μg protein, respectively. The preparations were stored at 4°C.

2.6. Preparation of oxidized and unoxidized BSA and ^{125}I-radiolabeling

A mixture of 10 ml of a solution of BSA at 0.5 mg/ml in 50 mM Tris-HCl (pH 7.8), 1.0 ml of 0.2 M H_2O_2 in the same buffer and 0.1 ml of a solution of HRP at 0.1 mg/ml in the same buffer was incubated at 37°C for 20 h. The mixture was then dialyzed against 10 mM sodium phosphate buffer (pH 8.0) to obtain oxidized BSA. BSA was similarly treated without the oxidant to obtain unoxidized BSA.

^{125}I-Labeling of BSA was carried out as described for that of spectrin using a 200 μl solution of 70 μg BSA and 250 μCi ^{125}I-Bolton-Hunter reagent. Spe-

cific activity of ^{125}I-oxidized and unoxidized BSA obtained was 0.77×10^6 and 2.6×10^6 cpm/μg protein, respectively. The ^{125}I-oxidized and unoxidized BSA preparations were mixed with unlabeled oxidized and unoxidized BSA, respectively, to reduce their specific radioactivity to 1/10. The preparations were stored at 4°C.

2.7. Degradation of ^{125}I-oxidized and unoxidized spectrin by oxidized and unoxidized erythrocyte membranes

A mixture of 20 μl of ^{125}I-oxidized or unoxidized spectrin (0.1 μg protein) solution in DPBS(−) and 100 μl of oxidized or unoxidized erythrocyte membrane suspension (25 μg protein) in DPBS(+) was incubated at 37°C for the indicated period. To the mixture were added 380 μl of a solution of BSA at 1 mg/ml as a carrier protein. To the mixture were added 500 μl of cooled 20% trichloroacetic acid, and the mixture was cooled on ice for 1 h. Radioactivity of the whole mixture was counted. After centrifugation at $320\times g$ for 10 min, trichloroacetic acid-soluble radioactivity of the 500 μl of the supernatant was counted. Radioactivities without added erythrocyte membrane suspensions were subtracted.

2.8. Degradation of ^{125}I-oxidized and unoxidized BSA by intact, unoxidized and oxidized erythrocyte membranes and intact erythrocyte cytosol

A mixture of 100 μl of erythrocyte membrane suspension (200 μg protein) in 0.1% NP-40/DPBS(+) and 5 μl of ^{125}I-oxidized or unoxidized BSA (0.1 μg) solution in 10 mM sodium phosphate buffer (pH 8.0) was incubated at 37°C for the indicated period. To the mixture were added 395 μl of a solution of BSA at 1 mg/ml as a carrier protein. For investigation of the effect of DFP, 1 μl of a solution of 0.2 M DFP was added to 100 μl of the erythrocyte membrane suspension. To the mixture were added 500 μl of cooled 20% trichloroacetic acid, and the mixture was cooled on ice for 1 h. Radioactivity of the whole mixture was counted. After centrifugation at $8000\times g$ for 10 min, trichloroacetic acid-soluble radioactivity of 500 μl of the supernatant was counted. Radioactivities without added erythrocyte membrane suspensions were subtracted.

A mixture of 100 μl of erythrocyte cytosol (16.8 mg hemoglobin) and 5 μl of ^{125}I-oxidized or unoxidized BSA (0.1 μg) solution in 10 mM sodium phosphate buffer (pH 8.0) was incubated at 37°C for 48 h. The mixture was similarly treated as described above.

2.9. [^{3}H]DFP-labeling of intact erythrocyte membranes and cytosol

To 20 ml of intact erythrocyte membrane suspension (100 mg protein) in DPBS(−), 100 μl of a solution of 1 mCi/ml (0.1 μmol/ml) [^{3}H]DFP in propylene glycol was added, and the mixture stirred at room temperature for 3 h. Unlabeled DFP was then added at a final concentration of 2 mM, and the mixture kept at 4°C for 15 h. The mixture was dialyzed against 1 mM EDTA. Specific activity of [^{3}H]DFP-labeled intact erythrocyte membranes was 27 600 cpm/mg protein. The labeled preparation was stored at −80°C until use.

For investigation of the effect of butyrylcholine iodide on [^{3}H]DFP-labeling of intact erythrocyte membranes, a mixture of 500 μl of intact erythrocyte membrane suspension (1.5 mg protein) in DPBS(−) and 500 μl of a solution of 5 M butyrylcholine iodide in DPBS(−) was cooled on ice for 30 min. To the mixture 5 μl of 1 mCi/ml (0.1 μmol/ml) [^{3}H]DFP in propylene glycol was added, and the mixture stirred at room temperature for 1 h. Unlabeled DFP was then added at a final concentration of 2 mM, and the mixture kept at room temperature for 30 min. The mixture was centrifuged at $16\,000\times g$ for 40 min at 4°C, and the membranes were washed once with DPBS(−) containing 0.25 M butyrylcholine iodide by centrifugation to be resuspended in the same buffer to make a total volume of 1.0 ml. The suspension was dialyzed against water.

To 200 μl of erythrocyte cytosol (3.4 mg hemoglobin) 20 μl of a solution of 1 mCi/ml (0.1 μmol/ml) [^{3}H]DFP were added, and the mixture was stirred at room temperature for 3 h. The mixture was treated as described above. Specific activity of [^{3}H]DFP-labeled intact erythrocyte cytosol was 34 400 cpm/mg hemoglobin. The labeled preparation was stored at −80°C until use.

2.10. Solubilization of intact erythrocyte membranes in 0.1% NP-40

To 15 ml of intact erythrocyte membrane suspension (75 mg protein), 15 ml of 100 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl, 2 mM EDTA and 0.2% NP-40 were added, and the mixture was stirred at 37°C for 1 h. The mixture was then centrifuged at 15 000×*g* and 4°C for 20 min to obtain the solubilized supernatant and the residue.

2.11. Gel filtration of intact erythrocyte membranes solubilized in 0.1% NP-40

To 2.0 ml of [^{3}H]DFP-labeled intact erythrocyte membranes solubilized in 0.1% NP-40 (1.6×10^5 cpm/3.1 mg protein), 8.0 ml of intact erythrocyte membranes solubilized in 0.1% NP-40 (18.6 mg protein) were added. The mixture was applied to a column (2.5 cm i.d.×130 cm) of Sepharose CL-6B equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl, 1 mM EDTA and 0.1% NP-40. The column was eluted with the same buffer at 4°C at a flow rate of 0.5 ml/min. The fractions were monitored by radioactivity and protein. The fractions were condensed using an Amicon membrane filter (PM 10).

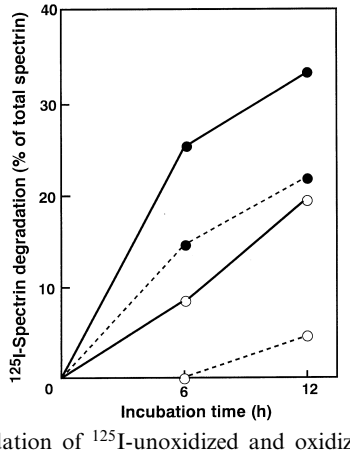

Fig. 1. Degradation of ^{125}I-unoxidized and oxidized spectrin by unoxidized and oxidized erythrocyte membranes. Unoxidized and oxidized spectrin obtained from erythrocytes treated without (○) or with X/XO/Fe(III) at 1 mM/10 mU ml^{-1}/0.1 mM (●) at 37°C for 3 h were labeled with ^{125}I and incubated at 37°C for the indicated period with erythrocyte membrane suspension obtained from erythrocytes treated without (- - -) or with the oxidant (———) at 37°C for 3 h. Degradation of ^{125}I-spectrin was examined by the release of trichloroacetic acid-soluble radioactivity. The data are the means of duplicate experiments.

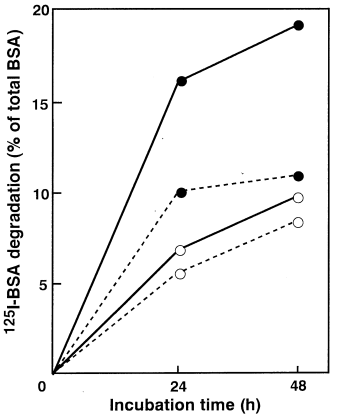

Fig. 2. Degradation of ^{125}I-unoxidized and oxidized BSA by unoxidized and oxidized erythrocyte membranes. Unoxidized and oxidized BSA obtained from BSA treated without (○) or with H_2O_2/HRP at 20 mM/1 μg ml^{-1} (●) at 37°C for 20 h were labeled with ^{125}I and incubated at 37°C for the indicated period with erythrocyte membrane suspension in 0.1% NP-40/DPBS(+) obtained from erythrocytes treated without (- - -) or with X/XO/Fe(III) at 1 mM/10 mU ml^{-1}/0.1 mM (———) at 37°C for 3 h. Degradation of ^{125}I-BSA was examined by the release of trichloroacetic acid-soluble radioactivity. The data are the means of duplicate experiments.

3. Results

It has been shown that erythrocyte membrane proteins become susceptible to degradation by membrane-bound serine protease activity after oxidative modification of the membranes [15]. At that time it was not clear whether the increased susceptibility due to oxidative stress reflected the increased proteolytic activity of enzymes or the increased degradability of oxidized proteins. To clarify these two possibilities, the following experiments were performed. Human erythrocytes were not treated (unoxidized) or oxidized with X/XO/Fe(III) at 37°C for 3 h. Erythrocyte membranes and spectrin were prepared from the unoxidized and the oxidized erythrocytes. Unoxidized and oxidized spectrin were radioiodinated with ^{125}I-Bolton-Hunter reagent, and incubated at 37°C for up to 12 h with the unoxidized and oxidized erythrocyte membrane suspensions. Degradation of ^{125}I-spectrin was examined by the release of trichloroacetic acid-soluble radioactivity (Fig. 1). While the extent of degradation of unoxidized spectrin in incubation

with unoxidized membranes was small (open circle, dotted line), the extent was larger in incubation with oxidized membranes (open circle, solid line). However, the extent of degradation of unoxidized spectrin by oxidized membranes was moderate as well as the extent of degradation of oxidized spectrin by unoxidized membranes (closed circle, dotted line). The extent of degradation of oxidized spectrin by oxidized membranes (closed circle, solid line) was the highest. It is likely that both proteolytic activity of the membranes and degradability of the substrate were increased by membrane oxidation.

Protein degradation by oxidized erythrocyte membranes was examined using BSA oxidized with H_2O_2/HRP. ^{125}I-Radiolabeled unoxidized and oxidized BSA were incubated at 37°C for up to 48 h with a suspension of unoxidized or oxidized erythrocyte membranes in 0.1% NP-40 (Fig. 2). While the extent of degradation of unoxidized BSA in incubation with unoxidized membranes was small (open circle, dotted line), the extent was slightly larger in incubation with oxidized membranes (open circle, solid line). The extent of degradation of unoxidized BSA by oxidized membranes was moderate as well as the extent of degradation of oxidized BSA by unoxidized membranes (closed circle, dotted line). The extent of degradation of oxidized BSA by oxidized membranes (closed circle, solid line) was the highest. The results are consistent with the results of Fig. 1, showing that both proteolytic activity in the membranes and degradability of the substrate were increased by oxidation.

Table 1
Degradation of ^{125}I-unoxidized and oxidized BSA by erythrocyte membranes and cytosol and its inhibition by DFP

Preparation from erythrocyte	^{125}I-BSA degradation (% of total BSA)			
	Unoxidized BSA		Oxidized BSA	
	−DFP	+DFP	−DFP	+DFP
Unoxidized membranes	6.3	6.1	10.4	4.8
Oxidized membranes	8.0	6.9	14.1	5.1
Intact membranes*	8.7	7.8	24.1	5.4
Intact cytosol	2.6	1.7	7.5	5.7

Unoxidized and oxidized BSA obtained from BSA treated without or with H_2O_2/HRP at 20 mM/1 μg ml^{-1} at 37°C for 20 h were labeled with ^{125}I and incubated at 37°C for 96 h with erythrocyte membrane suspension or its supernatant (*) in 0.1% NP-40/DPBS(+), or at 37°C for 48 h with erythrocyte cytosol in the absence or the presence of 2 mM DFP. Degradation of BSA was examined by the release of trichloroacetic acid-soluble radioactivity. The data are the means of duplicate experiments.

It has been shown previously that a serine protease inhibitor DFP strongly and a metalloprotease inhibitor EDTA slightly suppressed the degradation of proteins of oxidized erythrocyte membranes [15]. Inhibitory activity of DFP and EDTA in the degradation of unoxidized or oxidized BSA by erythrocyte membranes in 0.1% NP-40 was examined. The results are shown in Table 1. Unoxidized, oxidized, and intact membranes degraded oxidized BSA more efficiently than unoxidized BSA. While DFP only slightly inhibited the degradation of unoxidized BSA, it effectively inhibited the degradation of oxidized BSA. However, the degree of inhibition by DFP of oxidized BSA degradation was not complete, and the levels of degradation in the presence of the inhibitor were close to the level of unoxidized BSA degradation in the absence or presence of the inhibitor. Hence the increased protein degradation observed for oxidized BSA is likely to be caused by a serine protease, and the protein degradation uninhibitable by DFP, which is also observed for unoxidized BSA, may be caused by proteases of other types. The results suggest that the membrane serine protease preferentially degrades oxidized protein. EDTA (5 mM) did not inhibit BSA degradation in any combination of BSA and membrane preparations (data not shown), indicating that metalloproteases in the membrane did not contribute to the degradation of oxidized protein.

In order to characterize the serine protease in erythrocyte membranes, proteins that could be labeled with [^{3}H]DFP were pursued. Intact, unoxidized or oxidized erythrocyte membranes were incubated with [^{3}H]DFP at room temperature for 3 h to obtain [^{3}H]DFP-labeled membranes. The labeled membranes from intact erythrocytes were solubilized in 0.1% NP-40, and 56% of the radioactivity and 31% of protein were recovered in the solubilized supernatant. [^{3}H]DFP-labeled intact erythrocyte membranes, their 0.1% NP-40-solubilized fraction and the 0.1% NP-40-residual fraction were analyzed on SDS-PAGE under reducing conditions (Fig. 3A) and subsequent fluorography (Fig. 3B). Two radioactive protein bands at 72 and 80 kDa were detected by fluorography. Radioactivity at 72 and 80 kDa pro-

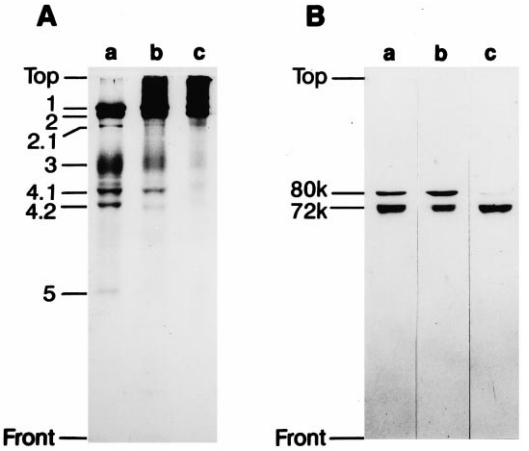

Fig. 3. SDS-PAGE (A) and subsequent fluorography (B) of [^{3}H]DFP-labeled intact erythrocyte membrane suspension (a), 0.1% NP-40-solubilized fraction of intact erythrocyte membranes (b), and 0.1% NP-40 residue of intact erythrocyte membranes (c). Protein bands in A were stained with Coomassie brilliant blue R-250.

tein bands was detected in the labeled membranes and their 0.1% NP-40-solubilized fraction, but radioactivity at the 80 kDa protein band did not appear in the 0.1% NP-40-residual fraction. The result indicates that 80 kDa protein was loosely bound to the membranes and readily solubilized by 0.1% NP-40. SDS-PAGE and subsequent fluorography of both [^{3}H]DFP-labeled unoxidized and oxidized erythrocyte membranes revealed both the radioactive protein bands at 72 and 80 kDa (data not shown).

It has been known that acetylcholine esterase, a serine enzyme, is present in human erythrocyte membranes, which is labeled by [^{3}H]DFP at a 72 kDa band on SDS-PAGE [22] and exists as multiform aggregates in 0.1% Triton X-100 [23]. Butyrylcholine iodide is known to block the active center of acetylcholine esterase and thus inhibit the labeling by [^{3}H]DFP [24]. [^{3}H]DFP-labeling of 72 kDa protein

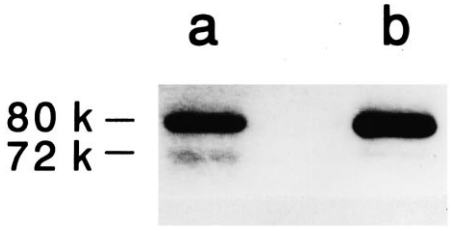

Fig. 4. SDS-PAGE and subsequent fluorography of [^{3}H]DFP-labeled intact erythrocyte membrane suspension in the absence (a) and the presence of butyrylcholine iodide (b). Loaded radioactivity: lane a, 700 cpm/lane, lane b, 3000 cpm/lane.

was completely inhibited by preincubation with butyrylcholine iodide, but the labeling of 80 kDa protein was not (Fig. 4). Hence, the 72 kDa protein was identified as acetylcholine esterase. The 80 kDa protein is most likely to be serine protease responsible for the degradation of oxidatively damaged proteins.

To isolate 80 kDa protein in membrane, [^{3}H]DFP-labeled intact erythrocyte membrane solution in 0.1% NP-40 mixed with unlabeled intact membrane solution was passed through a column of Sepharose CL-6B in the presence of 0.1% NP-40. Radioactivity and protein of each fraction were monitored (Fig. 5), and three radioactive peaks F I (molecular mass near to 4000 kDa), F II (molecular mass slightly above 67 kDa) and F III were obtained. High-molecular-weight F I and F II fractions were condensed by ultrafiltration and analyzed by SDS-PAGE and subsequent fluorography (Fig. 6). It was found that F I contained 72 kDa protein and F II contained 80 kDa protein. In the absence of SDS, 72 kDa protein must have been aggregated [23] and eluted in F I, a peak fraction with a higher molecular weight. By contrast, 80 kDa protein appears to exist as a monomer in 0.1% NP-40 and eluted in F II.

The presence of 80 kDa protein in cytosol of intact erythrocytes was investigated because it was readily solubilized from membranes into 0.1% NP-40. Intact erythrocyte cytosol was labeled with [^{3}H]DFP, and

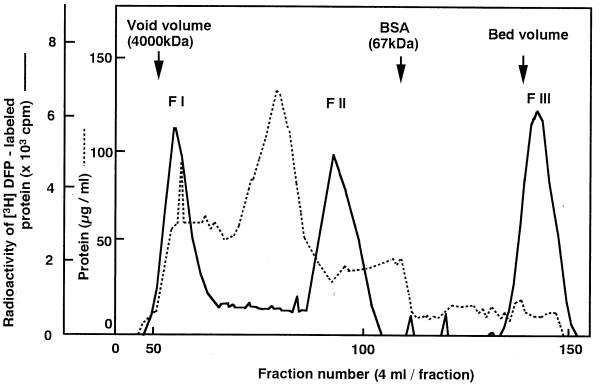

Fig. 5. Gel filtration of [^{3}H]DFP-labeled intact erythrocyte membranes through Sepharose CL-60. [^{3}H]DFP-labeled intact erythrocyte membranes solubilized in 0.1% NP-40 were mixed with unlabeled intact erythrocyte membranes solubilized in 0.1% NP-40. The mixture was applied to a column (2.5 cm i.d.×130 cm) of Sepharose CL-6B equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl, 1 mM EDTA and 0.1% NP-40. The column was eluted with the same buffer at 4°C at a flow rate of 0.5 ml/min.

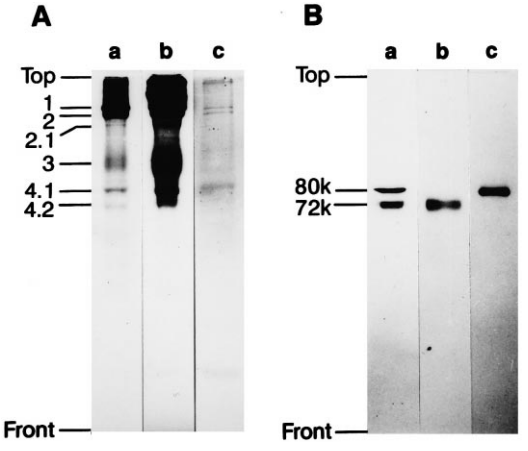

Fig. 6. SDS-PAGE (A) and subsequent fluorography (B) of gel fractions F I (b) and F II (c) from [^{3}H]DFP-labeled intact erythrocyte membranes (a) obtained in Fig. 5. Protein bands in A were stained with Coomassie brilliant blue R-250.

subjected to SDS-PAGE and subsequent fluorography. A single radioactive band at 80 kDa was observable (Fig. 7), suggesting that the same protein was present in intact erythrocyte cytosol. Proteolytic activities of cytosol toward ^{125}I-labeled unoxidized and oxidized BSA in the absence and presence of DFP were compared (Table 1, lower section). It was found that oxidized BSA was a better substrate and degradation was partially inhibited by DFP. Hence, the 80 kDa serine protease in oxidized erythrocyte membranes may be derived from erythrocyte cytosol.

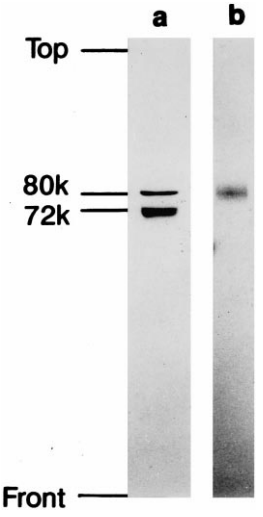

Fig. 7. Fluorography after SDS-PAGE of [^{3}H]DFP-labeled intact erythrocyte membranes (a) and cytosol (b).

4. Discussion

It has been shown in our previous study that erythrocyte membrane proteins become susceptible to degradation by membrane-bound serine protease activity after oxidative modification of the membranes [15]. In the present study, the presence of a serine protease responsible for preferential degradation of oxidatively damaged proteins was elucidated. It was found that the molecular mass of the serine protease is 80 kDa and the protease is loosely bound to the oxidized membranes. The protease in the oxidized membrane may be derived from erythrocyte cytosol.

Proteolytic activity of membranes from unoxidized and oxidized erythrocytes against unoxidized and oxidized spectrin, and against unoxidized and oxidized BSA was compared. Both proteolytic activity in the membranes and degradability of the substrates were increased, respectively, by membrane oxidation and substrate oxidation. Increase in the degradability of the substrates by oxidation may be due to the increased hydrophobic nature of the substrates, because it is known that hydrophobicity of proteins increases by oxidation [25–28]. Oxidation of proteins causes exposure of hydrophobic residues of the proteins to the surface of the molecules [27,28]. It is conceivable that the protease may preferentially hydrolyze the surface hydrophobic residues of the oxidatively modified protein molecules.

The proteolytic activity of the membranes toward oxidized proteins was inhibited by a serine protease inhibitor DFP, and two proteins in the membranes were radiolabeled with [^{3}H]DFP: 72 kDa (near to 4000 kDa on gel filtration) and 80 kDa (slightly larger than 67 kDa on gel filtration) proteins on analysis by SDS-PAGE under reducing conditions and subsequent fluorography. In erythrocyte membranes, the presence of several proteases including calpain [29], cathepsin E [30,31], acid proteinase [32] and multicatalytic proteinase [33] has been known. On the other hand, in erythrocyte cytosol proteases selective to oxidized hemoglobin or oxidized proteins are known [6,7]. Erythrocyte cytosolic proteases hitherto known show 670 kDa (on gel filtration) and 21.5–35.7 kDa protein (on SDS-PAGE under reducing conditions) or 700 kDa (on gel filtration) [6] and 23–32 kDa protein (on SDS-PAGE under reducing conditions) [7]. The 72 and 80 kDa proteins in the

present study were different from these proteases with respect to DFP susceptibility and molecular weight. Acetylcholine esterase, a serine enzyme, is known to be present in erythrocyte membrane [22–24]. It was found that the 72 kDa protein in the present study was acetylcholine esterase, because the molecular weight of the enzyme was similar to that of acetylcholine esterase and [^{3}H]DFP-labeling was inhibited by a specific inhibitor of acetylcholine esterase butyrylcholine iodide [24]. To our knowledge, the presence of the 80 kDa serine protease in erythrocyte membranes has not been hitherto known. This serine protease may preferentially degrade oxidatively modified membrane proteins.

The 80 kDa serine protease was loosely bound to erythrocyte membranes and could be released readily in a weak detergent NP-40. The proteolytic activity of membranes may have been increased by attachment of the enzyme present in cytosol to membranes when the cells were oxidized. The finding that the protein with the same molecular weight and enzyme activity was present in intact erythrocyte cytosol supports this hypothesis.

The 80 kDa serine protease may be present originally in erythrocyte cytosol, and when erythrocyte membranes become oxidized, the protease may adhere to the oxidized membranes to degrade oxidatively modified membrane proteins. In order to elucidate the function of the 80 kDa serine protease in erythrocyte cytosol and membranes, purification of the protease from erythrocyte cytosol is now under investigation.

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