OXIDATION AND OXIDATIVE CLEAVAGE OF TRYPTOPHANYL PEPTIDE BONDS DURING IODINATION

Nicholas M. Alexander

Department of Pathology, University of California, University Hospital, 225 West Dickinson Street, San Diego, California 92103

Received July 30, 1973

SUMMARY

Tryptophan peptides are oxidized to the oxindole by various chemical and peroxidase catalyzed iodination procedures in the pH range 4.0-7.5. This oxidation results in significant cleavage of tryptophanyl peptide bonds at pH 4-5. Thus, destruction of some biologically active peptides and proteins during iodination may be due to the modification of essential tryptophan residues.

INTRODUCTION

Polypeptide hormones are frequently radioiodinated for immunoassays and metabolic studies. Usually, the reaction mixture contains a few atoms of radioiodide per mole of peptide and several hundredfold excess of chloramine T at pH 7.5 (1,2). In water, chloramine T (the sodium salt of N-chloro-p-toluene sulfonamide) generates sodium hypochlorite, which oxidizes iodide to an active iodinating species, probably iodine monochloride (equations 1,2,3).

(1) $(p\text{-}CH_3\text{-}C_6H_4\text{-}SO_2N^-Cl)Na^+ + H_2O \rightleftarrows NaOCl + p\text{-}CH_3\text{-}C_6H_4\text{-}SO_2NH_2$

(2) $H_2O + NaOCl + NaI \longrightarrow ICl + 2NaOH$

Sum: $(p\text{-}CH_3\text{-}C_6H_4\text{-}SO_2N^-Cl)Na^+ + NaI + 2H_2O \longrightarrow ICl + 2NaOH + p\text{-}CH_3\text{-}C_6H_4\text{-}SO_2NH_2$

With this ratio of iodide to peptide, the tyrosyl residues are primarily converted to 3-monoiodotyrosine.

However, ICl and chloramine T are also potentially capable of reacting with several different amino acids besides tyrosine, e.g., tryptophan, methionine, histidine, cysteine and cystine (3-10). Thus, severe modifications in structure and activity of a polypeptide can occur during iodination by this procedure.

Some investigators have employed different, presumably milder, iodination methods to avert deleterious oxidative reactions. Among these methods are iodination with preformed ICl (11) and with I_2 generated by electrolysis (12). But these procedures yield iodinated peptides with lower specific activities when compared to the chloramine T method, especially with small amounts of protein (13). Enzymatic iodination of proteins (14-18) and cells (19) with H_2O_2 and a peroxidase is an apparently gentle iodination procedure that incorporates the desired amount of tracer into the product.

In this report, I present data which demonstrate that both chemical and enzymatic iodinating agents rapidly oxidize tryptophan over the pH range of 4-7.5. In acid pH, this oxidation leads to a significant cleavage of tryptophanyl peptide bonds whereas, much less peptide bond fission occurs at pH 7.5. Thus, the inactivation of some polypeptides and proteins during iodination may reflect oxidation of essential tryptophan residues.

MATERIALS AND METHODS

N-benzyloxycarbonyl-L-tryptophanyl-glycine (CBZ-Trp-Gly), and other peptides were purchased from Cyclo Chemical, Los Angeles, California. N-Iodosuccinimide was obtained from K and K Laboratories, Hollywood, California. Chloramine T was a product of Eastman Organic Chemicals, Rochester, N.Y. ICl was prepared according to Izzo et al. (20). Horseradish peroxidase, $A_{403}:A_{280}$ = 1.96 was purchased from Worthington Biochemical Corp., Freehold, N.J. Lactoperoxidase, $A_{410}:A_{280}$ = 0.59, was from Sigma Chemical Co., St. Louis, Mo. Oxidative cleavage of the peptides was determined by measuring the release of glycine with a quantitative ninhydrin method (21). Spectra were obtained with a Perkin-Elmer 202 dual beam spectrophotometer in 1 cm. cuvettes. Oxidative conversion of tryptophan to the oxindole was determined by disappearance of absorbance at 280 nm (22) 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 Patchornik et al. (22).

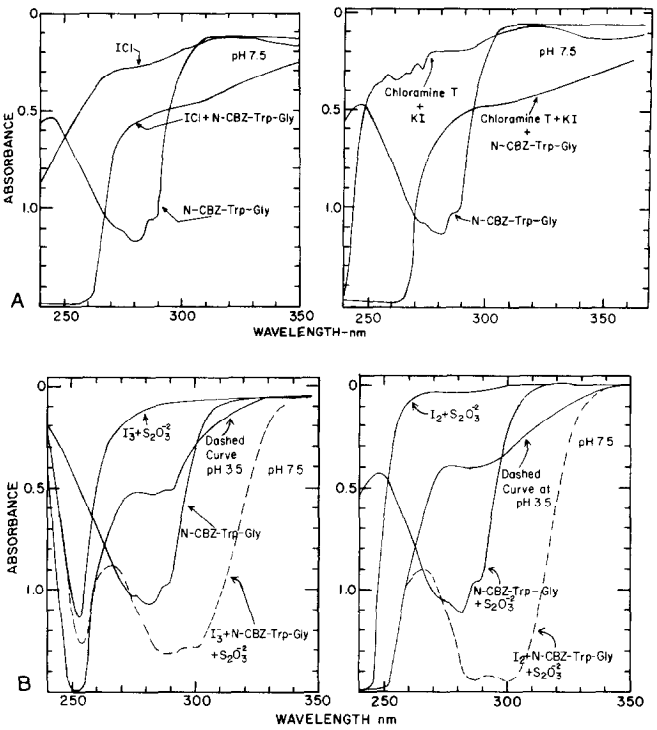

Figure 1A Oxidation of CBZ-Trp-Gly with ICl and chloramine T-KI.

All reaction mixtures contained 300 μmoles, pH 7.5 phosphate buffer and the following substances indicated above in a final volume of 3.0 ml: 300 μl of 2 mM CBZ-Trp-Gly dissolved in ethanol, 100 μl of 15 mM ICl, or 15 mM KI plus freshly prepared 15 mM chloramine T. The reaction was initiated by adding ICl or chloramine T last and the spectra were obtained at room temperature (20°-25°) 10 minutes later. Buffer was used in the blank cuvette.

Figure 1B Oxidation of CBZ-Trp-Gly with I_2 and I_3^-.

Conditions were the same as in Fig. 1A except 300 μl of 5 mM I_2 in ethanol or 300 μl of 5 mM I_3^- (5 mM I_2 in 20 mM KI) were used instead of ICl or KI plus chloramine T. The reactions were terminated after 10 minutes with 100 μl of 50 mM sodium thiosulfate. The pH of the complete reaction mixture represented by the dashed line was adjusted from 7.5 to 3.5 by adding 1 drop of 12 M HCl.

RESULTS AND DISCUSSION

The spectrum of N-CBZ-Trp-Gly before and after oxidation with ICl or chloramine T plus KI at pH 7.5 is shown in Figure 1A. A 2 1/2 molar excess of either iodination reagent induced a 50 to 60 percent reduction in absorbancy at 280 nm. The spectrum of the oxidized CBZ-Trp-Gly is essentially

identical to that reported for the oxindole derivative of CBZ-Trp derived with N-bromosuccinimide (23), and thus, probably represents conversion of the indole nucleus to 2-oxindole. The nearly identical spectral changes induced by ICl and chloramine T-KI indicate that iodide is converted to ICl by chloramine T, or that both reagents yield identical iodinating species, possibly hydrated I^+ (24 and see below). Neither ICl nor chloramine T-KI possessed spectra that interfered in the analysis (Figure 1A).

I_2 oxidizes N-CBZ-Trp-Gly as effectively as ICl and 1 1/2 to 2 times more effectively than I_3^- over a pH range of 4-7.5. The spectra of the tryptophan peptide after oxidation with I_2 and I_3^- are shown in Figure 1B. Thiosulfate was added to abolish the red-brown color of the halogens. The spectrum represented by the dashed line (Figure 1B) at pH 7.5 persisted after the addition of thiosulfate, but was irreversibly converted to the oxindole spectrum at pH 3.5 (any pH below 5 was effective). This spectrum was obtained only when CBZ-Trp-Gly, I_2 or I_3^-, and thiosulfate were all present (Figure 1B). Identical results were obtained when arsenite, metabisulfite, or mercaptoethanol replaced thiosulfate, or when ICl or chloramine T-KI were the iodinating agents. But the dashed line spectrum was not obtained with CBZ-Trp, Trp-Gly, or free tryptophan, even though all of these compounds were oxidized. Free tryptophan, but not the tryptophan peptides, formed a purple color with the iodinating agents that remained even after the addition of a reducing agent. When thiosulfate was mixed with tryptophan, or its peptides, before the addition of any iodinating agent, no oxidation occurred.

Three equivalents of I_3^- disappeared (determined by decrease in absorbance at 353 nm), for every mole of tryptophan oxidized. This stoichiometry is identical to the oxidation of tryptophan peptides by brominating agents (23).

Additional experiments (25) demonstrated that CBZ-Trp-Gly was oxidized (60-80%) to the oxindole either by chloramine T per se or by N-iodosuccinimide. The oxidations occurred within one minute over the pH range 4.0-7.5 with all of the iodinating agents and with chloramine T per se at pH 4.0-5.5. Lacto-

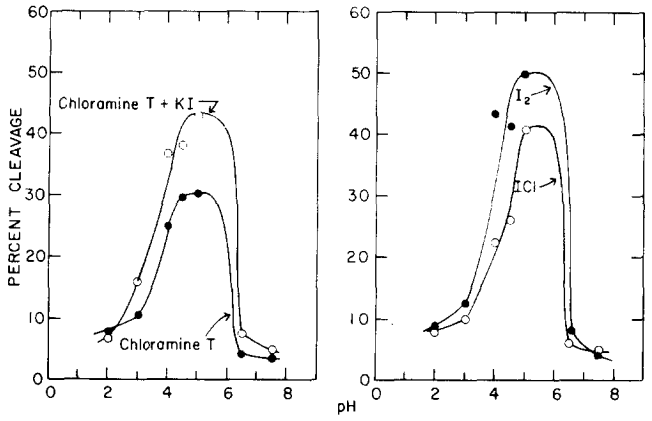

Figure 2 Oxidative cleavage of CBZ-Trp-Gly as a function of pH.

Each reaction mixture contained 90 μmoles buffer, 100 μl 2 mM CBZ-Trp-Gly in ethanol and 100 μl of a 5 mM solution (see Figures 1A and 1B) of each iodinating agent as indicated. The reaction was terminated with 100 μl of 50 mM sodium thiosulfate and analyzed for glycine. Each point represents the mean of duplicate analyses after correcting for the color with ninhydrin elicited by the peptide and chloramine T. These substances gave only 1-2% as much color as an equimolar amount of glycine. 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.

peroxidase (3.2 μg) and horseradish peroxidase (8.3 μg) produced an 80-90% oxidation of CBZ-Trp-Gly (0.2 mM) in the presence of 1 mM H_2O_2 and 1 mM KI. Omission of the peroxidase, H_2O_2 or KI abolished the oxidation. Optimum activity for the peroxidases was in the pH range 4.0-5.5.

The oxidative cleavage of CBZ-Trp-Gly, yielding free glycine, as a function of pH is depicted in Figure 2. Glycine formation was verified by thin layer chromatography. The cleavage occurs maximally at pH 4.5-5.5, although some fission (2-5%) was observed at pH 7.5. These results are similar to those reported with brominating agents (22,23,26,27). About 40-50% of the peptide is cleaved at pH 5 by chloramine T-KI, I_2 and ICl and 30% cleavage occurred with chloramine T alone. Doubling the concentration of the oxidants did not significantly increase the oxidative cleavage. The cleavage results with chloramine T and chloramine T-KI are minimum values because these agents (unlike I_2 and ICl) oxidize glycine to substances that do not yield a color with ninhydrin. Other experiments revealed that I_3^- failed to cleave the

peptide and 50% cleavage occurred with a fivefold excess of H_2O_2 plus KI and with either lactoperoxidase or horseradish peroxidase (25).

The oxidation of tryptophan by "active iodine" is not surprising in view of its reactivity towards electrophilic reagents (8). Oxidative cleavage of tryptophanyl peptides is promoted by iodinating agents with different oxidation potentials, but the presumed mechanism is the one suggested for Br^+ via the cyclized iminolactone (8,23,27). N-Iodosuccinimide, ICl, and chloramine T-KI probably generate I^+ because they induce the conversion of diiodophloretic acid to the dienone lactone, whereas I_2 and I_3^- do not (25,28). However, I_2 is at a higher oxidation potential than I_3^- based on the oxidation and fission results with CBZ-Trp-Gly. The peroxidases generate "active iodine" with an oxidation potential equivalent to either I^+ or I_2.

Several reports (4-6) have previously described the modification of tryptophan residues in proteins and polypeptides during iodination. If the tryptophan residues in a polypeptide are at the active site, they might be expected to be exposed and particularly susceptible to oxidation. An example is the inactivation of lysozyme by I_3^-, which readily converts tryptophan 108 in the active center cleft to the oxindole at pH 5.5 (9,10). Since I_3^- oxidizes CBZ-Trp-Gly to the oxindole directly (Figure 1B), it is unnecessary to postulate (8) the participation of histidine 15 in the oxidation of tryptophan 108 by I_3^- in lysozyme.

Another example is the heptadecapeptide, human gastrin I, where inactivation during iodination has been ascribed to oxidation of methionine 15 to the sulfoxide (3). Inasmuch as tryptophan 14 resides in the active C-terminal tetrapeptide sequence, its oxidation during iodination should also be considered as a possible explanation for the disappearance of gastrin activity.

Competition experiments with equimolar amounts of CBZ-Trp-Gly and CBZ-Tyr-Gly and a 2.5 molar excess of the various iodinating agents indicated that tryptophan oxidation proceeded rather selectively at pH 5.0, but that oxidation at pH 7.5 was reduced by 50%, or greater, because tyrosine was more

effectively iodinated at the higher pH. CBZ-Met-Gly and CBZ-His-Gly competed less effectively with CBZ-Trp-Gly for "active iodine" at either pH. Thus, some factors that affect tryptophan oxidation during iodination of a polypeptide or protein are: (1) pH, (2) the iodination reagent employed, (3) relative content of tryptophan and other amino acids, especially tyrosine, and (4) whether the reactive residues are buried or exposed. Studies with N-bromosuccinimide (29) have indicated that the rate and extent of oxidation of tryptophan in enzymes and proteins are affected by pH and the secondary structure of the protein. The same factors would be expected to play a role in the modification of tryptophan in peptides during iodination.

ACKNOWLEDGEMENTS

This work was supported by Grant No. AM-15778 from the U.S.P.H.S. The expert technical assistance of Mr. James F. Jennings is gratefully acknowledged.

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