Cleavage of Tryptophanyl Peptide Bonds in Cytochrome \(b_5\) by Cyanogen Bromide*

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JURIS OZOLS AND CRAIG GERARD
WITH THE TECHNICAL ASSISTANCE OF CONRAD STACHELEK
From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032

Quantitative cleavage of peptide bonds adjacent to tryptophanyl and methionyl residues in the polar moiety of cytochrome \(b_5\) was effected using cyanogen bromide in the presence of heptafluorobutyric and formic acids. Application of this method to native cytochrome \(b_5\), resulted in cleavage at tryptophanyl and methionyl residues in the polar and membrane-bound segments in high yield. Amino acid analysis of peptides isolated from such digests indicated that tyrosine was modified and the derivative eluted in a position preceding lysine; however, the color constant with ninhydrin remained unchanged. Hydrolytic acid hydrolysis of the phenylthiohydantoin derivative of the modified tyrosine regenerating the parent amino acid. Peptides containing the altered tyrosine remained susceptible to chymotryptic cleavage at this residue. Cleavage of methionyl bonds could be prevented by azzene blue-sensitized photodecomposition prior to cytochrome \(b_5\)/anhydrone heptafluorobutyric acid treatment. The conditions employed for photooxidation were selective for methionyl residues, and the yield and specificity of tryptophanyl cleavage by the cyanogen bromide/anhydrone heptafluorobutyric acid method was unaffected.

In previous studies we determined the covalent structures of the polar moiety (residues 1 to 98) of liver microsomal cytochrome \(b_5\) from several species (1-4). The elucidation of the entire primary structure of this hemoprotein, however, has been an extremely formidable task. Enzymatic hydrolysis of the segment comprising the COOH terminus of the molecule yielded peptides refractory to separation as well as sequence analysis (4, 5). Moreover, several well documented chemical cleavage procedures generated peptides from this region in yields of less than 10%. No significant cleavage of methionyl bonds could be demonstrated when native bovine, horse, or rat cytochrome \(b_5\) preparations were incubated with a large excess of cyanogen bromide (CNBr) using 70 or 80% formic acid as the solvent. Since the presence of oxidized methionine residues in these preparations could be ruled out, conditions were sought to expose and cleave the reversibly methionine residues. The use of cyanogen bromide for the specific cleavage of methionyl peptide bonds in peptides and proteins has been well documented (6-8). The advantages of this method are that the reagent is volatile and is active under a wide variety of solvent conditions such as hydrochloric, formic, or trifluoroacetic acids (6). Under such conditions many proteins are known to be cleaved at methionyl residues in yields exceeding 80% (8).

Tryptophanyl bonds in peptides may be cleaved by several methods, and a wide variety of oxidizing agents cleave with some specificity at peptide bonds adjacent to tyrosyl, prolyl, and histidyl residues (6, 7). While simple peptides are readily cleaved by these methods, such fragmentations, however, cannot necessarily be extended to large peptides and proteins.

In this communication we report that methionyl and tryptophanyl bonds are cleaved by CNBr in the presence of formic and heptafluorobutyric acids. Furthermore, the cleavage may be rendered specific for tryptophanyl residues by the modification of methionines by means of dye-sensitized photochemical oxidation in 84% acetic acid (9). This methodology provides peptides in high yield which are suitable for sequence analysis, and which may be separately studied for biological and immunological activity.

**EXPERIMENTAL PROCEDURES**

Cytochrome \(b_5\) preparations were isolated as previously described by Ozols (1, 10). Cytochrome \(c\), type III, was a product of Sigma. CNBr was purchased from either Pierce Chemical Co. (Rockford, Ill.) or Eastman Organic Chemicals (Rochester, N.Y.). Formic acid (88%) was a product of Baker and Adamson. Anhydrous HFBA, as well as other reagents for sequence analysis, was obtained from Beckman.

The amino acid compositions of the peptides and proteins were determined on acid hydrolysates of the samples with a Beckman 121 automatic amino acid analyzer, as described by Spackman et al. (11). Automated sequence analyses were performed on a Beckman 890C Sequencer. The peptide program 102974, supplied by the manufacturer, was employed to degrade CNBr digests or isolated peptides. The phenylthiohydantoin derivatives were identified by thin layer chromatography and quantitated by amino acid analysis following back conversion of derivatives to the corresponding amino acids by hydrolysis in hydroiodic acid vapor as described previously (4). Other experimental procedures have been described in earlier publications (4).

Cleavage of peptide bonds adjacent to tryptophanyl and methionyl bonds was performed as follows. To 100 to 500 nmol of hemochrome, 1.0 ml each of 88% formic acid and anhydrous HFBA were added. After addition of 700 mg of solid CNBr, the samples were

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held for 24 h in the dark. The reagent and solvents were removed with a stream of N₂ and the remaining white material was suspended in 10.0 ml of H₂O and lyophilized. The dried material was dissolved in 1.0 ml of 88% formic acid and a suitable sample (45 to 200 nmol) was applied directly to the Sequencer cup for quantitative NH₄⁺-terminal analysis. For gel filtration experiments, the lyophilized digest was dissolved in 50% acetic acid and applied to a column of Sephadex G-75 (2.9 × 105 cm) which had been equilibrated with 50% acetic acid. Gel filtration was performed at 20°C and fractions of 3 ml were collected at a flow rate of 13 ml/h. Peptide fractions were identified by the ninhydrin reaction after alkaline hydrolysis (10).

For selective cleavage at tryptophanyl bonds the protein preparation were photooxidized prior to CNBr/HFBA treatment by the following procedure. A sample of 300 to 500 nmol of either porcine cytochrome b₅, equine heart cytochrome c, or rabbit polaron cytochrome b₅ (residues 1 to 90) was dissolved in 2.0 ml of 84% acetic acid dissolved in 1.0 ml of 88% formic acid and transferred to a Pyrex test tube (1 x 10 cm) equipped with a chrome 6, (residues 1 to 90) was dissolved in 2.0 ml of 84% acetic acid and transferred quantitatively to the Sequencer cup. In Experiment 3, cleavage was performed in the absence of heptafluorobutyric acid (–HFBA).

### RESULTS AND DISCUSSION

The polar segment of rabbit cytochrome b₅ (residues 1 to 97) was chosen to confirm the applicability of the CNBr/HFBA method to the selective cleavage of tryptophanyl and methionyl peptide bonds for the following reasons: (a) this protein contains a single tryptophanyl and methionyl residue; (b) its entire primary structure is known (1); (c) the NH₂-terminus of this polypeptide is masked, thus simplifying sequence analysis of the digest. Sequencer analysis of several CNBr/HFBA digests of the rabbit preparation before and after photooxidation are shown in Table I. Only two sequences are generated from the digest. One sequence results from cleavage at tryptophan (position 26) and the yield obtained from the Sequencer indicates that the cleavage is quantitative. The second cleavage occurs at methionine (position 95), generating a dipeptide (Table I, Experiments 1 and 2). It is clear from these data that the CNBr/HFBA cleavage is confined to the tryptophanyl residue 26 and methionine residue 95. Omission of HFBA from the reaction mixture (Table I, Experiment 3) limits cleavage mainly to the methionine residue, but some cleavage at the tryptophanyl residue can be detected. CNBr/HFBA cleavage of the photooxidized rabbit preparation generated a single sequence corresponding to specific cleavage at Trp-26 (Table I, Experiment 4). Amino acid composition as well as sequence analysis (Table I, Experiment 4, Cycles 4 and 5) indicated clearly that histidyl residues were not modified.

Fig. 1 shows a typical gel filtration profile of the CNBr/HFBA-digested rabbit polaron segment. The first peak (CBI) represents the core peptide, residues 37 to 95; the second peak (CBI) contains the NH₂-terminal peptide, residues 1 to 26. The dipeptide (residues 96 to 97) is eluted in fractions corresponding to the included volume of the column. The amino acid composition of these peptides is shown in Table II. There is a good agreement between the amino acid analysis and values based upon sequence data. Interestingly, tyrosine obtained by acid hydrolysis of peptides CBI and CBII eluted in a position preceding the lysine peak. The color yield of tyrosine at this position, however, was essentially identical to the parent residue. The altered elution position of tyrosine after exposure to the CNBr/HFBA reaction mixture was confirmed with a standard mixture of amino acids. However, H1 hydrolysis of the PTH-derivative of tyrosine, obtained from Sequencer analysis of peptides isolated from CNBr/HFBA digests, yielded the parent molecule with unaltered chromatography behavior (Table I).

In order to determine whether peptides containing modified tyrosine are susceptible to enzymatic hydrolysis, chromatographic hydrolysis of peptide CBII (residues 1 to 26) was performed and at this position, however, was essentially identical to the parent residue. The altered elution position of tyrosine after exposure to the CNBr/HFBA reaction mixture was confirmed with a standard mixture of amino acids. However, H1 hydrolysis of the PTH-derivative of tyrosine, obtained from Sequencer analysis of peptides isolated from CNBr/HFBA digests, yielded the parent molecule with unaltered chromatography behavior (Table I).

![Fig. 1. Gel filtration of a CNBr/HFBA digest of 0.4 μmol of rabbit cytochrome b₅ fragment (residues 1 to 97) on a Sephadex G-75 column (2.9 x 105 cm) equilibrated with 50% acetic acid. Fractions of 3 ml were collected at a flow rate of 13 ml/h, and 100- to 200-μl aliquots were analyzed by the ninhydrin method (9).](http://www.jbc.org/)

### Table I

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino acids identified after HI hydrolysis of PTH</th>
<th>Yield</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
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<tr>
<td></td>
<td>nmol</td>
<td></td>
<td>nmol</td>
<td>nmol</td>
<td>nmol</td>
<td>nmol</td>
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<tr>
<td>1</td>
<td>Leucine + glutamic acid</td>
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<td>96 + 4</td>
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</tr>
<tr>
<td>2</td>
<td>Isoleucine + threonine</td>
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<td>3 + 5</td>
<td>33 + 1</td>
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</tr>
<tr>
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<td>Leucine</td>
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<td>46</td>
<td>7</td>
<td>90</td>
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</tr>
<tr>
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<td>Histidine</td>
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<tr>
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<td>22</td>
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<td>Leucine</td>
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<tr>
<td>11</td>
<td>Threonine</td>
<td>20</td>
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</tbody>
</table>

* Isoleucine is regenerated by HI hydrolysis as isoleucine + α-derivatives and threonine as α-aminobutyric acid.
Cleavage of Tryptophanyl Peptide Bonds

<table>
<thead>
<tr>
<th>Amino acid composition of peptides produced by CNBr-HFBA cleavage of rabbit cytochrome b$_5$ fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples were hydrolyzed for 24 h and duplicate analyses were performed on each hydrolysate. The number of residues per molecule of peptide determined from the complete sequence.</td>
</tr>
<tr>
<td>Amino acid</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Lysine</td>
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<tr>
<td>Histidine</td>
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<td>Arginine</td>
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<td>Aspartic acid</td>
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<td>Threonine</td>
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<td>Serine</td>
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<td>Glutamic acid</td>
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<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
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<tr>
<td>Alanine</td>
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<tr>
<td>Valine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine*</td>
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<td>Phenylalanine</td>
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<td>Tryptophan</td>
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<td>Methionine</td>
</tr>
<tr>
<td>Yield</td>
</tr>
<tr>
<td>Fraction</td>
</tr>
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</table>

* Tyrosine was calculated from the peak height of its derivative eluting from amino acid analysis column at a position preceding the lysine peak.

The digest was subjected to several cycles of automated sequence analysis (Table III). These results indicate that complete cleavage occurred at Tyr-11 and His-19 as reported previously. Thus, chymotryptic hydrolysis of the peptides obtained from CNBr/HFBA digests proceeded normally.

The usefulness and reliability of the methods described here in primary structure analysis of proteins is illustrated by the results obtained with native cytochrome b$_5$. Gel filtration of the porcine cytochrome b$_5$ digest gave five peptides (Fig. 2). Table IV gives the amino acid composition and yield of the peptides isolated. The purity of the peptides was confirmed by sequence analysis.

The usefulness and reliability of the methods described here in primary structure analysis of proteins is illustrated by the results obtained with native cytochrome b$_5$. Gel filtration of the porcine cytochrome b$_5$ digest gave five peptides (Fig. 2). Table IV gives the amino acid composition and yield of the peptides isolated. The purity of the peptides was confirmed by sequence analysis.

In order to demonstrate the general applicability of these methods to other proteins, cytochrome c was photooxidized and cleaved with CNBr/HFBA. Seven cycles of automated sequence analysis of the cytochrome c digest confirmed the aforementioned tryptophanyl bond cleavage and specificity. In addition, Table VI shows that the photooxidized methionyl residues are not cleaved by CNBr/HFBA and do not interfere with subsequent analysis and that the PTH-derivative may be identified by thin layer chromatography. These data are summarized in Tables VI and VII.

In summary, a method is presented which may serve as a useful adjunct to the well documented CNBr cleavage procedure. The specificity of the methods described here, as demonstrated in several cytochrome b$_5$ preparations and equine cytochrome c, is limited to tryptophanyl and methionyl peptide bonds, proceeds in a high yield, and may be limited to tryptophanyl bonds by methylene blue-sensitized photooxidation of methionyl residues. The strong denaturing characteristics of the solvent employed suggests its usefulness for cleavage of proteins insoluble or containing secondary structures in the solvents commonly used for chemical fragmentations.

Table VIII summarizes the type of Trp-X bonds that have been shown to be cleaved by the described method.

REFERENCES
5. Ozols, J. (1975) in First International Conference of Solid Phase Methods in Protein Sequence Analysis (Laursen, R. H., ed) pp. 221-228, Pierce Chemical Co., Rockford
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### Table III

<table>
<thead>
<tr>
<th>Residue</th>
<th>Identification</th>
<th>Result</th>
<th>Yield (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lysine</td>
<td>6.7</td>
<td>0.8 (1)</td>
</tr>
<tr>
<td>2</td>
<td>Histidine</td>
<td>4.8</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>3</td>
<td>Glutamic Acid</td>
<td>6.7</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>4</td>
<td>Lysine</td>
<td>6.7</td>
<td>0.8 (1)</td>
</tr>
</tbody>
</table>

### Table IV

<table>
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<tr>
<th>Amino Acid</th>
<th>Rt</th>
<th>H2O</th>
<th>Aet</th>
<th>D2O</th>
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</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>4.7</td>
<td>3.8</td>
<td>4.8</td>
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<tr>
<td>Histidine</td>
<td>4.6</td>
<td>3.0</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
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### Table V

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Rt</th>
<th>H2O</th>
<th>Aet</th>
<th>D2O</th>
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</thead>
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<td>Histidine</td>
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<tr>
<td>Glutamic Acid</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
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</tr>
</tbody>
</table>

### Figure B

**Fig. B**: Gel filtration of a CM-Sepharose column with native trypsinogen. A 0.2 ml sample on a column of Sephadex G-75 equilibrated in 50 mM acetic acid. Conditions for chromatography were as described in the legend of Fig. 1.

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**Note**: All values are in millimolar (mM) concentrations.
Cleavage of tryptophanyl peptide bonds in cytochrome b5 by cyanogen bromide.
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