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Enzymatic Removal of Oxidized Protein Aggregates from Erythrocyte Membranes[1]

Tomofumi Fujino, Ken Ando, Masatoshi Beppu, and Kiyomi Kikugawa[2]

School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392

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Erythrocytes oxidized or aged in the circulation undergo membrane protein aggregation and anti-band 3 autoantibody binding to the cell surface. When human erythrocytes were mildly oxidized *in vitro* with 0.1 mM Fe(III) at 37°C for 3 h, the aggregation of nonionic detergent $C_{12}E_8$-insoluble membrane protein and the binding of anti-band 3 IgG to the cell surface were increased. Incubation of membranes isolated from the oxidized cells increased the amount of protein aggregates by 5-fold after 6 h, while incubation for a further 12 h sharply decreased the amount of aggregates. In the presence of diisopropyl fluorophosphate (DFP), however, the increased amount of aggregates was maintained in the subsequent incubation. Western blot analysis of the aggregates using rabbit anti-band 3 showed that band 3 protein aggregates increased in the initial stage of incubation and decreased upon subsequent incubation, whereas the increased band 3 protein aggregates did not subsequently decrease when membranes were incubated in the presence of DFP. Incubation of the oxidized cells at 37°C for 18 h caused reduction of the membrane protein aggregates and the ^{125}I-anti-band 3 IgG binding to the cell surface, while incubation in the presence of DFP did not cause these reductions. The results suggest that the oxidation-induced cell membrane protein aggregates were probably removed by 80-kDa serine protease, namely, oxidized protein hydrolase (OPH), in the oxidized cell membranes [Fujino *et al.* (1998) *Biochim. Biophys. Acta* 1374, 47–54; (1998) *J. Biochem.* 124, 1077–1085; (2000) *Biochim. Biophys. Acta* 1478, 102–112], and as a result the increased anti-band 3 binding to the cell surface was reduced.

Key words: anti-band 3 binding, oxidized erythrocyte, oxidized protein hydrolase, protein aggregation.

Human erythrocytes undergo oxidative aging during circulation in the human body. Senescent erythrocytes undergo various oxidative modifications of cellular components including formation of oxidatively denatured hemoglobins (*1*, *2*), peroxidized lipids (*3*), fluorescent chromolipids (*4*, *5*), high-molecular-weight cross-linked membrane proteins (*6*, *7*), cross-linked complex of spectrin and hemoglobin (*8*), aggregates of band 3 protein (*1*, *9*), and advanced glycation end products (*10*). It has been shown that anti-band 3 autoantibody binds to *in vitro* oxidized erythrocytes (*11*) and also to *in vivo* senescent erythrocytes (*12*), and this event is caused by the aggregation of band 3 protein (*9*).

A multicatalytic proteolytic complex composed of multiple subunits has been shown to be responsible for the degradation of the oxidized intracellular proteins of erythrocytes (*13–15*). We have demonstrated the presence of an 80-kDa serine protease in the oxidized erythrocyte membranes that preferentially degrades oxidized proteins, namely, oxidized protein hydrolase (OPH) (*16–18*). On mild treatment of erythrocytes with ADP/Fe(III) at 37°C for 3 h, band 3 protein and spectrin are oxidized (*16*), and the amount of protein aggregates including band 3 protein aggregates is increased (*9*). Subsequent incubation of the isolated membranes results in extensive degradation of band 3 protein (*16*). These findings led us to identify OPH in the oxidized cell membranes, and it was found that this enzyme is identical with acylpeptide hydrolase (ACPH), whose biological function is still unknown (*19*). OPH is originally present in the cytosol and becomes adherent to the membranes when the cells are oxidized, and the membrane proteins become susceptible to degradation by the enzyme. The protease is characterized by its inhibition by a serine protease inhibitor, diisopropyl fluorophoshate (DFP).

In the present study, we examined whether the oxidation-induced erythrocyte membrane protein aggregates including band 3 protein aggregates were enzymatically removed, and whether the increased anti-band 3 binding to the cell surface was reduced. We found that the membrane protein aggregation and the anti-band 3 binding were reduced by the enzymatic activity in the membranes, suggesting that OPH played a role in removal of the oxidation-induced membrane protein aggregates and in preventing the anti-band 3 binding to the cell surface.

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[2] To whom correspondence should be addressed. Tel: +81-426-76-4503, Fax: +81-426-76-4508, E-mail: kikugawa@ps.toyaku.ac.jp

Abbreviations: BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; DPBS, Dulbecco's phosphate-buffered saline; DPBS(−), Ca^{2+}-, Mg^{2+}-free DPBS; OPH, oxidized protein hydrolase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SDS, sodium dodecylsulfate.

MATERIALS AND METHODS

Materials—Diisopropyl fluorophosphate (DFP) was obtained from Wako Pure Chemical Industries (Osaka). Bovine serum albumin (BSA) (γ-globlin–free and fatty acid–free), protein A–Sepharose CL-4B, Nonidet P-40 (NP-40), and *N*-acetylalanine *p*-nitroanilide (AANA) were obtained from Sigma Chemical (St. Louis, MO, USA). Protein A–horseradish peroxidase conjugate was obtained from Bio-Rad Laboratories (Richmond, CA). $Na^{125}I$ was obtained from ICN Pharmaceutical (Irvine, CA). Clear blot Membrane-P, a polyvinylidene difluoride (PVDF) microporous membrane for protein blotting, was obtained from Atto Co. (Tokyo). Non-ionic detergent, octaethylene glycol *n*-dodecyl monoether ($C_{12}E_8$), was obtained from Nikko Chemical Industry (Tokyo).

Analysis—Proteins were determined by the method of Lowry *et al.* (*20*) using BSA as a reference standard. Hemoglobin content was determined from its absorbance at 523 nm and its molecular extinction coefficient of 7,880 (*11*). Erythrocyte volume was calculated using the mean hemoglobin concentration at 339 g/liter erythrocyte (*21*). Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (*22*) using 25 mM Tris-HCl buffer (pH 8.3)/192 mM glycine with 0.1% SDS in a discontinuous buffer system with a 10% separating gel and a 4% stacking gel under reducing and nonreducing conditions at 10 mA for 2.5 h. Bromophenol blue was used as a top marker of the electrophoresis. ^{125}I-radioactivities were counted by use of an Aloka ARC 2000 autowell gamma-ray counter (Tokyo).

Preparation of Erythrocytes and Erythrocyte Membranes—Human blood withdrawn from a healthy donor using citrate-phosphate-dextrose as an anticoagulant was stored at 4°C for use within 4 days. Erythrocytes were collected from blood by centrifugation and washed using standard procedures as described previously (*9*). Erythrocyte membranes from unoxidized and oxidized erythroyctes were prepared by the method of Dodge *et al.* (*23*). For preparation of hemoglobin-free membranes, cells were lyzed, and membranes were centrifuged down and washed several times until the color of hemoglobins in the supernatant was completely removed.

Oxidized Erythrocytes—Oxidation of erythrocytes was performed as described previously (*16*). A 20% erythrocyte suspension in Dulbecco's phosphate-buffered saline (DPBS) was incubated with ADP (1.7 mM)/$FeCl_3$ (0.1 mM) at 37°C for 3 h. The reaction mixture was then centrifuged at 650 ×*g* for 5 min to remove the supernatant, and the cell pellet was washed three times with Ca^{2+}, Mg^{2+}-free DPBS (DPBS(−)), and resuspended in an appropriate buffer for use.

Determination of OPH Activity in Erythrocyte Membranes—Anti-OPH antibody (13 μg protein) prepared as described (*18*) was added to the erythrocyte membranes (150 μg protein), and the mixture was agitated at 4°C for 2 h. A 5-μl suspension of protein A–Sepharose CL-4B (100 mg/ml) in DPBS(−) containing 0.02% sodium azide was added to the membranes and the mixture was agitated at 4°C for 2 h. The protein A–Sepharose resin was washed three times with DPBS(−) containing 1% NP-40 by centrifugation (12,000 ×*g*, 3 min), and the immunoprecipitated OPH was recovered in a resin-bound form. OPH activity was measured as ACPH activity using *N*-acetylalanine *p*-nitroanilde (AANA) as a substrate (*19*). The resin-bound OPH was added to 1.0 ml of a solution of 1 mmol AANA in DPBS(−), and the mixture was incubated at 37°C for 20 min with shaking. Absorbance of *p*-nitroaniline at 405 nm was measured. The amount of *p*-nitroaniline released was determined from a calibration curve obtained with standard *p*-nitroaniline.

Incubation of Oxidized Erythrocytes and Their Membranes—The suspension of cell membranes obtained from oxidized or unoxidized erythrocytes in 1.0 ml of DPBS (3 mg protein) was incubated at 37°C for the indicated period. For investigation of the effect of DFP, 10 μl of 0.2 M DFP in ethanol was added to the suspension, and the suspension was incubated at 37°C after keeping at 4°C for 1 h. Membranes were collected in the usual manner.

Suspensions of oxidized or unoxidized erythrocytes (20%) in 5.0 ml of DPBS were incubated at 37°C for the indicated periods. For investigation of the effect of DFP, 50 μl of 0.2 M DFP in ethanol was added to the suspension, and the suspension was incubated at 37°C after keeping at 4°C for 1 h. The cells were washed and lyzed, and membranes were collected in the usual manner.

Isolation of Protein Aggregates in Erythrocyte Membranes—Non-ionic detergent $C_{12}E_8$-insoluble protein aggregates in erythrocyte membranes were isolated previously described (*1*, *9*). The whole membranes were treated with EDTA to prepare inside-out vesicles. To the vesicle suspension was added 5 volumes of 1% $C_{12}E_8$ solution, and the mixture was centrifuged at 70,000 ×*g* for 1 h. $C_{12}E_8$-insoluble membrane proteins were obtained as aggregates in the pellet. The aggregates were dissolved in 0.5 ml of a solution of 2% SDS, and the protein content was determined.

Detection of Band 3 Protein in Protein Aggregates by Western Blotting—The whole $C_{12}E_8$-insoluble aggregates isolated from the cell membranes were solubilized in 0.5 ml of 2% SDS and mixed with an equal volume of the electrophoresis buffer. A 10-μl aliquot of the solution was subjected to SDS-PAGE under reducing conditions. The gel was subjected to Western blotting according to the method of Towbin *et al.* (*24*). Band 3 protein in the aggregates transferred onto a PVDF membrane was detected as described previously (*16*). Briefly, after blocking with 1% BSA, the membrane was incubated with affinity-purified rabbit anti–human band 3 IgG at room temperature for 2 h, then with the Protein A-horseradish peroxidase conjugate. The peroxidase activity was detected with hydrogen peroxide and 4-chloro-1-naphthol.

Binding of ^{125}I-Labeled Anti–Band 3 IgG to Oxidized Erythrocytes—Human anti–band 3 IgG was isolated from normal adult plasma by anion exchange chromatography, and anti–band 3 IgG was purified from IgG fraction by affinity chromatography using a band 3–coupled Sepharose gel column by the method of Lutz *et al.* (*25*) as described previously (*26*). ^{125}I-labeling of anti–band 3 IgG was carried out by the chloramine T method (*27*).

^{125}I-anti–band 3 IgG binding assay was performed as described (*9*). A 100-μl solution of ^{125}I-anti–band 3 IgG (3.4 μg/ml, 230,000 cpm/μg) was added to 50 μl of 40% cell suspension in 1% BSA in DPBS, and the mixture was incubated at 4°C overnight. The cells were washed four times with DPBS(−) and resuspended in 100 μl of the same

buffer. The radioactivity of the suspension was counted. The amount of the cells was corrected by calculation of Hb content of the hemolysate of the sample cells as described (*9*). The data were expressed as ng of ^{125}I-anti–band 3 IgG bound per ml of the cells.

RESULTS

Human erythrocytes were oxidized with 1.7 mM ADP/0.1 mM Fe(III) at 37°C for 3 h under the same conditions described previously (*16*). As has been shown previously, levels of thiobarbituric acid–reactive substances were slightly increased by the treatment, whereas the cell deformability and osmotic fragility were unchanged. Hemoglobin was not converted into methemoglobin, and hemolysis was not observed.

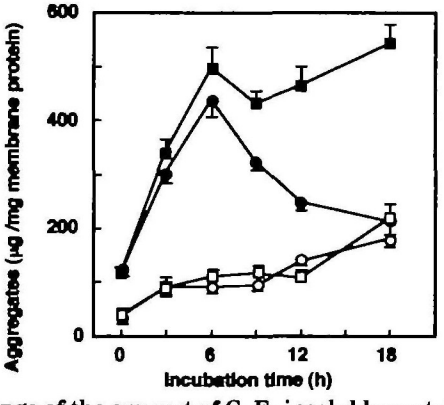

Fig. 1. **Change of the amount of $C_{12}E_8$-insoluble protein aggregates during the incubation of the membranes isolated from the oxidized erythrocytes.** Erythrocyte suspension (20%, 10 ml) was treated with 1.7 mM ADP/0.1 mM Fe(II) at pH 7.4 and 37°C for 3 h. Cell membranes (3 mg protein) from the oxidized and unoxidized cells were obtained. The cell membranes were incubated at 37°C in the absence and the presence of 2 mM DFP for the indicated period, and the amount of $C_{12}E_8$-insoluble membrane proteins was determined. The data are expressed as the mean ± SD of triplicate experiments. Membranes from oxidized cells in the absence (●) and presence of DFP (■); membranes from unoxidized cells in the absence (○) and presence of DFP (□).

Hemoglobin-free cell membranes were quickly isolated from the oxidized erythrocytes. Enzymatic activity of OPH in the oxidized erythrocyte membranes was determined as ACPH activity (*19*). The amount of *p*-nitroaniline released from *N*-acetylalanine *p*-nitroanilide (AANA) was 0.711 μmol/150 μg membrane protein. When the membranes were throughly washed by centrifugation 17 times by the usual procedure, the enzyme was still retained in the membranes and released 0.225 μmol *p*-nitroaniline/150 μg membrane protein. This result indicates that OPH was tightly bound to the oxidized erythrocyte membranes and was not readily released.

The cell membranes from the oxidized and the unoxidized cells were incubated at pH 7.4 and 37°C for up to 18 h. The cell membranes recovered were washed with non-ionic detergent $C_{12}E_8$, and the amount of insoluble aggregates of the membranes was determined according to the method described previously (*1*) (Fig. 1). In this treatment, aggregated membrane proteins were not solubilized, and non-aggregated proteins and fragmented proteins were solubilized and removed into the detergent solution. The amount of the aggregates was expressed relative to the amount of total protein in the membranes. Before the incubation, the amount of the aggregates in the membranes from the oxidized cells was about 3-fold larger than that of the aggregates in the membranes from the unoxidized cells, accounting for about 10% of the total protein in the membranes. Upon incubation, the amount of aggregates in the membranes from the unoxidized cells increased slightly in a time-dependent manner (Fig. 1, open circle), and the addition of DFP showed little effect (Fig. 1, open square). In the membranes from the oxidized cells, the amount of aggregates progressively increased during the first 6 h of incubation to reach a maximum of 0.5 mg/mg membrane protein (Fig. 1, closed circle). About 50% of the membrane proteins were aggregated under these conditions. In the subsequent 12 h of incubation, the amount of aggregates decreased sharply, reaching the level before the incubation (Fig. 1, closed circle). In the presence of DFP, however, the amount of aggregates in the membranes from the oxidized cells increased during the first 6 h of incubation but showed

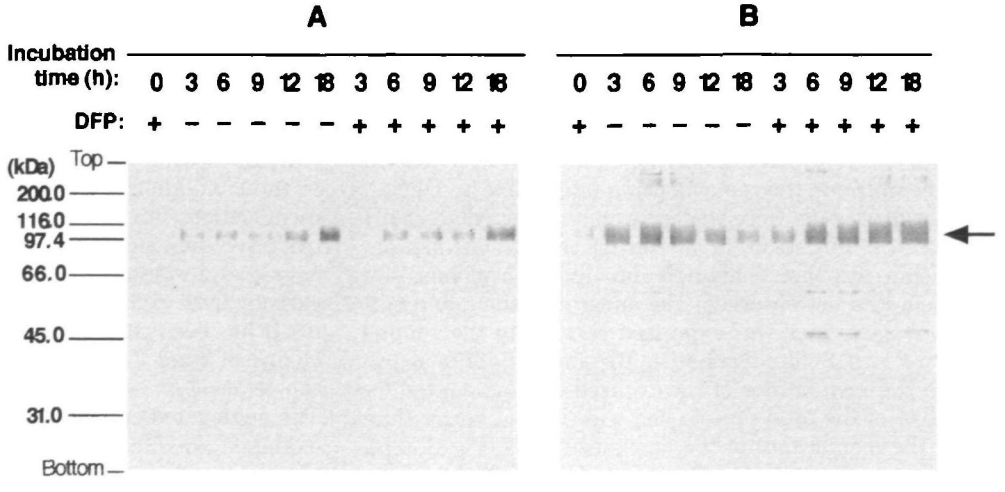

Fig. 2. **Change of the amount of band 3 protein in $C_{12}E_8$-insoluble protein aggregates during the incubation of the membranes isolated from the oxidized erythrocytes.** The whole $C_{12}E_8$-insoluble aggregates isolated from the cell membranes (Fig. 1) were subjected to SDS-PAGE under reduced conditions. The gels were subjected to Western blotting using rabbit anti–band 3 IgG. A: Aggregates in the membranes from the unoxidized cells incubated in the absence and the presence of DFP. B: Aggregates in the membranes from the oxidized cells incubated in the the absence and the presence of DFP. Arrows indicate the position of band 3 (monomer).

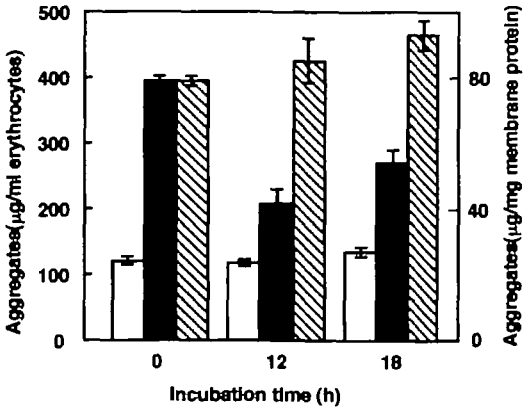

Fig. 3. **Change of the amount of $C_{12}E_8$-insoluble protein aggregates during the incubation of the oxidized erythrocytes.** Erythrocytes were oxidized as described. The oxidized and the unoxidized cells were incubated at pH 7.4 and 37°C in the absence or presence of 2 mM DFP for the indicated periods. Cell membranes were obtained, and the amount of $C_{12}E_8$-insoluble membrane proteins was determined. The data are expressed as the mean ± SD of triplicate experiments. The amount of the cells was corrected by calculation of hemoglobin content of the hemolysate of the sample cells. One milli liter of erythrocytes contain 5 mg of membrane protein, as determined by Lowry's method (*20*). Unoxidized cells (open column), oxidized cells in the absence of DFP (closed column), and oxidized cells in the presence of DFP (hatched column).

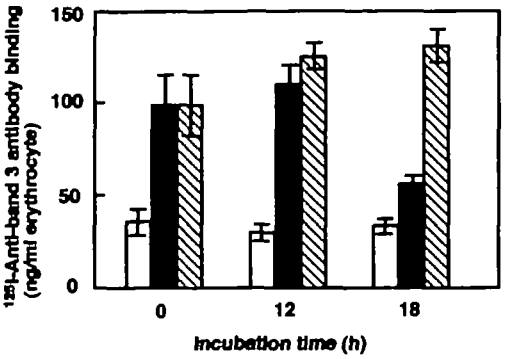

Fig. 4. **Changes of the binding of anti-band 3 IgG to erythrocytes during the incubation of the oxidized erythrocytes.** Erythrocytes were oxidized as described. The oxidized and the unoxidized cells were incubated at pH 7.4 and 37°C in the absence or presence of 2 mM DFP for the indicated periods. Cells were incubated with ^{125}I-labeled anti-band 3 IgG at 4°C overnight. The cell-bound radioactivity was counted. The data are expressed as the mean ± SD of ng of ^{125}I-anti-band 3 IgG bound per ml of the cells in triplicate determinations. The amount of the cells was corrected by calculation of hemoglobin content of the hemolysate of the sample cells. Unoxidized cells (open column), oxidized cells in the absence of DFP (closed column) and the oxidized cells in the presence of DFP (hatched column).

no decrease in the subsequent incubation (Fig. 1, closed square). Hence, the oxidation-induced protein aggregation was enhanced in the early stage of the incubation of the isolated membranes, and the increased aggregates were sharply decreased on subsequent incubation of the membranes, the decrease being inhibitable by a serine protease inhibitor, DFP.

The whole aggregates in the membranes obtained from the unoxidized and the oxidized cells were subjected to SDS-PAGE and subsequent Western blotting using rabbit anti–band 3. The amount of band 3 protein in the aggregates of the unoxidized cells gradually increased in both the absence and the presence of DFP (Fig. 2A). In the aggregates from the oxidized cells, the amounts of band 3 protein increased during the first 6 h of incubation in the absence and presence of DFP; but on further incubation, it decreased sharply to the level seen before the incubation in the absence of DFP, while showing no decrease in the presence of DFP (Fig. 2B). Hence, the oxidation-induced band 3 protein aggregation was enhanced in the early stage of incubation of the isolated membranes, and the increased aggregates were rapidly removed on subsequent incubation of the membranes, the removal being inhibitable by DFP.

The oxidized cells were washed well and incubated at pH 7.4 and 37°C for up to 18 h. During the cell incubation, hemoglobin was not converted into methemoglobin, and hemolysis was not observed. The amount of the detergent-insoluble aggregates was expressed relative to the volume of erythrocytes. Before incubation, the amount of the aggregates in the membranes of the oxidized cells accounted for about 10% of the total protein and was 3.5-fold larger than that in the membranes of the unoxidized cells (Fig. 3, 0 h). The amount of aggregates in the membranes of the unoxidized cells was little altered during the incubation (Fig. 3, open column), whereas the amount of the aggregates in the membranes of the oxidized cells was decreased after 12 or 18 h of incubation (Fig. 3, closed column). By contrast, the amount of aggregates in the membranes of the oxidized cells did not decrease during the incubation in the presence of DFP (Fig. 3, hatched column).

The binding of human anti–band 3 IgG autoantibody labeled with ^{125}I to the oxidized and the unoxidized cells was examined after incubation of cells for 18 h. Before the cell incubation, the binding to the oxidized cells was 3-fold higher than that to the unoxidized cells (Fig. 4, 0 h). The binding to the unoxidized cells was unchanged by the incubation (Fig. 4, open column). The increased binding to the oxidized cells was decreased to half after incubation for 18 h (Fig. 4, closed column). By contrast, the increased binding to the oxidized cells showed no decrease upon incubation in the presence of DFP (Fig. 4, hatched column).

These results indicate that the membrane protein aggregation induced by the oxidation was effectively reversed and the increased binding of anti–band 3 decreased by the incubation of cells, both being inhibitable by DFP.

DISCUSSION

Human erythrocyte membranes exposed to oxidative stress in the circulation are known to undergo various oxidative modifications during aging in the circulation (*1–10*). Upon oxidative damage, glycoproteins including band 3 protein aggregate to induce assembly of poly-*N*-acetyllactosaminyl carbohydrate chains of band 3 protein on the cell surface (*9*). It has been shown that aggregation of the carbohydrate chains of band 3 protein is an important event preceding the removal of senescent erythrocytes from the circulation. The aggregated carbohydrate chains generated by aging or oxidation are effectively recognized by anti–band 3 autoantibody (*12, 28*) as well as by macrophages (*29*). Aggregation of band 3 protein may be an important event for removal of effete or senescent erythrocytes from the circulation.

During our investigation on the proteolytic activity of *in vitro* oxidized erythrocyte membranes, we found an 80-kDa serine protease, namely oxidized protein hydrolase (OPH), that preferentially degrades oxidized proteins (*16–18*). OPH is originally present in the cytosol, but when the cells are oxidized, it adheres to the membranes and can degrade the membrane proteins (*18*). Isolated OPH was found to degrade oxidized or glycated proteins effectively (*18*). More recently, the primary structure of the isolated protease was found to be identical with that of acylpeptide hydrolase (ACPH), and OPH showed both endopeptidase activity for oxidized proteins and exopeptidase activity for acylated short chain peptides (*19*). The protease is characterized by inhibition by a serine protease inhibitor, DFP (*16–19*). Major enzymes in erythrocyte membranes that are inhibited and labeled by DFP are OPH and acetylcholine esterase (*17*).

The present study showed that the membrane proteins including band 3 protein in the isolated membranes from the oxidized cells readily aggregated upon incubation, and the amount of aggregated proteins increased from 10 to 50% of the total membrane protein. Nevertheless, the highly aggregated proteins were almost completely removed upon further incubation by the enzymatic activity due to a serine protease. The oxidation-induced aggregates of the cell membrane proteins (10% of the total membrane protein) were removed effectively by incubation of cells. This removal may be due to the endogenous OPH, because OPH was detected in the membranes as ACPH activity and the removal of aggregates was effectively prevented by DFP. As a consequence, the anti–band 3 binding to the cell surface augmented by the oxidation was reduced.

It has been suggested that common oxidized proteins are susceptible to proteolytic degradation by proteases. The hydrophobicity of proteins is increased by oxidation, and the increase in degradability of oxidized proteins may be due to the increased hydrophobicity (*30–33*). Aggregated membrane proteins in the oxidized erythrocytes may expose their hydrophobic residues, and endogenous OPH in the membranes would preferentially hydrolyze the surface hydrophobic residues of the aggregated proteins.

The present results suggest that OPH endogenously present in oxidized or aged erythrocyte membranes plays a role in removal of the oxidation-induced membrane protein aggregates and in reducing the oxidation-induced anti–band 3 binding enhanced during the aging process in the circulation. OPH can function in the cell membranes as a secondary defense system in removal of an unnecessarily large amount of protein aggregates produced by oxidation. Removal of a large amount of protein aggregates from the membranes may have some beneficial effects on cells in the circulation. Removal of the protein aggregates from the membranes results in the reduction of the anti–band 3 autoantibody binding and the macrophage recognition. There are several lines of evidence showing that the amount of the membrane protein aggregates and the level of the bound anti–band 3 autoantibody in senescent erythrocytes are higher than those in young erythrocytes (*12, 28*), and thus protein aggregates may not all be removed before the cell removal from the circulation. OPH may function as a regulator of the amount of the protein aggregates in the membranes of erythrocytes in the circulation, and thus may control the anti–band 3-dependent removal of erythrocytes from the circulation.

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