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Chemical Cleavage of Tryptophanyl and Tyrosyl Peptide Bonds via Oxidative Halogenation Mediated by *o*-Iodosobenzoic Acid†

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ABSTRACT: The procedure to cleave tryptophanyl peptide bonds in proteins using *o*-iodosobenzoic acid (IBA) [Mahoney, W. C., & Hermodson, M. A. (1979) *Biochemistry 18*, 3810–3814] has been reexamined in order to clarify the mechanism of the cleavage reaction. It was found that IBA, under the proposed experimental conditions of protein fragmentation (80% aqueous acetic acid/4 M guanidine hydrochloride), mediates the oxidative chlorination of the indole nucleus of tryptophan and of the phenol ring of tyrosine. The mechanisms of the peptide bond fissions are thought to be similar to those already proposed for other reagents of the positive halogen type (e.g., *N*-bromosuccinimide). Several tryptophanyl and tyrosyl peptides were cleaved in 24–83% yields. It was shown that halides are a necessary reactant for the IBA-mediated peptide bond fission to occur. Excess *p*-cresol added to the reaction mixture as a scavenger for tyrosine modification allows selective cleavage at tryptophan. Horse heart cytochrome *c*, which contains one tryptophan residue and four tyrosine residues, was not cleaved by IBA alone but extensively cleaved at both these amino acids when the reaction was carried out in the presence of 4 M guanidine hydrochloride. On the other hand, in the presence of *p*-cresol, cleavage at tryptophan occurred quite selectively, and protein fragments 1–59 and 60–104 were isolated after gel filtration in ~60% yield. It is concluded that incubation of IBA under acidic conditions and in the presence of halides involves a series of equilibrium reactions leading to the formation of halogenating species, including free halogen. In particular, modification and cleavage at tyrosine are not due to *o*-iodoxybenzoic acid, thought to be present in the commercially available IBA reagent [Mahoney, W. C., Smith, P. K., & Hermodson, M. A. (1981) *Biochemistry 20*, 443–448], but is an inherent property of the IBA/guanidine hydrochloride/acetic acid reagent. In fact, the purity of the IBA sample used throughout this study was determined by numerous criteria, including elemental analysis, iodometry, and thin-layer as well as column chromatography.

Several procedures have been explored in the past to cleave tryptophanyl peptide bonds in proteins (Witkop, 1961; Spande et al., 1970; Fontana & Toniolo, 1976; Fontana et al., 1980). Most of the reagents employed were of the positive halogen type, such as *N*-bromosuccinimide (NBS)[1] (Patchornik et al., 1960; Ramachandran & Witkop, 1967), 2,4,6-tribromo-4-methylcyclohexadienone (tribromocresol) (Burstein & Patchornik, 1972), *N*-chlorosuccinimide (Shechter et al., 1976; Lischwe & Sung, 1977), 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine (BNPS-skatole) (Omenn et al., 1970; Fontana, 1972; Fontana et al., 1973), and dimethyl sulfoxide (Me_2SO) in the presence of hydrobromic acid (Savige & Fontana, 1977c; Fontana et al., 1980). Presently, the most often used method for cleavage at tryptophan utilizes BNPS-skatole as a source of positive halogen. This reagent is highly selective, and yields of cleavage are satisfactory (up to 80%) [see Fontana et al. (1980) for references].

Recently, *o*-iodosobenzoic acid (IBA) has been reported to cleave specifically tryptophanyl peptide bonds in proteins with 70–100% yields (Mahoney & Hermodson, 1979). However, subsequent reports (Fontana et al., 1980; Wachter & Werhahn, 1980; Johnson & Stockmal, 1980) showed that the reagent was not as selective as orginally reported since peptide bond cleavage at tyrosine was demonstrated to occur in moderate to high yields when the procedure was applied to several proteins (cytochrome *c*, myoglobin, and actin). More recently (Mahoney & Hermodson, 1980; Mahoney et al., 1981), it was proposed that *o*-iodoxybenzoic acid, thought to be present as a contaminant in the commercially available sample of IBA, is responsible for the observed cleavage at tyrosine.

The results of the studies reported herein rule out the role of contaminants in the IBA-mediated peptide bond fissions and show that the reagent, under the proposed experimental conditions for protein fragmentation (80% aqueous acetic acid/4 M Gdn·HCl), mediates the oxidative chlorination of

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[1] Abbreviations used: IBA, *o*-iodosobenzoic acid; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; NBS, *N*-bromosuccinimide; Me_2SO, dimethyl sulfoxide; Oia, oxindolylalanine (2-hydroxytryptophan); Dia, dioxindolylalanine; Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; RNase, bovine pancreatic ribonuclease A (amino acids and amino acid derivatives have the L configuration).

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both the indole nucleus of tryptophan and the phenol ring of tyrosine with concomitant peptide bond cleavage.

Experimental Procedures

Materials. Tryptophan, tyrosine, and methionine were obtained from Fluka (Basel, Switzerland). The standard amino acid calibration mixture (2.5 μmol each in 1 mL of 0.01 N HCl) containing all the common amino acids except for tryptophan and cysteine was purchased from Pierce chemical Co. (Rockford, IL). The peptides Gly-Trp-Gly, Gly-Pro-Trp-Leu, Phe-Val-Gln-Trp-Leu, Gly-Tyr-Gly, Leu-Tyr-Leu, Val-Tyr-Val, and Lys-Tyr-Lys were gifts of Drs. E. Wünsch and L. Moroder (Max Planck Institut für Biochemie, Martinsried, Germany). *N*-Carbobenzoxy (Z)-Trp-Gly and Leu-Trp-Leu were synthesized in our laboratory.

Samples of oxindolylalanine (Oia) (Savige & Fontana, 1977a, 1980), dioxindolylalanine (Dia) diastereoisomers (α and β form) (Savige, 1975), and methionine sulfoxide (Savige & Fontana, 1980) were prepared as previously described. Methionine sulfone was prepared by performic acid oxidation of methionine (Hirs, 1967). Chromatographically pure standards of 3-chloro- and 3,5-dichlorotyrosine were prepared by chlorination of tyrosine with sodium hypochlorite in aqueous acetic acid. The halogenated tyrosine derivatives were purified by preparative chromatography on a Sephadex G-10 column equilibrated and eluted with 10% acetic acid. The order of elution from the column was tyrosine, 3-chlorotyrosine, and 3,5-dichlorotyrosine. Homogeneity of the standard samples was established by TLC, automatic amino acid analysis (see below), and on the basis of the spectral characteristics of the isolated compounds (Thompson, 1954).

Guanidine hydrochloride (Gdn·HCl) (Pierce, Sequenal grade), Me_2SO (Fluka), concentrated aqueous HCl (C. Erba, Milan, Italy), glacial acetic acid (Fluka), and *p*-toluenesulfonic acid (Pierce, Sequenal grade) were used as received. The 47% HBr (C. Erba) was stored in the dark with 0.1% phenol added to prevent formation of free bromine. NBS (C. Erba) was recrystallized from water and stored in a foil-covered container at −20 °C. BNPS-skatole was synthesized from NPS-skatole and NBS, and its homogeneity was established as previously described (Omenn et al., 1970). Tryptamine, *p*-cresol, and 2-mercaptoethanol were purchased from Fluka and used without further purification.

Sephadex G-10, G-25, and G-50 superfine were obtained from Pharmacia (Uppsala, Sweden). Bovine pancreatic ribonuclease A and horse heart cytochrome *c* (type III) were purchased from Sigma Chemical Co. (St. Louis, MO).

o-Iodosobenzoic Acid. IBA (quality-controlled reagent) was purchased from Pierce and used throughout this study: mp 225–228 °C dec; reported 209 (Meyer & Wachter, 1892), 226 (Askenasy & Meyer, 1893), and 231–232 °C (Baker et al., 1965). Anal. Calcd for $C_7H_5O_3I$: C, 31.8; H, 1.9; I, 48.1. Found: C, 32.08; H, 1.90; I, 47.60. IBA was titrated by iodometry (Hellerman et al., 1941) by transferring a sample (2–5 mg) dissolved in 2 mL of 80% aqueous acetic acid into a 2-mL solution of 5% KI. The released iodine was back-titrated with a solution of standard thiosulfate (0.02 N), with starch serving as indicator. This analysis indicated 99–100% purity of the reagent.

IBA was also synthesized by oxidation of *o*-iodobenzoic acid (Aldrich, Milwaukee, WI) with fuming nitric acid following essentially the procedure described in the literature (Meyer & Wachter, 1892; Askenasy & Meyer, 1893). The crude product was suspended in water and dissolved by alkalinization to pH 9.5 by slow addition of 2 N NaOH under stirring. Some undissolved material was filtered off, and then precipitation of IBA was achieved by acidification to pH 3 by addition of 5% aqueous acetic acid. The precipitate was collected by filtration, washed with water, and dried in a vacuum desiccator over P_2O_5. The analytical properties of this sample of IBA (melting point, elemental analysis, and chromatographic properties) were essentially identical with those observed with the sample of IBA purchased from Pierce.

o-Iodoxybenzoic acid was synthesized from IBA following the procedure described by Hartmann & Meyer (1895). The sample of *o*-iodoxybenzoic acid so obtained was contaminated by trace amounts of IBA. An analytically homogeneous sample was obtained by preparative chromatography on a Sephadex G-10 column equilibrated and eluted with 10% aqueous acetic acid.

Thin-layer chromatography (TLC) was performed on silica gel plates by using the eluants and spray reagents previously described (Savige & Fontana, 1980). TLC of *o*-iodo-, *o*-iodoso-, and *o*-iodoxybenzoic acid was carried out on cellulose plates (DC-Alufolien F-254, 0.1 mm, Merck AG, Darmstadt, Germany) with methanol/acetic acid/water (6:3:1 v/v) as eluent. The detection of components on the cellulose plates was obtained by illumination with ultraviolet light (quenching of fluorescence after exposure of the plates at 254 nm) and with the modified hypochlorite reagent (Von Arx et al., 1976). According to this test, the dried plate was first sprayed with aqueous hypochlorite and then with a mixture of 4,4′-(tetramethyldiamino)diphenylmethane–KI–ninihydrin in aqueous acetic acid solution. The compounds appear on the plate as dark blue spots.

Amino acid analyses were performed on a Jeol Model JLC-6AH automatic amino acid analyzer by using a single column system according to the manufacturer's instructions. With the single column system, Oia is eluted between histidine and arginine and Dia diastereoisomers as two discrete peaks before the basic amino acids, the β isomer being eluted before the α isomer (Savige, 1975). Chloro- and dichlorotyrosine elute before the lysine and after the histidine peak, respectively. Methionine sulfoxide diastereoisomers elute as a single peak preceding the peak of aspartic acid. Methionine sulfone coelutes with aspartic acid.

Some analyses were carried out with a C. Erba Model 3A-27 (Milan, Italy) amino acid analyzer by using a two column procedure (Spackman et al., 1958). Oia is eluted from the short column (0.9 × 12 cm) ahead of the lysine peak and just in front of tryptophan (Savige & Fontana, 1977a) when elution is carried out under standard conditions by using 0.2 M sodium citrate buffer, pH 5.23. Analyses of 3-chloro- and 3,5-dichlorotyrosine were carried out with the short column of the C. Erba analyzer.

Reaction of Free Amino Acids with IBA. The details of the reaction conditions are reported in the legends to figures (see Results and the supplementary material at the end of the paper).

Reaction of Ribonuclease with IBA. A mixture of 2 mg (0.14 μmol) of RNase and 2 mg (7.5 μmol) of IBA in 750 μL of 80% acetic acid was incubated at room temperature in the dark for 22 h. The modified protein was then separated from the reactants by gel filtration through a column (2 × 40 cm) of Sephadex G-25 equilibrated and developed with 10% acetic acid. The protein was eluted as a single peak, and the appropriate fractions were combined and lyophilized. An aliquot of the modified protein was subjected to acid hydrolysis with 3 N *p*-toluenesulfonic acid (Liu & Chang, 1971) and analyzed for its amino acid composition. The IBA modification of RNase was also carried out under identical conditions but in

the presence of 85 μmol of NaCl or of 85 μmol of NaCl and 50 μmol of *p*-cresol.

Cleavage of Tryptophanyl and Tyrosyl Peptides. The reaction conditions are illustrated by the following experiment: The peptides Gly-Pro-Trp-Leu and Lys-Tyr-Lys (0.5 μmol each) were reacted with IBA (10 μmol) in 0.5 mL of 80% acetic acid for 22 h at room temperature in the dark. Alanine (0.5 μmol) was added to the reaction mixture as an internal standard. The reaction was then terminated by the addition of 10 μL of 2-mercaptoethanol and the sample dried in a vacuum desiccator over NaOH pellets at room temperature. The residue was dissolved in citrate buffer, pH 2.2, and directly analyzed with the automatic amino acid analyzer. Yields of cleavage were determined on the basis of the recovery on the analyzer of leucine (cleavage at tryptophan) and lysine (cleavage at tyrosine).

Additional cleavage experiments of the above indicated peptides involved the use of NBS (Patchornik et al., 1960; Spande & Witkop, 1967), BNPS-skatole (Omenn et al., 1970; Fontana, 1972), and Me_2SO/HCl/HBr (Savige & Fontana, 1977c) (see Results for details).

Reaction of Horse Heart Cytochrome c with IBA. Reaction 1. Cytochrome *c* (20 mg, 1.6 μmol) was reacted with 20 mg (7.9 μmol) of IBA in 1.5 mL of 80% acetic acid for 22 h at room temperature in the dark. The reaction mixture was then applied to a Sephadex G-50SF column (2.4 × 140 cm) equilibrated and eluted with 10% formic acid. The protein was located in the effluent by spectrophotometric monitoring of the fractions at 280 nm. *Reaction 2.* The reaction was carried out as described under reaction 1, with the difference being that the solvent mixture contained 4 M Gdn·HCl. After 22 h at room temperature, the reaction mixture was diluted 1:5 with water, and the heme cleaved from the protein (Fontana et al., 1973) was removed by repeated extraction with ethyl acetate. The aqueous solution was then vacuum concentrated and applied to the Sephadex G-50SF column. *Reaction 3.* Cytochrome *c* (20 mg) was incubated for 22 h in a preformed mixture of 20 mg of IBA and 30 μL of *p*-cresol dissolved in 1.5 mL of 80% acetic acid containing 4 M Gdn·HCl. The reaction mixture was worked up as described under reaction 2.

Results

Homogeneity of o-Iodosobenzoic Acid. In view of the controversial issue of purity of IBA (Mahoney & Hermodson, 1979, 1980; Mahoney et al., 1981), it was of importance to develop suitable analytical methods to check unambiguously the quality of the reagent employed. The homogeneity of the commercially available reagent (Pierce) was established on the basis of several criteria, including elemental analysis and iodometry (see Experimental Procedures). The melting point of IBA (225–228 °C), although in agreement with some figures reported in the literature (from 209 to 232 °C), cannot be taken as a criterion of homogeneity [see Askenasy & Meyer (1893); Hartmann & Meyer, 1893; Baker et al., 1965]. TLC analysis (Figure 1) on a cellulose plate showed that IBA (R_f 0.82) is not contaminated by either *o*-iodo- (R_f 0.94) or *o*-iodoxybenzoic acid (R_f 0.54). Also, a Sephadex G-10 column eluted with 10% aqueous acetic acid can be conveniently utilized for the separation and analysis of these three compounds (Figure 2). The effective separation observed can be explained by the known selectivity of binding of low molecular weight aromatic compounds to tightly cross-linked dextran gels (Determann & Lampert, 1972; Nandi, 1976). The insert of Figure 2 shows the ultraviolet absorption spectra of the three compounds. Again, column chromatographic analyses indicate

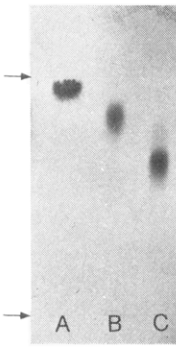


FIGURE 1: Thin-layer chromatography on a cellulose plate of *o*-iodobenzoic acid (A), IBA (Pierce Chemical Co.) (B), and *o*-iodoxybenzoic acid (C). The plate was developed with methanol/acetic acid/water (6:3:1, v/v), and then the compounds were localized by spraying the dried plates with the hypochlorite/potassium iodide dye reagent as described under Experimental Procedures.

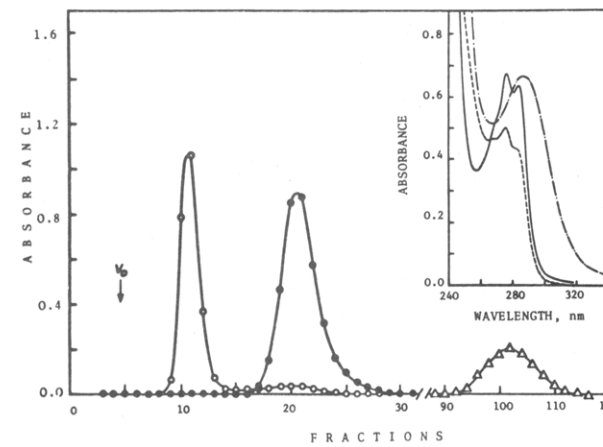


FIGURE 2: Chromatography on a Sephadex G-10 column (1.4 × 16 cm) equilibrated and eluted with 10% acetic acid of IBA (Pierce Chemical Co.) (●), *o*-iodoxybenzoic acid (○), and *o*-iodobenzoic acid (△). Sample (1–2 mg) were dissolved in 50% acetic acid (500 μL) and then applied to the column. Fractions of 2.4 mL were collected at a flow rate of 20 mL/h and then analyzed at 280 nm. V_0 indicates void volume of the column. (Insert) Ultraviolet absorption spectra of IBA (—), *o*-iodoxybenzoic acid (---), and *o*-iodobenzoic acid (–·–) in 10% acetic acid.

unambiguously the homogeneity of the IBA sample employed throughout this study.

Reaction of Amino Acids with IBA. Initial experiments on the reaction of IBA with free tryptophan were carried out without the addition of Gdn·HCl to the reaction mixture, considering that this reagent was employed by Mahoney & Hermodson (1979) as a component of the reaction mixture for protein fragmentation for its denaturing action on protein molecules, thus facilitating cleavage. Surprisingly, it was found by TLC analysis that when tryptophan was reacted with 2 equiv of IBA in 80% acetic acid, the modification of the amino acid occurred very slowly, if it occurred at all. On the other hand, when Gdn·HCl was added to the reaction mixture, tryptophan disappeared rapidly from the reaction mixture. The solution developed a pink color, precipitation of tar was observed, and oxindolylalanine (Oia) and dioxindolyalanine (Dia) diastereoisomers were identified in the reaction mixture by TLC, electrophoresis, and amino acid analysis (Savige & Fontana, 1980). Similar catalytic effects on the reaction were observed when other halides (NaCl and HCl) were used in combination with IBA. Typical amino acid chromatograms of the reaction of IBA with tryptophan are shown in Figure 3. When tryptophan was reacted for 1 h at room temperature with 3 equiv of IBA in 80% aqueous acetic acid, the recovery

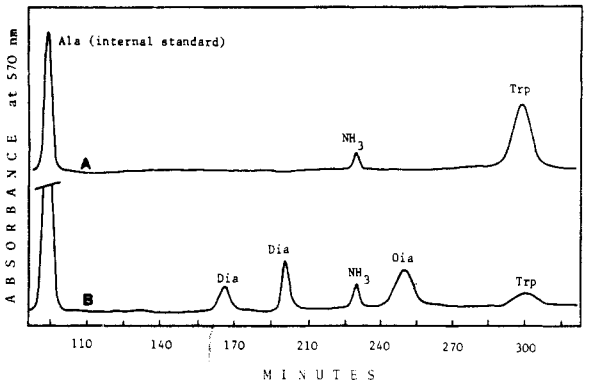


FIGURE 3: Amino acid chromatogram of the reaction mixture of tryptophan reacted with IBA. (A) Tryptophan (1 μmol) was incubated with IBA (3 μmol) in 80% aqueous acetic acid (200 μL). After 1 h at room temperature, the reaction was stopped by the addition of 2-mercaptoethanol (10 μL). (B) The reaction was conducted under the same reaction conditions as described under (A), with the only difference being that HCl (10 μmol) was added to the reaction mixture. Aliquots of the reaction mixtures [1/5 in (A) and 2/3 in (B)] were dried in a vacuum desiccator over NaOH pellets, and the residue was dissolved in citrate buffer, pH 2.2, and placed on the amino acid analyzer. Alanine (0.5 μmol) was added to the reaction mixtures as an internal standard.

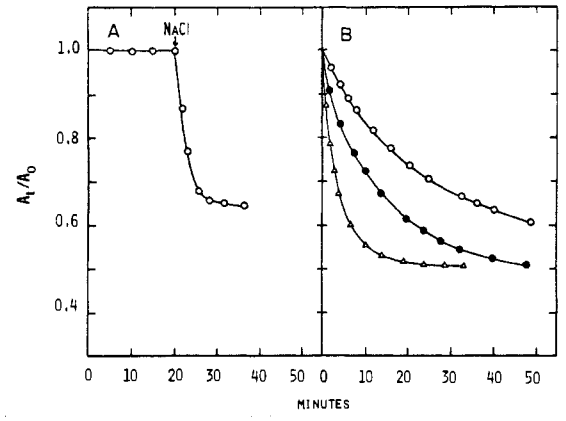


FIGURE 4: Time course of the modification of tryptophan by IBA, as followed by absorption measurements at 280 nm. For convenience, the absorption data have been reported as absorbance at the indicated time (A_t) over initial absorbance (A_0). (A) Tryptophan (5.5×10^{-5} M) and IBA (45×10^{-5} M) were dissolved in 80% acetic acid, and the absorption at 280 nm was followed as a function of time. After 20 min (arrow), to the cuvette was added NaCl at a concentration of 8.5×10^{-3} M. (B) Tryptophan (5.5×10^{-5} M) reacted with IBA (11.5×10^{-5} M) in 80% acetic acid in the presence of 5.7×10^{-3} M (○), 8.5×10^{-3} M (●), and 0.085 M NaCl (△). The temperature was 22 °C.

of the amino acid on the amino acid analyzer was quantitative. After 22-h reaction time, in different experiments, the recovery of tryptophan was variable (80–90%).

The participation of halide ions in the IBA reaction of tryptophan was clearly demonstrated by spectrophotometric monitoring of the reaction. Only in the presence of NaCl were a decrease in absorption at 280 nm and an increase near 250 nm (corresponding to oxindole formation) (Spande & Witkop, 1967) observed. Spectrophotometric readings at 280 nm allow a convenient method to follow the time course of the modification of tryptophan by IBA. From Figure 4, it is evident that the reaction occurs only in the presence of NaCl and that increasing concentrations of halide enhance the rate of tryptophan modification.

Tyrosine could be recovered quantitatively on the amino acid analyzer after reaction with a 20-fold molar excess of IBA in 80% aqueous acetic acid for 22 h at room temperature. On the other hand, when halides (Gdn·HCl, HCl, and NaCl) were

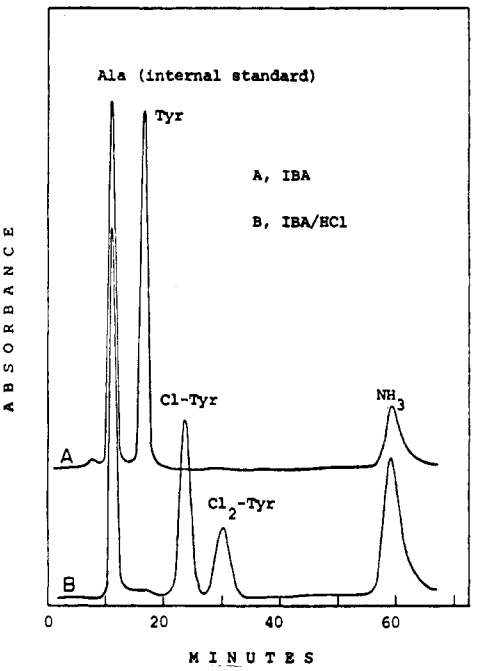


FIGURE 5: Elution pattern of the reaction mixture of tyrosine reacted with IBA from the short column of the amino acid analyzer. The column was eluted with citrate buffer, pH 5.23, according to Spackman et al. (1958). (A) Tyrosine (0.5 μmol) was reacted with IBA (10 μmol) in 80% aqueous acetic acid (400 μL) for 22 h at room temperature in the dark. (B) The reaction was performed as described under (A), with the only difference being that the reaction mixture contained HCl (10 μmol). Alanine (0.5 μmol) was added to the reaction mixtures as an internal standard.

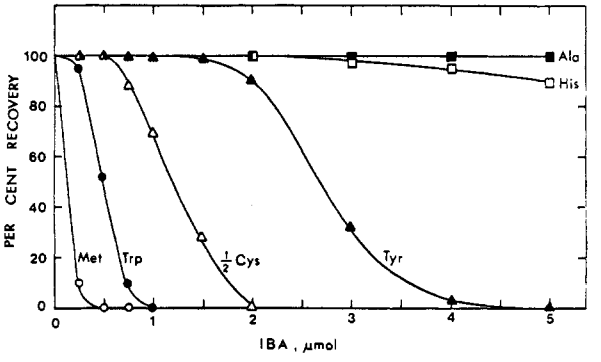


FIGURE 6: Competition between different amino acids for the IBA/HCl reagent. A mixture of tryptophan (0.5 μmol) and all common amino acids (0.25 μmol each), with the exception of cysteine, was reacted in 80% aqueous acetic acid (725 μL) containing 1 μmol of HCl with increasing amounts (0.25–5 μmol) of IBA for 22 h at room temperature in the dark. The percent recovery of free amino acids was determined by automatic amino acid analysis. Methionine (○); tryptophan (●); cystine (△); tyrosine (▲); histidine (□); all common amino acids (■).

included in the reaction mixture, 3-chloro- and 3,5-dichlorotyrosine were identified in the reaction mixture by automatic amino acid analysis. Figure 5 shows a typical result obtained when tyrosine is reacted with IBA with or without HCl added to the reaction mixture.

Figure 6 illustrates the destruction of tryptophan (0.5 μmol) and of the oxidation-sensitive amino acids (0.25 μmol each) when reacted in a mixture with increasing concentrations of IBA in the presence of HCl (1 μmol). Methionine and tryptophan react rapidly with the IBA/HCl reagent, while cystine and especially tyrosine require higher concentrations of reagent for their modification. It was found that cystine is partly converted to cysteic acid and methionine to methionine sulfone. Histidine appears to be the last amino acid to be

Table I: Reaction of Ribonuclease with IBA[a]

amino acid	residues/mole of RNase: theory	control, not treated	IBA	IBA/NaCl[b]	IBA/NaCl/p-cresol[c]
methionine	4	3.6	1.3	0	0
methionine sulfoxide	0	0	3.0	0	traces
methionine[d] sulfone	0	0	0	3.0	2.7
half-cystine	8	6.1	6.3	traces	2.5
cysteic acid	0	traces	traces	4.6	1.7
tyrosine	6	5.9	5.8	traces[e]	5.9
histidine	4	3.9	3.8	3.8	4.0

[a] Ribonuclease (0.14 μmol) was reacted with IBA (7.5 μmol) as described under Experimental Procedures. Hydrolyses of the protein were carried out with 3 N p-toluenesulfonic acid (Liu & Chang, 1971). The results are expressed as residues per mole of protein, and no corrections were made for low recovery or destruction of amino acids. Only the figures obtained for the oxidation and/or modification-sensitive amino acids are reported, whereas all other amino acids were recovered on the analyzer in essential agreement with theory. [b] 7.5 μmol of IBA and 85 μmol of NaCl. [c] 7.5 μmol of IBA, 85 μmol of NaCl, and 50 μmol and p-cresol. [d] Methionine sulfone coelutes with aspartic acid from the column of the amino acid analyzer (Jeol, Model JLC-6AH, single column procedure). The recovery of methionine sulfone was calculated by difference, assuming quantitative recovery of aspartic acid. [e] Peaks of 3-chloro- and 3,5-dichlorotyrosine appeared in the amino acid chromatogram.

modified by IBA. From the data shown in Figure 6, it appears that by limiting the excess of IBA one could achieve preferential modification of methionine and tryptophan. At high concentrations of the IBA/HCl reagent, partial destruction of amino acids occurs to products that no longer produce a color with ninhydrin. This parallels the NBS degradation of free amino acids described previously (Omenn et al., 1970; Burstein & Patchornik, 1972).

Reaction of Ribonuclease with IBA. To establish that the results with free amino acids were applicable in protein studies, it seemed desirable to perform tests on the reactivity of IBA with ribonuclease, a protein which does not contain any cysteine and tryptophan. Ribonuclease was allowed to react in 80% aqueous acetic acid solution with the reagent in a mole ratio of IBA to protein of 50:1, and then the modified protein, after separation from excess reagent by gel filtration on a Sephadex G-25 column, was subjected to acid hydrolysis with 3 N p-toluenesulfonic acid (Liu & Chang, 1971). This acid was used instead of the most common 6 N HCl since it appears that under this condition of hydrolysis methionine sulfoxide is not reduced back to methionine if the hydrolysis mixture is completely halide free (Savige & Fontana, 1977b, 1980). From the data shown in Table I, it appears that in the absence of added halide only the thioether group of methionine is oxidized to the sulfoxide derivative, while all other amino acids remain unchanged. On the other hand, when the reaction was performed in the presence of added NaCl, methionine, cystine, and tyrosine residues of ribonuclease were fully destroyed, and methionine sulfone, cysteic acid, and chlorinated tyrosine were recovered on the analyzer. When an excess of p-cresol was added to the reaction mixture as a scavenger for tyrosine modification, it was found that tyrosine was not modified by the reagent, methionine was still converted to methionine sulfone in high yields, and some cystine was not oxidized. Thus the results with ribonuclease are in essential agreement with the reactivities of the free amino acids with IBA. In particular, it is shown that added p-cresol can serve as an efficient scavenger for tyrosine modification.

Table II: Cleavage of Tryptophanyl and Tyrosyl Peptides by IBA[a]

peptide	cleavage product	cleavage (%)[b]: IBA	IBA/Gdn·HCl	IBA/NaCl
Z-Trp-Gly	Gly	19	60	
Leu-Trp-Leu	Leu	19	75	78
Gly-Trp-Gly	Gly	5	83, 59[c]	
Gly-Pro-Trp-Leu	Leu	0	66, 42[c]	68
Phe-Val-Gln-Trp-Leu	Leu	trace	61	
Gly-Tyr-Gly	Gly	trace	77	78
Val-Tyr-Val	Val	0	24	21
Leu-Tyr-Leu	Leu	0	27	14
Lys-Tyr-Lys	Lys	0	39	26

[a] Cleavage was performed by reacting separately each tryptophanyl peptide (0.2–0.5 μmol) in 80% acetic acid (400 μL) by using 5 mol of IBA/mol of peptide. Cleavage was also performed in the presence of 4 M Gdn·HCl or of 10 mg of NaCl. The four tyrosyl peptides (0.5 μmol each) were reacted in a mixture with IBA (30 μmol) in the desired solvent mixture (400 μL). The cleavage reaction was terminated after 22 h by addition of 10 μL of 2-mercaptoethanol. [b] Yields of cleavage were determined by automatic amino acid analysis of the released amino acid. [c] The reaction was terminated after 2 h.

Table III: Cleavage of Gly-Pro-Trp-Leu and Lys-Tyr-Lys by Various Reagents[a]

reaction conditions	cleavage product (%)[b]: Leu	Lys
IBA	5	0
IBA, Gdn·HCl	64	54
IBA, Gdn·HCl, p-cresol	66, 70[c]	5, 7[c]
NBS[d]	40	43
NBS, p-cresol[e]	38	0
BNPS-skatole[f]	80	0
Me_2SO/HCl/HBr[g]	82	0

[a] The peptides (0.5 μmol each) were reacted with IBA (10 μmol) in 80% acetic acid (500 μL) for 22 h at room temperature. The reaction was also carried out in the presence of 4 M Gdn·HCl or of 10 μmol of p-cresol. [b] Yields of cleavage were determined on the basis of the recovery on the analyzer of leucine and lysine (tryptophanyl and tyrosyl peptide bond cleavage, respectively). [c] The cleavage reaction was performed with 25 μmol of IBA. [d] 6 μmol of NBS and 80% acetic acid, 22 h. [e] 6 μmol of NBS, 10 μmol of p-cresol, and 80% acetic acid, 22 h. [f] 25 μmol of BNPS-skatole and 80% acetic acid, 48 h. [g] The cleavage reaction was performed following essentially the method previously described (Savige & Fontana, 1977c).

Cleavage of Tryptophanyl and Tyrosyl Peptide Bonds. As shown in Table II, IBA is able to cleave both the tryptophanyl and the tyrosyl peptide bonds when the reaction is conducted in 80% aqueous acetic acid containing 4 M Gdn·HCl for 22 h at room temperature with an excess of IBA. The yields of cleavage observed with a series of both tryptophanyl and tyrosyl peptides vary from 24% to 83%, cleavage at tryptophan being generally higher. From the data shown in Table II, it is also evident that NaCl can be used as an alternative source of halides to Gdn·HCl, with comparable yields of peptide bond fission. When the reaction is conducted without halides and over a 22-h reaction time, variable (but low) yields of cleavage at tryptophan are observed whereas cleavage at tyrosine *does not occur.*

Table III reports the cleavage yields obtained when both Gly-Pro-Trp-Leu and Lys-Tyr-Lys were cleaved in a mixture with IBA under different experimental conditions as well as with some other reagents previously proposed for protein fragmentation. It is seen that cleavage at tyrosine, which occurs in comparable yields to those observed at tryptophan,

can be greatly reduced if *p*-cresol is used as a scavenger for tyrosine modification. Analogously, NBS cleaves at both tryptophan and tyrosine, as expected from previous studies (Patchornik et al., 1960: Ramachandran & Witkop, 1967). Also, with this reagent, cleavage at tryptophan can be made selective if *p*-cresol is included in the reaction mixture. On the other hand, BNPS-skatole (Fontana, 1972) and Me_2SO/haloacid (Savige & Fontana, 1977c) cleave specifically at tryptophan in about 80% yields.

Cleavage of Cytochrome c. Horse heart cytochrome *c* was treated with IBA at a protein/reagent ratio of 1:1 (w/w) in 80% aqueous acetic acid for 22 h at room temperature, and then the reaction mixture was passed through a Sephadex G-50 column. Under these conditions, negligible protein fragmentation occurred since a main peak, identified as intact cytochrome *c* by amino acid analysis, was eluted from the column. On the other hand, when 4 M Gdn·HCl was included in the reaction mixture, the elution profile was complex, indicating extensive fragmentation and evidently not only at the level of the single tryptophan residue in position 59 of the polypeptide chain of 104 amino acid residues (expected two main peaks corresponding to fragments 1–59 and 60–104) but also at the level of the four tyrosine residues (Margoliash et al., 1961). When the cleavage reaction was carried out in the presence of *p*-cresol (cf. Experimental Procedures), fragmentation occurred quite selectively at tryptophan, and fragments 1–59 and 60–104 were isolated after gel filtration in ~60% yield. The heme group, which is covalently bound to cysteine residues of the protein via thioether bridges, was completely cleaved from the protein by IBA/4 M Gdn·HCl, analogously to other reagents of the positive halogen type (Fontana et al., 1973; Savige & Fontana, 1977c). The separation and analysis of the fragments derived from cytochrome *c* were carried out essentially as previously described (Fontana et al., 1973; Savige & Fontana, 1977c) (cf. supplementary material at the end of the paper).

Discussion

The most important observation emerging from this study is the participation of chloride ion in the IBA-mediated reactions with amino acids in aqueous acetic acid. IBA used in combination of a proper source of halide (Gdn·HCl, NaCl, and HCl) causes several modification reactions at the level of the side chains of the oxidation-sensitive amino acids; these side reactions are similar to those previously described for the very reactive halogenating agent NBS [cf. Ramachandran & Witkop (1967) and Spande et al. (1970) for reviews]. In fact, besides the aromatic amino acids (tryptophan, tyrosine, and, to a lesser extent, histidine), the sulfur amino acids are also extensively modified. In addition, the IBA/Gdn·HCl reagent shares in common with NBS (Omenn et al., 1970; Burstein & Patchornik, 1972) the property to react with free amino acids, leading to their degradation. These reactivities of both IBA/Gdn·HCl (or HCl) and NBS contrast with those observed with other milder reagents which have been proposed, subsequently to NBS, for protein fragmentation at the level of the tryptophanyl peptide bond (BNPS-skatole, tribromocresol, *N*-chlorosuccinimide, and Me_2SO/haloacid).

The modification of tryptophan by IBA in aqueous acetic acid occurs essentially only in the presence of added halide. Variable yields (0–19%) of peptide bond cleavage are observed only when the reaction is carried out over a long period of time (22 h). This could be considered as an indication of an intrinsic, slow reactivity of IBA toward the indole nucleus. It should be considered, however, that traces of halides in the solvent mixtures employed are difficult to avoid. Since oxi-

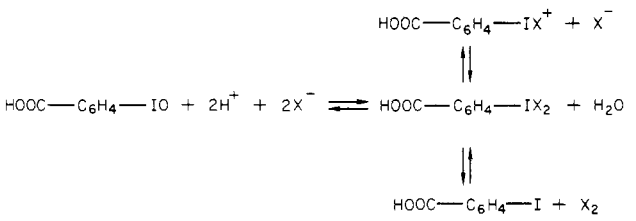
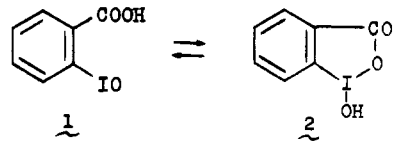


FIGURE 7: Proposed scheme of reactions of IBA incubated in acid solution and in the presence of halides.

dation of the indole nucleus of tryptophan to oxindole or dioxindole by positive halogen reagents involves halogenation–dehalogenation steps (Patchornik et al., 1960; Hinman & Bauman, 1964; Burstein & Patchornik, 1972; Savige & Fontana, 1977a; Fontana et al., 1980), catalytic amounts of halide would be sufficient to cause the IBA-mediated oxidation of the indole nucleus. The isolation of 3-chloro- and 3,5-dichlorotyrosine derivatives clearly proves that the IBA/Gdn·HCl reagent acts as a halogenating system. With tyrosine peptides, which require 3 equiv of active halogen for peptide bond fission to occur (Wilson & Cohen, 1963; Ramachandran & Witkop, 1967), cleavage is observed *only* in the presence of halides. Thus, cleavage at tyrosine is an inherent property of the IBA/Gdn·HCl reagent and not due to *o*-iodoxybenzoic acid (Mahoney et al., 1981). The absence of this reactive contaminant in the sample of IBA used throughout this study was proved unambiguously in a number of ways. For example, the determination of the oxidizing power of IBA by iodometry indicates 99–100% purity of the reagent. This method of analysis, which is the routine procedure to standardize the reagent (Hellerman et al., 1941), would permit unambiguous detection of components of higher oxidation states such as *o*-iodoxybenzoic acid, if present.

To explain the mechanism of the IBA-mediated halogenation, we propose that halogenating species are formed when IBA is incubated in aqueous acetic acid in the presence of halides as a result of the equilibrium reactions shown in Figure 7. In fact, evidences of the reactions shown in the scheme are available from the literature. First of all, halogen formation (iodine) by iodoso derivatives is used for their quantitative determination by iodometry (Hellerman et al., 1941). Treatment of IBA with HCl leads to the evolution of chlorine (Meyer & Wachter, 1892; Askenasy & Meyer, 1893). Iodobenzene dichloride, which has been used as a mild chlorinating agent, is in equilibrium in acetic acid solution with iodobenzene and chlorine (Keefer & Andrews, 1957, 1958). The possible existence in solution of the species $ArIX^+$ has been discussed (Keefer & Andrews, 1958).

There is evidence that IBA (**1**) might be in tautomeric



1 ⇌ **2**

equilibrium with the cyclic structure 1-hydroxy-1,2-benziodoxolin-3-one (**2**) (Meyer & Wachter, 1892; Askenasy & Meyer, 1893; Baker et al., 1965). In fact, the properties and reactions of IBA are quite different from those of its meta or para isomers. In particular, IBA is *stable to hot water*, which converts normal iodoso compounds into iodo and iodoxy compounds [see Baker et al. (1965) for additional references]. This finding contrasts with the proposal of Mahoney et al. (1981) that heating of IBA in aqueous solution causes the formation of *o*-iodoxybenzoic acid. In particular, these authors relie their claim on a literature reference (Lucas & Kennedy,

1955) which refers to a paper describing the disproportionation of *o*-iodosobenzene and not of IBA. The possible existence in solution of the iodoxolin ring system does not seem to impair, in the author's opinion, the proposed scheme of reactions shown in Figure 7.

The yields of cleavage of tryptophanyl peptides obtained with IBA/Gdn·HCl compare favorably with those observed with previously proposed reagents. However, cleavage occurs at both tryptophan and tyrosine in comparable yields, while with BNPS-skatole and Me_2SO/haloacid not a trace of cleavage of the tyrosyl peptide bond was observed. It was also shown in this study and by Mahoney et al. (1981) that cleavage at tryptophan can be made highly preferential if *p*-cresol is included in the reaction mixture as a scavenger for tyrosine modification. *p*-Cresol appears to compete with peptide- or protein-bound tyrosine for the halogenating agent(s) and most likely is converted by the IBA/Gdn·HCl reagent to polyhalogenated derivatives, including 2,4,6-trichloro-4-methylcyclohexadienone. This compound could have a reactivity toward amino acid side chains quite similar to that already described for the analogous bromo derivative, tribromocresol (Burstein & Patchornik, 1972), which was shown to cleave selectively at tryptophan and to cause only ortho halogenation of the phenol ring of tyrosine without peptide bond fission.

As a model protein for the IBA-mediated peptide bond cleavage, horse heart cytochrome *c* which has been used previously to test methods for cleavage at tryptophan (Fontana et al., 1973; Savige & Fontana, 1977c; Lischwe & Sung, 1977; Ozols & Gerard, 1977a,b), was used. Without added halides, IBA does not lead to noticeable protein cleavage, whereas in the presence of Gdn·HCl, cleavage occurs at both tryptophan and tyrosine, leading to a complex mixture of cleaved peptides. Excess, *p*-cresol added to the reaction mixture restricted cleavage at tryptophan, and fragments 1–59 and 60–104 were isolated after gel filtration in ~60% yields. Thus, the results obtained with model peptides have been essentially confirmed with a protein, and the yields of protein fragmentation at tryptophan by the IBA/Gdn·HCl reagent are comparable to those reported by Wachter & Werhahn (1980), far from being quantitative (Mahoney & Hermodson, 1979), but higher than those (13–22%) reported by Simpson et al. (1980).

In summary, the results of this study clarify the mechanisms of peptide bond fission at both tryptophan (Mahoney and Hermodson, 1979) and tyrosine (Johnson & Stockmal, 1980) mediated by IBA in presence of halides as well as the nature and extent of side reactions at the level of the side chain functions of other amino acid residues. Despite the limitations discussed above, the use of IBA as a reagent for protein fragmentation has found already useful practical applications in protein chemistry (cf. above for references). In fact, the yields of cleavage at tryptophan observed in this study with several tryptophanyl peptides (up to 83%) compare favorably with those obtained with other reagents (BNPS-skatole and Me_2SO/haloacid) presently often used for protein fragmentation at tryptophan. Apparently, the use of *p*-cresol as a scavenger for tyrosine modification and cleavage will permit achievement of selective and efficient cleavage at tryptophan.

Acknowledgments

We thank Dr. Daniela Sostero for carrying out some experiments reported here and Dr. Eloisa Celon-Mazzuccato for the elemental analyses.

Supplementary Material Available

Tables presenting the results of quantitative amino acid analysis of tryptophan, tyrosine, and methionine reacted with IBA under different experimental conditions, a table reporting the amino acid composition of fragments 1–59 and 60–104 of cytochrome *c*, figures showing the cleavage of tryptophanyl and tyrosyl peptide bonds as a function of time and equivalents of IBA, and figures showing elution profiles from a Sephadex G-50 column of cytochrome *c* reacted with IBA, IBA/4 M Gdn·HCl, IBA/4 M Gdn·HCl/*p*-cresol or dimethyl sulfoxide/haloacid (Savige & Fontana, 1977c) (5 pages). Ordering information is given on any current masthead page.

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Proteolytic Specificity of Hemorrhagic Toxin *a* Isolated from Western Diamondback Rattlesnake (*Crotalus atrox*) Venom†

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ABSTRACT: The proteolytic specificity of hemorrhagic toxin *a* from the venom of *Crotalus atrox* (western diamondback rattlesnake) has been investigated by using the oxidized B chain of bovine insulin and other peptides as substrates. The toxin appears highly specific for X-Leu bonds (cleaving the His_{10}-Leu_{11}, Ala_{14}-Leu_{15}, and Tyr_{16}-Leu_{17} bonds), with no detectable activity against the Gly-Phe, Phe-Phe, Phe-Tyr, and Leu-Tyr bonds also present in the insulin B chain. The X-Leu bond of the peptides Tyr-Gly-Gly-Phe-Leu, Phe-Ala-Leu, and Ala-Leu was also cleaved. The toxin seems to be a strict endopeptidase, in that the cleavage of the two most susceptible bonds, Ala_{14}-Leu_{15} and Tyr_{16}-Leu_{17}, are mutually exclusive; i.e., cleavage of either bond results in the other being too close to either the amino- or carboxyl-terminal of its respective fragment for the enzyme to be effective against it. The X-Met bond of Tyr-Gly-Gly-Phe-Met was cleaved, although a dipeptide Gly-Met was not hydrolyzed after 16 h of incubation. The substrates not hydrolyzed are furylacryloylglycyl-L-leucinamide, carbobenzoxy-L-glutamylglycine, carbobenzoxyglycyl-L-glutamic acid, benzoyl-L-arginine-*p*-nitroanilide, L-lysine-*p*-nitroanilide, $(L\text{-}Ala)_3$-*p*-nitroanilide, Gly-Met, Gly-Phe-Phe, Gly-Gly-Ala, TAME, and ATEE. The absence of hydrolytic activity against the last two substrates indicates that hemorrhagic toxin *a* does not possess trypsin- or chymotrypsin-like activity.

Hemorrhage is commonly induced by rattlesnake envenomation. Recently five hemorrhagic toxins with proteolytic enzyme activities were isolated from the venom of the western diamondback rattlesnake (*Crotalus atrox*) (Bjarnason & Tu, 1978). In the course of physical and chemical characterization of these toxins, all five were shown to be zinc-containing proteolytic enzymes. Light and electron microscopic studies by Ownby et al. (1978) have shown that exposure of endothelial cells to hemorrhagic toxin *a* results in lysis of the cells; these observations raise the possibility that cell membrane proteins may be the site of attack by the toxin. Proteolytic activity of each of the hemorrhagic toxins against the general protease substrate, dimethylcasein, has been measured by titration with TNBS[1] of the newly generated free amino groups (Bjarnason & Tu, 1978); the identities of the peptide bonds attacked had not, however, been determined prior to the present study. Determination of the bond specificity of the toxins is the logical next step in understanding the relationship of proteolytic and hemorrhagic activities. Since hemorrhagic toxin *a* (HT-*a*) is by far the most potent hemorrhage-producing agent of the five, it was considered the most interesting subject for specificity studies. The performic acid oxidized B chain of bovine insulin and other peptides were employed as substrates, and the fragments resulting from proteolysis of the oxidized B chain were purified by preparative thin-layer chromatography. From the amino acid compositions of the isolated fragments, we were able to identify the principal sites of attack of hemorrhagic toxin *a* on this substrate. Fragments of other peptides were identified by thin-layer chromatography.

Materials and Methods

Crude *C. atrox* venom was purchased as the lyophilized powder from Miami Serpentarium, Miami, FL. Hemorrhagic

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[1] Abbreviations used: HT-*a*, hemorrhagic toxin *a*; TNBS, trinitrobenzenesulfonic acid; Cbz-, carbobenzoxy-; FAGLA, furylacryloylglycyl-L-leucinamide; TAME, *p*-tolylsulfonyl-L-arginine methyl ester; ATEE, acetyl-L-tyrosine ethyl ester; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/81/0420-7004$01.25/0