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Partial Non-Cleavage by Cyanogen Bromide of a Methionine–Cystine Bond from Human Serum Albumin and Bovine α-Lactalbumin

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When human albumin was treated with CNBr, a fragment designated D was obtained and attributed to the absence from some of the albumin molecules of methionine at position 123 [Lapresle & Doyen (1975) *Biochem. J.* **151**, 637–643]. The present study shows that methionine-123 is converted into homoserine without cleavage of the subsequent methionine–cystine bond. With bovine α-lactalbumin, a further example of non-cleavage of a methionine–cystine bond with conversion of methionine into homoserine is reported.

Cleavage by CNBr of the six methionine residues of unreduced human serum albumin gives rise to three large fragments named B, C and A from the *N*- to the *C*-terminus of the albumin molecule (McMenamy *et al.*, 1971).

Another fragment, designated D, has been isolated and shown to be composed of fragment B linked to fragment C because the peptide bond after methionine-123 has not been cleaved by CNBr (Lapresle & Doyen, 1975). Residue 124 of human albumin is cystine (Behrens *et al.*, 1975; Meloun *et al.*, 1975) and the results in the present paper show that methionine-123 was converted into homoserine without cleavage of the methionine–cystine bond.

To study this point further, another protein, α-bovine lactalbumin, containing only one methionine residue followed by a cystine, was also studied. Its complete sequence has been established by Brew *et al.* (1970). Cleavage by CNBr of the one methionyl-peptide bond gives rise to two fragments: CB-1, which contains the first 90 residues, and CB-2, which contains the remaining 33 (Brew & Hill, 1970). In the present work, partial non-cleavage of this bond with conversion of methionine into homoserine was also observed.

Materials

Fragment D and its two chains were isolated from Squibb human albumin as described by Lapresle & Doyen (1975).

α-Lactalbumin from bovine milk was purchased from Sigma (St. Louis, MO, U.S.A.).

Agarose/polyacrylamide-gel plates for electrophoresis were from Industrie Biologique Française (Paris, France).

Methods

CNBr cleavage of α-lactalbumin was performed in 75% formic acid at a protein concentration of 17.6mg/ml and at two CNBr concentrations (either 8mg/ml or 35mg/ml). The reaction was allowed to proceed for 22h at room temperature. The solution was concentrated in a rotary evaporator and chromatographed on Sephadex G-25 equilibrated and eluted with 0.2% formic acid. The protein solution was freeze-dried.

For the reduction and alkylation of α-lactalbumin and CNBr-treated albumin, they were dissolved in 1M-Tris/HCl buffer, pH8.6, containing 8M-urea, EDTA (2mg/ml) and 2-mercaptoethanol (0.01ml/ml). The final protein concentration was 15mg/ml. The reaction mixture was left under N_2 for 4h at room temperature.

Iodoacetic acid was added to the mixture to give a 1.1 molar excess of alkylating agent relative to the thiol concentration. The solution was stored at 0°C in the dark for 1h, the pH being maintained at 8.6 with 5M-NaOH. The solution was immediately filtered in the dark on Sephadex G-25 equilibrated and eluted with aq. 0.02M-NH_3. The eluates containing peptides were collected and freeze-dried.

Gel filtration was performed on columns of Sephadex G-100 equilibrated with 0.1M-NH_4HCO_3.

Electrophoresis was performed in Tris/glycine buffer, pH8.7, and the peptides were detected by staining with Coomassie Blue as described by Uriel (1966).

Amino acid analysis was performed in a Technicon automatic analyser on fragments hydrolysed with 5.7M-HCl at 110°C for 18h. No corrections for losses were made. Homoserine lactone was converted into homoserine by the method of Ambler (1965).

Results

Amino acid compositions of fragment D and its chains

Reduction of fragment D gives rise to two chains, I and II, which can be separated by Sephadex G-100 chromatography. The larger one (chain I) contains the uncleaved bond 123–124. The amino acid compositions of fragment D and its chains I and II were determined and found to be the same as previously reported (Lapresle & Doyen, 1975), except for the amount of homoserine in fragment D and chain I. The results of several measurements of this residue are given in Table 1.

CNBr degradation of α-lactalbumin

CNBr treatment of α-lactalbumin followed by reduction and alkylation gives rise to three components, as shown by electrophoresis in agarose/polyacrylamide gel (Fig. 1). One of these has the same electrophoretic mobility as reduced and alkylated α-lactalbumin. Identical results were obtained with the two concentrations of CNBr examined.

These components are eluted in three fractions from Sephadex G-100 (Fig. 2). Fractions I and II were rechromatographed and eluted as a single peak. The three fractions were isolated uncontaminated by one another, as estimated by electrophoresis (Fig. 1). Fraction I is the component similar to uncleaved α-lactalbumin. It was obtained with a yield of 10%, the total recovery being about 50%.

Amino acid composition of α-lactalbumin and its CNBr fragments

The amino acid compositions of reduced and alkylated lactalbumin and of the three components

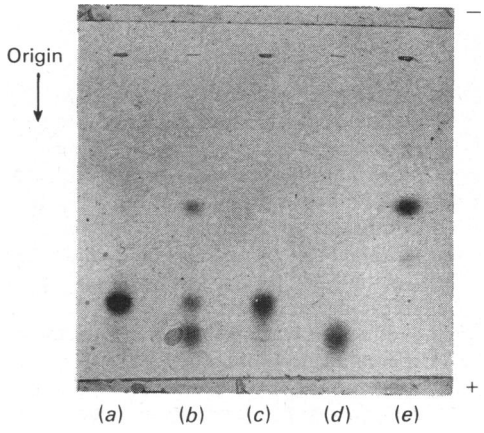

Fig. 1. *Agarose/polyacrylamide-gel electrophoresis of lactalbumin (reduced and alkylated) (a), CNBr-treated α-lactalbumin (reduced and alkylated) (b) and fractions I, II and III isolated by gel filtration of CNBr-treated α-lactalbumin (c, d, e)*
Electrophoresis was for 2h at 10V/cm.

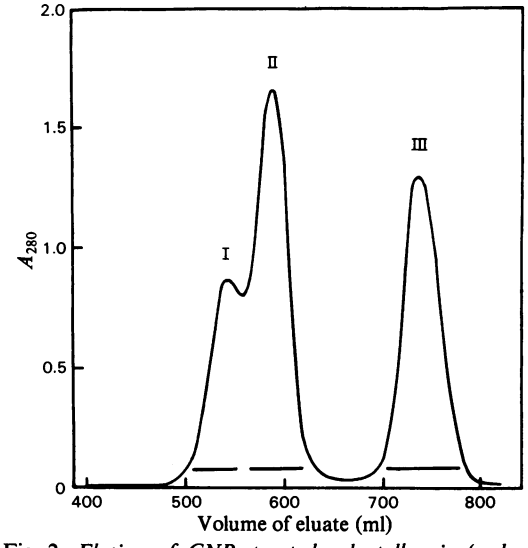

Fig. 2. *Elution of CNBr-treated α-lactalbumin (reduced and alkylated) from a column (2.5 cm × 200 cm) of Sephadex G-100 with 0.1 M-NH_4HCO_3*
Horizontal bars indicate regions that were pooled.

Table 1. *Homoserine and methionine contents of fragment D and its chains, and of α-lactalbumin and its CNBr fragments*
Results are expressed as mol of amino acid/mol of peptide. Molar contents were calculated with respect to isoleucine for fragment D and chains I and II, and with respect to alanine for lactalbumin and fractions I, II and III. The results for fragment D, chain I and fraction I are those of three different hydrolysates. The result for chain II is that of two different hydrolysates.

	Fragment D	Chain I	Chain II	Lactalbumin	Fraction I	Fraction II	Fraction III
Homoserine	2.62	1.45	0.47	0	0.63	0.96	0
	2.34	1.70	0.6		0.89		
	2.46	1.35			0.61		
Methionine	0	0	0	0.89	0.13	0	0
					0.17		
					0.14		

eluted from Sephadex were determined. Table 1 shows the results of measurements of methionine and homoserine. Fraction I has the same amino acid composition as reduced and alkylated α-lactalbumin, except for the amounts of homoserine and methionine. Fractions II and III have the same composition as the CB-1 and CB-2 fragments of Brew & Hill (1970), and correspond to the two fragments produced by cleavage of the methionylpeptide bond of α-lactalbumin. The presence of one homoserine residue in fraction II confirms that it represents the *N*-terminal fragment.

Discussion

In serum albumin, non-cleavage of the peptide bond between methionine-123 and cystine-124, which gives rise to fragment D, was attributed to the absence of methionine at position 123 from some of the albumin molecules (Lapresle & Doyen, 1975). Careful re-examination of the amino acid compositions of fragment D and its chain I, which contains the uncleaved peptide bond, showed that the number of homoserine residues had been under-estimated. The best integer for homoserine is 3 rather than 2 for fragment D, and 2 rather than 1 for chain I. This discrepancy can be ascribed to the difficulty in measuring small amounts of homoserine in fragment D, which consists of 297 residues, and in chain I, which consists of 210 residues. For chain II, with only 87 residues, the best integer is 1, as previously described (Lapresle & Doyen, 1975).

After CNBr treatment, fragment D has two homoserine residues and chain I has one homoserine residue at the *C*-terminus. Since they have one more homoserine residue, this indicates that methionine-123 has been converted into homoserine without cleavage of the adjacent peptide bond.

To investigate whether the same process would occur at another methionine–cystine bond, α-lactalbumin was cleaved by CNBr. It was reduced and alkylated after CNBr treatment, as had been done with human serum albumin. A certain amount of α-lactalbumin was not cleaved, and was eluted first from Sephadex. This amount represents 10% of the original lactalbumin, or about 20% if a correction is made for losses.

This uncleaved α-lactalbumin differs from the original lactalbumin molecule in the fact that it contains a small amount of methionine and almost one residue of homoserine, rather than one methionine and no homoserine. This result shows that fraction I consists of a small amount of lactalbumin unmodified by CNBr and a much greater number of lactalbumin molecules in which methionine had been converted into homoserine without cleavage of the subsequent peptide bond.

The conversion of methionine into homoserine without cleavage by CNBr of the subsequent peptide bond had already been reported for the acetylmethionyl-peptide (Carpenter & Shiigi, 1974) and acetylmethionyl-insulin or benzoylmethionyl-insulin (Saunders & Offord, 1977). This was attributed to a reverse reaction between methionine and acetyl or benzoyl groups via a dihydro-oxazine ring. It has been suggested (Carpenter & Shiigi, 1974) that, in proteins, such a mechanism would be more prone to occur when methionine is the *C*-terminal residue, which is not the case in human albumin and bovine α-lactalbumin.

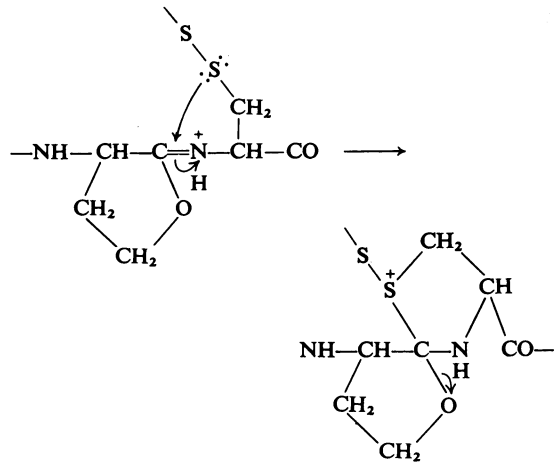

Fig. 3. *Diagram of the postulated intramolecular reaction between the sulphur atom of the cystine residue and the iminolactone formed during CNBr treatment*

Conversion of methionine into homoserine without cleavage of the subsequent bond by CNBr has also been reported for methionine–threonine and methionine–serine peptide bonds of several proteins (Narita & Titani, 1968; Cunningham *et al.*, 1968; Schroeder *et al.*, 1969; DeLange, 1970). This was attributed to an intramolecular reaction of the hydroxy group with the iminolactone (Schroeder *et al.*, 1969). A similar mechanism could be postulated for the methionine–cystine bond of human serum albumin and α-lactalbumin. The sulphur atom could react with the iminolactone, giving rise to a bicyclic structure as shown in Fig. 3. The opening of the first ring would release a homoserine side chain and the opening of the second ring would give either a thioester or a peptide linkage between homoserine and cystine-124. However, a more general mechanism, such as that proposed by Corradin & Harbury (1970) to explain the non-cleavage of methionine–glutamic acid and methionine–isoleucine bonds of cytochrome *c* with methionine conversion into homoserine, cannot be excluded.

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