Biochemistry

Volume 11, Number 25 December 5, 1972

Selective Chemical Cleavage of Tryptophanyl Peptide Bonds in Peptides and Proteins†

Yigal Burstein and Abraham Patchornik*

ABSTRACT: Tryptophanyl peptide bonds are selectively cleaved by 2,4,6-tribromo-4-methylcyclohexadienone (TBC). Tyrosyl and histidyl peptide bonds, which are usually cleaved by other brominating agents (such as *N*-bromosuccinimide, *N*-bromoacetamide, *etc.*) are not cleaved by this reagent. Other amino acids which are known to be sensitive to oxidation also react with TBC but do not bring about cleavage of peptide bonds. The nature of the reaction of TBC with several oxidation-sensitive amino acids was studied by titrimetric and spectrophotometric methods, and the oxidation products were identified. Tyrosine was converted to 3,5-dibromotyrosine, methionine was oxidized to methionine sulfoxide, cysteine was oxidized to cysteic acid, and tryptophan was most probably converted to a bromooxindole derivative. Optimal conditions for the cleavage reaction were found to be: 3 equiv of TBC, pH 3, for 5–15 min at room temperature. The cleavage reaction was studied on several tryptophan-containing peptides, and yields of cleavage were in the range of 40–60%. The method of cleavage was successfully applied to several proteins. In ribonuclease, a protein which does not contain any tryptophan residues, no cleavage of peptide bonds was observed. In lysozyme, selective cleavage of the expected tryptophanyl peptide bonds (yields of 5–60%) was obtained. The glucagon molecule (which contains a single tryptophan residue) was fragmented into two peptides in about 30% yield.

Oxidizing and brominating agents are widly used for the modification of amino acids side chains of proteins (Witkop, 1961; Spande and Witkop, 1967; Cohen, 1968; Stark, 1969; Spande *et al.*, 1970). Some of these modifications were used for the nonenzymatic cleavage of tryptophanyl peptide bonds in peptides and proteins. Periodate was used for the oxidative cleavage of tryptophan residues in myoglobin (Atassi, 1967). Ozone was used for the oxidation of tryptophan to *N*-formylkynurenine (Previero *et al.*, 1963; Previero and Bordignon, 1964), which was subsequently cleaved by hydrazinolysis (Morishita *et al.*, 1967), hydrolysis (Previero *et al.*, 1966a), or reduction (Previero *et al.*, 1966b). The most widely used method for the modification and cleavage of tryptophanyl peptide bonds is the NBS[1] method which was introduced by Patchornik *et al.* (1960). NBS is an extremely reactive agent which can cause modification, as well as cleavage, not only of tryptophan, but also of tyrosine (Schmir *et al.*, 1959) and histidine (Schmir and Cohen, 1961; Shaltiel and Patchornik, 1963) residues. In order to obtain selective cleavage of tryptophanyl peptide bonds a milder brominating agent was needed. Funatsu *et al.* (1964) suggested that NBS in 8 M urea could be used for the preferential cleavage of tryptophanyl peptide bonds. Recently BNPS-skatole was used for the selective modification and cleavage of the single tryptophan residue in staphylococcal nuclease (Omenn *et al.*, 1970).

We wish to report here the use of a mild brominating agent 2,4,6-tribromo-4-methylcyclohexadienone (TBC) for the selective modification and cleavage of tryptophanyl peptide bonds in peptides and proteins.

Materials

TBC (a modification of the method of Fries and Oehmke, 1928). To a solution of 5.4 g of *p*-cresol (50 mmoles) in 100 ml of glacial acetic acid containing 12.3 g of sodium acetate (150 mmoles), 24 g of bromine (150 mmoles) was added, with constant stirring in an ice–water bath. After 30 min, water was added and the precipitate was filtered off, washed with cold water, dried, and crystallized from ethyl acetate. The yield

† From the Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel. *Received June 12, 1972.* A preliminary report was presented at the 37th meeting of the Israel Chemical Society, (Burstein *et al.*, 1967). This research was supported in part by Grant No. AM05098 from the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used are: TBC, 2,4,6-tribromo-4-methylcyclohexadienone; NBS, *N*-bromosuccinimide; NBU, *N*-bromourea; Z, benzyloxycarbonyl; Ac, acetyl; 8CM-lysozyme, lysozyme derivative in which all four disulfide bonds were reduced and carboxymethylated; RNase, bovine pancreatic ribonuclease; BNPS-skatole, bromine adduct of 2-(2-nitrophenylsulfenyl)3-methylindole; Dnp, dinitrophenyl.

was 25 g (73%), mp 105°. *Anal.* Calcd for C_7H_5BrO: Br, 69.9 (69.56); neutral equiv (oxidative bromine), 348 (345). The following tryptophanyl and tyrosyl peptide derivatives were prepared by coupling Z-tryptophan[2] or Z-tyrosine with the corresponding amino acid methyl or ethyl ester hydrochlorides *via* dicyclohexylcarbodiimide in the presence of triethylamine, and then saponifying the ester group with NaOH in aqueous dioxane: Z-Trp-Gly (mp 158°), Z-Trp-Ala (mp 154–5°), Z-Trp-Phe (mp 128–129°), Z-Tyr-Gly (mp 99–101°), Z-Tyr-Ala (mp 173–174°), Z-Tyr-Phe (mp 170–172°). Phlorethylglycine was prepared according to Schmir *et al.* (1959, mp 176–177°).

The following peptide derivatives were a gift of Dr. M. Wilchek: Z-His-Leu, Z-His-Val-OBzl, Z-His-Phe, Z-Trp-Leu, Z-Cys(S-Me)-OMe, bis(Z-Phe)-Cys-$(OMe)_2$. Ac-Trp-NH_2 and Ac-Tyr-NH_2 were a gift of Dr. M. Fridkin. Bis(Z)-Cys-$(OMe)_2$ and Cys-Cys-Ala were a gift of Mr. E. Bondi.

Trp-Gly, Trp-Ala, Trp-Phe, Tyr-Gly, Tyr-Ala, Tyr-Phe, His-Gly, His-Ala, and His-Phe were purchased from Miles-Yeda (Rehovot, Israel). DL-Ala-DL-Met, 3-iodotyrosine, and 3,5-diiodotyrosine were obtained from Nutritional Biochemical Corp. Oxidized glutathione was a product of Schwarz. Bovine pancreatic ribonuclease A and hen egg-white lysozyme were purchased from Sigma. Proline iminopeptidase was a gift of Mr. D. Mlynar of the Biophysics Department in our institute. Dnp-amino acids and glucagon were products of Mann Research Laboratories, Inc. The standard amino acid mixture was purchased from Spinco-Beckman and to it an equivalent amount of tryptophan was added. NBS was crystallized from water. Urea was crystallized from ethanol; freshly prepared solutions were used.

Methods

Amino acid analyses were performed with a Beckman Model 120C automatic amino acid analyzer. Hydrolysis of proteins and peptides was performed in evacuated sealed tubes in 6 N HCl at 105° for 22 hr (Spackman *et al.*, 1958; Spackman, 1963). Analysis of tryptophan residues in proteins was performed according to Spies and Chambers (1949).

Protein concentrations were determined by amino acid analysis of solutions of known absorptivity and then checked routinely by measurement of absorbance at 280 nm.

Ultraviolet absorption spectra were obtained with a Cary 14 recording spectrophotomoter; nmr spectra were obtained with a Varian 60 recording spectrophotometer.

Determination of Yields of Cleavage in Dipeptides. The method is illustrated by the following experiment. Z-Trp-Gly (5 μmoles/ml) was dissolved in the desired solvent. To this stirred solution at room temperature, a solution of TBC or NBS in dioxane or dimethylformamide was added and the reaction was terminated by the addition of excess thiosulfate or indolepropionic acid. A sample of 0.1 ml of the terminated reaction mixture was diluted tenfold with citrate buffer (pH 2.2) and analyzed on the amino acid analyzer. Since N-terminal tryptophanyl, tyrosyl, and histidyl peptides are not cleaved by NBS (Wilchek *et al.*, 1967; Wilchek and Witkop, 1967), such peptides were acetylated with acetic anhydride prior to cleavage; any *O*-acetyltyrosine residues thus formed were hydrolyzed by exposing the acetyl-peptide solution to pH 13 for 30 min.

Reaction of Free Amino Acids with TBC. To a number of samples of free amino acids (0.25 μmole/ml, in 65% acetic acid), increasing amounts of TBC (0–40 μmoles) in 100 μl of dioxane were added. Each sample was equally divided into two portions. After 15 min at room temperature, excess sodium thiosulfate was added to one portion, and excess indolepropionic acid to the other. The solvent was evaporated and the residue was redissolved in citrate buffer (pH 2.2) and analyzed for its amino acid content. Basic amino acids were analyzed in the "thiosulfate" fraction, and acidic and neutral amino acids in the "indolepropionic acid" fraction.

Titrimetric Monitoring of the Reaction between TBC and Several Amino Acids and Peptide Derivatives. To a solution of an amino acid or a peptide derivative in 80% acetic acid (5 μmoles/9.9 ml), a solution of TBC in dioxane (50 μmoles/0.1 ml) was added. After indicated times, 1–2 ml of the reaction mixture was transferred into a solution of 2 ml of 10% KI. The released iodine was back-titrated with a solution of 0.02 N sodium thiosulfate, starch serving as indicator (according to Kolthoff and Sandell, 1952). Bromide titrations were carried out in the same manner by transferring samples of the reaction mixture to a solution of ethanol–0.5 M nitric acid (4 ml) and titrating the bromide ions with $Hg(ClO_4)_2$, using diphenylcarbazone as indicator (Lapin and Zamanov, 1955).

Modification of Tryptophanyl Residues in Proteins. To a solution of 2 μmoles of the protein (8CM-lysozyme, lysozyme, and RNase) in 2 ml of 80% acetic acid, 120 μmoles of TBC in 25 μl of dioxane was added. The reaction was allowed to proceed at room temperature, with constant stirring, for 15 min. Two portions of 0.1 ml were transferred to hydrolysis tubes and excess TBC in one tube was destroyed with sodium thiosulfate, and in the other tube with indolepropionic acid. Both samples were hydrolyzed and analyzed for their amino acid composition. The "thiosulfate" fraction was analyzed for basic amino acids and the "indolepropionic acid" fraction was analyzed for acidic and neutral amino acids.

Cleavage of Tryptophanyl Peptide Bonds in 8CM-lysozyme. Excess of TBC in the remaining above-mentioned reaction mixture was destroyed with sodium thiosulfate and the solvent was evaporated and redissolved in 1.5 ml of water.

DETERMINATION BY THE Dnp METHOD. One milliliter was brought to pH 8 with sodium bicarbonate, treated with fluorodinitrobenzene, and hydrolyzed according to the procedure summarized by Frankel-Conrat *et al.* (1955). The mixture of Dnp-amino acids thus formed was separated by two-dimensional thin-layer chromatography on 20 × 20 cm silica gel plates, developed with toluene–pyridine–2-chloroethanol–0.8 M ammonia (10:3:6:6, v/v, upper phase) in the first dimension and chloroform–methanol–acetic acid (95:5:1, v/v) in the second dimension. Acid-soluble Dnp-amino acids were separated by high-voltage paper electrophoresis in 10% acetic acid, 60 V/cm for 3 hr. The amount of each Dnp-amino acid was determined by elution of the yellow spots and spectrophotometric assay (Frankel-Conrat *et al.*, 1955). The results were corrected for hydrolytic and chromatographic losses.

DETERMINATION OF THE CLEAVAGE OF THE Trp–Pro BOND. The cleavage reaction mixture (0.5 ml; 0.5–1.0 μmole) was brought to pH 8 with 0.1 M Veronal buffer (pH 8), and 50 μl of 0.05 M $MnCl_2$ and 100–200 μg of the enzyme proline iminopeptidase were added. The reaction mixture was incubated at 37° for 24 hr and the reaction was stopped by boiling for 2 min. To 1 ml of the reaction mixture, 2.5 ml of acidic nin-

[2] The amino acids, peptides, and peptide derivatives have the L configuration, unless otherwise stated.

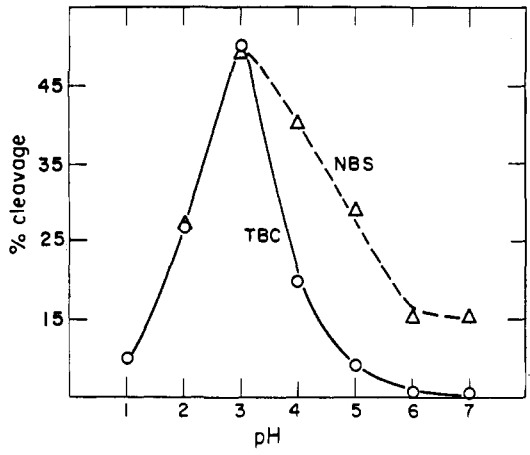


FIGURE 1: Effect of pH on the cleavage of Z-Trp-Gly. The reaction was carried out with 3 moles of TBC (○) or NBS (△) per mole of peptide for 10 min at room temperature. The solvents for the reactions were prepared by adding pyridine to 25% acetic acid. The experiments at pH 1–2 were performed in 0.1 M solutions of KCl–HCl. Yields of cleavage were determined by automatic amino acid analysis of the released glycine.

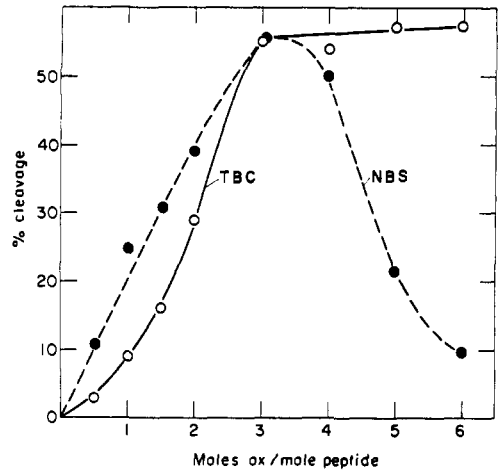


FIGURE 2: Cleavage of Z-Trp-Gly as a function of addition of TBC (○) or NBS (●). The reaction was performed in 60% acetic acid for 10 min at room temperature. Yields of cleavage were determined by automatic amino acid analysis of the released glycine.

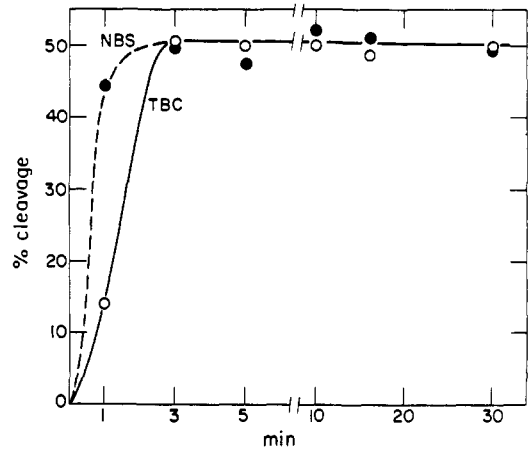


FIGURE 3: Release of glycine with time from Z-Trp-Gly. The reaction was performed in 60% acetic acid with 3 moles of TBC (○) or NBS (●) per mole of peptide, at room temperature. The reaction was stopped by addition of excess sodium thiosulfate; yields of cleavage were determined by automatic amino acid analysis of the released glycine.

hydrin solution (75 mg of ninhydrin in 1.5 ml of glacial acetic acid and 1.0 ml of 70% orthophosphoric acid) was added. The mixture was boiled for 1 hr and proline was determined spectrophotometrically at 530 nm (Sarid *et al.*, 1959, 1962).

Cleavage of the Tryptophanyl Peptide Bond in Glucagon. To a solution of 0.5 μmole of glucagon in 2.0 ml of 70% acetic acid, 25 μmoles of TBC in 50 μl of dioxane was added. The reaction was allowed to proceed at room temperature, with constant stirring, for 15 min. Then the mixture was applied onto a Sephadex G-25 column (1 × 50 cm) and eluted with 0.1 M acetic acid at a rate of 60 ml/hr, 4 ml/fraction. Fractions were analyzed spectrophotometrically at 280 nm. The glucagon (tubes 10–12) and the ninhydrin-positive peak (tubes 13–16) were eluted in separate peaks prior to the reagent.

Results

When Z-tryptophanylglycine was treated with TBC in acidic medium, glycine was liberated. In order to determine optimal conditions for cleavage, tryptophanyl dipeptide models were treated with various molar ratios of TBC or NBS in different solvents, in different hydrogen ion concentrations for varying times. Figure 1 illustrates the pH dependency of cleavage at room temperature. From this figure it is evident that the highest yields of cleavage can be obtained at pH 3. The same yield was obtained when the reaction was carried out in 60–80% acetic acid. Three moles of TBC per mole of peptide brought about optimal cleavage (Figure 2). When Z-Trp-Gly was treated with 3 moles of TBC or NBS per mole of peptide in 60% acetic acid and the reaction was allowed to stand at room temperature, we found that the cleavage reaction reached its highest value after 3–5 min (Figure 3). We therefore chose to carry out the cleavage reaction in 60–80% acetic acid, with 3 moles of TBC/mole of peptide for 10–15 min at room temperature. By this procedure up to 50% cleavage was obtained with several tryptophanyl dipeptide models (Table I).

Selectivity of Reagent. To compare the brominating properties of TBC to those of NBS, an amino acid mixture was reacted with these reagents in 60% acetic acid. As shown in Table II, after exposure to excess TBC or NBS for 15 min at room temperature, tryptophan, tyrosine, histidine, cystine, and methionine were completely destroyed, but in contrast to NBS, TBC does not degrade other amino acids. Figure 4 illustrates the destruction of the oxidation-sensitive amino acids as a function of TBC concentration. As can be seen from this figure, methionine and tryptophan were oxidized rapidly, while cystine, histidine, and especially tyrosine required higher concentrations of TBC for oxidation. By limiting the excess of TBC one could minimize the oxidation of cystine, histidine, and tyrosine.

Modification of Amino Acid Residues in Proteins. Lysozyme, 8CM-lysozyme, and ribonuclease were treated with 60 equiv of TBC in 60% acetic acid (optimal conditions for tryptophanyl peptides cleavage). After 15 min at room temperature, the reaction was stopped and excess reagent was destroyed (see Methods). As expected, The oxidation-sensitive amino acids were damaged by TBC. The amino acid composition of the modified proteins is summarized in Table III. As can be

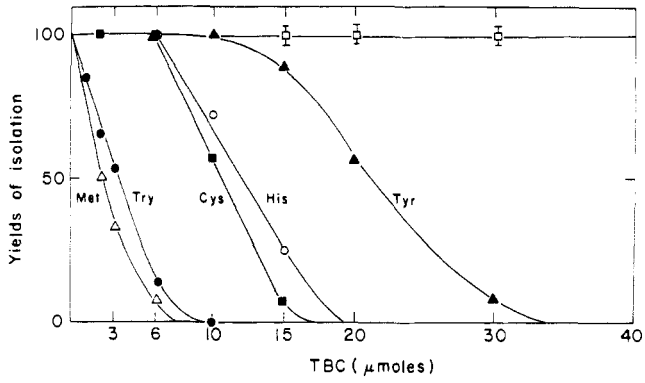


FIGURE 4: Competition between different amino acids for TBC. A mixture of 18 amino acids, 0.25 μmole of each (see text), was reacted with increasing amounts of TBC in 60% acetic acid for 15 min at room temperature. Yields of isolation of the free amino acids were determined by automatic amino acid analysis: methionine (△), tryptophan (●), cystine (■), histidine (○), tyrosine (▲), and all other amino acids (□).

TABLE I: Yields of Cleavage of Tryptophanyl Peptides.[a]

Peptide	Cleavage Product	Cleavage (%)[b]
Z-Trp-Phe	Phe	45
Z-Trp-Ala	Ala	47
Z-Trp-Leu	Leu	44
Ac-Trp-Phe	Phe	46
Ac-Trp-Ala	Ala	48
Ac-Trp-Gly	Gly	49

[a] Cleavage was performed in 60% acetic acid, 3 moles of TBC/mole of peptide, for 15 min at room temperature. [b] Yields of cleavage were determined by automatic amino acid analysis.

TABLE II: Recovery of Amino Acids after Incubation with TBC or NBS.[a]

Amino Acid	Recovery (%)[b] TBC	NBS
Tryptophan	0	0
Lysine	97	75
Histidine	0	0
Arginine	99	72
Aspartic acid	98	74
Threonine	100	86
Serine	99	58
Glutamic acid	100	71
Proline	99	85
Glycine	100	74
Alanine	98	72
Half-cystine	0	0
Valine	99	80
Methionine	0	0
Isoleucine	99	83
Leucine	100	78
Tyrosine	2	0
Phenylalanine	101	81

[a] The reaction mixture contained 0.25 μmole of each amino acid and 15 μmoles of the brominating agent, in 60% acetic acid for 15 min. [b] As recovered by automatic amino acid analysis.

seen from this table, histidine in the native molecule is protected from TBC to a certain extent, and only in the unfolded molecule is it completely modified. Tryptohpan is modified quantitatively and so is tyrosine. Cystine is not completely oxidized. About 15% of the original cystine can be recovered and only 20–35% of it appear as cysteic acid. The rest was not identified and probably reached some intermediate oxidation stages. Methionine is recovered quantitatively as is S-CM-cysteine, but in separate experiments we found out that TBC oxidized these amino acids to the corresponding sulfoxides (as was demonstrated by iodometric titrations and by amino acid analysis). These sulfoxides were reduced back to the original thioethers under the hydrolysis conditions (Ray and Koshland, 1960).

Titrimetric Monitoring of the Reaction of TBC with Several Oxidation-Sensitive Amino Acids. In order to follow this reaction the appropriate amino acid derivative or peptide was treated with TBC. Excess oxidative bromine (TBC) was back-titrated iodometrically, and the amount of bromide ions which had been liberated into the solution was titrated, in another sample, with $Hg(ClO_4)_2$. The results of these titrations are summarized in Table IV and in Figures 5 and 6.

As can be seen from Table IV, the reaction between cystine residues and TBC proceeds through several steps of oxidation, consuming 2, 4, or 5 equiv of TBC depending on the nature of the peptide.

Tyrosine residues consumed 2 equiv of TBC which were not liberated into the solution as bromide ions. Since 3-iodotyrosine absorbed only 1 equiv of TBC and 3,5-diiodotyrosine did not absorb any, one can assume that tyrosine residues were brominated by TBC at positions 3 and 5 of the phenol moiety to form 3,5-dibromotyrosine. The formation of this derivative was also demonstrated by its amino acid analysis: peak position at 47 min (lysine 57 min) on the medium column (15 cm) of the automatic amino acid analyzer (pH 5.28).

Methionine residues consumed only 1 equiv of TBC to form a sulfoxide derivative. This derivative was identified by amino acid analysis and could be further oxidized (by NBS) to methionine sulfone or reduced back (by thioglycolic acid or thiosulfate) to methionine.

The reaction between NBS and histidine was investigated by Shaltiel and Patchornik (1963), who suggested the possibility of several side reactions. They found that 3 equiv of NBS was first consumed by the imidazole ring and then released into the solution as bromide ions. As can be seen from Figure 5, the reaction between TBC and hisitidine is similar but much slower. The presence of a base (sodium acetate) enhanced only the early stages of the reaction, but the presence of a strong acid ($HClO_4$) slowed it markedly.

Tryptophan is modified quite readily. In 5–10 min the bromination reaction is completed (Table IV), and 3 atoms of bromine are consumed by the indole nucleus. Two of these atoms are released into the solution as bromides at a rate similar to that of the bromination reaction (Figure 6).

Selectivity of the Cleavage. Since tryptophanyl (Patchornik *et al.*, 1960), tyrosyl (Schmir *et al.*, 1959) and histidyl (Shaltiel

TABLE III: Modification of Amino Acid Residues in Proteins by TBC.[a]

Amino Acid[b]	Ribonuclease			Lysozyme			8CM-lysozyme	
	Calcd[c]	Found	After TBC	Calcd[d]	Found	After TBC	Found	After TBC
Tryptophan				6	5.9	0	5.9	0
Lysine	10	9.9	10.0	6	5.9	5.9	5.9	5.9
Histidine	4	3.9	2.2	1	0.9	0.9	1.0	0
Arginine	4	4.0	4.0	11	11.0	11.1	10.9	11.0
Aspartic acid	15	14.7	14.6	21	20.8	20.9	21.1	21.3
Threonine	10	9.7	9.7	7	6.9	7.0	6.9	7.1
Serine	15	13.3	13.2	10	8.8	8.9	8.9	9.0
Glutamic acid	12	12.1	12.2	5	5.1	5.0	5.0	4.9
Proline	4	3.9	4.1	2	2.1	2.1	1.8	2.0
Glycine	3	3.2	3.1	12	11.9	12.2	12.0	12.1
Alanine	12	*12.0*	*12.0*	*12*	*12.0*	*12.0*	*12.0*	*12.0*
Half-cystine	8	7.2	1.1	8	7.2	1.1		
Valine	9	9.0	8.9	6	5.7	5.6	5.8	5.6
Methionine	4	3.8	3.7	2	1.9	1.9	1.8	1.8
Isoleucine	3	2.1	2.2	6	5.5	5.6	5.8	5.6
Leucine	2	1.9	1.9	8	7.9	8.0	7.8	7.8
Tyrosine	6	5.4	0	3	2.7	0	2.8	0
Phenylalanine	3	2.9	2.9	3	3.1	3.0	3.0	2.8
CM-cysteine							7.8	7.2
Cysteic acid		tr	2.7		tr	1.4	tr	tr

[a] The reaction was performed in 60% acetic acid for 15 min at room temperature with 60 equiv of TBC. [b] After hydrolysis in 6 N HCl, 22 hr 110°; not corrected. [c] According to Smyth *et al.* (1962). [d] According to Canfield (1963). [e] Determined colorimetrically before acid hydrolysis.

and Patchornik, 1963) peptide bonds are cleaved by NBS under acidic conditions, it was necessary to test the selectivity of the cleavage reaction by TBC. This was done by using equimolar mixtures of tryptophanyl, tyrosyl, and histidyl dipeptides (Table V). The extent of cleavage of each of the peptides could be determined simultaneously since the dipeptides were chosen so that a different amino acid was liberated from each of the peptides in the mixture. As can be seen from this table, only tryptophanyl peptide bonds were cleaved by TBC whereas tyrosyl and histidyl peptide bonds remained intact.

Cleavage of Tryptophanyl and Tyrosyl Peptide Bonds by NBS in Urea. Funatsu *et al.* (1964) have reported that NBS

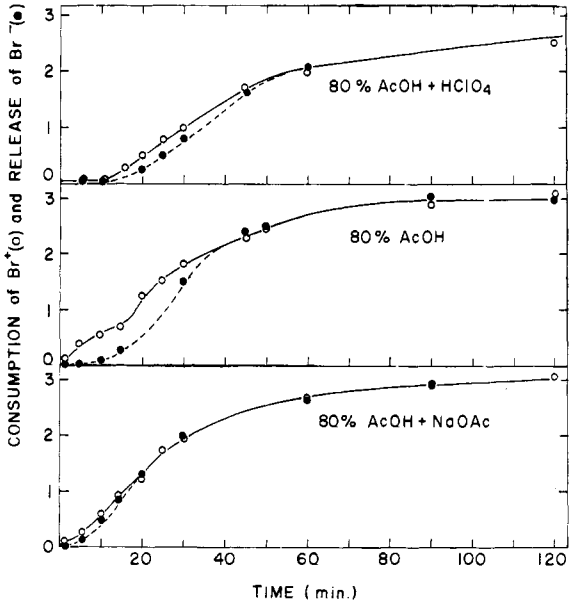


FIGURE 5: Titrimetric monitoring of the reaction of Z-His-Gly with TBC. The peptide (0.5 μmole/ml) was treated with 10 equiv of TBC in 80% acetic acid (middle curve); in 80% acetic acid containing 10 equiv of $HClO_4$ (upper curve); in 80% acetic acid containing 10 equiv of sodium acetate (lower curve). The titrations were carried out as described under Methods. Consumption of oxidative bromine (○); release of bromide (●).

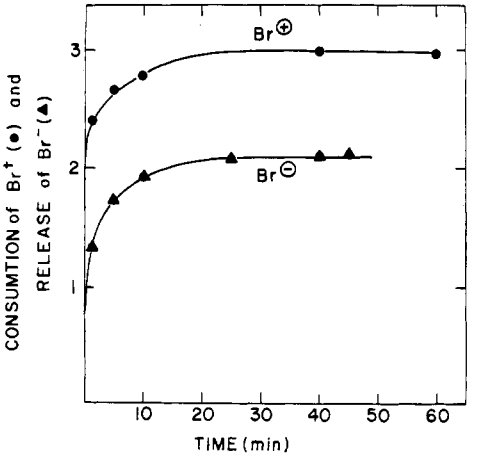


FIGURE 6: Titrimetric monitoring of the reaction of Ac-Trp-NH_2 with TBC. The reaction was performed in 80% acetic acid and the titrations were carried out as described in Figure 5. Consumption of oxidative bromine (●); release of bromide (▲).

TABLE IV: Bromination of Oxidation-Sensitive Amino Acids by TBC.[a]

Peptide	Equiv of TBC Consumed after 5 min	10 min	20 min	30 min
Cystine				
Bis(Z)-Cys-$(OMe)_2$	3.3	3.5	3.8	3.85
Bis(Z-Phe)-Cys-$(OMe)_2$	1.4	1.7	2.0	1.95
Cys-Cys-Ala	3.0	4.3	4.5	4.85
Oxidized glutathione	5.0	5.0	5.1	4.9
Tyrosine				
Ac-Tyr-NH_2	1.80	2.01	1.92	2.0
3-Iodotyrosine	0.90	1.00	0.95	1.05
3,5-Diiodotyrosine	0.02	0.07	0.03	0.05
Phloretylglycine	1.85	1.95	2.00	2.05
Methionine				
Methionine	0.53	0.72	0.98	1.06
DL-Ala-DL-Met	1.09	1.06	1.00	0.97
Z-Cys(*S*-Me)-OMe	1.10	1.06	1.01	1.01
Histidine				
Z-His-Phe	0.4	0.8	1.4	1.8
Z_2-His-Val-OBzl	0.3	0.8	1.0	1.9
Histidine	0.5	1.1	1.3	1.7
Tryptophan				
Z-Trp-Gly-OBzl	2.45	2.60	2.85	2.95
Ac-Trp-NH_2	2.80	2.95	3.05	3.00
Ac-Trp-Ala	2.90	2.85	2.95	3.05
Ac-Trp-Phe	2.75	2.90	2.95	3.00

[a] To 5 μmoles of the amino acid derivative in 9.9 ml of 80% acetic acid, 50 μmoles of TBC in 0.1 ml of dioxane was added. Fractions (2 ml) were taken out of the reaction mixtures at indicated times and excess TBC was determined titrimetrically as described under Methods.

TABLE V: Cleavage of Mixtures of Tryptophanyl, Tyrosyl, and Histidyl Peptides.[a]

Peptide Mixture[b]	Cleavage Product	Cleavage (%)[c] NBS	TBC
Ac-Trp-Gly	Gly	47	49
Ac-Tyr-Ala	Ala	45	1
Ac-His-Phe	Phe	26	0
Ac-Trp-Ala	Ala	48	48
Ac-Tyr-Phe	Phe	51	1
Ac-His-Gly	Gly	27	0
Ac-Trp-Phe	Phe	50	46
Ac-Tyr-Gly	Gly	62	1
Ac-His-Ala	Ala	20	1
Z-Trp-Phe	Phe	52	49
Z-Tyr-Gly	Gly	48	2
Z-His-Leu	Leu	26	0

[a] In 80% acetic acid, for 15 min at room temperature. [b] To an equimolar mixture of three dipeptides, 9 equiv of NBS or TBC was added. [c] Determined by automatic amino acid analysis.

TABLE VI: Cleavage of Mixtures of Tryptophanyl and Tyrosyl Peptides by NBS in Urea.[a]

Peptide[b]	Cleavage Product	Cleavage (%)[c] No Urea	Urea 0.2 M	0.4 M
Phloretylglycine	Gly	60	61	47
Z-Trp-Leu	Leu	44	43	40
Ac-Tyr-Ala	Ala	44	43	37
Ac-Trp-Phe	Phe	45	44	41
Z-Tyr-Phe	Phe	51	51	42
Z-Trp-Gly	Gly	47	47	45

[a] NBS was dissolved in 60%, acetic acid–8 M urea 10 min prior to reaction. [b] Equimolar mixture of the two peptides with 6 equiv of the brominating agent, for 15 min at room temperature. [c] Determined by automatic amino acid analysis.

in 8 M urea (NBU) reacts with tyrosine and its derivatives at a rate 200 times more slowly than does NBS in aqueous solutions. As it is not always desirable to use a high concentration of urea in the reaction mixture, we tested the cleavage reaction with NBS at low concentrations of urea (0.2–0.4 M). It can be seen from Table VI that urea concentrations of up to 0.4 M had hardly any effect on the cleavage next to tryptophan residues, and only a very small effect on the cleavage next to tyrosine residues. Another way to check the cleavage next to tyrosine residues, is to follow the increase of the optical density at 260 nm, which is characteristic for the formation of the intermediate dibromodienone–spirolactone (Schmir and Cohen, 1961). This method was used in order to follow the effect of higher concentrations of urea (up to 2 M) on the cleavage of tyrosyl residues by NBS. As can be seen from Figure 7, the rate of the dibromodienone–spirolactone formation of phlorethylglycine by NBS is decreased markedly by increasing the urea concentration in the reaction mixture. When a similar experiment with Ac-Trp-Leu was carried out, hardly any effect of urea could be observed, as was demonstrated by Funatsu *et al.* (1964).

Cleavage of Tryptophanyl Peptide Bonds in 8CM-lysozyme. When 8CM-lysozyme was incubated with 60 equiv of TBC in acetic acid, cleavage of tryptophanyl peptide bonds occurred in addition to modification. For a comparison, RNase, a protein which does not contain tryptophan residues, was incubated with TBC under the same conditions. The released new amino terminals were dinitrophenylated, hydrolyzed, separated, identified, and determined quantitatively by a spectrophotometric assay according to the procedures summarized by Frankel-Conrat *et al.* (1955), Randerath (1966), and Burstein and Patchornik (1965). Amino-terminal proline was determined by the enzyme proline iminopeptidase (Sarid *et al.*, 1959, 1962). The results are summarized in Table VII. From this table it seems that tyrosyl peptide bonds in proteins are not cleaved by TBC, and the expected tryptophanyl peptide bonds in 8CM-lysozyme were cleaved in 5–60% yield. The cleavage of the Trp (62)–Trp (63) bond in this protein was not determined.

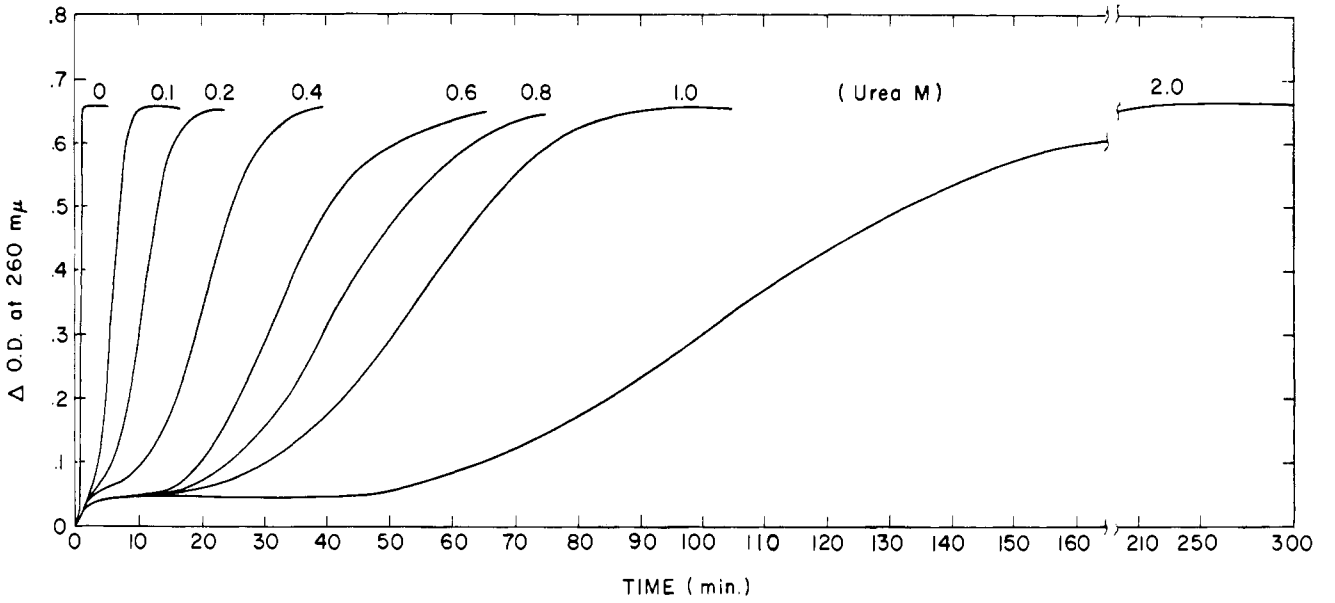


FIGURE 7: Spectrophotometric "titrations" of phloretylglycine with NBS in different urea concentrations. The reactions were performed in 0.1 M acetate buffer (pH 3.5) in the spectrophotometer cell. To 3 ml of the peptide solution (6.5×10^{-5} M) in urea, 1 μmole of NBU was added. The differences in the optical density at 260 nm were recorded as a function of time.

TABLE VII: Cleavage of Tryptophanyl Peptide Bonds in 8CM-lysozyme.[a]

Split Bond	Amino Terminal Determined	Cleavage (%)
	a. In RNase	
Tyr (25)–Cys	Not determined	
Tyr (73)–Gln	Glu	tr
Tyr (76)–Ser	Ser	0
Tyr (92)–Pro	Pro[b]	0
Tyr (97)–Lys	Lys[c]	68
Tyr (115)–Val	Val	0
	b. In 8CM-lysozyme	
Trp (28)–Val Trp (108)–Val	Val	125
Trp (62)–Trp	Not determined	
Trp (63)–*S*-CMCys	CMCys	5
Trp (111)–Arg	Arg	31
Trp (123)–Ile	Ile	47
	Lys[c]	64

[a] In 80% acetic acid for 15 min, at room temperature with 60 equiv of TBC. The new amino terminals were determined by the Dnp method as described under Methods. [b] Determined by proline iminopeptidase. [c] Lysine is the amino-terminal residue of the enzyme.

TABLE VIII: Amino Acid Composition of the COOH-Terminal Tetrapeptide Released from Glucagon upon Cleavage with TBC.[a]

Amino Acid	Theory[b]	Found
Aspartic acid	1	1.0
Threonine	1	0.95
Methionine	1	0.97
Leucine	1	1.05

[a] Glucagon was treated with 5 equiv of TBC in 80% acetic acid for 20 min at room temperature. The tetrapeptide was isolated by gel filtration and hydrolyzed. [b] According to Bromer *et al.* (1957).

Cleavage of the Tryptophanyl Peptide Bond in Glucagon. When glucagon was incubated with 5 equiv of TBC in 80% acetic acid for 20 min at room temperature, cleavage of the tryptophanyl bond occurred. The released carboxy-terminal tetrapeptide was isolated by gel filtration and hydrolyzed. Its amino acid analysis is presented in Table VIII. The yield of isolation was 30% and no other peptide bond was split.

Rearrangement Product of TBC. TBC is stable for months when kept at −20°, while at 4° it decomposes after several weeks and at room temperature after several days. It was found that the decomposition product did not contain oxidative bromine although it still contained three bromine atoms per molecule and had the same elemental analysis as that of TBC. Anhydrous titration with NaOMe revealed 2 equiv of acidic groups, compared to one acidic group in TBC. The nuclear magnetic resonance spectrum of the new compound revealed benzylic hydrogens at 4.38 ppm, phenolic hydrogen at 5.95 ppm (exchangeable in D_2O), and aromatic hydrogens at 7.49 ppm (Figure 8), in a ratio of 2:1:2, respectively. All these data indicate that the rearrangement product is 2,6-dibromo-4-bromomethylphenol. Its melting point, 150°, is in agreement with that reported in the literature, mp 149–150° (Auwers, 1903).

Discussion

The results of these studies with amino acid derivatives, peptides, and proteins indicate that TBC is a much more selective agent than NBS. NBS and other brominating agents,

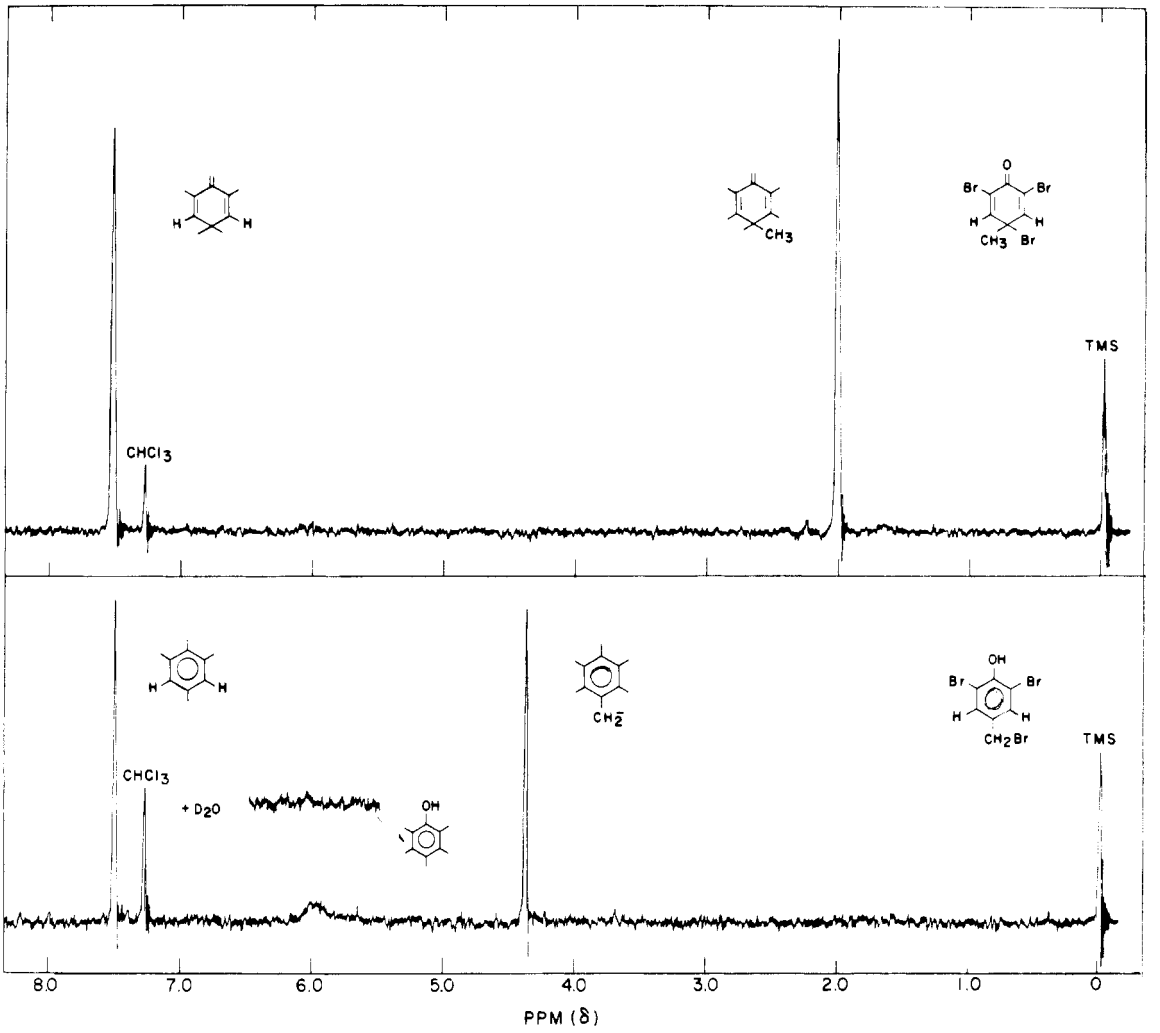


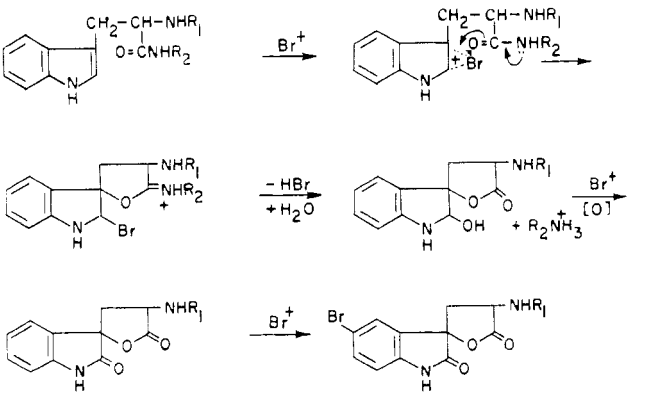
FIGURE 8: Nmr spectra of TBC (upper curve) and its rearrangement product (lower curve) in $CDCl_3$.

such as *N*-bromoacetamide, *N*-bromophthalimide, or aqueous bromine, were found to cleave tryptophanyl (Patchornik *et al.*, 1958a,b; 1960; Ramachandran and Witkop, 1959; Lawson *et al.*, 1960), tyrosyl (Schmir *et al.*, 1959), and histidyl (Shaltiel and Patchornik, 1963) peptide bonds under acidic conditions. TBC, although capable of modifying these residues, will cleave only tryptophanyl peptide bonds while tyrosyl and histidyl peptide bonds remain intact.

TBC, in contrast to NBS, does not react with free amino groups, and therefore excess TBC can be used in the cleavage reaction, when the expected modification of other bromination-sensitive amino acids can be tolerated.

According to the reaction mechanism suggested by Patchornik *et al.* (1960), 3 equiv of oxidative bromine participate in the modification-cleavage reaction (Scheme I). Two equivalents of bromine brominate the indole nucleus and undergo spontaneous debromination through a series of oxidation and hydrolysis reactions. These reactions lead to the formation of an oxindole derivative, which promotes the cleavage reaction. The third bromine atom forms a stable 5-bromooxindole derivative. Under acidic conditions, TBC reacts with tryptophan derivatives in a similar manner. TBC (3 equiv) is consumed by tryptophan derivatives, of which only 2 can be titrated as bromide ions. The pH profile of the TBC-cleavage reaction, as well as the optimal pH value and the yields of cleavage of this reaction, are very similar to those of the

SCHEME I



NBS-cleavage reaction. These findings lead us to believe that the mechanism of the cleavage reaction of tryptophanyl peptide bonds with TBC could be very similar to the one with NBS. TBC reacts with other oxidation-sensitive amino acids. Thioether side chains such as of *S*-carboxymethylcysteine or methionine are oxidized to sulfoxides. If present, sulfhydryl groups must be protected, as in selective modification with many other classes of reagents. Cysteine, methionine, and

tryptophan are very sensitive to oxidation with TBC and in a polypeptide chain, no cleavage of tryptophanyl peptide bonds without oxidation of cysteine and methionine could be obtained. However, the sulfoxides of methionine and *S*-carboxymethylcysteine could be reduced back to the corresponding thioethers with thioglycolic acid or with thiosulfate.

The second group of oxidation-sensitive amino acids consists of cystine, histidine, and tyrosine; among this group, tyrosine is the least bromination-sensitive amino acid. By using limited amounts of TBC, oxidation of these amino acids could be minimized.

The reaction of cystine peptides with TBC proceeds through several steps of oxidation. These steps were demonstrated when an oxidation reaction with TBC, a bulky oxidation agent, was carried out with a series of cystine peptides. When a sterically *unhindered* disulfide bond such as in cystine, oxidized glutathione or Cys-Cys-Ala was oxidized with TBC, 5 equiv of TBC was consumed and 2 residues of cysteic acid were formed (as was shown in the case of cystine oxidation). A *slightly hindered* disulfide bond, such as in bis(benzyloxycarbonyl)cystinedimethyl ester, consumed only 4 equiv of TBC, and a relatively *hindered* disulfide bond, such as in bis(benzyloxycarbonylphenylalanyl)cystinedimethyl ester reacts with only 2 equiv of TBC. Such distinct steps of oxidation of disulfide bonds in cystine peptides were suggested by Wilson and Cohen (1963) and Wilchek *et al.* (1966) for the oxidation of cystine with NBS.

Imidazoles are oxidized by oxidation reagents (Cohen, 1968). Shaltiel and Patchornik (1963), who investigated the reaction of histidine with NBS, found that 3 equiv of NBS was needed in order to obtain optimal cleavage of histidyl peptide bonds. We found that histidyl peptides are not cleaved by TBC, and the modification reaction which involved 3 equiv of TBC was much slower than the one with NBS. The fact that bromination reactions of the imidazole ring were slowed in the presence of a strong acid and were accelerated in the presence of a general base indicates that several dehydrobromination intermediate steps are involved in the modification of histidyl residues by TBC.

TBC brominates the phenol moiety of tyrosine residues; 2 equiv of TBC is consumed and 2,6-dibromotyrosine is formed. No bromination of the phenol ring at position 4 occurs, and therefore no dienone is formed and no cleavage of tyrosyl peptide bonds was observed.

The modification-cleavage reaction was tried with several model compounds, peptide and with several proteins such as glucagon, lysozyme, 8CM-lysozyme, and ribonuclease. In all cases tryptophanyl peptide bonds were the only ones which were cleaved by TBC.

TBC is poorly soluble in water and most of the cleavage reactions were performed in acetic acid (50–80%). This solvent will dissolve many proteins and peptides. If aqueous solutions are used, the reagent may be dissolved in an organic solvent and then added to the protein solution.

These studies indicate that TBC is a highly selective reagent for the cleavage of tryptophanyl peptide bonds in peptides and proteins. Considering the fact that proteins, in general, contain a relatively small number of tryptophan residues, TBC may be of possible general utility for fragmentation of polypeptides and proteins, especially for sequence analysis.

References

Atassi, M. Z. (1967), *Arch. Biochem. Biophys. 120*, 56.

Auwers, K. (1903), *Ber. 36*, 1883.

Bromer, W. W., Sinn, L. G., and Behrns, O. K. (1957), *J. Amer. Chem. Soc. 79*, 2807.

Burstein, Y., and Patchornik, A. (1965), *Israel J. Chem. 3*, 97.

Burstein, Y., Wilchek, M., and Patchornik, A. (1967), *Israel J. Chem. 5*, 65.

Canfield, R. E. (1963), *J. Biol. Chem. 238*, 2691.

Cohen, L. A. (1968), *Annu. Rev. Biochem. 37*, 698.

Frankel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 359.

Fries, K., and Oehmke, G. (1928), *Ann. 462*, 1.

Funatsu, M., Green, N. M., and Witkop, B. (1964), *J. Amer. Chem. Soc. 86*, 1846.

Kolthoff, I. M., and Sandell, E. B. (1952), Textbook of Quantitative Inorganic Analysis, New York, N. Y., The MacMillan Co., p 585.

Lapin, L. N., and Zamanov, R. K. (1955), *Zh. Anal. Khim. 10*, 364.

Lawson, W. B., Patchornik, A., and Witkop, B. (1960), *J. Amer. Chem. Soc. 82*, 5918.

Morishita, M., Sowa, T., Sakiyama, F., and Narita, K. (1967), *Bull. Chem. Soc. Japan 40*, 433.

Omenn, G. S., Fontana, A., and Anfinsen, C. B. (1970), *J. Biol. Chem. 245*, 1897.

Patchornik, A., Lawson, W. B., Gross, E., and Witkop, B. (1960), *J. Amer. Chem. Soc. 82*, 5923.

Patchornik, A., Lawson, W. B., and Witkop, B. (1958a), *J. Amer. Chem. Soc. 80*, 4747.

Patchornik, A., Lawson, W. B., and Witkop, B. (1958b), *J. Amer. Chem. Soc. 80*, 4748.

Previero, A., and Bordignon, E. (1964), *Gazz. Chim. Ital. 94*, 630.

Previero, A., Coletti-Previero, M. A., and Jollès, P. (1966a), *Biochem. Biophys. Res. Commun. 22*, 17.

Previero, A., Coletti-Previero, M. A., and Jollès, P. (1966b), *Biochim. Biophys. Acta 124*, 400.

Previero, A., Scoffone, E., Benassi, C. A., and Pajetta, P. (1963), *Gazz. Chim. Ital. 93*, 849.

Ramachandran, L. K., and Witkop, B. (1959), *J. Amer. Chem. Soc. 81*, 4028.

Randerath, K. (1966), Thin Layer Chromatography, New York, N. Y., Academic Press, p 116.

Ray, W. J., and Koshland, Jr., D. E. (1960), *Brookhaven Symp. Biol. 13*, 135.

Sarid, S., Berger, A., and Katchalski, E. (1959), *J. Biol. Chem. 234*, 1740.

Sarid, S., Berger, A., and Katchalski, E. (1962), *J. Biol. Chem. 237*, 2207.

Schmir, G. L., and Cohen, L. A. (1961), *J. Amer. Chem. Soc. 83*, 723.

Schmir, G. L., Cohen, L. A., and Witkop, B. (1959), *J. Amer. Chem. Soc. 81*, 2228.

Shaltiel, S., and Patchornik, A. (1963), *J. Amer. Chem. Soc. 85*, 2799.

Smyth, D. G., Stein, W. H., and Moore, S. (1962), *J. Biol. Chem. 237*, 1845.

Spackman, D. H. (1963), *Fed. Proc., Fed. Amer. Chem. Soc. 22*, 244.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spande, T. F., and Witkop, B. (1967), *Methods Enzymol. 11*, 498.

Spande, T. F., Witkop, B., Degani, Y., and Patchornik, A. (1970), *Advan. Protein Chem. 24*, 97.

Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.

Stark, G. R. (1969), *Advan. Protein Chem. 24*, 261.
Wilchek, M., Spande, T. F., Milne, G. W. A., and Witkop, B. (1967), *J. Amer. Chem. Soc. 98*, 3349.
Wilchek, M., and Witkop, B. (1967), *Biochem. Biophys. Res. Commun. 26*, 296.
Wilchek, M., Zioudru, C., and Patchornik, A. (1966), *J. Org. Chem. 31*, 2865.
Wilson, J. G., and Cohen, L. A. (1963), *J. Amer. Chem. Soc. 85*, 560.
Witkop, B. (1961), *Advan. Protein Chem. 16*, 221.

Characteristics of the Reversible Heat, Solvent, and Detergent Denaturation of Leucine Binding Protein†

Kenneth Berman† and Paul D. Boyer*

ABSTRACT: The conformation and binding capacity of leucine binding protein from *Escherichia coli* was measured over a range of temperatures and solvent compositions. At temperatures less than 60° the apparent dissociation constant (K_d) followed a linear Arrhenius relationship and the protein showed no fluorescence changes indicative of conformational changes. Above 60° large increases in K_d were accompanied by quenching and shift of protein fluorescence typical of gross denaturation. Presence of 0.16 mM L-leucine increased the transition temperature by about 10°. The changes were almost completely but slowly reversible. Increases in methanol, ethanol, and dioxane concentration did not produce appreciable changes in binding until relatively high concentrations were reached where pronounced fluorescence changes occurred, except that with dioxane a largely competitive inhibition of leucine binding was noted. Significantly, optical rotatory dispersion measurements indicated 20 vol % dioxane nearly doubled the helical content of the protein with little change in binding capacity. Presence of leucine did not prevent the optical rotatory dispersion changes induced by dioxane. Addition of sodium dodecyl sulfate decreased the total binding capacity and increased K_d. Gel electrophoresis experiments gave no evidence for interconvertible forms with differing binding capacities. These and other results do not give support to active transport models based on conformational changes of binding proteins.

In the past few years, various low molecular weight proteins have been isolated from bacteria, particularly *Salmonella typhimurium* and *Escherichia coli*, which specifically bind certain amino acids, sugars, or inorganic ions. Roles for these proteins as carriers in the active transport of their ligands is suggested by a number of criteria, summarized by Pardee (1968) and by Kaback (1970). Similar considerations have been given by Penrose *et al.* (1970) for leucine binding protein of *Escherichia coli*, studies of which are reported here.

How metabolic energy may be coupled to active transport is unknown. No enzyme activity, modification in binding, or other response to the presence of ATP has been found associated with these proteins. One possibility for participation of the binding proteins in active transport is that energy-linked conformation changes are correlated with the release of the ligand. Support for such a possibility comes from the detection by Boos and Gordon (1971) of interconvertible forms of galactose binding protein. These forms had properties under physiological conditions like native protein, but had pronounced differences in binding capacity.

Such interconvertible forms have not been reported for other binding proteins. Penrose *et al.* (1970) found no evidence of ligand-induced conformation changes as assessed by optical rotatory dispersion (ORD) and fluoresence probes. They did demonstrate that leucine binding protein will undergo reversible denaturation with respect to activity and physical properties when treated with urea or guanidine hydrochloride, or exposed to high temperature or extremes of pH. The conditions they used were such that gross denaturation of the binding protein had likely occurred, that is, the disruption of many hydrophobic interactions and hydrogen bonds, followed by removal of the denaturing condition and regaining of the native protein properties. For example, the binding protein was exposed to 100°, then cooled, and assayed at room temperature. It is likely, however, that any conformation changes associated with the release of the bound substance as part of the transport process would be quite limited. A slight displacement of interacting portions of the molecule, or of even one or a few groups might suffice. Extensive changes characteristic of gross denaturation would not be necessary for changes in binding activity.

The present paper presents data on the binding capacity of leucine binding proteins over graded temperature ranges and with various solvent compositions, with use of protein fluorescence and optical rotatory dispersion as criteria for conformation changes and denaturation.

Materials

Crystalline leucine binding protein was prepared as described by Penrose *et al.* (1968) but using the osmotic shock procedure of Anraku (1968) to allow the preparation of larger amounts of protein. The protein concentration was determined using a value of 0.65 as the optical density of 1 mg/ml at 280 nm and a molecular weight of 35,000 (Penrose *et al.*,

† From the Department of Chemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received June 5, 1972.* Supported in part by Grant GM-11094 of The Institute of General Medical Sciences, U. S Public Health Services.

‡ Postdoctoral Fellow of the Institute of General Medical Sciences, U. S. Public Health Service.