Comparison of the Effects of Ozone on the Modification of Amino Acid Residues in Glutamine Synthetase and Bovine Serum Albumin*

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Ozone is one of the most toxic pollutants in the atmosphere. Brief exposure to concentrations of ozone below 1 ppm leads to damage of the lung, especially to regional damage of bronchio-lar ciliated cells and alveolar epithelial type I cells. Prolonged exposure leads to inhibition of ciliogenesis and type 2 cell maturation, to inflammation, fibrosis, enhanced collagen synthesis, and to increased sensitivity to bacterial infection (for review, see Mehlman and Borek (1), Menzel (2), and Stokinger (3)). Pryor (4) has argued that ozone reacts with the alveolar epithelial type I cells so rapidly that it cannot cross the lunglining fluid layer except in patchy areas where the lower airways are virtually uncovered. This accounts for the observation that tissue damage by ozone is largely restricted to the lung. Nevertheless, exposure of rats to ozone has been shown to elicit a number of extrapulmonary effects, including a decrease in visual acuity (5), defects in the desaturation of oxyhemoglobin in skin capillaries (6), changes in cardiac protein metabolism (7), increased lipid peroxidation in heart and brain tissue, and the elevation of peroxide scavengers in these tissues (8).

Because of its pronounced cytotoxicity, recent studies have focused on the fact that ozone can give rise to a number of other reactive oxygen species including OH, H₂O₂, O₂, RCOO⁻, O₅⁻, and singlet oxygen (9–15). In some circumstances, these secondary reactive oxygen species might be more damaging than ozone itself. Thus, the reaction of ozone with polyunsaturated fatty acids in membrane lipids leads to the generation of relatively stable lipid peroxides, which can subsequently give rise to tissue-damaging alkoxy radicals and alkyl radicals (12, 15, 16). Lipid-independent mechanisms must also be considered since ozone reacts directly with some amino acids (17, 18), with proteins (see Ref. 1 for review), and with nucleic acids (19, 20).

In the case of free amino acids and amino acid residues in proteins, cysteine, methionine, tryptophan, phenylalanine, and histidine, residues are particularly sensitive to oxidation by ozone; other amino acids are fairly resistant to ozone (17, 21). Kynurenine and N-formylkynurenine have been identified as major products of free tryptophan and of tryptophan residues in proteins (22). Cysteic acid and cystine are major products of cysteine oxidation (2, 22, 23). Methionine sulfoxide is a major product of methionine oxidation (17). Dihydroxyphenylalanine was produced in low yields during the oxidation of tyrosine by ozone. Ammonia and an amino acid tentatively identified as proline are products of histidine oxidation at pH 4.6 (17).

The rates at which the aromatic and sulfur amino acid residues of proteins are oxidized by ozone varies widely from one protein to another, suggesting that the susceptibility to oxidation is dependent upon the size and amino acid composition as well as the secondary and tertiary structure. In the present study, we compare the ability of ozone to oxidize amino acid residues in Escherichia coli glutamine synthetase (GS) and bovine serum albumin (BSA). These two proteins are similar with respect to subunit size (50 versus 67 kDa), they contain nearly equal numbers of Trp, Tyr, His, and Phe residues per subunit, and X-ray crystallographic data are available for GS (24) and human serum albumin (25), which is highly homologous to BSA. The GS was selected also because the results could be compared with those derived from extensive studies on the oxidation of the enzyme by metal-catalyzed oxidation (MCO) systems. In addition, we investigated the ozone-mediated oxidation of a series of tripeptides, each of which contained two alanine residues and one histidine residue that was present in either the N-terminal, middle, or C-terminal position.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (BSA) was obtained from Sigma. Glutamine synthetase (GS) was from E. coli YMC 10/pgln6, which overproduces the enzyme. The enzyme was purified by the zinc-induced aggregation procedure as described (26). GS activity was measured by the γ-glutamyl transferase assay (27). [1-¹³C]Histidine, 0.38 mCi/μmol, was from DuPont NEN.

Carbonyl Group Assay—The presence of carbonyl groups was measured by the 2,4-dinitrophenylhydrazine technique as modified for anal-

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1 The abbreviations used are: GS, glutamine synthetase; BSA, bovine serum albumin; MCO, metal-catalyzed oxidation; OPA, o-phthalaldehyde; NBT, nitroblue tetrazolium; DOPA, 3,4-dihydroxyphenylalanine; HPLC, high performance liquid chromatography.
ysis using reverse phase high performance liquid chromatography to separate excess reagent from the protein hydrazine derivatives (28).

Ozonolysis—Prior to ozonolysis, protein solutions were buffer-exchanged by passage through a PD-10 gel filtration column (Sephadex G-25) from Pharmacia, eluted with 10 mM potassium phosphate buffer, pH 7.4, and adjusted to a final concentration of 3.0 mg/ml. Ozone was produced by passing 100% oxygen through a Sander model 25 ozonizer purchased at a local aquarium shop. Quantitation of the ozone was accomplished by titrimetric measurement of the I$_2$ produced when the ozone-oxygen mixture was passed through a solution of 1 x KI at pH 7.4, using 0.001 M Na$_2$S$_2$O$_3$ as titrant, or by the bleaching of indigo-trisulfonate (29). The rate of bubbling of the ozone-oxygen mixture through the reaction mixture was adjusted to yield a constant supply of ozone for the duration of the experiment. The supply of ozone was varied from 50 to 500 nmol/min depending upon the experiment. As standard procedure, 1,875 ml of the 10 mM phosphate buffer, pH 7.4, was passed with the ozone-oxygen mixture for 10 min; then, 0.625 ml of the protein stock solution was added to yield a mixture containing 0.75 mg of protein/ml, and the bubbling was continued at a constant rate. At various times, aliquots were removed and analyzed for spectral changes, total amino acid composition, quinoprotein content, and carboxyl content.

Amino Acid Analysis—Aliquots of reaction mixtures were subjected to acid hydrolysis as described previously (30). Amino acids in the hydrolysate were converted to their o-phthalaldehyde (OPA) derivatives, and these were separated by high pressure liquid chromatography on a J&J 15-cm C$_18$ column and quantified as described previously (30). Tryptophan is destroyed by acid hydrolysis; therefore, alkaline hydrolysates were used for measurement of tryptophan (31). Quantitation of proline was achieved by first derivatizing the primary amino acids with OPA, then the secondary amino acids were converted to their Fmoc (N-9-fluorenylmethoxycarbonyl) derivatives, prior to separation by HPLC (32). The HPLC was performed on a Hewlett-Packard model 1090 instrument equipped with a Hewlett-Packard 1046 programmable fluorescence detector.

As found in previous studies (33) with other proteins, the aromatic and sulfur-containing amino acid residues of BSA and GS are most sensitive to ozone modification. In both proteins, the order of susceptibility to oxidation is Met > Trp > His > Tyr > Phe (Table I). In addition, as noted by Mudd et al. (17), some cysteine residues are converted to cysteic acid residues. Under our conditions, only 0.6 out of 4 residues in GS and 3 of 35 residues in BSA were converted to cysteic acid residues during a 120-min exposure to ozone at a rate of 500 nmol per min. None of the other amino acid residues were modified at a significant rate upon exposure of the proteins to ozone at levels varying from 70 to 800 nmol/min (data not shown).

From the data in Fig. 1 and other data not shown, it is evident that the modification of a given kind of aromatic amino acid residue is a nearly linear function of time until at least 60 to 90% of the residues have been oxidized. Also, as shown in Table I, the specific rate constant $k$ = (number of residues
Modification of Glutamine Synthetase and BSA by Ozone

Fig. 1. Ozone-induced changes in the number of tyrosine, histidine, and aspartic acid residues of BSA and GS. Mixtures (2.5 ml) containing 10 mM potassium phosphate buffer, pH 7.4, and 0.75 mg/ml BSA (closed symbols) or GS (open symbols) were bubbled with an ozone/oxygen mixture yielding 545 nmol of ozone/min. At times indicated, aliquots were removed and the amino acid composition of the proteins was determined. The time-dependent changes in the number of tyrosine (triangles) and histidine (squares) residues per subunit is indicated in the lower half of the figure. The time-dependent increase in the number of aspartic acid residues in BSA (closed circles) and GS (open circles) is indicated in the upper portion of the figure.

Modified per min) / (total number of residues present in the protein) for each aromatic amino acid is about 1.5–2.0 times higher for residues in BSA than in GS. Since GS and BSA contain nearly equal amounts of each of the aromatic amino acids (Table I), this decrease in susceptibility to oxidation by ozone likely reflects differences in the primary, secondary, tertiary, or quaternary structures. In this regard, it is noteworthy that each of the 12 identical subunits of GS are arranged in two superimposed hexagonal arrays, whereas BSA exists as a monomer.

Histidine Residues Are Converted to Aspartic Acid Residues—The ozone-mediated oxidation of BSA and GS leads to an increase in the number of aspartic acid residues or to residues that are converted to aspartic acid during acid hydrolysis (Fig. 1). The possibility that the aspartic acid is derived from histidine residues is suggested by the fact that there is a nearly stoichiometric relationship between the number of histidine residues lost and the number of aspartic residues formed. This probably involves conversion of the histidine residues to aspartic acid or to a-oxohistidine residues, which may be converted to aspartic acid upon acid hydrolysis (see "Discussion").

To confirm that ozone promotes conversion of histidine residues to aspartic acid or to a derivative that yields aspartic acid upon acid hydrolysis, a protein preparation in which all of the histidine sources was uniformly labeled with 14C was prepared by growing a histidine-requiring auxotrope of E. coli on a medium containing uniformly labeled [14C]histidine as the sole histidine source. The [14C]histidine-labeled protein fraction from cell-free extracts of the organism was then exposed to ozone under our standard conditions. As shown in Fig. 2, the treatment with ozone led to a nearly linear, time-dependent decrease in the amount of [14C]-labeled histidine and was accompanied by a nearly stoichiometric (approximately 80%) increase in the amount of [14C]-labeled aspartate that was present following acid hydrolysis of the ozone-treated protein. Conversion of histidine residues to aspartate was confirmed also by the demonstration that the histidine residue in a tripeptide containing one His residue and two Ala residues was converted to aspartic acid following exposure to ozone and acid hydrolysis.

Oxidation of Tripeptides—To determine how the location of histidine residues in the polypeptide chain affects its susceptibility to oxidation by ozone and the kinds of products formed, we investigated the oxidation of tripeptides containing two residues of alanine (A) and a single residue of histidine (H) that was present in either the C-terminal, middle, or N-terminal position. As shown in Fig. 3A (open symbols), the rate of histidine loss was independent of its position in the tripeptide. However, the fraction of modified histidine that was recovered as aspartic acid in acid hydrolysates of the ozone-treated peptide varied depending upon its location in the peptide. Thus, the yields of aspartic acid from HAA, AAH, and AHA were 32, 88, and 88%, respectively. Moreover, for each tripeptide, the fraction of histidine that was converted to aspartic acid was constant throughout the time course of ozone exposure (Fig. 3B). These results confirm that ozone promotes conversion of histidine residues to a derivative that appears as aspartic acid following acid hydrolysis.

The fact that a histidine residue in the internal position of the peptide is stoichiometrically recovered as aspartic acid explains why the histidine residues in BSA and GS are converted almost entirely to aspartic acid. In contrast, aspartic acid accounts for only 32% of the products formed from a histidine residue occupying the N-terminal position of the peptide. An examination of the ultraviolet absorption spectra of the peptides shows that ozone treatment leads, in the case of HAA, to products possessing much higher absorption in the range of 360 nm than products obtained from either AHA or AAH (Fig. 4). Finally, it appears significant that little or no carbonyl compounds could be detected among the products obtained from any of the histidine-containing tripeptides.

Conversion of Histidyl Residues to D-Aspartate—Racemization of L-aspartyl or L-asparaginyl residues occurs in mamma-
lian proteins with the content of the D-isomer increasing as a function of animal age (36). Since ozonolysis of histidyl residues of proteins and of histidyl peptides gave rise to aspartate, we examined the possibility that some of the aspartate formed might be the D-isomer. No D-aspartic acid was detected in BSA or GS exposed to ozone for 10 min, except for the basal level due to acid hydrolysis. Moreover, no D-aspartic acid was recovered in acid hydrolysates of the ozone-treated AHA tripeptide. However, D-aspartic acid accounted for 8 and 17%, respectively, of the aspartic acid derived from the ozone-treated HAA and AAH tripeptides. The effect of time of exposure to ozone was examined for Ala-Ala-His. Racemization was 17% regardless of the length of exposure (5–10 min).

Effect of Ozone Treatment on GS Activity—Under our conditions, when GS is exposed to a constant supply of ozone (545 nmol/min), the loss of catalytic activity and the number of histidine residues lost are both linear functions of time, and complete loss of activity is not obtained until 65% of the histidine residues (10 residues/subunit) have been modified (Fig. 5). This is in contrast to the site-specific inactivation of GS by metal-catalyzed oxidation systems where the loss of activity is associated with the oxidation of only one histidine residue (His-269) that is present at the catalytic site (37, 38). It is, therefore, evident that the histidine residue at the catalytic site is no more sensitive to ozone oxidation than at least 10 other histidine residues in the enzyme.

Generation of Carbonyl Groups—When proteins are exposed to ionizing radiation (39–41) or to MCO systems (38, 42, 43), the side chains of some amino acids are converted to carbonyl derivatives. Ozone reacts readily with compounds possessing ethylenic double bonds to form ozonides, which upon decomposition may lead to carbon-carbon bond cleavage yielding either two aldehydic or one aldehyde and a carboxyl group. Aldehydes have, in fact, been identified among the proteolytic degradation products of protein fractions from lungs of rabbits following their exposure to ozone (44). It is therefore not surprising that carbonyl groups are generated during the exposure of BSA and GS to ozone (Fig. 6). Under our conditions, the rate of carbonyl group formation in BSA is 3 to 4 times faster than in GS. After 10 min, the yield of carbonyl groups in BSA and GS was 11 and 3.3 eq per subunit, respectively. The sources of these carbonyl groups were not determined. Ozone-induced generation of carbonyl groups in human serum albumin was reported previously by Cross et al. (45).

Generation of DOPA-like Derivatives—As illustrated in Fig. 7, during exposure of GS and BSA to ozone there is a
time-dependent increase in the level of quinoprotein formation as determined by the NBT assay. Both the rate and amplitude of quinoprotein formation are greater with BSA than with GS. Significantly, the level of quinoprotein reaches a maximum that coincides with completed destruction of tyrosine, suggesting that the quinoprotein formation involves conversion of tyrosine residues to DOPA derivatives. The level of such derivatives corresponds to about 0.3 to 0.5 mol of quinoprotein per mol of GS and BSA, respectively. In fact, there is an almost linear relationship between the loss of tyrosine residues and the amount of quinoprotein formed (inset, Fig. 7).

**DISCUSSION**

The demonstration that several kinds of amino acid residues in BSA are more rapidly oxidized by ozone than are in GS confirms results of earlier workers (21) showing that the sensitivity of a given kind of amino acid residue to oxidation by ozone varies from one protein to another. However, the conclusion that this variability is due to differences in the primary, secondary, tertiary, and quaternary structures of the proteins is strengthened by the results presented here, because the subunits of BSA and GS are of comparable size and contain nearly equal numbers of each of the aromatic amino acids.

Three observations support the conclusion that the histidine residues in protein and peptides are converted to aspartyl residues or to derivatives that are converted to aspartic acid upon acid hydrolysis. 1) The ozone-induced loss of histidine residues in BSA and GS is accompanied by a nearly stoichiometric increase in the amount of aspartic acid present in acid hydrolysates of the ozone-treated proteins. 2) The histidine lost during oxidation of the tripeptide, Ala-His-Ala, is recovered in acid hydrolysates as aspartic acid. 3) When proteins in which all histidine residues are uniformly labeled with 14C are treated with ozone, the labeled histidine which is lost is almost quantitatively (80%) recovered in acid hydrolysates as 14C-labeled aspartic acid.

Of particular interest is the finding that the ozone-dependent loss of GS activity is directly proportional to the total number of histidine residues that are modified (Fig. 5), over a range of histidine residues representing 75% of all histidine residues in the protein. This is in sharp contrast to the fact that, when GS is exposed to the ascorbate/FeIII/O2 MCO system, the loss of activity is correlated with the loss of a single histidine residue (His-269) (36, 38). Thus, the inactivation of GS by ozone involves a more or less random attack of histidine residues; His-269 is no more susceptible to ozone attack than at least 10 other histidine residues in the molecule (and possibly even less so if oxidation of any of the other residues causes any loss of catalytic activity).

In earlier studies, Farber and Levine (37) presented evidence indicating that in the oxidation of GS by the ascorbate/FeII/O2 MCO system His-269 is converted to an asparaginyl residue, which upon acid hydrolysis would be converted to aspartic acid. In the meantime, prompted by the report of Uchida et al. (47) that the histidine moiety of N-benzoylhistidine is converted to 2-oxohistidine by the CuII/H2O2/MCO system, Levine and co-workers2 reinvestigated the metal-catalyzed oxidation of GS using more advanced technology and have confirmed that the oxidation of His-269 does indeed give rise to 2-oxohistidine.

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2 J. Sahakian and R. L. Levine, unpublished data.
which under some conditions can be converted to aspartic acid
by acid hydrolysis. Accordingly, we suspected that the oxidation
of histidine residues by ozone would also yield 2-oxohistidine
as the primary product. Thus, if 2-oxohistidine is formed,
it can only be a transitory intermediate that is rapidly con-
verted further to asparaginase or aspartate.

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