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Identification of the Cleavage Sites of Oxidized Protein That Are Susceptible to Oxidized Protein Hydrolase (OPH) in the Primary and Tertiary Structures of the Protein[1]

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Amino acid sequences in H_2O_2-oxidized bovine serum albumin (BSA) that are susceptible to proteolytic cleavage by oxidized protein hydrolase (OPH) were investigated. When oxidized BSA was treated with OPH, low-molecular-weight fragments (54, 46, 24, 22, 20, and 8 kDa) were produced as analyzed by SDS-PAGE. N-Terminal amino acid sequence analysis of these fragments indicated that oxidized BSA was cleaved by OPH at three major sites, Leu218-Ser219, Tyr410-Thr411, and Phe506-Thr507, at an early stage of the proteolytic degradation. In the three-dimensional structure of BSA deduced by computer modeling, these cleavage sites were found to be located slightly inside the BSA molecule, in positions not easily accessible by OPH. The influence of oxidation on the tertiary structure of BSA was then investigated by hypothetically replacing all the four methionine and two tryptophan residues with their oxidized forms, methionine sulfoxide and *N'*-formyl-kynurenine, respectively. The three-dimensional structure of the hypothetically oxidized BSA indicated that all the three cleavage sites in the protein could become more exposed to the solvent than in unoxidized BSA. These results suggest that, upon oxidation of BSA, the amino acid sequences that are potentially cleavable by OPH but present inside the molecule become exposed on the surface and susceptible to proteolysis by OPH. This is the first report demonstrating the cleavage sites of oxidized protein by oxidized protein-selective protease, suggesting the possible mechanism of oxidized protein-selective degradation by the enzyme.

Key words: computer modeling, oxidative stress, oxidized protein, oxidized protein hydrolase, protein degradation, three-dimensional structure of protein.

Primary antioxidant defence systems including antioxidants and antioxidative enzymes, which prevent generation of free radicals or break radical chain reactions, are known to protect living cells from oxidative damage (*1–4*). Secondary antioxidant defence systems including proteases that preferentially degrade oxidatively damaged proteins are also known to exist within cells (*5*). So far, preferential degradation of oxidatively damaged proteins by certain proteases has been demonstrated in reticulocytes, erythrocytes, and *Escherichia coli* (*6–18*). It is, however, not known why oxidatively damaged proteins are susceptible to proteolysis by these proteases. We previously demonstrated the presence of an 80-kDa serine protease, oxidized protein hydrolase (OPH), in human erythrocytes, which preferentially degrades oxidatively damaged membrane proteins and is inhibited by diisopropylfluorophosphate (DFP) (*17, 18*). The protease is originally present in cytosol and becomes adherent to the cell membranes when the cells are oxidized (*19*). More recently, this enzyme has been identified as acylpeptide hydrolase (ACPH) (*20*), and was found to be also present in human cell lines, rat tissues and human/rat plasma (*21*).

In the present study, we determined the amino acid sequences in oxidized protein that are susceptible to proteolysis by OPH, using oxidized bovine serum albumin (BSA) as a substrate. Three-dimensional structural study of unoxidized and oxidized BSA by computer modeling indicated that the cleavable sequences are located near, but not on, the surface of the protein molecule, and that these sites become exposed to the surface upon oxidation of the protein. The results suggested that oxidation of the protein results in exposure of potentially cleavable sites inside the molecule to the surface by changes in the tertiary structure, and that this renders the protein susceptible to proteolysis by OPH.

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Abbreviations: ACPH, acylpeptide hydrolase; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue R-250; CD, circular dichroism; GST, glutathione S-transferase; HRP, horseradish peroxidase; HSA, human serum albumin; OPH, oxidized protein hydrolase; PVDF, polyvinylidene difluoride.

MATERIALS AND METHODS

Materials—Bovine serum albumin (BSA) (γ-globulin–free and fatty acid–free) was obtained from Sigma Chemical (St. Louis, MO, USA). Coomassie brilliant blue (CBB) was purchased from Nacalai Tesque (Kyoto).

Analysis—Protein concentrations were determined by the method of Lowry *et al.* (*22*) using BSA as a reference standard. Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (*23*) 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 conditions, or the method of Shagger and Jagow (*24*) using 200 mM Tris-HCl buffer (pH 8.9) as an anode buffer and 100 mM Tris-HCl buffer (pH 8.25)/ 100 mM tricine with 0.1% SDS as a cathode buffer in a discontinuous buffer system (tris-tricine SDS-PAGE) with a 16.5% separating gel, a 10% spacer gel, and a 4% stacking gel under reducing conditions. The extent of oxidation of tryptophan residues in BSA was assessed by measuring decrease in fluorescence intensity of tryptophan residues (*25*) (Ex. 280 nm, Em. 345 nm), using a Hitachi 650-60 fluorescence spectrometer (Tokyo).

Preparation of Oxidized BSA—Oxidized BSA was prepared by treating BSA with various concentrations of H_2O_2 (0.2–200 mM) in the presence of horseradish peroxidase (HRP) for 20 h according to the method previously described (*18*). Unoxidized BSA was prepared under the same conditions without H_2O_2.

Preparation of Recombinant ACPH (rACPH) with GST Tag as OPH—Glutathione S-transferase (GST)–tagged recombinant ACPH (rACPH) bound to glutathione-Sepharose 4B resin was prepared from the cDNA of human erythroleukemic cell line K562 according to the method previously described (*20*). This Sepharose-bound rACPH was used as OPH in the present study.

Time-Course Studies on the Degradation of Oxidized BSA by OPH—A solution of unoxidized BSA (10 μg) or oxidized BSA (oxidized with 20 mM H_2O_2/HRP) (10 μg) was incubated with OPH (4 μg protein) in 60 μl of 10 mM sodium phosphate buffer (pH 8.0) at 37°C for various periods up to 96 h. The reaction mixture was centrifuged at 4°C and 8,000 ×*g* for 1 min to obtain the supernatant; then 10 μl of the supernatant (1.7 μg protein) was applied to Tris-tricine SDS-PAGE, and the protein bands were stained with CBB.

Preparation of Oxidized BSA Fragments for Amino Acid Sequencing—Preparation of large fragments (54, 46, 24, and 22 kDa) of oxidized BSA: A solution of BSA (20 μg) oxidized with 20 mM H_2O_2/HRP was incubated with OPH (8 μg protein) in 120 μl of 10 mM sodium phosphate buffer (pH 8.0) at 37°C for 48 h. The reaction mixture was centrifuged at 4°C and 8,000 ×*g* for 1 min to obtain the supernatant, then 40 μl of the supernatant (6.8 μg protein) was subjected to SDS-PAGE using Laemmli's buffer system. The separated protein was transferred to a polyvinylidene difluoride (PVDF) microporous membrane by electrophoresis at 200 mA for 2 h, then stained with CBB. The stained bands (3 mm × 5 mm) at 54, 46, 24, and 22 kDa were excised and destained with a solution of acetic acid–methanol–water (1:2:7 by volume).

Preparation of small fragments (20, 14, and 8 kDa) of oxidized BSA: A solution of BSA (20 μg) oxidized with 20 mM H_2O_2/HRP was incubated with OPH (8 μg protein), and the fragments were isolated by the procedure described above except that incubation was prolonged up to 72 h and that SDS-PAGE was performed using the Tris-tricine buffer system.

Amino Acid Sequencing of Oxidized BSA Fragments—For N-terminal amino acid sequencing of each of the oxidized BSA fragments isolated on the PVDF membrane, the membrane was directly introduced into the protein sequencing systems (a 490 PROCISE protein sequencing system (Perkin Elmer, Nowalk, CT, USA) for 54, 46, 24, and 22 kDa fragments, and a HPG1005A Protein Sequencing System (Hewlet Packard, Palo Alto, CA, USA) for 20, 14, and 8 kDa fragments).

CD Spectra—CD spectra of unoxidized and oxidized BSA were measured with a Jasco J-720 spectropolarimeter. Cuvettes with 1 mm path-length were used in the far-UV region (200–250 nm). Temperature was kept at 37°C, and the sample concentration was 0.1 mg/ml in 10 mM sodium phosphate buffer (pH 8.0) containing 0.14 M NaCl (PBS).

Computer Modeling of the Three-Dimensional Structure of BSA—The three-dimensional structure of the BSA molecule was built using a homologous protein structure as a template. In selecting the template structure, we first performed sequence alignment in order to search for the most homologous protein whose three-dimensional structure had already been determined experimentally. The alignment was carried out with the BLAST program (*26, 27*) for the Protein Data Bank database, which showed that the primary structure of human serum albumin (HSA) was most homologous with that of BSA (score, 919; identities, 74%; and positives, 85%). Therefore, the corresponding atomic coordinates of HSA (1A06) were used as a template structure in the subsequent modeling procedure. From the sequence alignment, a total of 136 out of 583 residues of BSA were found to be different from HSA. We deleted Val116 in 1A06, and replaced several other amino acid residues with appropriate ones. After adding hydrogen atoms, we calculated the molecular dynamics near the mutated residues in order to relax the local energetic hindrances. Among the resultant transient structures, the most energetically stable one was adopted, and the subsequent energy minimization calculation was carried out. Then, the whole molecule was energy-minimized to obtain the final structure. All the calculations were performed with the SYBYL molecular modeling system (Tripos, USA) using the cutoff distance of 10 angstrom, dielectric constant of 4r, and otherwise default parameter-settings. Molecular graphics images were produced using the MidasPlus program (UCSF Computer Graphics Lab.) (*28, 29*).

RESULTS

Characteristics of Oxidized BSA Preparations—BSA was oxidized with various concentrations of H_2O_2 in the presence of HRP according to the method previously described (*18*). These oxidized BSA preparations were subjected to Tris-tricine SDS-PAGE (Fig. 1). There was no loss of 66-kDa protein band of BSA in the preparations oxidized with H_2O_2 at 20 mM or less. On the other hand, there was a little loss of the 66-kDa band of BSA in the preparation oxi-

dized with 100 mM H_2O_2, with production of a small quantity of higher-molecular-weight protein, and there was a remarkable loss of this band in the preparation oxidized with 200 mM H_2O_2 with production of a large quantity of higher-molecular-weight protein. These observations suggested that a structural alteration of the BSA molecule including intermolecular covalent cross-linking took place at H_2O_2 concentrations over 20 mM. We then determined the extent of oxidation of tryptophan residues by measuring fluorescence of tryptophan at excitation 280 nm/emission 345 nm. There was a significant loss of tryptophan fluorescence in the oxidized BSA preparations (Fig. 2). In the BSA preparation oxidized with 20 mM H_2O_2, the fluorescence of tryptophan residues was decreased by 70%. The results suggest that some, if not extensive, structural changes took place in BSA oxidized with 20 mM H_2O_2. Since this oxidized BSA preparation appeared to be moderately oxidized and adequate for examination of proteolytic degradation by OPH, we used this preparation in the following experiments.

Susceptibility of Oxidized BSA to Degradation by OPH—Degradation of oxidized BSA by OPH was analyzed using Tris-tricine SDS-PAGE. Unoxidized and oxidized BSA (20 mM H_2O_2) were incubated with OPH at pH 8.0 and 37°C for up to 96 h, and the supernatant of each reaction mixture was subjected to Tris-tricine SDS-PAGE. The 66-kDa protein band of unoxidized BSA was not decreased during the incubation with OPH (Fig. 3A), while that of oxidized BSA was gradually decreased, and lower-molecular-weight fragments of 54, 46, 24, 22, 20, 14, and 8 kDa appeared (Fig. 3B). The results clearly demonstrate that oxidized BSA is degraded by OPH and produces discrete lower-

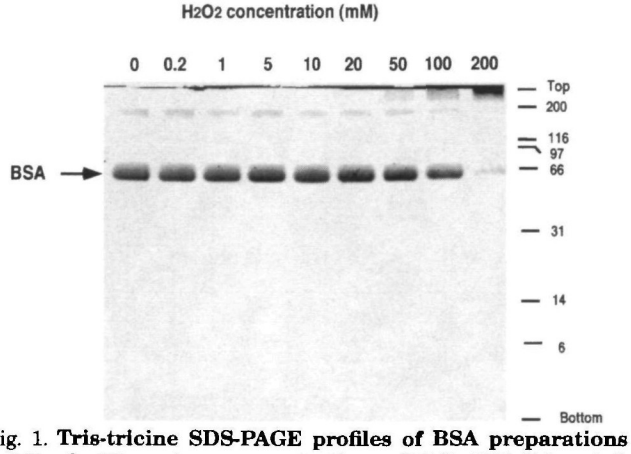

Fig. 1. **Tris-tricine SDS-PAGE profiles of BSA preparations oxidized with various concentrations of H_2O_2.** BSA (0.5 mg/ml) was oxidized with H_2O_2 (0.2, 1, 5, 10, 20, 50, 100, and 200 mM)/horseradish peroxidase (HRP) for 20 h, as described previously (*19*). A part of each oxidized BSA preparation (2 μg protein each) was subjected to Tris-tricine SDS-PAGE, and the proteins were stained by CBB. The numerals at the right side of the figure indicate the molecular masses (kDa) of standard proteins.

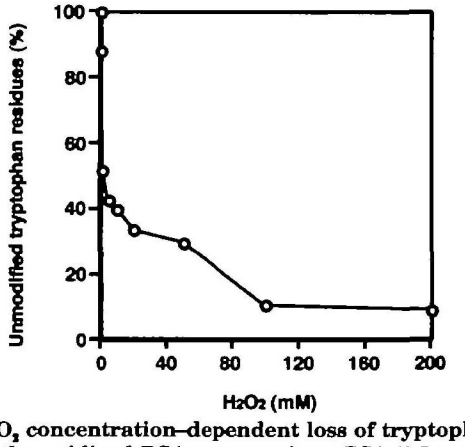

Fig. 2. **H_2O_2 concentration–dependent loss of tryptophan residues in the oxidized BSA preparations.** BSA (0.5 mg/ml) was oxidized with H_2O_2 (0.2, 1, 5, 10, 20, 50, 100, and 200 mM)/HRP for 20 h, and the extent of oxidation of tryptophan residues of BSA was assessed by measuring decrease in fluorescence intensity of tryptophan residues (Ex. 280 nm, Em. 345 nm). The percentage of unmodified tryptophan residues in the oxidized BSA preparations relative to the unoxidized BSA preparation is represented.

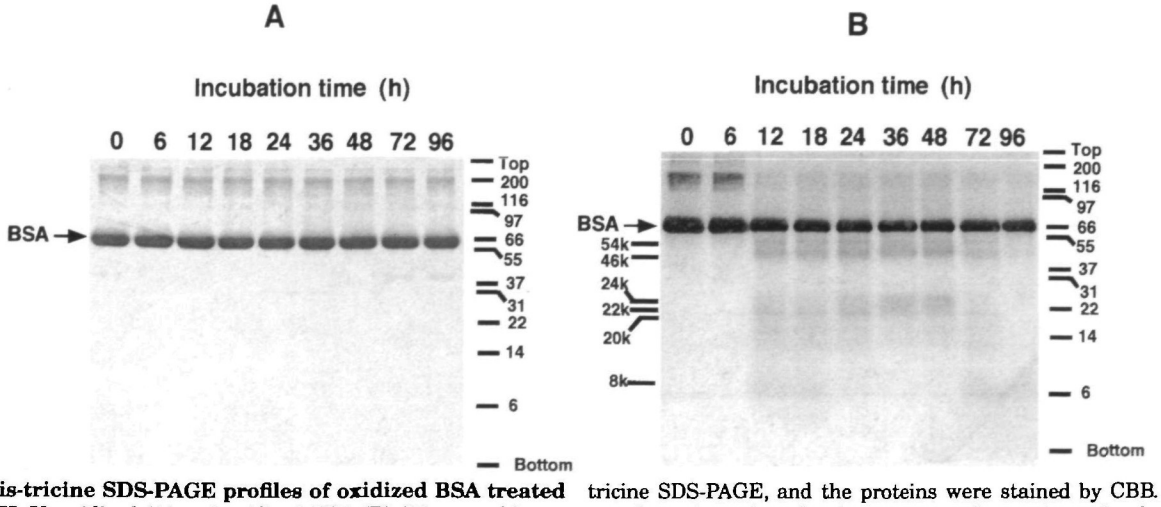

Fig. 3. **Tris-tricine SDS-PAGE profiles of oxidized BSA treated with OPH.** Unoxidized (A) and oxidized BSA (B) (10 μg each) was treated with OPH (4 μg protein) at pH 8.0 and 37°C for the indicated period. The supernatant of the reaction mixture was subjected to Tris-tricine SDS-PAGE, and the proteins were stained by CBB. The numerals at the right side of the figure indicate the molecular masses (kDa) of standard proteins.

molecular-weight fragments of 54, 46, 24, 22, 20, 14, and 8 kDa. It should be noted that the preparation oxidized with 200 mM H_2O_2 was not degraded at all by OPH (data not shown), which indicates that excessively oxidized BSA is not susceptible to the proteolytic degradation.

Sites of Cleavage in Oxidized BSA Fragments—To examine the position of the OPH cleavage sites in the primary structure of BSA, N-terminal sequencing of oxidized BSA fragments produced by OPH was performed. Oxidized BSA (20 mM H_2O_2) was incubated with OPH at pH 8.0 and 37°C for 48 h. After SDS-PAGE of the supernatant of the reaction mixture, the protein was transferred to PVDF membrane, and the protein on the membrane was stained with CBB. The stained protein bands at 54, 46, 24, and 22 kDa were excised and destained. The membranes were subjected to N-terminal sequencing, and the N-terminal partial sequences of these bands were determined (Table I). Oxidized BSA was similarly digested with OPH for a longer period (72 h) in order to obtain larger amounts of smaller peptides (20, 14, and 8 kDa bands). After Tris-tricine SDS-PAGE of the supernatant of the reaction mixture, the protein was transferred to PVDF membrane, and the protein on the membrane was stained with CBB. The stained bands at 20, 14, and 8 kDa were excised and destained. The membranes were subjected to N-terminal sequencing, and the N-terminal partial sequences of 20 and 8 kDa bands were determined (Table I). Then, the location of these N-terminal sequences in the known primary sequence of BSA (*30*) was assigned (Table I). The results indicated that oxidized BSA was cleaved at three sites, Leu218-Ser219, Tyr410-Thr411, and Phe506-Thr507, in its primary structure at an early stage of degradation.

Location of the Cleavage Sites in the Three-Dimensional Structure of Oxidized BSA—To determine why these cleavage sites are attacked by OPH only when BSA is oxidized, we hypothesized that the cleavage sites are not exposed on the surface of the unoxidized BSA molecule, but become exposed when the molecule is oxidized. We first tried to locate these sites in the three-dimensional structure of BSA. Figure 4 shows the model structure of BSA con-

TABLE I. **N-Terminal amino acid sequences of proteolytic fragments of oxidized BSA.** The oxidized BSA (oxidized with 20 mM H_2O_2) fragments obtained by OPH treatment were separated by SDS-PAGE or Tris-tricine SDS-PAGE and transferred to PVDF membrane. Each fragment on the membrane was excised and introduced into a protein sequencing system. The residues represented by "X" are residues that could not be determined. The residue X* was deduced to be Thr based on the known amino acid sequence of BSA.

Fragments	N-Terminal amino acid sequence	Corresponding amino acid residues in BSA
54 kDa	DTXKXEIA	1–8
46 kDa	SQKFPKAEFV	219–228
24 kDa	DTXKSEIAXXF	1–11
22 kDa	SQKFPKAEFVE	219–229
20 kDa	X*RKVPQ	411–416
8 kDa	TFHADI	507–512

Fig. 4. **Ribbon representation of the BSA molecule constructed by computer modeling.** Leu218 and Ser219 are colored in yellow, Tyr410 and Thr411 in red, and Phe506 and Thr507 in cyan. Other residues are represented only with main chain atoms according to their secondary structures.

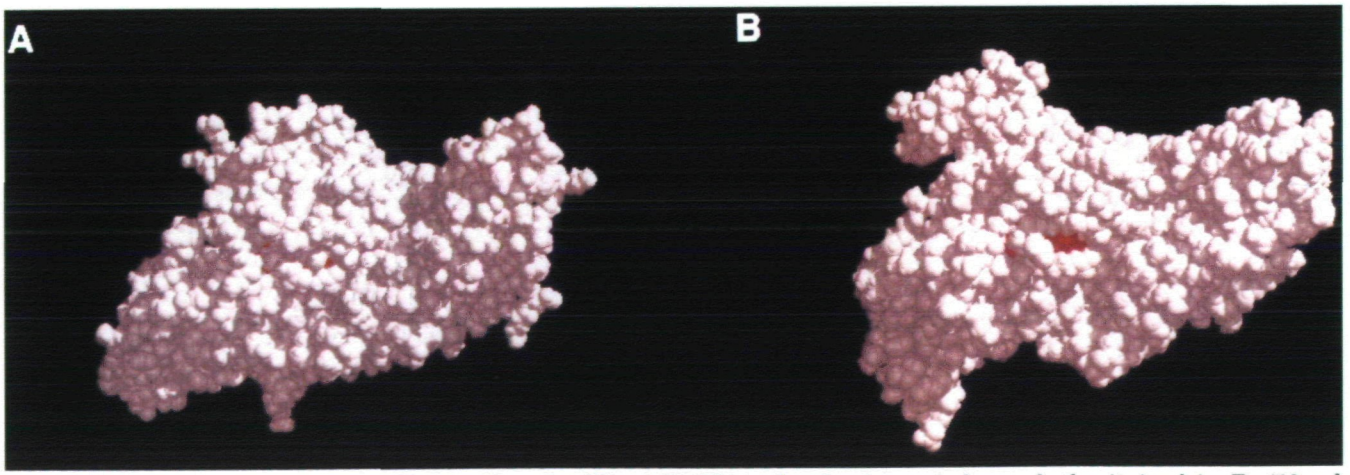

Fig. 5. **Space-filling representations of intact (A) and oxidized (B) BSA molecules.** As a typical example, the site involving Tyr410 and Thr411 is shown; these residues are colored in red. The other two cleavage sites are on the back of the molecule and cannot be seen in this figure.

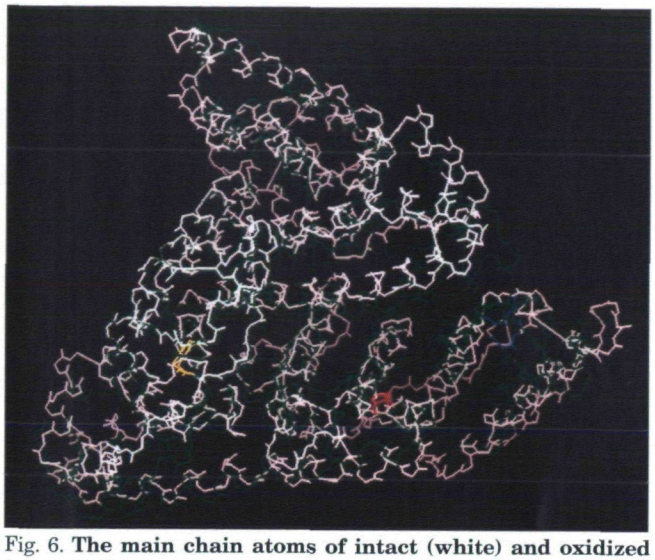

Fig. 6. **The main chain atoms of intact (white) and oxidized (green) BSA molecules.** Two molecules are superimposed to minimize the rms differences from the main chain atoms. In each molecule, Leu218 and Ser219 are colored in yellow, Tyr410 and Thr411 in red, and Phe506 and Thr507 in cyan.

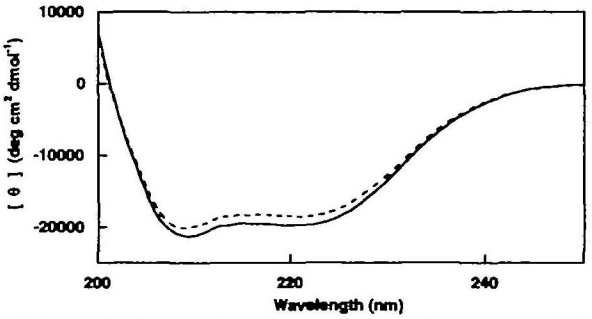

Fig. 7. **Far-UV CD spectra of intact (solid line) and oxidized (dashed line) BSA structures.** Secondary structure contents in intact BSA were: 82% of helix, 18% of β-Sheet, and 0% of other structures, as estimated by the CONTIN program (*41*).

structed by computer modeling using the three-dimensional structure of human serum albumin (HSA) as a template. Like the template, the constructed structure was rich in α-helices, and had no β-sheet structures. Of the three sites cleaved by the protease, Leu218-Ser219 and Tyr410-Thr411 were located in the marginal region of α-helices, while Phe506-Thr507 was not involved in regular secondary structures. All three sites appeared to be inaccessible from outside the molecule, although Phe506-Thr507 was relatively close to the surface. To investigate the influence of oxidation on the molecular structure of BSA, we hypothetically replaced all the methionine residues with sulfoxide (*31*) and all the tryptophan residues with *N'*-formylkynurenine (*32*), then optimized the structure energetically. Comparison of the structures of intact and oxidized BSA indicated that the three cleaved sites became more exposed to the solvent upon oxidation, although the overall structure remained mostly unchanged (Figs. 5 and 6). Far-UV CD spectra of the two molecules showed that both had mary helices and that the secondary structure of oxidized BSA were almost the same as that of intact one (Fig. 7), which was consistent with the above results.

These results support our hypothesis that, upon oxidation of BSA, the cleavable sites are exposed on the surface of the protein, thus becoming susceptible to proteolysis by OPH.

DISCUSSION

In the present study, we attempted to locate the cleavage sites in oxidized BSA that are susceptible to proteolysis by OPH, using a BSA preparation moderately oxidized by 20 mM H_2O_2 as a substrate. The preparation severely oxidized by 200 mM H_2O_2 including intermolecular cross-linking was not degraded by OPH at all, while the preparation oxidized by 20 mM H_2O_2 was degraded by OPH. Therefore, the latter preparation was used exclusively as a substrate for OPH. Lower-molecular-weight fragments of oxidized BSA (54, 46, 24, 22, 20, and 8 kDa) produced by the action of OPH were successfully isolated, and their location in the primary sequence of BSA was identified. The results of N-terminal sequence analysis of these fragments revealed that oxidized BSA was cleaved by OPH at least three sites, Leu218-Ser219, Tyr410-Thr411, and Phe506-Thr507, at an early stage of degradation. This is the first identification of the sites of cleavage in oxidized protein by oxidized protein-selective protease. It is interesting to note that OPH clearly functioned as an endopeptidase toward oxidized BSA, although the enzyme is identical with ACPH (*20*), which is known to be an exopeptidase (*33*, *34*). The cleavage specificity of OPH appears to be similar to that of chymotrypsin, a typical serine endopeptidase, which generally cleaves its substrate protein at the C-terminal side peptide bonds of tyrosine, phenylalanine, and tryptophan residues, and occasionally at those of leucine and some other residues (*35*). Thus, OPH may be classified as a chymotrypsin-type enzyme in terms of its cleavage specificity, although it is known to be structurally a homolog of prolyl oligopeptidase (*36*).

We investigated the location of the cleavage sites in the tertiary structure of BSA. The three-dimensional structure of BSA was obtained by replacing several amino acid residues in that of the HSA molecule. Most of the replaced residues were located in the regions of residues 110–135, 155–165, and 181–190, in which 73% of the total residues were different in both molecules. These regions are located close to each other, but far from the OPH cleavage sites, and involved in the binding of fatty acids in the HSA molecule (*37*). Therefore, it is feasible that BSA and HSA had almost the same structure in other regions, since more than 81% residues in these regions were identical in the two molecules. The three cleavage sites were found to be located slightly inside the BSA molecule, in positions not easily accessible by OPH. To investigate the influence of oxidation on the tertiary structure of BSA, we hypothetically replaced all the methionine and tryptophan residues with methionine sulfoxide and *N'*-formyl-kynurenine, respectively. The three-dimensional structure of the hypothetically oxidized BSA indicated that all the OPH-cleavage sites became more exposed to the solvent upon oxidation. The degree of exposure of the cleavage sites increased in the order Leu218-Ser219 < Tyr410-Thr411 < Phe506-Thr507. In this computer modeling, we assumed that only methionine and tryptophan residues were oxidized and

that all these residues were oxidized simultaneously. However, it seems more likely that these residues were oxidized heterogeneously, molecule by molecule, and that certain other residues were also oxidized. In the oxidized BSA preparation used in this study, approximately 70% of the tryptophan residues was estimated to have been oxidized. Davies and his colleagues indicated previously that all amino acids in BSA were potentially susceptible to modification by the hydroxyl radical, although tryptophan was one of the most susceptible residues (*38*), and that the oxidation resulted in a gross conformational change (*39*). Taken together, these findings suggest that the increased susceptibility of BSA to degradation by OPH upon oxidation is due to a conformational change that brings the cleavage sites to the surface of the molecule, where they are easily accessible by OPH. Not all BSA molecules undergo such conformational change, since a significant amount of the 66-kDa BSA band remained undegraded (Fig. 3B). This is probably due to insufficient oxidation of the molecules.

Pacifici and his colleagues (*40*) reported previously that the increased susceptibility of BSA to degradation by proteasome upon oxidation was accompanied by increased hydrophobicity of the protein substrate, indicating that hydrophobicity may be a signal for the degradation of oxidized proteins. They also suggested that the hydrophobic region of the substrate, the possible proteasome recognition site, could become exposed to the molecular surface upon oxidation. It is feasible that OPH recognizes hydrophobic regions of the substrate, since the three cleavage sites in BSA identified in this study are surrounded by hydrophobic environments in the deduced three-dimensional structure of the molecule. This recognition selectivity of OPH for hydrophobic regions may distinguish OPH from chymotrypsin, which has similar cleavage specificity for amino acid sequences but little selectivity for oxidized proteins (*19*).

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