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OZONATION OF LYSOZYME IN THE PRESENCE OF OLEATE IN REVERSE MICELLES OF SODIUM DI-2-ETHYLHEXYLSULFOSUCCINATE

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Ozone is shown to react with lysozyme in reverse micelles formed by 0.1 M sodium di-2-ethylhexylsulfosuccinate and 1.2-3 M water (pH 7.4) in isooctane solvent. The reaction of ozone is assessed by the oxidation of tryptophan residues in the protein to N-formylkynurenine. Cosolubilization of oleate in lysozyme-containing reverse micellar solutions at concentrations of 0.5-10 mM results in a progressive inhibition (19% to 82%) of the oxidation of tryptophan residues with a concentration for 50% inhibition around 2 mM. At this concentration of oleate, the magnitude of inhibition is independent of the micelle size and concentration, the overall interfacial area of reverse micelles, and the amount of ozone employed. These findings are discussed in terms of competitive reactions of ozone with unsaturated fatty acids and proteins in the lung lining fluid and in biological membranes.

Ozone is the most important oxidant formed in photochemical smog. A number of investigators have studied the reaction of ozone with unsaturated fatty acids (1-8) and proteins (9-16). The data indicate that ozone has a high reactivity toward both proteins and unsaturated fatty acids (UFA) when they are studied separately. However, Mudd and his coworkers have reported that ozonation of red cell membranes (1,17) and glycophorin A incorporated into egg-phosphatidylcholine liposomes (18) results in oxidation of proteins and not the UFA present in the lipid bilayer. Cross *et al.* (19,20) report that ozonation of human blood plasma brings about oxidative damage to the proteins and not to the lipids that are present.

We have previously reported absolute rate constants for reaction of ozone with amino acids and model compounds (21). The rate of reaction of ozone with thiols (such as, cysteine and GSH) is about 1,000-times faster than with double bonds (21). However, ozone reacts with other amino acid side-chain functionalities (such as, indole, $-SCH_3$, imidazole, and phenol) at about the same rate as with double bonds (21,22). Therefore, it is intriguing that ozone is reported to selectively react with proteins when red cell membranes, which consist of approximately half protein and half lipid, are ozonized (1,17).

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Reverse micelles formed by sodium di-2-ethylhexylsulfo-succinate ["Aerosol OT", (AOT)] and trace amounts of water in hydrocarbon solvents are a novel system in which the reactions of ozone with proteins and UFA can be studied. With this system, changing the molar ratio of H_2O/AOT allows one to exert control over the size of the dispersed water pool, which can be made to accommodate large proteins (> 200,000 Daltons) (23). Proteins are held in the inner cavity of reverse micelles with a monolayer cover of hydrated surfactant molecules (24,25). The UFA, introduced in the form of sodium or potassium salts, are sequestered at the micellar interface with the head groups facing inward towards the water pool and the hydrocarbon tails away from it into the bulk organic solvent (26). The solutions of proteins and UFA in reverse micelles are homogeneous and optically clear and are amenable to spectrophotometric measurements. The results presented in this communication demonstrate that oleate inhibits oxidative damage to lysozyme by undergoing a sacrificial reaction with ozone.

MATERIALS AND METHODS

Hen egg-white lysozyme (E.C. 3.2.1.17, 3x crystallized, dialyzed and lyophilized, approx. 95% protein, 46,200 units/mg solid), diethylenetriaminepentaacetic acid (DTPA), 5,5',7-indigotrisulfonic acid (potassium salt, approx. 75%), oleic acid (sodium salt, approx. 99% by capillary GC), and AOT were purchased from Sigma Chemical Company (St. Louis, MO); potassium phosphate monobasic from J.T. Baker (Pillipsburg, NJ); and nanograde 2,2,4-trimethylpentane (or isooctane) and sodium phosphate dibasic heptahydrate from Mallinckrodt (Paris, KY). All chemicals were used without further purification.

Generation of ozone. Ozone was generated by passing dried air at a constant flow (20 mL/min) through a Welsbach ozonator producing a silent electrical discharge at 40-60 V. The output of ozone was estimated by bubbling the gas stream through a glass-frit into a 5 mL solution of 2 mM indigotrisulfonate in 0.02 M phosphoric acid for 5 min. The extent of dye bleaching was measured by following the change in absorbance at 600 nm ($\Delta\varepsilon$ = 20,000 M^{-1} cm^{-1}) (27). At a constant voltage, the rate of ozone production (given in μmol/min) did not vary by more than 5% during the course of an experiment, as assessed by repeated measurements based on the dye-bleaching method (27).

Solubilization of lysozyme in reverse micelles of AOT can be achieved a standard injection method (25). Aqueous solutions of lysozyme (3.88-7.76 mM) were first prepared in 0.02 M sodium/ potassium phosphate buffer, pH 7.4 containing 0.04 mM DTPA. (DTPA was included to minimize reactions by adventitious metal ion impurities during or after ozonation.) A small aliquot (10-180 μL) of the concentrated protein sample in phosphate buffer was then injected into a 4 mL solution of 0.125 M AOT in isooctane as the solution was continuously stirred on a vortex mixer, and stirring was continued until the solution became homogeneous and optically transparent. The molar ratio of H_2O/AOT was adjusted to 12, 20, 25, or 30 by adding H_2O in the form of 0.02 M phosphate buffer, pH 7.4 containing 0.04 mM DTPA. The final volume was then brought to 5 mL by adding required amounts of isooctane. In certain assays, sodium oleate was cosolubilized with lysozyme in the reverse micellar solution at concentrations of 0.5-10 mM. The protein solutions, including those prepared in the presence of oleate, were optically clear with OD ≤ 0.1 at 350 nm, did not tend to precipitate even after 3-5 h of standing at room temperature, and the absorbance of replicates at 280 nm did not vary by more than 5%, indicating the proteins were completely taken up by the reverse micelles

Ozonation of proteins in reverse micelles. Known amounts of ozone (0.53-3.54 μmol) in a stream of air (20 mL/min) were bubbled through a glass-frit into a 5 mL solution of reverse micelles containing protein and various levels of oleate over a period of 5 min at room temperature. The extent of oxidation of tryptophan (Trp) residues to N-formylkynurenine (NFK) was estimated spectrophotometrically at 320 nm ($\Delta\varepsilon$=3,750 M^{-1} cm^{-1}) (9). Each assay was carried out in either triplicate or quadruplicate.

RESULTS

Influence of the concentration of lysozyme on its ozonation.

Lysozyme was solubilized in reverse micellar solutions of 0.1 M AOT/2 M H_2O at a final concentration of 14 to 252 μM, following the method of Grandi *et al.* (25). Figure 1 shows NFK formation when a 5 mL solution of reverse micelles containing different amounts of lysozyme was allowed to react with 1.91 μmol of ozone. The yields of NFK increase with increasing concentrations of the protein until about 140 μM and then plateau. Since lysozyme has 6 Trp residues per molecule (28), the total amount of Trp in a 5 mL reaction mixture containing 140 μM lysozyme corresponds to 4.2 μmol. Thus, Trp is not the limiting reagent, and the plateau in Fig. 1 is a consequence of complete reaction of ozone and not the lack of Trp residues. The maximum yield of NFK is 0.65 moles per mole of ozone employed (Fig. 1). Since the reaction between ozone and Trp has a stoichiometry of 1:1 (29), about 65% ozone reacts with Trp and 35% with other amino acid residues in lysozyme.

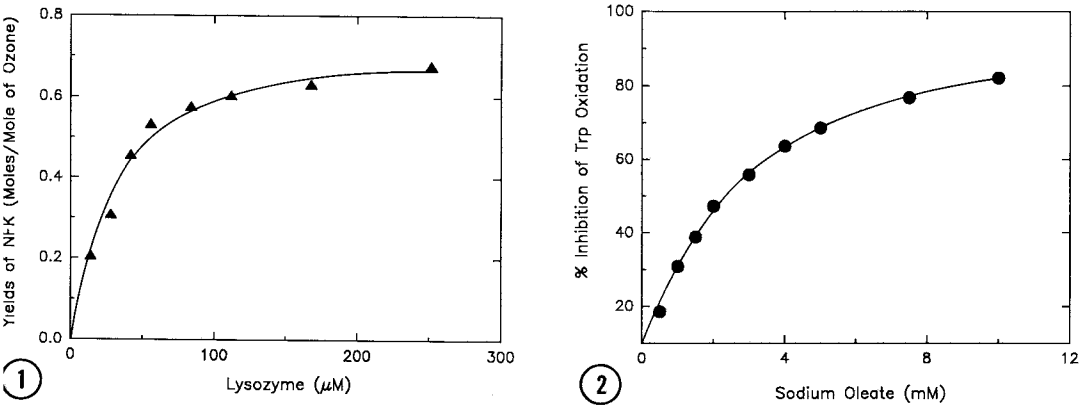

Fig. 1. Influence of the concentration of lysozyme on its ozonation. Lysozyme was solubilized in reverse micelles of 0.1 M AOT/2 M H_2O in isooctane solvent at concentrations of 14-252 μM. Using air as a carrier gas at a flow of 20 mL/min, 1.91 μmol of ozone was bubbled through a glass-frit into a 5 mL solution of the protein in reverse micelles over a period of 5 min. The yields (moles/mole of ozone) of the Trp oxidation product, NFK were estimated spectrophotometrically at 320 nm ($\Delta\varepsilon$=3,750 M^{-1} cm^{-1}) (9). Other details are as given in the Methods section and in the text.

Fig. 2. Effect of oleate on ozonation of lysozyme in reverse micelles. Oleate (0.5-10 mM) was cosolubilized with lysozyme (140 μM) in reverse micelles of 0.1 AOT/2 M H_2O in isooctane solvent. A 5 mL solution of reverse micelles containing admixtures of oleate and lysozyme was exposed to 3.54 μmol of ozone and the oxidation of lysozyme-Trp residues to NFK was estimated as described in the legend to Fig. 1.

Ozonation of lysozyme in the presence of oleate.

Optically clear solutions of lysozyme (140 μM) in reverse micelles of 0.1 M AOT containing 2 M H_2O and 0.5-10 mM oleate were allowed to react with 3.54 μmol of ozone, and the yield of oxidized Trp residues was estimated as described above. Control assays, which omit lysozyme, do not show appreciable changes in the absorbance at 320 nm following ozonation. Incubation of lysozyme with previously ozonized solutions of oleate or with 1 mM hydrogen peroxide do not result in a measurable change in absorbance at 320 nm even after 30 min. This suggests that the changes in absorbance observed when mixtures of oleate and lysozyme are ozonized reflect a direct reaction of ozone with the Trp residues.

As shown in Figure 2, oleate at a concentration of 0.5 mM results in 19% inhibition of the oxidation of Trp residues, and the inhibition increases to 82% as the concentration of oleate is increased to 10 mM. To test whether the observed inhibition by oleate is dependent on the amount of ozone used, inhibition studies were performed at different levels of ozone (0.53-2.13 μmol), keeping the concentrations of lysozyme (140 μM) and oleate (2 mM) constant. In all cases, oleate brought about an identical inhibition of Trp oxidation (mean=44%, SD= 3.3%, n=6).

Lack of influence of micelle size, concentration, and interfacial area.

Lysozyme (140 μM) was solubilized in reverse micelles made with 0.1 M AOT and different amounts of water (1.2-3 M), with or without inclusion of oleate at a final concentration of 2 mM. The literature (24) shows that going from a H_2O/AOT ratio of 12 to 30 changes the mean inner radius of micelles from 22 to 49 Å and the fractional volume of the dispersed water pool increases from 0.021 to 0.053 mL per mL of reverse micellar solution. The fractional volume of the surfactant monolayer, on the other hand, remains constant at 0.04 mL per mL of reverse micellar solution at all ratios of H_2O/AOT (24) (Table 1).

Aliquots (5 mL each) of lysozyme-containing reverse micellar solutions were ozonized as described earlier using 2.94 μmol ozone. As shown in Table 1, in the absence of oleate, the fractional reaction of ozone with the Trp residues (F_{Trp}) does not change when the ratio of H_2O/AOT is increased from 12 to 30. Inclusion of oleate (2 mM) results in an identical magnitude of inhibition of Trp oxidation at all molar ratios of H_2O/AOT (Table 1). Similarly, when the concentration of AOT is changed from 0.075 M to 0.25 M with the ratio of H_2O/AOT held constant at 30, the % inhibition of Trp oxidation by 2 mM oleate remains essentially unchanged (mean=45%, SD=1.3%, n=4).

DISCUSSION

Revere micellar systems offer a convenient media for solubilizing mixtures of polar, semipolar and apolar compounds, and such diverse molecular species as oleate and lysozyme can be solubilized without altering the optical transparency of the system. Since (i)

Table 1. Lack of effect of the micelle size and concentration on the fraction of ozone that reacts with the tryptophan residues in the presence and absence of oleate in reverse micelles[a]

	Fractional volume, mL/mL, of the reaction mixture[b]		%			
H_2O/AOT	Water pool	Monolayer	Occupancy[c]	Oleate, mM	F_{Trp}[d]	% inhibition
12	0.021	0.039	18	0	0.60±0.013	
				2	0.33±0.006	45
20	0.035	0.040	40	0	0.59±0.002	
				2	0.32±0.008	46
30	0.053	0.040	79	0	0.60±0.003	
				2	0.32±0.009	47

[a] The reaction mixture in a final volume of 5 mL contained 0.7 μmol of lysozyme, 0.1 M AOT, 1.2-3 M H_2O, and indicated amounts of oleate (0 or 2 mM) in isooctane solvent. Amount of ozone employed was 2.94 μmol.

[b] Calculated assuming an aggregation number of AOT of 131, 282 and 560 per micelle; an inner radius of reverse micelles of 22.1, 33.9 and 48.9 Å; an outer radius of reverse micelles of 31.4, 43.7 and 59.0 Å at H_2O/AOT molar ratios of 12, 20 and 30, respectively (24).

[c] Refers to the fraction of reverse micelles that contain 1 molecule of lysozyme solubilized per micelle.

[d] F_{Trp} refers to moles of NFK formed per mole of ozone employed.

ozonized oleate does not have any absorption at 320 nm, (ii) addition of ozonized oleate or hydrogen peroxide does not bring about the oxidation of Trp residues, and (iii) oxidation of amino acid residues other than Trp does not result in any appreciable change in absorbance at 320 nm, measurement of Trp oxidation provides a valuable tool for analyzing the protein-UFA competition in ozone-mediated reactions.

Our results indicate that oleate competes for the reaction of ozone with lysozyme and inhibits the oxidation of Trp residues by a sacrificial mechanism (Fig. 2). The inhibition of Trp oxidation by oleate, which is an indirect measure of the oxidation of oleate itself, is evident under varied conditions of micelle size and concentration (Table 1), overall interfacial area of reverse micelles, and amount of ozone employed. The ratio of oleate to lysozyme used here ranges from 0.08 to 1.5 (w/w). This encompasses the composition of lung lining fluid as well as biological membranes isolated from diverse species of animals (30,31). Unsaturated fatty acids appear to be a potential target for ozone reaction in the lung; Rabinowitz and Bassett (32) found an enhanced recovery of glutaric (15-fold) and nonanoic (118%) acids in the lung lipids isolated from rats exposed to 2 ppm ozone for 4 h. Their assay involved oxidation of the isolated lung lipids with hydrogen peroxide, which converts the aldehydic products to carboxylic acids, and subsequent saponification to liberate the fatty acids from the glycerol backbone; the glutaric and nonanoic acids therefore are markers of the

ozonation of arachidonic and oleic acids, respectively (32). We find aldehydic products (heptanal, nonanal and 3β-hydroxy-5α-secocholestan-6-aldehyde) from ozonation of palmitoleate, oleate and cholesterol, respectively in lung lavage isolated from rats exposed to 1.3 ppm ozone for 12 h (unpublished observations).

Banerjee and Mudd (18) reported that ozonation of glycophorin A incorporated into egg-phosphatidylcholine (PC) liposomes results in the oxidation of protein-methionine residues and not the UFA present in the lipid bilayer. Based on the average number of double bonds per PC molecule (1.5), the molar ratio of PC to glycophorin A (150:1), the molar ratio of ozone to glycophorin A (10:1), and the number of methionine residues per glycophorin molecule (2) (18), we calculate that in the system used by Banerjee and Mudd the molar ratio of double bonds to methionine residues to ozone is 113 to 1 to 5. Under these conditions, even if all the ozone employed were to react with double bonds, the change in concentration of UFA will be less than 5%. This change will be distributed among three major UFA present in egg-PC, oleic acid, linoleic acid, and arachidonic acid. This relatively small decrease may be within the experimental error of an assay based on the disappearance of initial reactants.

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