Oxidative Cleavage of Tryptophanyl Peptide Bonds during Chemical- and Peroxidase-catalyzed Iodinations*

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SUMMARY

Tryptophanyl peptide bonds are oxidatively cleaved by “active iodine” that is generated with H2O2, iodide, and a peroxidase (donor: H2O2, oxidoreductase, EC 1.11.1.7). Complete oxidation of tryptophan derivatives and peptides to the oxindole occurs within 2 min at pH 3 to 5 with either lactoperoxidase or horseradish peroxidase, and a 2.5-fold molar excess of iodide plus H2O2. Under identical conditions, simple tryptophan peptides are cleaved 30 to 40% in 10 min at pH 5.0, and the rate of cleavage is parallel to, but less than, the rate of oxidation. All three constituents (iodide, H2O2, and peroxidase) are required for oxidation and fission to proceed.

Iodinating reagents yield similar results, except that they oxidize tryptophan derivatives and peptides (Z-Trp-X, X-Trp-X) over the pH range of 2 to 11. Fission of the tryptophan peptide proceeds in acid media with a maximum at pH 5.0, and no cleavage occurs in alkaline media. A 2.5-fold molar excess of positive halogen reagents, ICl, iodide oxidized with chloramine-T, and N-iodosuccinimide completely oxidize tryptophan peptides at pH 5.0 within 1 min, whereas oxidation by I2 and I3 is 2 and 8 times slower, respectively. Chloramine-T per se oxidizes tryptophan peptides at about the same rate as I2, below pH 6.5, but is unreactive above this pH. All of the iodinating reagents induce complete oxidation within 1 min at pH 9.5. Thirty to forty percent of a tryptophan peptide (e.g., Z-Trp-Gly or Z-Trp-Leu) is cleaved in 10 min at pH 5 by a 2.5- to 10-fold molar excess of all the reagents, except for I2 which promotes half as much cleavage. With all of the iodinating reagents, the rate of cleavage is parallel to, but less than, the rate of oxidation. Iodometric titrations and pH profiles suggest that the oxidation and oxidative cleavage of tryptophan peptides during iodination proceeds via the mechanism proposed for brominating agents (Patchornik, A., Lawson, W. B., Gross, E., and Witkop, B. (1960) J. Amer. Chem. Soc. 82, 5923). Two equivalents of I+ or I2 react with proteins, substitution and oxidation reactions occur with amino acid side chains. Tyrosine and histidine undergo substitution to form mono- and diiodinated derivatives, while oxidative transformations occur with tryptophan, methionine, cysteine, cystine, and possibly serine and threonine residues (1-11). Furthermore, it has been discovered that N-iodosuccinimide iodinates and oxidatively cleaves tyrosyl peptides (12) in a manner identical to the oxidative fission promoted by N-bromosuccinimide (13), which also cleaves tryptophan and histidine peptides (14, 15). In a recent report (16) from this laboratory, we found that several different iodinating agents (ICl, chloramine-T-KI, I2, I3-, peroxidase-catalyzed iodination) oxidize tryptophan to the oxindole and that this oxidation results in significant fission of tryptophan peptide bonds at pH 4 to 5. The oxidation and oxidative cleavage of tryptophan peptides by various chemical and peroxidase-catalyzed iodination procedures have been more thoroughly studied and are the subject of this communication.

EXPERIMENTAL PROCEDURE

Materials—All N-benzyloxy carbonyl peptides were obtained from Cyclo Chemical, Los Angeles, Calif. nL-Tryptophan and tryptophan tripeptides were products of Schwarz-Mann and β-(indole-3)-propionic acid was supplied by Calbiochem. N-Iodosuccinimide and 2-oxindole (indolin-2-one) were purchased from K & K Laboratories, Hollywood, Calif. N-Bromosuccinimide and chloramine-T were products of Eastman Organic Chemicals. Solutions of chloramine-T, N-iodosuccinimide, and N-bromosuccinimide were freshly prepared before use. Iodine monochloride (20 mm in 1 M HCl and 2 M NaCl) was prepared as described by Izzo et al. (17); preparation of ICl requires KI rather than KCl as inadvertently printed in the reference. A stock solution of 15 mm I2 was prepared in ethanol, and I2· consisted of 15 mm I2 dissolved in 45 mm KI. Chloramine-T is the sodium salt of N-chloro-p-toluenesulfonamide and generates sodium hypochlorite in water (18). Horseradish peroxidase (A403:A250 = 1.96) was purchased from Worthington Biochemical Corp., Free-
hold, N.J. Lactoperoxidase (A414A294 = 0.59) was a product of Sigma Chemical Co., St. Louis, Mo. Stock solutions of the peroxidases (1 mg per ml) were prepared in water and stored frozen. They were freshly diluted as required. Amberlite XAD-2, a co-polymer of styrene and divinylbenzene was obtained from Mallinckrodt Chemical Works, St. Louis, Mo.

Cleavage of Peptides—Oxidative cleavage of peptides was determined by measuring the release of free amino acid with ninhydrin (18). Glycine was used as the standard when Z-Trp-Gly cleavage was measured and leucine for Z-Trp-Leu. Glycine was confirmed in reaction mixtures by thin layer chromatography on silice gel with 1-butanol-glacial acetic acid-water (4:1:1). The Rf of glycine is 0.16 and of Z-Trp-Gly is 0.81.

Spectral Analysis and Triptophan Oxidation—Spectra were obtained with a Perkin-Elmer 202 dual beam spectrophotometer in 1-cm quartz cuvettes. Oxidative conversion of tryptophan to the oxindole was determined by the disappearance of absorbance at 280 nm (14, 15) in a Gilford model 240 spectrophotometer. When the molar disappearance of tryptophan was calculated, the decrease in absorbance was multiplied by 1.31 according to Patichnik et al. (15) to correct for the residual absorbance by the oxindole.

Titrimetric Analysis of Extent of Reaction of Iodinating Agents with Peptides and Triptophan Derivatives—The reaction mixture, in a total volume of 30 ml, contained 10 ml of 0.3 M acetate buffer (pH 5.0), 3 ml of 0.01 M meproporphyrin acid dissolved in 95% ethanol, and 150 µmoles of iodinating agent. When other triptophan derivatives were used, they were dissolved in water if possible. The iodinating agent was added last to initiate the reaction. After thorough mixing, 5 ml were withdrawn at 2, 5, 10, 30, and 60 min and immediately pipetted into a solution containing 1 ml of 2 M HCl and 1 ml of 10% (w/v) KI. This procedure quenches the reaction by forming I2 with the residual, unreacted iodinating agent or chloramine-T, and is titrated with 0.01 M NaSO4 using starch as an indicator. A control reaction mixture containing everything but the peptide was treated in an identical manner to determine the amount of iodinating agent initially present. The difference in titrations between the control and the complete reaction mixtures represents the amount of iodinating agent consumed by the tryptophan compound.

RESULTS

Oxidation and Oxidative Cleavage of Z-Trp-Gly by Iodination with Peroxidases—The ultraviolet absorption spectra of Z-Trp-Gly before and after oxidation with active iodine generated by H2O2 and horseradish or lactoperoxidase are shown in Fig. 1. The peroxidases induce a 60 to 70% reduction in absorbance within 10 min at 280 nm with a 2.5 molar excess of H2O2 and KI. The spectrum of the oxidized Z-Trp-Gly is nearly identical to that reported for the oxindole derivative of Z-Trp-Gly (15) and Z-Trp-Gly (16). If enzyme, iodide, or H2O2 are omitted from the reaction mixture, no alteration in the spectrum of the peptide occurs. Lactoperoxidase activity was destroyed by heating at 100° for 5 min, but horseradish peroxidase activity was unpaired. No absorbance peaks for I2 at 287 or 350 nm are seen in the mixtures containing iodide, H2O2, and peroxidase (Fig. 1) because excess thiosulfate was added to reduce the yellow color of I2 and I2− which would accumulate in the absence of peptide (19).

Lactoperoxidase is 2.5 times more efficient on a weight basis than horseradish peroxidase. The oxidation of Z-Trp-Gly by the peroxidases is identical to that previously reported with chemical iodinating agents (16). Other derivatives that were tried and found to be susceptible to oxidation included Z-Trp, Trp-Gly, m-tryptophan, and indolepropionic acid.

Z-Trp-Gly oxidation as a function of pH is shown at the top of Fig. 2. The maximum rate with horseradish peroxidase is at pH 3 and at pH 5 with lactoperoxidase. These pH profiles are similar to the pH activity curves for iodide oxidation by the peroxidases.

The pH profiles for the peroxidase-catalyzed oxidative fission with H2O2 and iodide are shown at the bottom of Fig. 2. Maximum cleavage proceeds at pH 4 to 5 with lactoperoxidase, identical to the pH optimum for oxidation. With horseradish peroxidase, oxidative fission peaks at pH 4 to 5, out of phase with the tryptophan oxidation pH curve. This difference is probably explained by the fact that the rate of fission is 3 to 4 times faster at pH 5 than at pH 3 (16). Moreover, oxidative fission was measured after 10 min, whereas the oxidation was measured after 1 min.

The kinetics of Z-Trp-Gly oxidation at pH 5 by a 2.5-fold molar excess of H2O2 and KI with lactoperoxidase and horseradish peroxidase is shown in Fig. 3. Lactoperoxidase oxidizes 90 to 95% of the peptide to the oxindole in 2 min, whereas horseradish peroxidase promotes nearly complete oxidation after about 10 min.

The rates of oxidative fission of Z-Trp-Gly by the peroxidases (Fig. 4) are parallel to, but less than, the rates of oxidation. Lactoperoxidase cleaves 37% of the peptide after 10 min. Glycine formation was verified by thin layer chromatography. Thus, under these conditions, peptide bond fission is about one-third as efficient as oxindole formation. Doubling the H2O2 and iodide concentrations increased the cleavage to 50%. Similar results were obtained with Z-Trp-Leu.

Oxidation and Oxidative Cleavage of Triptophan Peptides by ICI, Chloramine-T-KI, Chloramine T, N-Iodosuccinimide, I2, and I−—The effect of pH on the oxidation of Z-Trp-Gly by various iodinating agents and chloramine-T is shown in Fig. 5. N-Iodosuccinimide, ICI, and chloramine-T-KI had nearly identical pH profiles with 90 to 100% oxidation (equivalent to 77% decrease in absorbance) occurring between pH 3 to 5 and 9.5 to 10.5, whereas two-thirds as much oxidation is seen over the pH range 5.5 to 8.5. Chloramine-T probably oxidizes iodide to ICI (16), and between pH 2 and 7.5 the order of addition of chloramine-T...
FIG. 2. Oxidation and oxidative cleavage of Z-Trp-Gly by peroxidases as a function of pH. Each reaction mixture, in a final volume of 3.0 ml, contained 300 μmoles of buffer, 100 μl of 2 mM Z-Trp-Gly in ethanol, 1.5 μmoles of KI, 12.5 μg of horseradish peroxidase (HRP), or 5.0 μg of lactoperoxidase (LP) as indicated. The reactions were initiated with 100 μl of 15 mM H₂O₂ and were performed at room temperature (23°C). When oxidation (upper curves) was measured, the reactions lasted for 1 min and were terminated with 100 μl of 50 mM Na₂S₂O₃ and 50 μl of 6 M HCl before obtaining the absorbance at 280 nm. When oxidative cleavage (lower curves) was measured, the reactions lasted 10 min at 23°C and were terminated with 100 μl of 50 mM Na₂S₂O₃. One milliliter of the oxidative cleavage reaction mixtures was analyzed with ninhydrin and corrected for the small color with ninhydrin elicited by the complete reaction mixtures in which thiosulfate was added before H₂O₂ to prevent cleavage. Each point represents the mean of duplicate analyses that agreed within 5% of each other. Phosphate buffer was employed at pH 4.0, 4.5, 5.0; 0.01 M HCl at pH 3.0, 6.5, 7.5; acetate buffer at pH 2.0.

FIG. 3 (left). Rate of Z-Trp-Gly oxidation by peroxidases. Conditions were the same as described in the upper portion of Fig. 2 for lactoperoxidase (LP) and horseradish peroxidase (HRP) in pH 5.0 acetate buffer. Each time point represents the mean of duplicate analyses which agreed within 5% of each other. Phosphate buffer was employed at pH 3.0, 6.5, 7.5; acetate buffer at pH 4.0, 4.5, 5.0; 0.01 M HCl at pH 2.0.

FIG. 4 (right). Rate of oxidative cleavage of Z-Trp-Gly by peroxidases. Conditions were the same as described for the lower portion of Fig. 2 in pH 5.0 acetate buffer. Each time point represents the mean of duplicate analyses which agreed within 5% of each other. LP, lactoperoxidase; HRP, horseradish peroxidase.

FIG. 5. Oxidation of Z-Trp-Gly by various iodinating agents as a function of pH. Each reaction mixture, in a volume of 3.0 ml, contained 300 μmoles of buffer, 300 μl of 2 mM Z-Trp-Gly in ethanol, and 1.5 μmoles of iodinating agent or chloramine-T. The reaction lasted for 1 min at 23°C and was terminated with 50 μl of 0.1 M Na₂S₂O₃ and 50 μl of 6 M HCl. All points are the mean of duplicate analyses. The same buffers listed in Fig. 2 were employed, along with pH 5.0 Tris-HCl; pH 9.5, 10.5 carbonate. NIS, N-iodosuccinimide; and iodide to the buffered solution of Z-Trp-Gly was not critical. However, if chloramine-T is added to a buffered solution of Z-Trp-Gly and iodide above pH 7.5, diminished oxidation of the peptide is observed at pH 8.5 (dashed line), and no oxidation occurs at pH 9.5 and 10.5. When chloramine-T and iodide are premixed in distilled water and then added to a buffered solution of the peptide at pH 7.5 or greater, the solid line (Fig. 5) was obtained. Chloramine-T optimally oxidizes Z-Trp-Gly at pH 5.0, but is unreactive at pH 6.5 or above (Fig. 5). However, chloramine-T still effectively oxidizes iodide at pH 7.5 and less so at pH 8.5, as indicated by the curves with chloramine-T and KI. I₂ and I₃⁻ have similar pH curves, except that I₃⁻ is less reactive than I₂ below pH 7.5. Very little oxidation with I₃⁻ is seen here in acid media because the reaction time was only one minute. I₂ and I₃⁻ may be converted to a common intermediate in alkali. This intermediate may be IO⁻, but cannot be either IO₃⁻ or IO₄⁻ since these substances do not oxidize Z-Trp-Gly even after 10 min. I₃⁻ dissociates to I₂ and I⁻, although the equilibrium greatly favors I⁻ (19).

The rate of oxidation of Z-Trp-Gly by a 2.5 molar excess of each iodinating agent was measured at pH 5.0 and pH 7.5 (Fig. 6). ICl, chloramine T KI, and N-iodoacetic acid completely oxidize the peptide within 1 min at pH 5.0, whereas chloramine-T and I₂ require 5 and 10 min, respectively. Two-thirds of the peptide is oxidized by I₃⁻ at pH 5.0 after 10 min. A slower rate of oxidation is seen at pH 7.5 with all of the agents, except for I₃⁻, which is more reactive at pH 7.5 than at pH 5.0, and chloramine-T by itself is inactive toward Z-Trp-Gly at pH 7.5. Similar results were observed with other tryptophan compounds, including Z-Trp-Leu, Z-Trp, benzoyl-Trp, indolepropionic acid, Ac-Trp-NH₂, Gly-Trp-Gly, Glu-Trp-Glu, Lys-Trp-Lys, and Leu-Trp-Leu.

1. N. M. Alexander, unpublished observations.
2. The abbreviation used is: Ac-Trp-NH₂, N-acetyltryptophan amide.
As shown in Fig. 7, the rate of oxidative cleavage of Z-Trp-Gly at pH 5.0 by the various iodinating agents is parallel to, but less than, the rate of oxindole formation (Fig. 6), resembling the results obtained with the peroxidases (Figs. 3 and 4). Glycine formation in each reaction mixture was verified by thin layer chromatography. N-Iodosuccinimide, I$_2$, chloramine-T, ICl, and chloramine-T-KI have similar rate curves and promote 27 to 36% oxidative fission of the peptide in 10 min. The extent of cleavage in those reaction mixtures containing chloramine-T is probably underestimated because this agent oxidizes glycine to a form that no longer produces a color with ninhydrin. A 5-fold excess of chloramine-T over glycine destroys about 15% of the amino acid in 10 min. I$_2$ displays a slower rate than the other iodinating agents but cleaves one-fourth of the peptide at 10 min. The rate of cleavage by I$_2$ doubles if the reaction is performed at 37° rather than room temperature. A previous statement (16) that I$_2$ does not oxidatively cleave Z-Trp-Gly was in error because glycine was not detected by thin layer chromatography at that time.

The oxidative cleavage of Z-Trp-Gly by varying concentrations of the iodinating agents and chloramine-T with a fixed amount of Z-Trp-Gly is shown in Fig. 8. Cleavage increases with greater oxidant concentrations and reaches a maximum when the ratio is 2 to 2.5 with N-iodosuccinimide, chloramine-T-KI, and chloramine-T. There is a moderate decrease in cleavage at high concentrations of these agents, probably because they destroy glycine (as indicated above). Oxidative cleavage with I$_2$, ICl, and I$_2$ increases quickly up to 2 to 2.5 moles of oxidant and continues to increase to a lesser extent up to a ratio of 10, except for I$_2$, which declines slightly. Part of the greater cleavage with I$_2$ appears to be a solvent effect, since the halide is dissolved in ethanol and increasing concentrations of ethanol were in those reaction mixtures containing increasing amounts of iodine. This interpretation is supported by the finding that cleavage with a 5-fold molar excess of ICl increased from 37 to 53% when the ethanol content of the reaction mixture was changed from 10 to 30% by volume.

Other experiments in which the ratio of the oxidant to Z-Trp-
excess thiosulfate prior to the addition of 1311C1. This adsorbed activity to the column with a reaction mixture that contained XAD-2, after correct, ion for nonspecific adsorption of radioac-

for 10 min and the unreacted halide was reduced to iodide with

stance that is adsorbed by a column (15

formed. Z-Trp-Gly was incubated with a 5-fold excess of lalIC1

oxindole. To determine whether the reaction of more than 2

moles of ICI with Z-Trp-Gly represented covalent attachment of

1 Br+ atom is incorporated into the indole. Indolepropionic

acid and N-benzoyl tryptophan each reacted with 2 moles of ICI

in 30 min, contained 300 nmoles of pH 5.0 acetate buffer, 300 μl of 2 max Z-Trp-Gly, and the necessary volume of 15 mM iodinating

agent or chloramine T (Chlor. T) to give the ratio of oxidant to

Z-Trp-Gly listed on the abscissa. ICl was neutralized to pH

5.0 with NaOH just before use. The reactions lasted for 10 min

at 23° and were terminated with 50 μl of 0.1 M Na2S2O3. One

milliliter was analyzed with ninhydrin and corrected for any

color elicited with ninhydrin by the complete reaction mixture

in which Na2S2O3 was added prior to the oxidants. Each point

is the mean of duplicate analyses which agreed within 5% of each

other. NIS, N-iodosuccinimide.

Gly was increased, as in Fig. 8, also showed that the oxidation of

Z-Trp-Gly linearly reached a maximum at a ratio of 2 to 2.5, where nearly complete oxidation occurred.

**Stoichiometry and Kinetics of Reaction of Iodinating Agents with**

**Tryptophan, Tyrosine, Histidine, and Methionine Peptides**—Using an iodimetric titration procedure, data on the rate and extent of reaction of the iodinating agents, chloramine-T, and N-bromo-
succinimide with oxidation-sensitive amino acids is presented in

Table I. With the exception of 13, as was found in the control titration mixture.

Two moles of I2 react with each mole of Z-Trp-Gly and

whereas this molar ratio increases to 2.4 with ICI and to 2.9 with

chloramine-T (Table I). The results with chloramine-T may

refect either chlorination or oxidation of Z-Trp-Gly beyond the

oxindole. To determine whether the reaction of more than 2

moles of ICI with Z-Trp-Gly represented covalent attachment of

iodine to the peptide, an experiment with labeled ICl was per-

formed. Z-Trp-Gly was incubated with a 5-fold excess of [13]ICl

for 10 min and the unreacted halide was reduced to iodide with

excess thiosulfate. Radioactivity is incorporated into a sub-

stance that is adsorbed by a column (15 × 25 mm) of Amberlite

XAD-2, after correction for nonspecific adsorption of radioac-

tivity to the column with a reaction mixture that contained

excess thiosulfate prior to the addition of [13]ICl. This adsorbed

substance presumably represents peptide containing covalently

linked iodine, since XAD-2 adsorbs Z-Trp-Gly, but not iodide.

Similar results were obtained with Z-Trp and Z-Trp-Leu with peroxidase-catalyzed iodinations. Further experimentation is required to determine whether iodine is attached to the indole or benzoxycarbonyl moieties of Z-Trp-Gly.

Two moles of I2 or N-iodosuccinimide react with Z-Trp-Gly and

only 0.9 mole of I2 reacts after 30 min. N-Bromosuccinimide

reacts to the extent of 4.1 moles per mole of Z-Trp-Gly in 30 min,

**Table I.**

| Iodinating or oxidizing reagent | Compound | Oxidant consumed |
|---------------------------------|----------|------------------|
| 7 min | 5 min | 10 min | 15 min |
| ICI | Ac-Trp-NH₂ | 1.6 | 1.7 | 1.6 | 2.0 |
| | Chloramine-T-KI | 1.8 | 1.6 | 1.6 | 1.7 |
| | Chloramine-T | 1.9 | 1.9 | 1.9 | 1.8 |
| | I₂ | 1.8 | 1.9 | 2.0 | 2.0 |
| NIS | Ac-Trp-NH₂ | 1.8 | 1.9 | 1.9 | 1.9 |
| NBS | Ac-Trp-NH₂ | 2.8 | 2.8 | 3.0 | 3.2 |
| I₂⁺ | Ac-Trp-NH₂ | 0.1 | 0.2 | 0.3 | 0.6 |
| | ICl | 1.4 | 1.5 | 1.6 | 2.0 |
| | | 1.8 | 1.8 | 2.0 | 2.2 |
| | Chloramine-T | 1.7 | 1.9 | 1.9 | 2.0 |
| | IPA | 1.4 | 1.7 | 2.0 | 2.0 |
| | | 2-Oxindole | 0 | 0 | 0 | 0 |
| | | 2-Oxindole | 0 | 0 | 0 | 0 |
| NBS | 2-Oxindole | 0.3 | 0.7 | 1.2 | — |
| | | Z-Trp-Gly | 1.9 | 2.1 | 2.3 | 2.4 |
| | | Z-Trp-Gly | 1.9 | 1.8 | 1.8 | 1.9 |
| | | Z-Trp-Gly | 2.0 | 2.2 | 2.5 | 2.6 |
| | | Z-Trp-Gly | 1.5 | 1.6 | 1.7 | 1.9 |
| | NBS | Z-Trp-Gly | 2.0 | 2.0 | 2.0 | 2.0 |
| | NBS | Z-Trp-Gly | 2.8 | 3.3 | 3.3 | 4.1 |
| | | Z-Trp-Gly | 0.2 | 0.3 | 0.5 | 0.9 |
| ICl | Z-Trp | 1.9 | 2.1 | 2.2 | 2.4 |
| | Z-Trp | 1.6 | 1.8 | 1.9 | 2.1 |
| | I₂ | Z-Trp | 2.4 | 2.5 | 2.6 | 2.7 |
| | | Z-Trp | 2.4 | 2.5 | 2.6 | 2.7 |
| | I₂⁺ | Z-Trp | 2.8 | 2.9 | 3.0 | — |
| ICl | Z-Tyr-Gly | 0.6 | 0.7 | 1.0 | 1.4 |
| | Z-Tyr-Gly | 2.0 | 2.1 | 2.1 | 2.2 |
| ICl | Z-Met-Gly | 0.9 | 0.9 | 1.0 | 1.1 |
| | Z-Met-Gly | 1.1 | 1.2 | 1.2 | 1.3 |
| ICl | Z-His-Gly | 0 | 0 | 0 | 0 |
| | Z-His-Gly | 0 | 0 | 0.2 | 0.1 |
| I₂⁺ | L-Cysteine | 2.1 | 2.2 | 2.3 | 2.5 |
| | L-Cysteine | 1.8 | 1.9 | 2.0 | 2.0 |

* An insoluble precipitate is formed during the reaction.

* A precipitate of I₂ is formed that dissolves with KI and titrates

as 13, as was found in the control titration mixture.

* Titration aliquot was lost.
which is not surprising in view of the oxidation potential of this 
Br⁺ agent at pH 5 (13).

Free tryptophan reacts rapidly with I₂ or ICl and consumes 2.7 
and 3.0 moles of each oxidant, respectively, after 30 min (Table 
1). Analysis with Amberlite XAD-2 of reaction mixtures con-
taining [¹²⁷I]Cl or [¹²⁷I]₂ and tryptophan (or Ac-Trp-NH₂) indicated 
that very little iodine was covalently linked to tryptophan. It 
thus appears, that with iodination, free tryptophan is oxidized 
beyond the oxindole and explains our earlier finding (16) that 3 eq 
of I⁻ disappeared for every mole of tryptophan.

Additional results in Table 1 demonstrate that Z-Tyr-Gly, 
Z-Met-Gly, and cysteine, but not Z-His-Gly, react rapidly with 
ICI or I₂ at pH 5.0. Free glycine was not detected with ninhy-
drin in those reaction mixtures that contained any of the benzyl-
loxycarbonyl peptides and thus, no peptide bond cleavage occurs. 
Inasmuch as diiodophloretylglycine and Z-Tyr-Gly are cleaved 
by N-iodosuccinimide (12), the failure to detect fission of Z-Tyr-
Gly by ICl was unexpected; however, cleavage of diiodophloretyl-
glycine is promoted by ICl.¹ We may have failed to detect 
cleavage of Z-Tyr-Gly with ICl because a precipitate (Z-I₂Tyr-
Gly and possibly its dieneone lactone) settled out of the reaction 
mixture, thus preventing further reaction with ICl.

Competition experiments at pH 5.0 revealed that no inhibition 
of fission of Z-Trp-Gly occurred in 10 min when equimolar 
amounts of Z-Met-Gly, Z-His-Gly, and Z-Tyr-Gly were mixed 
separately with Z-Trp-Gly and a 5-fold excess of ICI. At pH 
7.5, Z-Tyr-Gly partially inhibited the oxidation of Z-Trp-Gly 
because tyrosine is more effectively iodinated at an alkaline pH. 
Further experimentation is necessary to assess fully the relative 
rates of reaction of the sensitive amino acids with the iodinating 
agents.

**DISCUSSION**

The oxidation and oxidative cleavage of tryptophanyl peptides 
during iodination probably occurs by the mechanism suggested 
for brominating agents (14, 15, 20). The reaction scheme is 
shown in Fig. 9 and is illustrated with Z-Trp-Gly (I). Two 
equivalents of active iodine (I₂ or I⁺) convert tryptophan to the 
oxindole (IV) via oxidative and hydrolytic reactions over a wide 
pH range. At pH 5.0 the oxindole cyclizes to an iminolactone 
(V), which spontaneously hydrolyzes to the lactone (VI) and 
glycine. No significant hydrolysis occurs above pH 7, because 
iminolactone formation is hindered. A third iodine equivalent 
of ICl, but not I₂, becomes covalently linked to Z-trypto-
phan derivatives either on the indole or benzoxycarbonyl 
moieties, a conclusion supported by iodimetric titration data and 
labeling experiments. It would appear that the iodine is bound 
to the benzoxycarbonyl portion because ICl does not iodinate 
2-oxindole, whereas N-bromosuccinimide does (Table 1). 
Moreover, derivatives such as Ac-Trp-NH₂, indolepropionic acid, 
and benzoyltryptophan react with only two equivalents of ICl or I₂ 
(free tryptophan is an exception and has been discussed above). 
These results indicate that the oxidation potential of ICl is less 
than that of the brominating agents, since 3 eq of N-bromo-
succinimide react with either Z-Trp-Gly or Ac-Trp-NH₂ (14, 15, 
20, 21). The lower oxidation potential of iodinating agents is 
also reflected in the finding that histidiyl peptides are oxidatively 
cleaved at pH 5 during bromination (13, 22), but not during 
iodination. In fact, little or no iodination of histidine with ICl 
or I₂ occurs at pH 5.0 in 10 min, although iodoalbinic formation 
proceeds readily at an alkaline pH (7).

Iodinating and brominating agents react similarly toward 
ytrosine, methionine, and cysteine. Some iodinating agents, 
such as N-iodosuccinimide (12) and ICl, or chloramine-T-KI', 
oxidatively cleave diiodotyrosyl and diiodophloretyl peptide 
bonds, whereas, I₂ and I⁺ substitute onto the phenolic ring, but 
do not cleave these peptides. Differences between brominating 
agents in effecting the oxidative cleavage of tyrosyl peptides 
have also been reported (13, 20). Thus, ICl, chloramine-T-KI, 
and N-iodosuccinimide appear to be similar to N-bromosuc-
cinimide in this respect, while I₂ and I⁺ behave more like 2,4,6-
trichloro-4-methylcyclohexadienone (20). Whether the iodin-
ating agents could be employed as selective agents for peptide 
cleavage in the same manner as the brominating agents remains 
to be established. Methionine is oxidized during iodination to 
the sulfoxide, and cysteine oxidation probably ultimately 
proceeds to cysteic acid.

The oxidation and oxidative cleavage of simple tryptophan 
peptides during iodination cannot necessarily be extrapolated to 
larger peptides and proteins that possess secondary structures 
(16, 23). But numerous examples of tryptophan oxidation in 
various proteins have been described. Lysozyme is a noteworthy 
example in which essential tryptophan residues (Nos. 62 and 
108) are readily oxidized at pH 4.7 to 5.5 to the oxindole by a 
small molar excess of I⁻ per mole of enzyme (24, 25). This 
finding indicates that exposed tryptophan residues may be par-
ticularly susceptible to oxidation during iodination. Modifica-
tion of tryptophan residues during iodination in alkaline 
media has also been reported for casein (2), thyroglobulin (2), 
albumin (3), and γ-globulin (4). Thus, the destruction of bi-
ologically active peptides and proteins undergoing chemical or 
peroxidase catalyzed iodination for metabolic studies and radio-
imunoassays (26-32) may be due to the modification of es-
tential tryptophan residues. As previously discussed (1, 16, 23), 
the extent and quality of iodination of proteins is dependent on 
several factors: (a) pH, (b) temperature, (c) iodinating agent, 
(d) concentration of iodinating agent, (e) nature of the protein.

The peroxidase-catalyzed oxidation and oxidative cleavage of 
tryptophan peptides with iodo and H₂O₂ establishes a new 
property for this class of enzymes, but the relevance of this 
phenomenon to biological systems remains to be determined.

A possible, pertinent example is the bactericidal (33, 34) and 
virucidal (35) action of myeloperoxidase or lactoperoxidase 
with iodide and H₂O₂, which could be explained by the oxidation 
of tryptophan residues in the bacterial or viral proteins by peroxi-
dase-generated active iodine. This possibility seems attractive 
since optimum killing activity (33, 34) and tryptophan peptide 
oxidation and cleavage with peroxidase-iodide-H₂O₂ both proceed 
at pH 5.0. However, modification of other amino acid residues
(e.g. tyrosine (33), methionine, cysteine, and cystine) on the bacterial or viral surfaces could also account for the killing process. Parenthetically, oxidation of tryptophan would be expected by the 'active chlorine' generated with a myeloperoxidase-chloride-H\textsubscript{2}O\textsubscript{2} mixture that is employed in bactericidal studies (33, 36).

A peroxidase in the thyroid gland oxidizes iodide with H\textsubscript{2}O\textsubscript{2} for iodotyrosine and iodothyronine synthesis (37). Results with a partially purified beef thyroid peroxidase preparation indicate that it oxidizes Z-Trp-Gly to the oxindole in the same manner as lactoperoxidase.1 A priori, no relationship between tryptophan oxidation and iodotyrosine or thyroid hormone synthesis is obvious at this time. However, it is conceivable that the peroxidase-catalyzed cleavage of tryptophanyl (and possibly diiodotyrosyl) peptide bonds might assist in the intracellular hydrolysis of thyroglobulin, an essential step for the release of free thyroid hormones from the thyroid into the blood plasma for transport to the peripheral tissues. This hydrolysis is primarily accomplished by intracellular proteases and peptidases, but peroxidase may assist this process by forming smaller peptides from thyroglobulin or thyroglobulin fragments, thus, enhancing digestion by proteolytic enzymes.

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