The Action of Cyanogen Bromide on Horse-Heart Cytochrome *c* and Horse-Heart Myoglobin

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1. The effects of cyanogen bromide on horse-heart cytochrome *c* and horse-heart myoglobin have been investigated. Cytochrome *c* yielded four fragments, of which two were haemopeptides. The two colourless peptides had amino acid compositions corresponding to those that are expected, on the basis of the sequence proposed for horse-heart cytochrome *c* by Margoliash, Smith, Kreil & Tuppy (1961), from cleavage at both methionine residues. Of the two haemopeptides, one was isolated and shown to be that derived from cleavage at only one methionine residue, that nearer to the *C*-terminus of the peptide chain. 2. Myoglobin also gave four peptides, three of which accounted for the total amino acid content of the intact protein. The fourth fragment arose by cleavage at a single methionine residue, that nearer the *C*-terminus. Characterization of this fourth fragment made it possible to deduce the order of arrangement of the fragments in the intact molecule.

Determination of the amino acid sequence of a protein requires selective methods for splitting the long polypeptide chain into smaller fragments in which the sequence can be determined by established techniques. The study of the larger proteins has placed a greater emphasis on the need for more selective methods of protein degradation.

The use of enzymes, particularly trypsin, has met with great success with sequences of up to 120 amino acid residues, but beyond this limit serious difficulties arise, among them the difficulty of separating complex mixtures of oligopeptides. This difficulty increases with the length of the polypeptide chain to be analysed. Attempts to extend the use of trypsin by restricting its action to arginyl bonds has met with some success (Goldberger & Anfinsen, 1962; Merigan, Dreyer & Berger, 1962), but there is a need for a variety of such methods with varying specificity. Search has therefore been made for chemical reagents of high specificity. The most promising of these is cyanogen bromide, which was shown by Gross & Witkop (1961) to cause specific cleavage of methionyl bonds. As most proteins contain relatively few methionine residues, this reagent appears to offer the best method for the preliminary fragmentation of long peptide chains.

When the present work was initiated, the potential of the method had already been revealed by Gross & Witkop's (1962) study of ribonuclease, which

helped to remove an ambiguity that existed between two published sequences for this enzyme (Anfinsen, Sela & Tritch, 1956; Spackman, Stein & Moore, 1960). Subsequently it has been used successfully to elucidate sequence problems with other small proteins. It has been used to cleave sperm-whale myoglobin (Edmundson, 1963), where it helped to surmount the problem of insoluble tryptic core. It has been used by Hofmann (1964) on bovine trypsinogen and provided information that, when taken with the results of other methods, permitted the formulation of a working sequence for trypsinogen (Walsh, Kauffman, Sampath Kumar & Neurath, 1964). Craven, Steers & Anfinsen (1964) are applying the method to sequence work on β -galactosidase. It has been used by Ambler (1963) to further degrade methionine-containing tryptic peptides in his sequence work on *Pseudomonas* cytochrome *c*-551. Koshland, Strumeyer & Ray (1962) have used cyanogen bromide to locate the reactive methionine residue in chymotrypsin after photo-oxidation.

The present paper reports on the action of cyanogen bromide on horse-heart cytochrome *c* and horse-heart myoglobin. The sequence of horse-heart cytochrome *c* has been determined by Margoliash, Smith, Kreil & Tuppy (1961). Cleavage with cyanogen bromide at the two methionine residues should therefore give rise to three fragments of known composition. Horse-heart myoglobin, which also contains two methionine residues (Tristram, 1949; Åkeson & Theorell, 1960), would similarly be expected to give three fragments.

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EXPERIMENTAL

Cyanogen bromide. This was obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Cytochrome c. Horse-heart cytochrome *c* of 100% purity was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Myoglobin. This was prepared from fresh horse heart by the method of Åkeson & Theorell (1960). The two fractions of myoglobin that were obtained after CM-cellulose chromatography were combined, dialysed against distilled water and freeze-dried. Globin was prepared by dissolving 100 mg. of myoglobin in 5 ml. of water, removing undissolved material by centrifugation and adding 50 ml. of acetone containing 1% (v/v) of conc. HCl. The globin precipitate was centrifuged down, washed several times with acid acetone and dried in a desiccator.

Sephadex. Sephadex for gel filtration was obtained from Pharmacia, Uppsala, Sweden.

Trypsin. A crystalline preparation, with little chymotryptic impurity, was obtained from the British Drug Houses Ltd., Poole, Dorset.

Cleavage with cyanogen bromide. The conditions used were those of Gross & Witkop (1961). A sample (10 μ moles) of the protein was dissolved in 10 ml. of 0.1 N-HCl and stirred with a 30-fold molar excess of CNBr for 24 hr. at room temperature. Excess of CNBr and volatile side products of the reaction were removed by freeze-drying.

Electrophoresis on cellulose acetate. Cellulose acetate strips were obtained from the Oxoid Division of Oxo Ltd., London. The separation was carried out in the Shandon low-voltage electrophoresis apparatus. Buffers used were pyridine-acetic acid-water, pH 3.4 (10 ml. of pyridine, 100 ml. of acetic acid and 890 ml. of water) and pH 6.5 (100 ml. of pyridine, 10 ml. of acetic acid and 890 ml. of water), and barbitone, pH 9.0 (163 ml. of 25 mM-barbitone, 100 ml. of 0.5 M-barbitone sodium and 737 ml. of water). Electrophoresis was continued for 1 hr. with a current of 30 mA. After electrophoresis the strips were stained in a 0.1% solution of Ponceau S in 0.2 N-acetic acid and then washed in 5% (v/v) acetic acid. Proteins then appeared as deep-red bands.

Filtration on Sephadex. The dimensions of the columns used were 100 cm. $\times$ 2 cm. for Sephadex G-50 and 150 cm. $\times$ 1.75 cm. for Sephadex G-75. The eluting solvent was 0.2 N-acetic acid and its rate of flow was adjusted, by a screw clip on the column outlet, to 10–20 ml./hr. Fractions (2.5 ml.) were collected in an automatic fraction collector.

The extinction of these fractions at 280 μ m (measured in a Unicam SP. 500 spectrophotometer) was taken as a measure of protein concentration. In the separations of cytochrome *c* peptides, the extinction was also measured at 415 μ m. When necessary, assays with ninhydrin were carried out on 100 μ l. samples after alkaline hydrolysis.

Fractions containing the desired peptide fragments were pooled and freeze-dried.

Determination of amino groups. Alkaline hydrolysis of the 100 μ l. samples was carried out by the method of Hirs, Moore & Stein (1956). The ninhydrin colour was developed by using the method of Yemm & Cocking (1955) and the extinction was determined at 570 μ m in a Unicam SP. 600 spectrophotometer.

Acid hydrolysis. A weighed quantity of the protein or peptide was sealed in a test tube ($\frac{3}{8}$ in. diam.) with 2 ml. of

6 N-HCl and hydrolysed for 24 hr. at 115°. After hydrolysis the HCl was removed in a vacuum desiccator at room temperature.

Amino acid analysis. Analyses were performed on an amino acid analyser built to the original design of Spackman, Stein & Moore (1958) and modified as described by Piez & Morris (1960). During the work on myoglobin, contamination of either the buffers or the environment of the machine gave rise to a large ammonia plateau, which, in some analyses, made calculation of the quantity of lysine impossible. In the analysis of haem peptides from cytochrome *c* the cysteine residues, originally attached to the haem, were recovered as cysteic acid. Under the conditions used for hydrolysis these residues appeared to undergo a quantitative oxidation.

Peptide maps. After digestion of the protein or peptide in 50 mM-phosphate buffer, pH 7.0, with a fiftieth part (by wt.) of trypsin for 2 hr. in a water bath at 37°, approx. 1.5 mg. of material was applied to Whatman no. 1 chromatography paper for 'fingerprinting'. This was carried out by a modification of the method of Ingram (1958). Electrophoresis was carried out in pyridine-acetic acid-water, pH 6.5 (10:1:89, by vol.), for 1 hr. in a Shandon high-voltage electrophoresis apparatus with a potential gradient of 60 v/cm. The paper was then dried and the second dimension was developed overnight in an upward direction with butanol-acetic acid-water (40:6:15, by vol.). The peptide spots were located with a ninhydrin-collidine dip (600 ml. of ethanol, 100 ml. of acetic acid, 40 ml. of collidine and 1 g. of ninhydrin). The dried papers were dipped, dried and placed in an oven at 80–100° for 5 min.

Determination of N-terminal amino acids. The fluorodinitrobenzene technique of Sanger (1945) was used as a qualitative method. Reaction with fluorodinitrobenzene was carried out in the reaction cell of a Radiometer pH-stat as described by Levy (1954). When the reaction was complete, acidification of the reaction mixture caused precipitation of the DNP-protein or DNP-peptide. It was washed with water, ethanol and ether and, after being dried, was hydrolysed with 1 ml. of 6 N-HCl in a sealed tube at 115° for 8 hr. After hydrolysis, the ether-soluble DNP-amino acids were extracted and dinitrophenol was removed by the method of Mills (1952). Two-dimensional chromatography of the ether- and water-soluble DNP-amino acids was carried out in the solvent systems of Koch & Weidel (1956).

RESULTS

Cytochrome c. Cytochrome *c* was allowed to react with cyanogen bromide and the product was freeze-dried. A sample was subjected to electrophoresis on cellulose acetate at pH 9 and five bands were observed, three being coloured red. This indicated that fission was incomplete and that some unchanged cytochrome *c* was present. Attempts to isolate these five fractions on a larger scale, by electrophoresis in a cellulose column, were unsuccessful. The reaction mixture was therefore fractionated by chromatography on Sephadex. Passage through a column of Sephadex G-50 brought about a partial separation. Fractions from the eluate from this column were further refined by repeated

Fig. 1. Diagram showing the sequence of chromatographic separations used in the isolation of peptide fractions from CNBr-treated cytochrome *c*. Graphs are plots of extinction at 280 $m\mu$ (—) and 415 $m\mu$ (---) against tube no. The initial separation was made with Sephadex G-50. The eluting solvent used in all the separations was 0.2N-acetic acid and the dimensions of the columns were 100 cm. $\times$ 2 cm. for Sephadex G-50 and 150 cm. $\times$ 1.75 cm. for Sephadex G-75. Fractions enclosed in a bracket were pooled, freeze-dried and dissolved in 2–4 ml. of 0.2N-acetic acid for application to the next column.

passage through columns of Sephadex G-50 or Sephadex G-75 until symmetrical peaks were obtained. The course of this fractionation is summarized in Fig. 1. The final fractions so obtained were freeze-dried and samples were subjected to amino acid analysis. The results of these analyses are compared in Table 1 with the compositions of peptides expected on the basis of the amino acid sequence proposed for cytochrome *c* by Margoliash *et al.* (1961).

The absence of methionine from fraction C_1 suggested that it should contain the sequence 1–65, but in most other respects its amino acid composition agreed better with that of the sequence 1–80. As this sequence contains about 80% of the amino acid residues in the cytochrome *c* molecule, precautions were taken to ensure that fraction C_1 did not contain any unchanged protein. Comparison

of tryptic 'fingerprints' of fraction C_1 and cytochrome *c* showed that such contamination must be slight. This was confirmed by electrophoresis on cellulose acetate strips. At pH 3.4 and at pH 9, fraction C_1 gave a single band whereas a mixture with cytochrome *c* gave two separate bands.

When cyanogen bromide-treated cytochrome *c* was treated with fluorodinitrobenzene, glutamic acid and isoleucine were identified as *N*-terminal residues, which is in accordance with the sequence given by Margoliash *et al.* (1961).

Myoglobin. The peptide map of the products of cleavage of myoglobin by cyanogen bromide (Fig. 2) indicates that five components were present. If unchanged myoglobin were present, this is one more than can be expected from a protein containing two methionine residues. The cleavage products were separated, by chromatography on Sephadex

Table 1. *Amino acid composition of peptides obtained from cytochrome c*

The observed values are compared with those expected on the basis of the amino acid sequence published by Margoliash *et al.* (1961).

	Amino acid composition (residues/molecule)						
	Peptide C ₁	Sequence 1-65	Sequence 1-80	Peptide C ₂	Sequence 81-104	Peptide C ₃	Sequence 66-80
Cys (half)*	1.9	2	2	0	0	0	0
Asp	6.0	5	6	2.1	2	1.0	1
Thr	7.5	7	8	1.8	2	0.8	1
Glu	9.3	7	9	3.1	3	2.0	2
Pro	2.9	2	4	0	0	2.0	2
Gly	10.4	10	11	1.1	1	1.0	1
Ala	4.1	3	3	2.8	3	0	0
Val	2.9	3	3	0	0	0	0
Met	0	0	1	0	0	0	0
Ile	3.5	2	3	2.9	3	0.9	1
Leu	4.1	3	4	2.1	2	0.9	1
Tyr	1.3	1	3	0.6	1	0.7	2
Phe	3.1	3	3	0.9	1	0	0
Lys	15.5	11	14	5.1	5	3.0	3
His	2.9	3	3	0	0	0	0
Arg	1.2	1	1	1.0	1	0	0

* Recovered as cysteic acid.

Fig. 2. Peptide map obtained from CNBr-treated myoglobin by electrophoresis followed by chromatography on filter paper. Details are given in the text.

G-75, into four fractions, the first of which proved to be unchanged myoglobin (Fig. 3). Fractions 1 and 2 were purified by further passages through columns of Sephadex G-75, yielding peptide fractions M₁ and M₂. Material from the fourth peak was separated by chromatography on Sephadex G-50 into peptide fractions M₃ and M₄.

Amino acid analyses of the four fractions and of intact horse-heart myoglobin are shown in Table 2. The *N*-terminal residue of each peptide is also shown in Table 2.

Fig. 3. Diagram of chromatographic separation of peptides obtained from myoglobin by treatment with CNBr. The ordinate gives the extinction at 280 mμ (—) and at 570 mμ after alkaline hydrolysis and reaction with ninhydrin (----). Fractions were grouped for further purification as indicated by the lines above the curves.

Table 2. Amino acid composition of myoglobin and the peptides obtained from it by reaction with cyanogen bromide

	Amino acid composition (residues/molecule)					
	Myoglobin	Peptide M ₁	Peptide M ₂	Peptide M ₃	Peptide M ₄	Peptides M ₂ +M ₃ +M ₄
Asp	9.8	8.7	4.0	3.9	2.9	10.8
Thr	5.9	5.7	3.1	1.0	1.5	5.6
Ser	3.7	3.3	2.7	0.19	0.5	3.4
Glu	20.1	16.6	6.9	8.9	4.2	20.0
Pro	3.7	3.6	2.9	1.1	0.20	4.2
Gly	14.8	12.3	6.9	5.7	3.0	15.6
Ala	14.2	11.5	8.2	4.3	4.0	16.5
Val	7.3	6.9	3.1	3.9	0.7	7.7
Met	1.9	0	0	0	0	0
Ile	8.4	7.0	5.1	2.3	1.3	8.7
Leu	17.0	13.4	8.0	5.9	3.9	17.8
Tyr	1.0	0.73	0.72	0.21	0.64	1.6
Phe	6.9	4.9	2.2	2.9	2.2	7.3
Lys	19.3	—	11.0	—	—	—
His	11.1	9.8	7.2	3.0	0.9	11.1
Arg	2.0	1.4	0.15	1.0	1.0	2.15
<i>N</i> -Terminal residue ...	Gly	Gly	Ser	Gly	Lys	

DISCUSSION

Cytochrome c. Agreement between our analyses of peptides C₂ and C₃ and the sequences 81–104 and 66–80 from the proposed amino acid sequence of cytochrome *c* given by Margoliash *et al.* (1961) is almost complete. The values obtained for tyrosine are the most discrepant, the tyrosine values for all peptides being lower than expected. The explanation for this is not clear, but the observation of a reaction of cyanuric halides with bound tyrosine in proteins by Kurihara, Horinishi & Shibata (1963) may be of some significance, since cyanuric bromide could arise by decomposition of cyanogen bromide.

With respect to fraction C₁, it seems clear that this represents the sequence up to the second methionine residue at position 80. Agreement between the observed amino acid composition and that calculated from the amino acid sequence proposed for cytochrome *c* by Margoliash *et al.* (1961) is not entirely satisfactory, probably owing to the presence of some impurity in the peptide. A peptide corresponding to the sequence 1–65 must be present in the unfractionated mixture of cleavage products, since sequences from residue 66 onwards were found. However, it was not isolated and could have been lost in the portions of column effluent discarded in the process of fraction cutting.

Myoglobin. Our analyses for the intact protein are in close agreement with published values (Tristram, 1949; Boardman & Adair, 1956; Holleman & Biserte, 1959; Åkeson & Theorell, 1960). There are no data with which to compare the ana-

lyses of the peptides, but the addition of the individual values for peptide fractions M₂, M₃ and M₄ gives an amino acid composition very similar to that of myoglobin. The small differences are most probably due to incomplete purification of the peptides. For tyrosine, it appears that the analysis of the protein is in error, for the results obtained with the peptides suggest the presence of two tyrosine residues. This is more in keeping with the conclusions of the other workers quoted above. Our results do, however, show that myoglobin has been selectively split into three peptides M₂, M₃ and M₄; by the evidence of its *N*-terminal residue peptide M₃ must occupy the *N*-terminal position in the intact protein. The additional peptide, M₁, appears to have been formed by cleavage at only one methionyl bond. Table 2 suggests that peptide M₁ contains the sequences present in peptides M₂ and M₃. The fact that peptide M₁ has the same *N*-terminal residue as myoglobin and peptide M₃ establishes the sequence of the three peptides in myoglobin as M₃–M₂–M₄. The isolation of peptide M₁ indicates that, of the two methionyl bonds in myoglobin, the methionyl-lysine bond is split more readily than the methionyl-serine bond. This is the reverse of what is found in the cleavage of ribonuclease by cyanogen bromide (Gross & Witkop, 1962).

Edmundson (1963) obtained three fragments from sperm-whale myoglobin that accounted for all amino acids of the complete protein. A comparison of the amino acid compositions of the two myoglobins and of their *N*-terminal, middle and *C*-

Table 3. Comparison of peptides M_1 , M_3 and M_4 with the corresponding peptides from sperm-whale myoglobin

The amino acid values are corrected to the nearest integer. S.W., Sperm-whale myoglobin; H.H., horse-heart myoglobin.

	Amino acid composition (residues/molecule)							
	Whole protein		Peptides					
	S.W.	H.H.	S.W.	M_3	S.W.	M_2	S.W.	M_4
Asp	8	10	3	4	3	4	2	3
Thr	5	6	2	2	3	3	0	1
Ser	6	4	2	1	4	3	0	1
Glu	19	20	8	9	7	7	2	3
Pro	4	4	1	1	3	3	0	0
Gly	11	13	3	6	6	7	2	3
Ala	17	14	4	4	10	8	3	4
Val	8	7	4	4	3	3	0	1
Met	2	2	—	—	—	—	—	—
Ile	9	8	2	2	6	5	1	1
Leu	18	17	7	6	8	8	3	4
Tyr	3	2	0	0	1	1	2	1
Phe	6	7	3	3	2	2	1	2
Lys	19	19	5	*	10	11	4	*
His	12	11	4	3	8	7	0	1
Arg	4	2	2	1	1	0	1	1

* Present but not determined.

terminal peptides (Table 3) shows that the respective peptides are of the same order of size. The similarity in the compositions of the two proteins is also evident in the peptides. It remains to be seen whether the similarity of methionine distribution is coincidental or whether it is indicative of a greater similarity between the structures of the two proteins.

The fact that cleavage of these two proteins, myoglobin and cytochrome *c*, was incomplete was disappointing, for with larger proteins incomplete cleavage leads to considerable difficulty in separation of the fragments, as has been found in a parallel investigation of the action of cyanogen bromide on serum albumin (G. Leaf & J. A. Black, unpublished work). Gross & Witkop (1962) attributed incomplete cleavage of ribonuclease to partial oxidation of the methionine residues of the protein, for cyanogen bromide does not react with methionine sulfoxide or sulphone residues (Koshland *et al.* 1962). If this were the cause of incomplete cleavage in the present work we should have detected either methionine sulphone or methionine in the hydrolysates of the intact proteins and of the peptides C_1 and M_1 , for Ray & Koshland (1960) report that the sulfoxide reverts to methionine under conditions used for hydrolysis. As neither methionine nor the sulphone was found in hydrolysates of peptides C_1 and M_1 , it is concluded that the methionine residues underwent reaction with cyanogen bromide but that this was

not followed by cleavage. The fate of these methionine residues was not determined.

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