Oxidation of Protein Sulfhydryls by Products of Peroxidase-Catalyzed Oxidation of Thiocyanate Ion†

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ABSTRACT: Lactoperoxidase or myeloperoxidase catalyzed the oxidation of thiocyanate ion by hydrogen peroxide in the presence of proteins. Protein sulfhydryls were oxidized by the products of oxidation of thiocyanate. Similar oxidation of sulfhydryls was obtained by adding thiocyanogen, $(SCN)_2$, or hypothiocyanite ion, $OSCN^-$, to the proteins. The results were consistent with peroxidase-catalyzed oxidation of thiocyanate to these thiocyanate analogues of the halogens or hypohalite ions. Oxidation of thiocyanate by 1 mol of hydrogen peroxide resulted in oxidation of about 1 mol of sulfhydryls of bovine serum albumin. Sulfhydryls were oxidized to the sulfenyl thiocyanate derivative, as indicated by incorporation of the intact thiocyanate moiety into a derivative of sulfhydryls. Also, the derivative reacted with the sulfenyl-specific reagent, 4,4′-bis(dimethylamino)thiobenzophenone (thio-Michler's ketone), to release thiocyanate and yield the mixed disulfide derivative. The sulfenyl thiocyanate derivative was in equilibrium with sulfenic acid and free thiocyanate ion. An equilibrium constant of 0.3 mM was obtained for hydrolysis of sulfenyl thiocyanate. Thiocyanate could also be displaced by exchange with iodide, sulfite, or cyanide ions. An equilibrium constant of 0.03 was obtained for displacement of thiocyanate to yield the sulfenyl iodide derivative. Oxidation of albumin sulfhydryls was proportional to hydrogen peroxide and independent of thiocyanate concentration over a wide range. Because thiocyanate was released from sulfenyl thiocyanate at low thiocyanate concentrations, and the released thiocyanate could be reoxidized, 1 thiocyanate ion could participate in oxidation of many sulfhydryls. Unreacted $OSCN^-$ accumulated when the amount of $OSCN^-$ formed exceeded the amount required to oxidize all the albumin sulfhydryls. The excess $OSCN^-$ then slowly oxidized the sulfenyl derivatives. Whereas complete oxidation of albumin sulfhydryls could be obtained at low thiocyanate concentrations, about 1 mol of sulfhydryls per mol of β-lactoglobulin was resistant to oxidation regardless of hydrogen peroxide or thiocyanate concentrations. Also, the excess $OSCN^-$ did not oxidize sulfenyl derivatives of β-lactoglobulin.

Lactoperoxidase-catalyzed oxidation of thiocyanate (SCN^-) in milk and saliva contributes to the antimicrobial activity of these fluids (Wright & Tramer, 1958; Jago & Morrison, 1962; Zeldow, 1963; Mickelson, 1966; Oram & Reiter, 1966a,b; Morrison & Steele, 1968; Steele & Morrison, 1969; Hogg & Jago, 1970; Hamon & Klebanoff, 1973; Bjorck et al., 1975; Reiter et al., 1976; Hoogendoorn et al., 1977). In addition, SCN^- is an antithyroid substance and the oxidation of SCN^- is catalyzed by thyroid peroxidase (Barker, 1936; Wood & Williams, 1949; Coval & Taurog, 1967). Oxidation of protein sulfhydryls by oxidized forms of SCN^- may account for these antimicrobial and antithyroid activities (Mickelson, 1966; Hoogendoorn et al., 1977; Aune et al., 1977).

Lactoperoxidase catalyzes the incorporation of SCN^- into a derivative of protein sulfhydryls (Aune et al., 1977). In the absence of a protein substrate, lactoperoxidase-catalyzed oxidation of SCN^- results in accumulation of hypothiocyanite, $OSCN^-$ (Hoogendoorn et al., 1977; Aune & Thomas, 1977). The aims of the present study were to identify the derivative of sulfhydryls, to determine the stoichiometry of sulfhydryl oxidation, and to determine whether $OSCN^-$ is responsible for sulfhydryl oxidation by the lactoperoxidase, hydrogen peroxide (H_2O_2), SCN^- system.

Materials and Methods

Lactoperoxidase purified from bovine milk (Morrison & Hultquist, 1963) was provided by Dr. M. Morrison. Myeloperoxidase purified from human leukemic granulocytes was provided by Drs. M. Morrison and J. Naskalski. Horseradish peroxidase, albumin[1], β-lactoglobulin, catalase, dithiothreitol, *N*-ethylmaleimide, and Nbs_2 were obtained from Sigma Chemical Co., $K^{35}SCN$ (15.6 Ci/mol) was from Amersham Corp., $NaS^{14}CN$ (10 Ci/mol) was from ICN Pharmaceuticals, Inc., and 4,4′-bis(dimethylamino)thiobenzophenone was from Eastman Kodak Co. Sodium borohydride was used to reduce Nbs_2 to Nbs (Aune & Thomas, 1977).

Synthesis of thiocyanogen, $(SCN)_2$, either unlabeled or radioactively labeled in the sulfur or carbon portion was performed as described previously (Aune et al., 1977). The $OSCN^-$ ion was obtained from $(SCN)_2$ by extracting 1 mL of 2 mM $(SCN)_2$ in carbon tetrachloride (CCl_4) with 10 mL of 0.1 M potassium hydroxide, then neutralizing with 1 M monobasic potassium phosphate. Alternatively, the $OSCN^-$ ion was obtained by incubating 30 nM lactoperoxidase with 1 mM KSCN and 0.2 mM H_2O_2 in 10 mM potassium phosphate buffer, pH 6.6, for 5 min. Oxidation of Nbs to Nbs_2 was used to quantitate $OSCN^-$ (Aune & Thomas, 1977).

Modification of Proteins. Reactions were carried out in 1 mL total volume containing albumin (10 mg; 150 nmol of protein; 60 to 90 nmol of sulfhydryl) or β-lactoglobulin (2.6 mg; 75 nmol of protein; 120 nmol of sulfhydryl). Either $OSCN^-$ or $(SCN)_2$ was added in 10 to 20-nmol increments 1 min apart. Alternatively, the proteins were incubated with 0.1 μM lactoperoxidase and SCN^-, with H_2O_2 added in 10-

†From the St. Jude Children's Research Hospital, Memphis, Tennessee 38101. *Received November 3, 1977.* This work was supported by Research Grant DE 04235, Research Center Grant CA 08040, and Multidisciplinary Training Grant CA 05176 from the National Institutes of Health and by ALSAC.

[1]Abbreviations used: albumin, bovine serum albumin; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, 5-thio-2-nitrobenzoic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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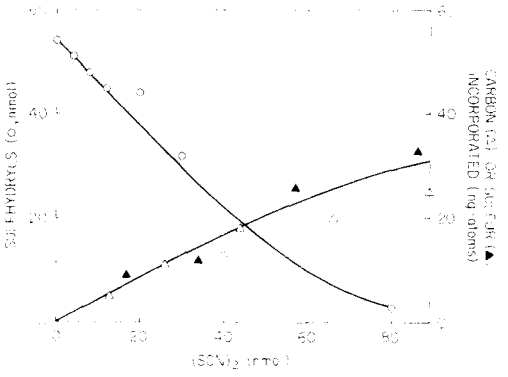


FIGURE 1: Oxidation of albumin sulfhydryls by $(SCN)_2$. Albumin was incubated with the indicated amounts of $(SCN)_2$. Sulfhydryls (○) and incorporation of radioactivity from $(^{35}SCN)_2$ (▲) or $(S^{14}CN)_2$ (△) were measured.

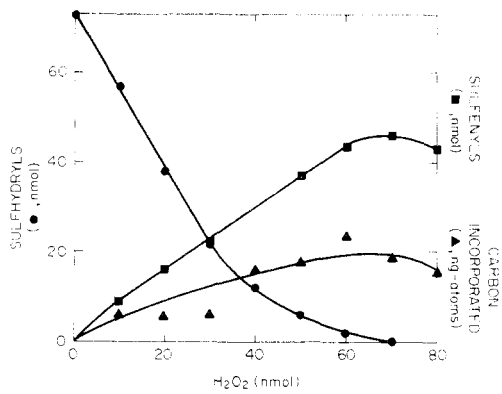


FIGURE 2: Lactoperoxidase-catalyzed oxidation of albumin sulfhydryls. Albumin was incubated with 0.1 nmol of lactoperoxidase, 1 μmol of SCN^-, and the indicated amounts of H_2O_2. Sulfhydryls (●), sulfenyl derivatives (■), and incorporation of radioactivity from $S^{14}CN^-$ (▲) were measured.

or 20-nmol increments. Incubations were continued for 10 min after the last addition.

Incorporation of Radioactivity. Aliquots of 0.1 mL of reaction mixtures were mixed with 1 mL of cold 10% (w/v) trichloroacetic acid and then filtered through nitrocellulose filters (Millipore Corp.). The filters were washed in 5% trichloroacetic acid and then dissolved in scintillation fluid (Bray, 1960), and the radioactivity was determined in a liquid scintillation spectrometer.

Acetone Precipitation. Proteins were washed with acetone prior to determination of sulfhydryls or sulfenyl derivatives. To 1 mL of reaction mixture containing albumin, 9 mL of cold acetone was added. After centrifugation at 6000*g* for 10 min at 4 °C, the pellet was suspended in 3 mL of acetone and centrifuged again. The pellet was dried at 37 °C and then resuspended in 1 mL of water. Reaction mixtures containing β-lactoglobulin were treated similarly but received 7 mL of acetone and 2 mL of 0.1 M lithium chloride.

Sulfhydryl Determination. Resuspended proteins were diluted with 2 mL of a solution containing 0.1 M Tris, 10 mM EDTA, and hydrochloric acid to adjust to pH 8. Solutions were made 0.17 mM in Nbs_2 and 0.7% (w/v) in $NaDodSO_4$ and then incubated at 37 °C for 1 h. Absorbance at 412 nm was measured. Molar extinction coefficients of 13 600 and 230 were assumed for Nbs (Ellman, 1959) and Nbs_2, respectively.

Determination of Sulfenyl Derivatives. Protein sulfenyl derivatives were determined by reaction with 4,4′-bis(dimethylamino)thiobenzophenone to yield the blue-green mixed disulfide derivative (Jirousek & Soodak, 1974). Resuspended albumin was diluted with 0.1 mL of 1 M acetate buffer, pH 5.1, 0.01 mL of 10% (w/v) $NaDodSO_4$, and 0.2 mL of 2 mM 4,4′-bis(dimethylamino)thiobenzophenone in dimethyl sulfoxide and then incubated 15 min at 0 °C. Resuspended β-lactoglobulin was treated similarly but received 0.05 mL of 10% $NaDodSO_4$ and was incubated at 37 °C. The mixtures were passed over Sephadex G-25 columns (1.5 × 40 cm) equilibrated and eluted with 0.1 M acetate buffer, pH 5.1, and the blue-green product was collected. After chromatography, 0.05 mL of 10% $NaDodSO_4$ was added to fractions containing β-lactoglobulin. Absorbance at 630 nm was measured and a molar extinction coefficient of 70 720 was assumed (Jirousek & Soodak, 1974).

Results

Sulfhydryl Oxidation per Mol of $(SCN)_2$, $OSCN^-$, or H_2O_2. Figure 1 shows that about 1 mol of albumin sulfhydryls was oxidized per mol of $(SCN)_2$. Also, the sulfur and carbon portions of $(SCN)_2$ were incorporated in a 1:1 ratio. In other experiments, about 1 mol of sulfhydryls was oxidized per mol of $OSCN^-$, and the sulfur and carbon portions of $OSCN^-$ were incorporated into albumin.

Figure 2 shows that identical oxidation of albumin sulfhydryls was obtained with the lactoperoxidase system. About 1 mol of sulfhydryls was oxidized per mol of H_2O_2. The carbon portion of SCN^- was incorporated into albumin. Equal incorporation of sulfur was observed in other experiments. Sulfhydryl oxidation was accompanied by formation of sulfenyl derivatives. These results were consistent with the lactoperoxidase-catalyzed oxidation of SCN^- to yield 1 mol of $(SCN)_2$ or $OSCN^-$ per mol of H_2O_2. Oxidation of 1 mol of sulfhydryls per mol of $(SCN)_2$ or $OSCN^-$ yielded a sulfenyl derivative, which contained the intact SCN moiety.

At high sulfhydryl concentrations, slightly more than 1 mol of sulfhydryls was oxidized per mol of H_2O_2. Also, the yield of sulfenyl derivatives was lower than the amount of oxidized sulfhydryls. Therefore, a small portion of sulfhydryls was oxidized to another form, possibly a disulfide derivative.

Properties of the Bound SCN Moiety. Further evidence was obtained that protein sulfhydryls were involved in the formation of sulfenyl derivatives and the incorporation of SCN^-. First, the highest yields of sulfenyl derivatives and of bound SCN were obtained when all the sulfhydryls were oxidized. Second, the SCN moiety was released upon reduction of the sulfenyl derivatives with sulfhydryl compounds such as dithiothreitol, or with 4,4′-bis(dimethylamino)thiobenzophenone. Third, blocking of sulfhydryls by reaction with *N*-ethylmaleimide prevented the formation of sulfenyl derivatives and prevented the incorporation of SCN^- into a form removed by reducing agents. The ability of these agents to displace both sulfur and carbon confirmed that SCN^- was incorporated into a sulfenyl thiocyanate derivative, R–S–SCN, rather than into an alkyl or aryl thiocyanate, R–SCN.

Sulfenyl compounds of the form R–S–X undergo exchange reactions (Kharasch, 1961), where Y^- represents the exchanging nucleophile.

$$R\text{–}S\text{–}X + Y^- \rightleftharpoons R\text{–}S\text{–}Y + X^-$$

Consistent with this equation, nonradioactive SCN^- could displace the bound radioactive SCN moiety, and vice versa. Exchange was complete within the shortest time interval obtained using the filtration procedure. Similarly, the addition of 0.1 M cyanide (CN^-), sulfite (SO_3^{2-}), or iodide (I^-) caused

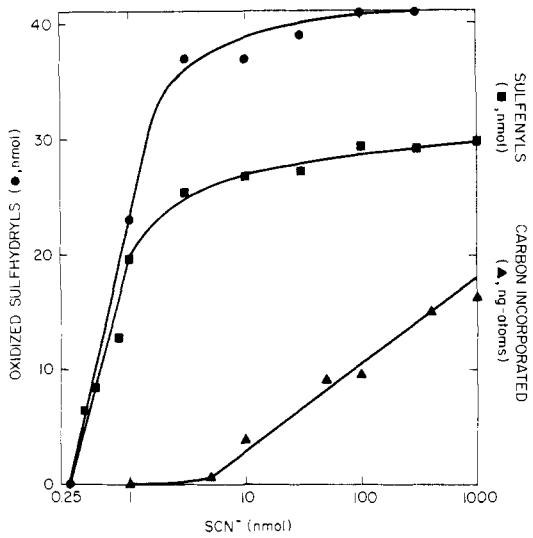


FIGURE 3: Effect of SCN^- concentration on lactoperoxidase-catalyzed oxidation of albumin sulfhydryls. Albumin was incubated with 0.1 nmol of lactoperoxidase, 40 nmol of H_2O_2, and the indicated amounts of SCN^-. The change in the amount of sulfhydryls (●), the formation of sulfenyl derivatives (■), and the incorporation of radioactivity from $S^{14}CN^-$ (▲) were measured.

quantitative release of bound ^{35}SCN or $S^{14}CN$. An equilibrium constant of 0.03 was obtained for displacement of SCN by I^-. A number of other anions, including cyanate (OCN^-), sulfate (SO_4^{2-}), fluoride (F^-), chloride (Cl^-), bromide (Br^-), nitrate (NO_3^-) and acetate did not cause release of bound SCN. This specificity indicated that absorption of SCN^- to a modified form of the protein could not account for incorporation of SCN^-. Instead, these results were consistent with exchange of certain anions with the bound SCN moiety of the sulfenyl thiocyanate derivative.

Sulfhydryl Oxidation vs. *SCN^- Concentration.* Figure 3 shows oxidation of albumin sulfhydryls by the lactoperoxidase system with a constant amount of H_2O_2 and varying amounts of SCN^-. Loss of sulfhydryls was equal to H_2O_2 at SCN^- concentrations as low as 3 nmol per 1 mL of reaction mixture. This concentration of SCN^- was more than an order of magnitude lower than the concentration of H_2O_2 or of oxidized sulfhydryls. Therefore, at low SCN^- concentrations, each SCN^- ion participated in the oxidation of at least 10 sulfhydryls. This ratio of sulfhydryls oxidized per SCN^- ion could be increased by adding more H_2O_2.

Figure 3 also shows that sulfenyl derivatives were obtained in amounts slightly less than the amount of oxidized sulfhydryls. Therefore, about 10 sulfenyl derivatives could be formed per SCN^- ion. As the SCN^- concentration was increased, the loss of sulfhydryls and the yield of sulfenyl derivatives remained constant, but incorporation of the carbon portion of SCN^- increased. In other experiments, identical incorporation of sulfur was observed. As measured with a SCN^--specific electrode (Orion Research, Inc.), the loss of SCN^- from solution was similar to the incorporation of SCN^- shown in Figure 3. The bound $S^{14}CN$ moiety was released upon addition of dithiothreitol or excess nonradioactive SCN^-.

Results shown in Figure 3 indicated the presence of two sulfenyl derivatives, one of which contained the bound SCN moiety and one of which did not. The following experiments were undertaken to determine the relationship between the two derivatives. First, at a high and a low concentration of SCN^-, equal amounts of H_2O_2 were added resulting in production of nearly equal amounts of sulfenyl derivatives, but different amounts of bound SCN. Dialysis of these samples resulted in complete removal of both free SCN^- and bound SCN with no loss of sulfenyl derivatives. Second, varying amounts of $S^{14}CN^-$ were added to the dialyzed proteins. Incorporation of $S^{14}CN^-$ was obtained, and the amount of incorporation was about the same as shown in Figure 3. Therefore, the two sulfenyl derivatives were in equilibrium, and the amount of bound SCN depended on the free SCN^- concentration. An equilibrium constant of 0.3 mM was calculated for the release of SCN^-, assuming that the amount of sulfenyl derivatives equaled the sum of sulfenyl thiocyanate and sulfenic acid.

Results similar to those in Figure 3 were obtained with the myeloperoxidase, H_2O_2, SCN^- system. In contrast, no sulfhydryl oxidation, formation of sulfenyl derivatives, or incorporation of SCN^- was obtained with horseradish peroxidase. Horseradish peroxidase does not catalyze the oxidation of SCN^- (Sorbo & Ljunggren, 1958).

Lower Limit of SCN^- Concentration for Sulfhydryl Oxidation. As shown in Figure 3, when the initial SCN^- concentration was in the range of 0.3 to 3 nmol per 1 mL of reaction mixture, the amount of oxidized sulfhydryls was lower than the amount of H_2O_2 added. When the amount of SCN^- was less than 0.3 nmol, no oxidation of sulfhydryls was obtained. Increasing the lactoperoxidase concentration 10-fold or extending the incubation to 4 h did not result in increased oxidation of sulfhydryls. However, when 1 μmol of SCN^- was added after a 1-h incubation with these low SCN^- concentrations, sulfhydryl oxidation resumed and the amount of sulfhydryls oxidized was equal to the amount of H_2O_2 added before the 1 h incubation. Therefore, the inability to oxidize sulfhydryls at low SCN^- concentrations was not due to irreversible inactivation of lactoperoxidase or to a competing reaction that consumed H_2O_2.

The limitation to sulfhydryl oxidation was related to the conversion of lactoperoxidase to the compound II form. Formation of lactoperoxidase compound II was indicated by the diminution and shift in the Soret band from 412 to 430 nm and the appearance of increased absorbance at 538 and 570 nm. When the initial KSCN concentration was less than 3 μM, lactoperoxidase was converted to the compound II form within 15 s from the time of addition of H_2O_2. At higher KSCN concentrations, compound II was not observed. The concentration of SCN^- required to prevent formation of compound II was independent of lactoperoxidase concentration in the range of 0.1 to 1 μM, and independent of albumin concentration in the range from 0.1 to 25 mg/mL.

Oxidation of β-Lactoglobulin Sulfhydryls. Protein sulfhydryls were not all equally susceptible to oxidation. As shown in Figure 4, about 1 mol of sulfhydryls per mol of β-lactoglobulin was not oxidized. A portion of the sulfhydryls of β-lactoglobulin was oxidized and oxidation of this class of sulfhydryls was qualitatively identical with that obtained with albumin. Incorporation of SCN^- and formation of sulfenyl derivatives paralleled sulfhydryl oxidation. However, sulfhydryl oxidation was not proportional to H_2O_2. Similar results were obtained with $(SCN)_2$ or $OSCN^-$. Therefore, the limited oxidation of β-lactoglobulin sulfhydryls was not due to limited oxidation of SCN^- by lactoperoxidase.

Accumulation of $OSCN^-$ in the Presence of Proteins. Unreacted $OSCN^-$ was detected when $OSCN^-$ was added to proteins, or when lactoperoxidase-catalyzed oxidation of SCN^- was carried out in the presence of proteins. The unreacted $OSCN^-$ was quantitated by measuring the oxidation of Nbs to Nbs_2. In the absence of denaturing agents, the proteins reacted very slowly with Nbs_2, so that Nbs oxidation could be measured in the presence of the proteins. On the other

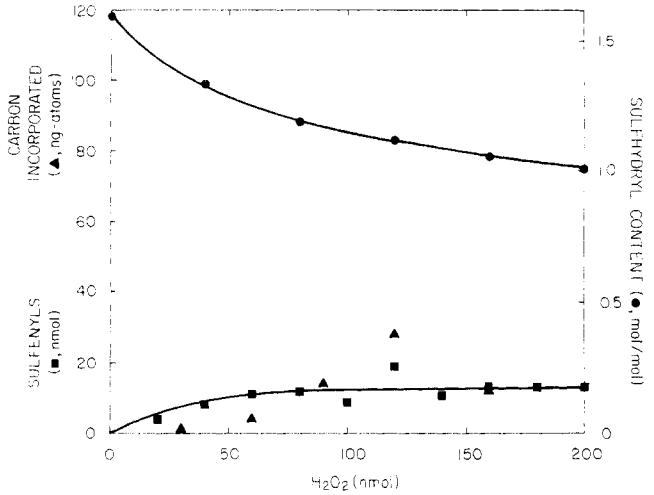


FIGURE 4: Lactoperoxidase-catalyzed oxidation of β-lactoglobulin sulfhydryls. β-Lactoglobulin was incubated with 0.1 nmol of lactoperoxidase, 1 μmol of SCN^-, and the indicated amounts of H_2O_2. Sulfhydryls (●), sulfenyls (■), and incorporation of radioactivity from $S^{14}CN^-$ (▲) were measured.

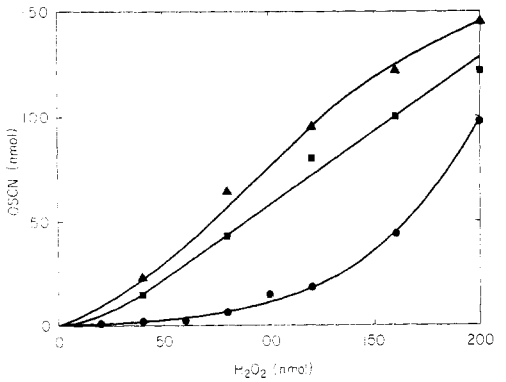


FIGURE 5: Accumulation of $OSCN^-$. β-Lactoglobulin (▲), albumin reacted with *N*-ethylmaleimide (■), or albumin (●) were incubated 15 min at 25 °C in 1 mL of total volume with 0.1 nmol of lactoperoxidase, 1 μmol of SCN^-, and the indicated amounts of H_2O_2. Unreacted $OSCN^-$ was measured.

hand, determinations of protein sulfhydryls were carried out after precipitation of the proteins with acetone so as to remove any unreacted $OSCN^-$. This procedure was essential for the determination of β-lactoglobulin sulfhydryls because excess $OSCN^-$ oxidized the Nbs obtained by reaction of Nbs_2 with sulfhydryls.

Figure 5 shows that in the presence of β-lactoglobulin, $OSCN^-$ was observed at all H_2O_2 concentrations. Adding catalase prior to adding Nbs had no effect on the subsequent oxidation of Nbs. Therefore, Nbs was oxidized by $OSCN^-$, and not by unreacted H_2O_2. The yield of $OSCN^-$ was almost 1 mol per mol of H_2O_2. Therefore, very little of the $OSCN^-$ was consumed by reaction with β-lactoglobulin. Much different results were obtained with albumin. Accumulation of $OSCN^-$ was observed only when the amount of H_2O_2 exceeded the amount of albumin sulfhydryls. When the albumin sulfhydryls were blocked by reaction with *N*-ethylmaleimide, $OSCN^-$ was observed at lower H_2O_2 concentrations. Therefore, the SCN^- oxidation products were consumed by reaction with albumin sulfhydryls. When all the sulfhydryls were oxidized, $OSCN^-$ accumulated rather than reacting with other functional groups of albumin.

When the amount of H_2O_2 added was less than the amount required to oxidize all the albumin sulfhydryls, the sulfenyl

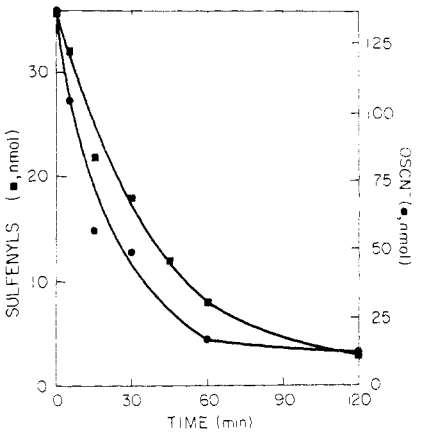


FIGURE 6: Oxidation of albumin sulfenyl derivatives. Albumin was incubated with 0.1 nmol of lactoperoxidase and 1 μmol of SCN^- with 0.2 μmol of H_2O_2 added in 20-nmol increments. Following the last addition of H_2O_2, the incubation was continued and sulfenyls (■) and $OSCN^-$ (●) were measured at the indicated times.

derivatives were stable. Also, when albumin was precipitated with acetone to remove excess $OSCN^-$ and was then suspended in buffer, the sulfenyl derivatives were stable. On the other hand, Figure 6 shows that albumin sulfenyl derivatives were lost during a long-term incubation with excess $OSCN^-$. About 1 to 2 h of incubation were required for complete loss of the sulfenyl derivatives. Figure 6 also shows that the disappearance of $OSCN^-$ paralleled the loss of sulfenyl derivatives. In the absence of albumin and starting with a $OSCN^-$ concentration of 150 μM, only about 5% of the $OSCN^-$ decomposed during a 2-h incubation. Therefore, the rate of disappearance of $OSCN^-$ was greatly increased in the presence of albumin. In contrast, the rate of disappearance of $OSCN^-$ was not increased in the presence of β-lactoglobulin.

These results suggested that excess $OSCN^-$ was consumed in a slow reaction that resulted in oxidation of the albumin sulfenyl derivatives. The amount of $OSCN^-$ that was consumed was larger than the amount of sulfenyl derivatives. However, there was no evidence for a slow reaction with $OSCN^-$ with other functional groups of the protein. The slow disappearance of $OSCN^-$ was not accompanied by an increase in the incorporation of $OSCN^-$ into derivatives of aromatic amino acid residues.

Discussion

Identical oxidation of protein sulfhydryls is obtained with $(SCN)_2$, $OSCN^-$ or the lactoperoxidase, H_2O_2, SCN^- system. The chemical species responsible for sulfhydryl oxidation by the lactoperoxidase system may be $(SCN)_2$, $OSCN^-$, or hypothiocyanous acid, HOSCN. Each of these species has the same number of oxidizing equivalents, so that the same stoichiometry and the same derivatives of sulfhydryls would be obtained with each. The oxidation of SCN^- is followed by oxidation of albumin sulfhydryls to the sulfenyl thiocyanate derivative, as illustrated with $OSCN^-$.

$$SCN^- + H_2O_2 \xrightarrow{\text{lactoperoxidase}} OSCN^- + H_2O$$

$$OSCN^- + \text{protein-SH} \rightarrow \text{protein-S-SCN} + OH^-$$

Sulfhydryl compounds are known to react with $(SCN)_2$ to yield sulfenyl thiocyanates (Bacon, 1961). Formation of sulfenyl thiocyanates is usually followed by disulfide formation.

$$R\text{-}S\text{-}SCN + R\text{-}SH \rightarrow R\text{-}S\text{-}S\text{-}R + SCN^- + H^+$$

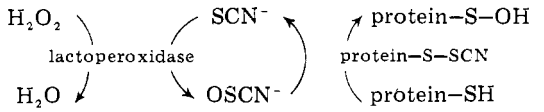
However, sulfenyl derivatives of many proteins are sterically restrained from forming inter- or intramolecular disulfide bonds (Allison, 1976).

The albumin sulfenyl thiocyanate derivative is in equilibrium with sulfenic acid and free SCN^-.

$$\text{protein-S-SCN} + H_2O \rightleftarrows \text{protein-S-OH} + SCN^- + H^+$$

Reversible hydrolysis accounts for the loss of the bound SCN moiety upon dialysis without loss of sulfenyl derivatives, and for the incorporation of SCN^- into sulfenyl thiocyanate as the SCN^- concentration is raised.

Release of SCN^- from sulfenyl thiocyanate is favored at low SCN^- concentrations. When SCN^- is released, it can be reoxidized and participate in the oxidation of another sulfhydryl.



As illustrated with $OSCN^-$, the oxidation of sulfhydryls to sulfenic acids does not consume SCN^-. Therefore, the amount of sulfhydryls oxidized does not depend on the amount of SCN^-.

Oxidation of protein sulfhydryls by the lactoperoxidase, H_2O_2, SCN^- system is analogous to oxidation by the peroxidase, H_2O_2, I^- system (Thomas & Aune, 1977). The oxidation of albumin sulfhydryls is complete at low I^- or SCN^- concentrations, as a result of the turnover of I^- or SCN^-. However, sulfhydryl oxidation is not entirely independent of I^- or SCN^- concentrations. Depletion of I^- and competition by other peroxidase substrates limit sulfhydryl oxidation at low I^- concentrations. Similarly, depletion of SCN^- could result from accumulation of unreacted $OSCN^-$, or incorporation of SCN^- into derivatives of aromatic amino acid residues (Aune et al., 1977). Competition by other peroxidase substrates is indicated by the conversion of the peroxidases to their compound II forms (Thomas & Aune, 1977).

Both the sulfenyl thiocyanate and sulfenyl iodide derivatives of albumin undergo reversible hydrolysis (Thomas & Aune, 1977). Also, the two derivatives are interconvertible because I^- exchanges with the bound SCN moiety. The relative ability of anions to exchange with bound SCN appears related to their "softness". Soft Lewis species have large atomic radii, low effective nuclear charge, and high polarizability (Ho, 1975). Soft Lewis acids form strong bonds with soft Lewis bases, whereas a hard-soft combination is destabilized. The order of decreasing softness of $CN^- > I^- > SO_3^{2-} > SCN^- > Br^- > Cl^- > F^-$ > acetate was determined in an experimental system, although factors other than softness can influence reactivity in a particular reaction (Ho, 1975). This order is generally consistent with the relative ability of anions to exchange with albumin sulfenyl thiocyanate. However, a high ratio of I^- to SCN^- is required to displace the bound SCN moiety. It was reported that SCN^- displaces I^- from β-lactoglobulin sulfenyl iodide (Cunningham & Neunke, 1960; Cunningham, 1964). Exchange of SCN^- for I^- was proposed and an equilibrium constant of 10.5 was calculated. The reciprocal of the equilibrium constant for displacement of SCN^- by I^- from albumin sulfenyl thiocyanate gives a similar value of 33.

The oxidation of sulfhydryls by $(SCN)_2$ is analogous to oxidation by iodine, I_2 (Thomas & Aune, 1977). However, I_2 oxidizes all the sulfhydryls of β-lactoglobulin, but $(SCN)_2$ does not. Although $(SCN)_2$ is a better oxidant than I_2 (Walden & Audrieth, 1928), $(SCN)_2$ or its hydrolysis products may be too large or too polar to have access to all the sulfhydryls of β-lactoglobulin.

The accumulation of unreacted $OSCN^-$ in the presence of proteins indicates that $OSCN^-$ is poorly reactive toward β-lactoglobulin sulfhydryls and other functional groups of proteins. Rather than reacting with other functional groups of albumin, $OSCN^-$ slowly oxidizes the sulfenyl derivatives. About 3 to 4 $OSCN^-$ ions are consumed per sulfenyl derivative. The oxidation of a sulfenyl derivative to a sulfonic acid would require 4 oxidizing equivalents, or only 2 $OSCN^-$ ions. These results may indicate a more complex mechanism than direct oxidation of each sulfenyl derivative by 2 $OSCN^-$ ions.

Although $OSCN^-$ reacts only with albumin sulfhydryls, incubation of albumin with the lactoperoxidase, H_2O_2, SCN^- system results in modification of tyrosine, tryptophan, and histidine residues (Aune et al., 1977). Similar modification is obtained by adding $(SCN)_2$ in CCl_4 to aqueous solutions of albumin. In contrast, $OSCN^-$ does not react with tyrosine or tryptophan, although it does react with histidine at high histidine concentrations (Aune & Thomas, unpublished results). These observations suggest that lactoperoxidase-catalyzed oxidation of SCN^- yields $(SCN)_2$ and that $OSCN^-$ is obtained by hydrolysis of $(SCN)_2$.

On the other hand, hydrolysis of $(SCN)_2$ at neutral pH does not yield a stable solution of $OSCN^-$. The $OSCN^-$ ion can be obtained from $(SCN)_2$ by extracting the $(SCN)_2$ solution with dilute base, then neutralizing (Hoogendoorn et al., 1977; Aune & Thomas, 1977). It was proposed that hydrolysis of $(SCN)_2$ at neutral pH yields HOSCN, and that HOSCN is much less stable than $OSCN^-$ (Hoogendoorn et al., 1977). This proposal implies that lactoperoxidase catalyzes the oxidation of SCN^- directly to $OSCN^-$, and that $(SCN)_2$ or HOSCN are not intermediates.

One explanation that could resolve this apparent contradiction would be that $OSCN^-$ is the major product of SCN^- oxidation, and that $(SCN)_2$ or HOSCN are minor products. The yield of $OSCN^-$ is somewhat less than 1 mol per mol of H_2O_2, particularly at higher H_2O_2 concentrations (Hoogendoorn et al., 1977; Aune & Thomas, 1977), which could indicate rapid decomposition of a small amount of $(SCN)_2$ or HOSCN. The chemical species which modifies tyrosine and tryptophan may be a decomposition product of $(SCN)_2$ or HOSCN. For example, cyanogen thiocyanate, NC–SCN, may be formed during the decomposition of $(SCN)_2$ (Wilson & Harris, 1961; Hughes, 1975). The derivatives of tyrosine and tryptophan contain the carbon portion of the SCN moiety, but do not contain the sulfur portion (Aune et al., 1977). Also, the yield of these derivatives is very low. These results would be consistent with the involvement of NC–SCN, or a similar reactive decomposition product.

Oxidation of SCN^- to $OSCN^-$ can account for sulfhydryl oxidation and modification of histidine by the lactoperoxidase system. The oxidation of albumin sulfenyl derivatives clearly involved $OSCN^-$, and not any short-lived intermediates in the oxidation of SCN^-; oxidation of sulfenyl derivatives was slow and occurred after the oxidation of SCN^- was complete.

Peroxidase-catalyzed oxidation of sulfhydryls mediated by products of SCN^- oxidation may account for the antimicrobial and antithyroid activities of SCN^-. Sulfhydryl groups that are not sterically hindered are oxidized to disulfides. Sulfhydryls of at least two proteins are oxidized to sulfenyl derivatives. In addition, the sulfenyl derivatives can be slowly oxidized by $OSCN^-$. The poor reactivity of $OSCN^-$ toward other functional groups and the poor reactivity toward sulfhydryls of

certain proteins could make the antibiological activities of SCN^- highly specific.

Acknowledgments

We thank Drs. M. Morrison and G. Schonbaum for helpful discussions and Ms. Kate Pera for technical assistance.

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Mechanism of Inactivation of Ornithine Decarboxylase by α-Methylornithine†

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ABSTRACT: Ornithine decarboxylase from *Lactobacillus* 30a is gradually inactivated by treatment with α-methylornithine, but activity is restored by treatment of the inactivated enzyme with pyridoxal phosphate. Inactivation of the enzyme is associated with formation of pyridoxamine phosphate and 5-amino-2-pentanone. α-Methylornithine is decarboxylated by the enzyme about 6000 times more slowly than is ornithine under the same conditions. These observations provide an explanation for the previously observed inhibition of ornithine decarboxylase by α-methylornithine [M. M. Adbel-Monem, N. E. Newton, and C. E. Weeks (1974), *J. Med. Chem. 17,* 447]: α-Methylornithine undergoes a decarboxylation-dependent transamination as a result of incorrect protonation of the quinoid intermediate which is formed by decarboxylation of the enzyme-bound pyridoxal phosphate–substrate Schiff base. This protonation produces inactive enzyme. Decarboxylation of ornithine by this enzyme produces a small amount of 4-aminobutanal, presumably also by decarboxylation-dependent transamination.

Ornithine decarboxylase (EC 4.1.1.7) catalyzes the decarboxylation of ornithine to form putrescine. This is the first step in polyamine synthesis, and it is ordinarily rate determining. In mammalian systems ornithine decarboxylase appears to have an important regulatory function (Russell, 1970), and the enzyme is turned over very rapidly (Russell and Snyder, 1969; Jänne and Raina, 1969). The level of ornithine decarboxylase increases during rapid cell growth (Russell, 1970), although the significance of this phenomenon is not understood. The enzymes from rat liver (Ono et al., 1972; Friedman et al., 1972; Heller et al., 1975) and from rat prostate (Jänne and Williams-Ashman, 1971) have been purified, but the levels of the enzyme in most mammalian tissues are so low that little work with the purified enzyme has been reported. The mam-

† From the Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received October 7, 1977.* This work was supported by Grant NS-07657 from the National Institutes of Health.

0006-2960/78/0417-1010$01.00/0