Fragmentation of Proteins with *o*-Iodosobenzoic Acid: Chemical Mechanism and Identification of *o*-Iodoxybenzoic Acid as a Reactive Contaminant That Modifies Tyrosyl Residues†

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ABSTRACT: *o*-Iodoxybenzoic acid, a disproportionation product of *o*-iodosobenzoic acid, has been identified as a contaminant in most preparations of *o*-iodosobenzoic acid capable of both modifying and cleaving certain tyrosyl residues. A new synthetic approach for the production of *o*-iodosobenzoic acid containing low amounts of *o*-iodoxybenzoic acid combined with preincubation of the reagent with *p*-cresol to destroy the remaining *o*-iodoxybenzoic acid prior to the reaction with a polypeptide allows preparation of reagent solutions in which tyrosyl residues remain intact during tryptophanyl bond cleavage. In addition, the product produced by the action of *o*-iodosobenzoic acid upon tryptophanyl bonds has been identified as *N*-acyldioxindolylalanine. It is inferred from that structure that the chemical reaction proceeds via a two-step oxidation of the tryptophanyl residue followed by formation of an iminospirolactone which hydrolyzes, cleaving the peptide chain. Small peptides ending with dioxindolylalanine can be coupled to aminopropyl glass in high yield and are suitable for solid-phase Edman degradation.

The limitation in yield inherent to the chemistry of the automated Edman degradation prevents the unambiguous identification of more than 30–60 NH_2-terminal residues for a given polypeptide. Consequently, overlapping fragments generated by the selective cleavage of polypeptide chains at several different residues or sequences must be obtained to determine a complete protein sequence.

We recently introduced a procedure for cleaving proteins specifically at the carboxyl side of tryptophanyl residues (Mahoney & Hermodson, 1979, 1980), which, by virtue of its high yields and controllable side reactions, has already aided in the sequence analyses of several proteins (Orr et al., 1979; Nute & Mahoney, 1980; Mahoney & Nute, 1980; Isihara & Hogg, 1980; Simpson et al., 1980; Bitar et al., 1980). Originally, we observed no irreversible side reactions at any residue under standard reaction conditions, although at very high concentrations of *o*-iodosobenzoic acid tyrosine was destroyed as inferred by amino acid analyses. Upon further experience with various commercial sources of *o*-iodosobenzoic acid, certain lots of the reagent were identified that were capable of both modifying tyrosyl residues and cleaving tyrosyl peptide bonds in many but not all proteins. In addition, certain proteins had unusually susceptible tyrosyl bonds which were cleaved in low yield by all reagent preparations. One such protein that had a very susceptible tyrosyl bond was sperm whale myoglobin where one out of three tyrosyl residues (Tyr-103) was unusually sensitive. The yield of cleavage at this tyrosyl–leucyl bond was ~30%, while quantitative cleavage yields occurred at the two tryptophanyl residues.

We report here that a disproportionation product of *o*-iodosobenzoic acid, *o*-iodoxybenzoic acid, is the agent responsible for tyrosine modification and cleavage. Approaches for the synthesis of *o*-iodosobenzoic acid free of *o*-iodoxybenzoic acid and chemical approaches to consume contaminating *o*-iodoxybenzoic acid prior to the cleavage procedure are described. Identification of the product of the reaction at tryptophan is also reported, and from that we deduce the probable reaction mechanism. We further demonstrate that the initial product of the cleavage reaction is a lactone suitable for coupling small peptides ending in the modified tryptophanyl residues to solid phase supports.

Experimental Procedures

Materials. Bovine trypsinogen was obtained from Worthington Biochemical Corp. Aminopropyl glass, trifluoroacetic acid, and solid-phase sequenator chemicals were obtained from Pierce Chemical Co. Tryptophanylglycine was purchased from Sigma Chemical Co.

N-Acetyltryptophanylglycine was prepared by incubating the dipeptide with acetic anhydride–water–pyridine (1:1:0.8) for 1 h at 0 °C according to Shechter et al. (1976). The product was purified by high-performance liquid chromatography on a reversed-phase support (LiChrosorb C8, Brownlee Laboratories) using a Varian Model 5000 liquid chromatograph equipped with a Vari-Chrom detector.

Sperm whale myoblobin was obtained from Sigma, and apomyoglobin was prepared according to the procedure of Teale (1959). The protein was applied to a column of Sephadex G-75 (2.5 × 90 cm) equilibrated and developed with 10% (v/v) acetic acid. The apomyoglobin was recovered by lyophilization.

Peracetic acid was prepared by heating a mixture of 30 mL of 30% hydrogen peroxide and 300 mL of acetic acid for 5 h at 70 °C. Acetic acid was purified by distillation following oxidation for 18 h with CrO_3 at refluxing temperature. All other reagents used were of the highest commercial quality available.

Cleavage of N-Acetyltryptophanylglycine with o-Iodosobenzoic Acid. *N*-Acetyltryptophanylglycine was dissolved in 70% acetic acid containing twice as much *o*-iodosobenzoic acid as dipeptide by weight. The reaction was allowed to proceed overnight in the dark at room temperature. Following the addition of ethanethiol (1 mol/mol of *o*-iodosobenzoic acid) the mixture was lyophilized. The cleavage products were subsequently purified by high-performance liquid chromatography on the same system as used for the *N*-acetyltryptophanylglycine above.

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High-Resolution Mass Spectrometry. Analyses were performed on a GEC-AE1 MS9 double-focusing instrument according to the manufacturer's instructions. Probe temperature was set at 135 °C (I_A = 100 μA; V_I = 70 eV; V_A = 8 kV; electron multiplier = 160 V).

Synthesis of o-Iodosobenzoic Acid. *o*-Iodobenzoic acid (Aldrich Chemical Co., Milwaukee, WI) was purified by two recrystallizations from a filtered solution of 50% (v/v) ethanol in water containing activated carbon (Darco G-60). The mixture was heated until the *o*-iodobenzoic acid, at a concentration of 1 g/8 mL, was completely dissolved. The final yield was 70%. Material from two other suppliers and material made from anthranilic acid (Wachter, 1893; Baker et al., 1965) were dark brown in color and often contained free iodine. This material was best purified by formation of the ethyl ester, distillation, and saponification (Baker et al., 1965). *o*-Iodobenzoic acid of acceptable quality consists of colorless to slightly off-white crystalline needles, has a melting point of 162–163 °C, shows no reaction with potassium iodide–starch paper, gives a negative test for salicylate with Fe^{3+}, and assays >99.9% by titration.

In a well-ventilated hood 120 mL of reagent grade 90% nitric acid (J. T. Baker, Phillipsburg, NJ) was added to a 250-mL Erlenmeyer flask equipped with a teflon-coated stirring bar and an external cooling bath maintained at 15 °C with tap water. *o*-Iodobenzoic acid (7.5 g) was added in several portions with stirring over a 10-min period. Upon the final addition of *o*-iodobenzoic acid the mixture was stirred for an additional 10 min. The initial additions produced a small amount of heat, and the mixture darkened to a yellow-gold color. The reaction mixture was then slowly poured onto 500 g of ice and 100 mL of water precipitating the product. Filtration on a sintered glass funnel followed by four washes with 100 mL each of chilled (4 °C) deionized water followed by five washes of 100 mL each of acetone yielded 5.5–6.0 g (75%) of *o*-iodosobenzoic acid after vacuum or tray drying at room temperature. The acetone effectively removed untreated *o*-iodobenzoic acid.

o-Iodosobenzoic acid prepared in this manner consisted of typically white to slightly off-white leaflets melting at 226 °C with decomposition. Material with improved color and crystalline appearance could be obtained from recrystallization from 2 L of water heated to 95 °C, but this caused formation of iodoxybenzoic acid (see Results). Consequently, this final recrystallization was omitted. *o*-Iodoxybenzoic acid was synthesized by the method of Hartmann & Meyer (1893).

o-Iodosobenzoic Acid Cleavage. Protein fragmentation was accomplished as described previously (Mahoney & Hermodson, 1979, 1980). *o*-Iodosobenzoic acid (10 mg) was dissolved in 80% acetic acid, 4 M in guanidine hydrochloride. Myoglobin or trypsinogen (5 mg) was added, and the reaction was allowed to proceed at room temperature in the dark for 24 h. The reaction was stopped by the addition of dithioerythritol (5 mg). The peptides were separated from the reactions by gel filtration through a column (2.5 × 25 cm) of Bio-Gel P-2 equilibrated and developed with 0.05% (v/v) trifluoroacetic acid. The peptides were recovered by lyophilization. In those applications where a *p*-cresol preincubation was required, variable amounts (see figure legends) of *p*-cresol were added to the reaction mixture. Following 2–4 h of preincubation at room temperature the protein was added, and the reaction was allowed to proceed for an additional 24 h.

Attachment of Peptides to Aminopropyl Glass. The unfractionated cleavage products of the *o*-iodosobenzoic acid digestion of trypsinogen and the purified NH_2-terminal 40-residue peptide were covalently attached to aminopropyl glass by the procedure of Wachter & Werhahn (1979).

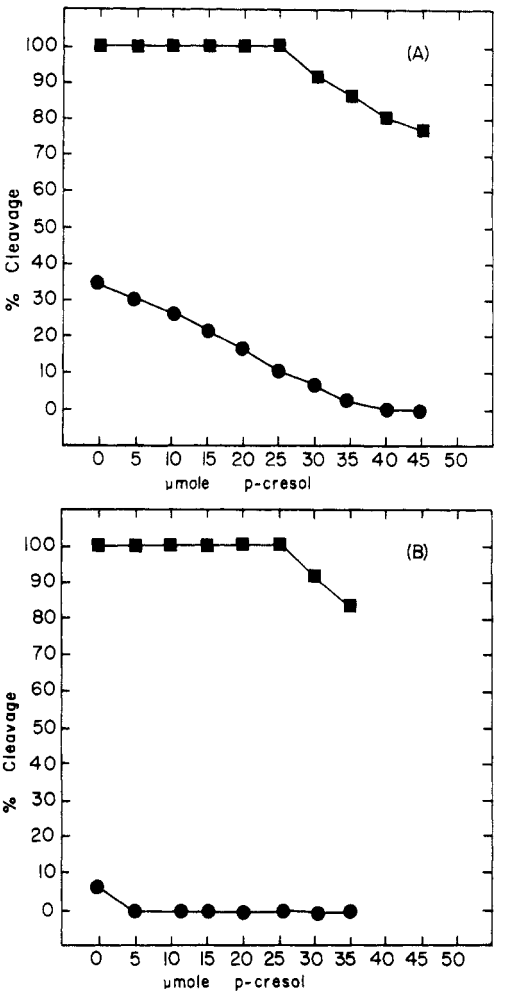

FIGURE 1: (A) Yields of cleavage at tryptophanyl residues and at Tyr-103 in sperm whale myoglobin using crude *o*-iodosobenzoic acid. Cleavage at tryptophanyl residues (■); cleavage at Tyr-103 (●). Myoglobin (5 mg) was reacted with 10 mg (38 μmol) of *o*-iodosobenzoic acid with or without *p*-cresol at the amounts indicated on the figure. (B) The cleavage of myoglobin using *o*-iodosobenzoic acid synthesized from highly purified *o*-iodobenzoic acid by low temperature (15 °C) oxidation with fuming nitric acid. Cleavage at tryptophanyl residues (■); cleavage at Tyr-103 (●). Reaction is as in (A).

Solid-Phase Sequence Analysis. Solid-phase sequence analysis was performed with a Sequemat Model 12 sequencer according to manufacturer's instructions.

Liquid-Phase Sequence Analysis. Unfractionated digests were analyzed in a Beckman Model 890 C sequencer as previously described (Mahoney & Hermodson, 1979). Identification of the Pth-amino acids was accomplished by high-performance liquid chromatography on either a Bioanalytical Systems (West Lafayette, IN) or a Varian Model 5000 liquid chromatograph by a modification (Nute & Mahoney, 1980) of the method of Zimmerman et al. (1977). Both chromatographs were equipped with integrators to quantitate the Pth-amino acids.

Amino Acid Analysis. Analyses were performed on a Durrum D-500 amino acid analyzer according to the manufacturer's instructions.

Results

The preincubation of *o*-iodosobenzoic acid with various amounts of *p*-cresol has a dramatic effect on the amount of cleavage at tyrosyl residues (Figure 1). No tyrosine modification or cleavage was observed with the best preparations

Table I: Yield of Cleavage at Tryptophanyl and Tyrosyl Residues in Myoglobin Using Various Iodoso and Iodoxy Compounds[a]

reagent	yield of cleavage at tryptophan (%)	yield of cleavage at tyrosine (%)
iodosobenzene[b]	20	25
iodosobenzene diacetate[b]	40	30
iodoxybenzoic acid	70	50
iodosobenzoic acid prepn		
I	>95	35
II	>95	30
III	>95	8
IV	>95	0
V	>95	25
VI	>95	35

[a] The various preparations of *o*-iodosobenzoic acid are as follows: (I) a typical commercial preparation; (II) synthesized as described under Experimental Procedures followed by recrystallization from hot water containing *p*-cresol; (III) synthesized as described under Experimental Procedures without a final recrystallization; (IV) as in (III) with a *p*-cresol preincubation (0.2 mol of *p*-cresol/mol of *o*-iodosobenzoic acid); (V) as in (IV) except following the *p*-cresol incubation reagent was heated at 110 °C for 18 h prior to the cleavage reaction; (VI) as in (IV) except following the *p*-cresol incubation 2 mL of peracetic acid was added to the cleavage solution simultaneously with addition of protein. The cleavage reaction was performed as described in the legend to Figure 1. [b] Since iodosobenzene and iodosobenzene diacetate are both capable of undergoing disproportionation, tyrosine cleavage could be due to contamination with the respective iodoxy compounds.

of *o*-iodosobenzoic acid when the reagent was treated with 0.1 molar equivalents of *p*-cresol prior to reaction with the protein, but several commercial preparations of *o*-iodosobenzoic acid required considerably more *p*-cresol to eliminate tyrosyl modification. In those cases so much cresol was required that the yield of tryptophanyl bond cleavage was also affected (Figure 1A).

In an attempt to determine the nature of the contaminant responsible for tyrosyl modification, we added *p*-cresol during the preparation of *o*-iodosobenzoic acid. When reagent recrystallized from hot water containing *p*-cresol was tested on myoglobin, cleavage at both tryptophan and tyrosine was observed in approximately 95% and 30% yields, respectively (Table I). This observation strongly suggested that the reactive contaminant was something other than I^+ or I_2, since oxidized iodine would react faster with the cresol at elevated temperature than at room temperature. Reagent prepared at low temperature was then examined. The yields of cleavage at tryptophan remained unaffected, while those at tyrosine were reduced to 8% (Figure 1B; Table I). The addition of *p*-cresol to reagent prepared in this manner prevented modification at tyrosyl residues at concentrations far lower than those required to affect cleavage yields at tryptophan.

Since elevated temperatures appeared to increase the amount of the reactive contaminant and since disproportionation of *o*-iodosobenzoic acid had been shown to occur with heating (Lucas & Kennedy, 1955), *o*-iodoxybenzoic acid was added to solutions of *o*-iodosobenzoic acid prepared at low temperature. An increase in the cleavage yields at tyrosyl residues occurred commensurate with the amounts of *o*-iodoxybenzoic acid (Figure 2). The cleavage yield of ~55% was the maximum obtained for Tyr-103–Leu-104 in myoglobin regardless of the amount of *o*-iodoxybenzoic acid used.

o-Iodosobenzoic acid which had been heated after synthesis at reduced temperature or which had been treated with peracetic acid also showed increased side reaction at tyrosine (Table I). Thus this series of experiments strongly suggested

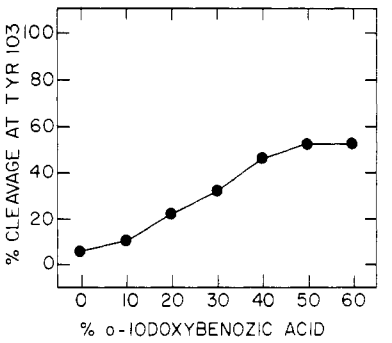

FIGURE 2: The yield of cleavage at Tyr-103 in sperm whale myoglobin with *o*-iodosobenzoic acid in the presence of added *o*-iodoxybenzoic acid. The reaction was accomplished as described in Figure 1 in the absence of *p*-cresol.

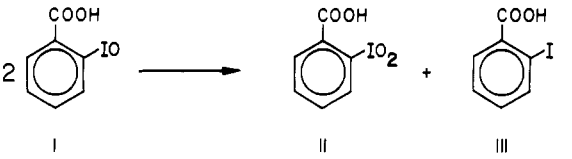

FIGURE 3: Disproportionation of two molecules of *o*-iodosobenzoic acid (I) produces one molecule of *o*-iodoxybenzoic acid (II) and one molecule of *o*-iodobenzoic acid (III).

Table II: Amino Acid Compositions of Sperm Whale Myoglobin[a]

amino acid	I[d]	II[e]	III[f]
Asx	7.8 (8)	8.1	8.0
Thr	4.7 (5)	4.7	4.6
Ser	5.2 (6)	5.3	5.3
Glx	18.2 (19)	18.9	18.7
Pro	3.9 (4)	3.9	3.9
Gly	11.0 (11)	11.2	11.0
Ala	17.2 (17)	17.1	17.1
Val	7.9 (8)	7.9	8.0
Met	1.8 (2)	1.7	1.8
Ile[b]	8.1 (9)	7.9	8.2
Leu	17.7 (18)	18.2	18.1
Tyr	2.9 (3)	0.8	2.8
Phe	6.1 (6)	5.8	5.9
Lys	19.1 (19)	18.7	18.8
His	11.8 (12)	11.9	12.2
Arg	3.7 (4)	3.9	4.2
Cys	0 (0)	0	0
Trp[c]	ND (2)	ND	ND

[a] Determined after hydrolysis in glass-distilled 6 N HCl at 110 °C for 24 h. Values in parentheses show compositions as determined by amino acid sequence. [b] Ile values are low due to the incomplete hydrolysis of the Ile-111–Ile-112. [c] ND, not determined. [d] (I) Amino acid composition of uncleaved myoglobin. [e] (II) Amino acid composition of myoglobin cleaved with a typical commercial preparation of *o*-iodosobenzoic acid without *p*-cresol. [f] (III) Amino acid composition of myoglobin cleaved with *o*-iodosobenzoic acid synthesized as described under Experimental Procedures by using reagent preincubated for 2 h in the presence of 0.2 mol of *p*-cresol/mol of *o*-iodosobenzoic acid.

that the contaminant causing the modification and cleavage of tyrosine residues was *o*-iodoxybenzoic acid, a disproportionation product of *o*-iodosobenzoic acid (Figure 3).

Table II presents the amino acid compositions of unfractionated digests of myoglobin. These data demonstrate that tyrosine can be oxidized without leading to cleavage of the peptide, since two to the three residues of tyrosine are lost with only 30% cleavage at one of them. The *p*-cresol treatment of the best reagent preparations totally eliminates tyrosine modification (Table II, column III).

Iodosobenzene, iodosobenzene diacetate, and iodoxybenzoic acid were also tested as reagents for cleaving myoglobin (Table

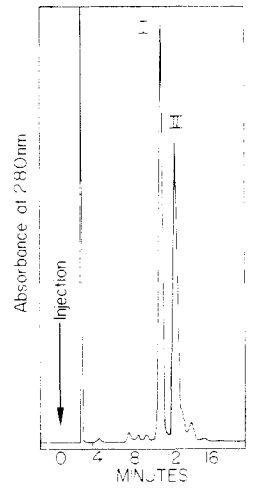

FIGURE 4: High-performance liquid chromatographic separation of the products of the cleavage of *N*-acetyltryptophanylglycine with *o*-iodosobenzoic acid. Elution from a Zorbax ODS (Dupont) 4.6 × 250 mm (5 μm) column was accomplished by using 9% aqueous acetonitrile containing acetic acid (0.1 M). Temperature was maintained at 40 °C; flow rate was 1 mL/min.

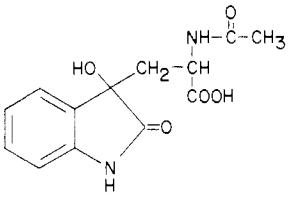

FIGURE 5: *N*-Acetyldioxindolylalanine.

I). None gave particularly high cleavage yields at tryptophan, and all modified and cleaved tyrosyl residues in substantial yield.

Mass spectral analyses of *o*-iodosobenzoic samples from any type of preparation showed only two major peaks, one corresponding to iodobenzoic acid and one to iodoxybenzoic acid. Essentially no iodosobenzoic acid was observed due to disproportionation of the reagent on the probe of the instrument which had to be heated to 233 °C to generate the spectrum. This confirmed the sensitivity of the reagent to heating and further supported the identification of *o*-iodoxybenzoic acid as the reactive contaminant.

Following purification of the *N*-acetyltryptophanylglycine and cleavage of the dipeptide with *o*-iodosobenzoic acid, the products of the reaction were isolated by liquid chromatography (Figure 4). The two major peaks were collected and recovered by lyophilization. Mass spectrographic analysis of peak I produced a major ionization peak at mass 278.088, which is consistent with the structure $C_{13}H_{14}N_2O_5$ (expected mass 278.0902). Peak II (Figure 4) showed no mass peak above 160 and was not further characterized.

The most reasonable structure for the product with the formula $C_{13}H_{14}N_2O_5$ was deduced to be *N*-acetyldioxindolylalanine (Figure 5). It this were so, the internal lactone should be readily produced by treatment of the product with anhydrous trifluoroacetic acid, and the lactone should couple readily to aminopropyl glass (Figure 6). To test this hypothesis, we coupled the mixture of tryptophanyl peptides from bovine trypsinogen and the purified NH_2-terminal 40-residue tryptophanyl peptide of that molecule to aminopropyl glass (Table III). Sequence analysis showed that only the peptides containing residues 1–40 and 200–222 were successfully coupled from the mixture, even though all five expected peptides were observed in about equimolar yield when

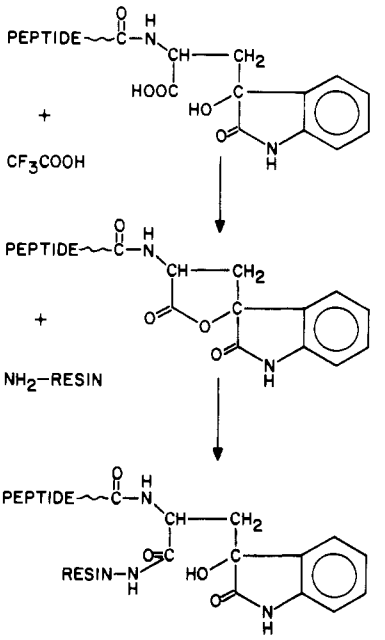

FIGURE 6: Reaction scheme for the formation of dioxindolylalanine spirolactone followed by reaction with an aminated support to immobilize the peptide via an amide linkage.

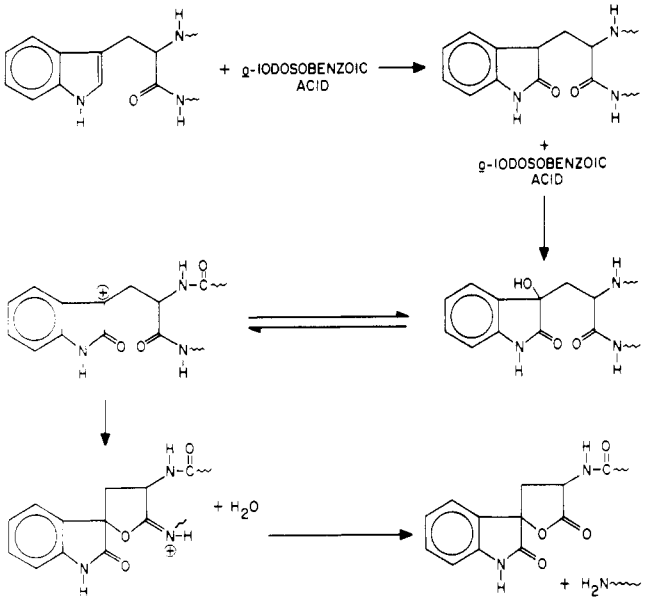

FIGURE 7: Proposed reaction mechanism of *o*-iodosobenzoic acid at tryptophanyl residues leading to scission of the peptide chain.

Table III: Yield of Coupling of Tryptophanyl Peptides to 3-Aminopropyl Glass Prior to Solid-Phase Edman Degradation on Trypsinogen[a]

total unfractionated digest	
1	20%
2	18.4%
N-terminal 40 residues	
1	83%
2	79%

[a] Yield of coupling was determined by amino acid analysis of an aliquot of the washed and dried product compared with that of the unretained material.

the mixture was subjected to liquid-phase sequence analysis. Thus, the two large peptides did not couple to aminopropyl glass, while the 21- and 40-residue fragments were immobilized in high yield. The short C-terminal peptide lacked the derivatized residue, and thus could not couple to the support.

The successful coupling of the smaller peptides provides additional support for the proposed reaction mechanism which is shown in Figure 7.

Discussion

Preincubation of properly prepared *o*-iodosobenzoic acid with a small amount of *p*-cresol prevents modification and partial cleavage of tyrosyl residues in polypeptides while still allowing quantitative cleavage at tryptophanyl residues. At present we are uncertain what factors lead to only certain tyrosine residues becoming modified and cleaved in the absence of *p*-cresol. In myoglobin only one of three tyrosyl residues is cleaved at low yield, but in a neutral protease peptide a Tyr–Gly and a Tyr–Phe bond are cleaved in almost quantitative yield, while a Tyr–Asn bond remains intact (A. Boosman, unpublished data). On the other hand, there are 18 tyrosyl residues in bovine serum albumin including Tyr–Leu, Tyr–Phe, and Tyr–Gly bonds (Dayhoff, 1976), but only Trp–X bonds are cleaved (Mahoney & Hermodson, 1979). Several other reports (Fontana et al., 1980; Wachter & Werhahn, 1980; Johnson & Stockmal, 1980) indicate low to moderate yields of cleavage at certain tyrosyl residues in various proteins (cytochrome *c*, myoglobin, and actin). Again some tyrosyl residues are affected, others are not, and no pattern is obvious with respect to the sequence surrounding these tyrosines. Due to the low yield of cleavage and the uncertain specificity of the reaction, it does not seem practical at this time to attempt to generate peptides by cleavage of tyrosyl residues with *o*-iodoxybenzoic acid.

A number of synthetic methods have been published for producing *o*-iodosobenzoic acid and the *o*-iodobenzoic acid used as starting material (Meyer & Wachter, 1892; Askenasy & Meyer, 1893; Boeseken & Schneider, 1932; Twiss & Heinzelmann, 1950; Baker et al., 1965; Hartmann & Meyer, 1893). However, these reports do not take into account the possibility of a disproportionation reaction of the product. The purity of *o*-iodosobenzoic acid prepared by various procedures is difficult to assess, since *o*-iodosobenzoic and *o*-iodoxybenzoic acid are not clearly distinguished by infrared spectroscopy, chromatographic techniques, or oxidizing potential (Mahoney & Hermodson, 1980). The value and nature of the melting point (gaseous decomposition or deflagration) as well as the nature of the crystal form (leaflets or needles) do appear to have qualitative significance in terms of *o*-iodoxybenzoic acid content. The best preparations of *o*-iodosobenzoic acid in terms of specific tryptophan cleavage are colorless leaflets melting at 226 °C with gaseous decomposition as opposed to preparations high in *o*-iodoxybenzoic acid content which are often needles deflagrating at ~233 °C. The most consistently suitable material is that produced from very pure *o*-iodobenzoic acid at low temperature, since most methods for purifying the final product only increase the amounts of the disproportionation products.

The *p*-cresol presumably reduces the *o*-iodoxybenzoic acid contaminant. Other reagents, such as sulfhydryl reagents, can also reduce iodoxy compounds, but they reduce iodoso compounds as well. Susceptibility of the iodoso compounds to phenols may also be indicated by the fall in yields of tryptophanyl bond scission at high cresol concentrations. Thus, a combination of careful synthesis and *p*-cresol treatment of *o*-iodosobenzoic acid of high quality ensures reliable digestion of tryptophanyl bonds without side reactions at tyrosyl residues.

High-quality reagent appears to be very stable on the shelf. We have kept one lot at room temperature for over 6 months with no noticeable change in effectiveness or increase in side reactions.

The proposed reaction mechanism is essentially identical with those proposed for tryptophanyl bond cleavage by other reagents (Savige & Fontana, 1977b; Fontana et al., 1980; Ramachandran & Witkop, 1967; Shechter et al., 1976), except that no halogenated intermediate is formed. The demonstration in this work that the reaction proceeds normally in the absence of guanidine hydrochloride and thus in the absence of any halide eliminates the possibility (Fontana et al., 1980) that halides are a necessary reactant. The tryptophanyl residue must be exposed to the reagent in solution, however, and for many proteins that may require a denaturant like guanidine hydrochloride. We have found that high concentrations of urea adversely affect the cleavage yields for reasons which are not readily appearent (W. J. Ray and W. C. Mahoney, unpublished observations), and so urea is not recommended as the denaturant.

Earlier work shows that the modification and cleavage reaction is slow since more than 16 h are required for complete reaction and destruction of the reagent at any time during the reaction stops further cleavage from occurring (Mahoney & Hermodson, 1979). There are four separate steps in the proposed mechanism, two oxidative steps, a cyclization of the carbonyl oxygen on the oxidized indole nucleus, and finally hydrolysis of the iminolactone. The first oxidative step to produce the oxindolyl derivative can occur under rather mild oxidative conditions (Savige & Fontana, 1977a) and may thus be fairly rapid in the presence of *o*-iodosobenzoic acid. The second oxidative step is likely to be rate limiting, since once it occurs the cleavage reaction would take place in the aqueous acid whether or not reagent is present. Since no further cleavage occurs when reagent is destroyed, we conclude that one of the oxidative steps, probably the second, is rate limiting.

The demonstration that relatively small peptides produced by this method can be attached simply and specifically to aminopropyl glass via the C termini provides an additional approach for solid-phase protein sequence analysis. Like the peptides generated by cyanogen bromide, the size of the peptide is inversely correlated to coupling yield (Laursen et al., 1975). Since solid phase sequence analysis is most amenable to relatively short peptides anyway, this is not a significant problem.

Acknowledgments

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