Isolation and Properties of a Fragment of Human Serum Albumin Demonstrating the Absence of a Methionine Residue from some of the Albumin Molecules

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(Received 23 June 1975)

1. A fragment designated D was isolated from human serum albumin degraded by CNBr. Its properties show that it is made up of the B and C fragments isolated by McMenamy *et al.* (1971) (*J. Biol. Chem.*, **246**, 4744–4750). 2. Reduction of fragment D gives rise to two chains, one of which consists of the second subfragment of reduced fragment B linked to fragment C by an amino acid different from methionine. It thus demonstrates the existence of albumin molecules from which the second methionine residue located between fragments B and C is missing.

Cleavage by CNBr of the six methionine residues of unreduced human serum albumin gives rise to three large fragments, which have been named A, B, and C. Reduction of these fragments showed that A is made up of four subfragments, B of two subfragments and C of one peptide chain (McMenamy *et al.*, 1971). Similar results have been obtained by Meloun & Kusnir (1972) and Kusnir & Meloun (1973) who used a different nomenclature.

Fragment A which corresponds approximately to one-half of the albumin molecule is separated by gel filtration on Sephadex G-100 from fragments B and C which each correspond to approximately one-quarter of the molecule and are eluted together. Fragments B and C have been separated by chromatography on CM-cellulose. Chromatography of fragment A on CM-cellulose permits the isolation of a contaminating component which was supposed to be composed of unsplit human serum albumin (McMenamy *et al.*, 1971).

Results reported in the present paper show that this component is a fragment of human albumin which has been designated fragment D. Study of its properties indicates that it is made up of fragments B and C that are not cleaved by CNBr degradation because one of the subfragments of B is linked to fragment C through an amino acid residue different from methionine. It thus demonstrates the existence of albumin molecules differing from the others by the lack of one methionine residue.

Experimental

Materials

Human serum albumin was either 100% pure (by electrophoresis) crystallized albumin, pH 5.1, from Mann Assayed Biochemicals (New York, N.Y.,

U.S.A.) or Squibb fraction V prepared by ethanol fractionation and kindly given by the American Red Cross. Mercaptalbumin (human) [crystallized 100% pure (by electrophoresis)] was from Mann Assayed Biochemicals.

CNBr and 2-mercaptoethanol were purchased from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.). Ethyleneimine monomer was from Fluka (Buchs, Switzerland); it was redistilled and kept in the presence of KOH. Maleic anhydride was from Merck (Darmstadt, Germany). *N*-Bromosuccinimide (RP) was from Carlo Erba (Milano, Italy).

CM-cellulose (Whatman CM-32) was used in columns kept at 10°C. Polyacrylamide–agarose plates for electrophoresis (5.5%, w/v, of acrylamide) were from Industrie Biologique Française (Paris, France). Cellulose-coated t.l.c. plates (without fluorescence indicators) were from Merck.

Rabbit anti-(human serum albumin) serum was prepared as follows. Rabbits were immunized by subcutaneous injections of 2 mg of human serum albumin (Mann Assayed Biochemicals) with complete Freund's adjuvant and 2 months later, by injections of 2 mg of albumin intradermally the first day, intramuscularly the second day and intravenously the third day. The rabbits were bled 7 days later. The sera of six rabbits were pooled.

Methods

CNBr reaction. Human serum albumin either from Mann or Squibb fraction V and mercaptoalbumin were dialysed against distilled water and freeze-dried. A portion (0.5 g) of freeze-dried material was treated with ethyleneimine and cleaved with CNBr as described by Meloun & Kusnir (1972). Fragment D and reduced fragment D, reduced as described by

Fig. 1. Chromatography of CNBr-cleaved fragments of unreduced human serum albumin

A column (2.5 cm $\times$ 250 cm) of Sephadex G-100 was eluted with 0.174 M-formic acid and 0.026 M-ammonium formate at pH 2.86 as described by Meloun & Kusnir (1972).

Adelstein & Kuehl (1970), were treated with CNBr under the same conditions as were used for albumin.

Preparation of fragment D. Human albumin treated with CNBr was concentrated in a rotary evaporator and passed through a column (2.5 cm $\times$ 250 cm) of Sephadex G-100 equilibrated with 0.174 M-formic acid and 0.026 M-ammonium formate at pH 2.86 as described by Meloun & Kusnir (1972). The CNBr-cleaved fragments were eluted in two zones, I and II (Fig. 1). Zone I was dialysed against 5 mM-potassium phosphate buffer, pH 6.55, and applied to a column (1.5 cm $\times$ 40 cm) of CM-cellulose CM-32 equilibrated in the same buffer. Fragment D was not adsorbed under these conditions and was eluted by passage of the same buffer. Fragment A was retained on CM-cellulose and could be eluted by the addition of 0.7 M-NaCl (Fig. 2). Eluates containing fragment D were dialysed against 0.01 M-sodium phosphate buffer, pH 3.25, containing 0.05 M-NaCl and applied to a column (1.5 cm $\times$ 40 cm) of CM-cellulose CM-32 equilibrated in the same buffer. Fragment D was eluted by a 0.05–0.5 M-NaCl gradient (Fig. 3). It was dialysed against distilled water and freeze-dried.

Preparation of fragments A, B and C. Fragments A, B and C were prepared from zones I and II (Fig. 1) as described by McMenamy *et al.* (1971) with slight modifications. Zones I and II were dialysed against 0.01 M-sodium phosphate buffer, pH 3.25, containing 0.05 M-NaCl and applied to a column (1.5 cm $\times$ 40 cm) of CM-cellulose CM-32 equilibrated in the same buffer. Fragment A was eluted from zone I and fragments B and C were eluted from zone II by a gradient of 0.05–0.5 M-NaCl.

Electrophoresis in polyacrylamide-agarose plates. Electrophoresis was performed in Tris-glycine buffer,

Fig. 2. Fractionation of fragments D and A by CM-cellulose chromatography

A column (1.5 cm $\times$ 40 cm) of CM-cellulose CM-32 was eluted with 5 mM-potassium phosphate buffer, pH 6.55, as described in the text. The fractions between the vertical bars were pooled for fraction D.

pH 8.7, and the peptides were detected by staining with Coomassie Blue, as described by Uriel (1966).

Ultracentrifugation. Fragment D or human albumin (9 mg/ml) was dissolved in 0.01 M-sodium phosphate buffer, pH 3.25, containing 0.05 M-NaCl. These solutions were centrifuged at $20 \pm 0.1^\circ\text{C}$ and at 259 700 g in an analytic ultracentrifuge (Beckman model E). The sedimentation coefficients were calculated by the method of Schachman (1959). Fragment D was centrifuged under the same conditions at 54 690 g and its molecular weight was measured by the method of Yphantis (1960).

Isolation of the chains from fragment D. Fragment D (90 mg) was reduced with 2-mercaptoethanol, alkylated with iodoacetic acid, maleoylated and filtered on Sephadex G-100. The techniques used were those used by Kusnir & Meloun (1973) for isolating the chains of component N which corresponds to the B and C fragments of McMenamy *et al.* (1971).

Demaleylation of the chains of fragment D. Chain I of fragment D was demaleylated by the technique of Kusnir & Meloun (1973) for chain II-Ala or chain III-Cys. Chain II of fragment D was demaleylated by the technique used by Kusnir & Meloun (1973) for chain I-Asp.

Amino acid analysis. Fragments were hydrolysed with 5.7 M-HCl at 110°C for 18 h. Amino acid analyses

Fig. 3. CM-cellulose chromatography of fragment D

A column (1.5 cm $\times$ 40 cm) of CM-cellulose CM-32 was equilibrated with 0.01 M-sodium phosphate buffer, pH 3.25, containing 0.05 M-NaCl. Fragment D was eluted with a 0.05–0.5 M-NaCl gradient in the same phosphate buffer. —, E_{230} ; ----, NaCl gradient.

were performed on a Technicon automatic analyser. No corrections for losses were made. Homoserine lactone was converted into homoserine by the method of Ambler (1965). Tryptophan was determined with *N*-bromosuccinimide by the method of Spande & Witkop (1967). Amino acid analysis was also performed on fragment D oxidized with performic acid as described by Hirs (1956) to transform methionine into methionine sulphone.

***N*-Terminal analysis.** The *N*-terminal amino acid of fragment D and its demaleylated chains I and II were determined by the fluorodinitrobenzene method of Sanger & Thompson (1953) under the conditions described by Kasper *et al.* (1965). Dinitrophenylated peptides were hydrolysed for 16 h at 105°C. Dnp-amino acids were identified by two-dimensional chromatography as described by Fraenkel-Conrat *et al.* (1955). The chromatography was performed on cellulose-coated plates by the ascending method for both directions. The plates were desiccated at 60°C and the yellow spots corresponding to Dnp-amino acids were suspended in 1 ml of 1 M-NaOH at 60°C for 30 min. After centrifugation, the E_{360} of the supernatant was determined. The amount of each Dnp-amino acid was calculated by using the extinction coefficients of Fraenkel-Conrat *et al.* (1955). Correction for destruction of amino acids during hydrolysis was calculated from the recovery of similar Dnp-amino acids simultaneously hydrolysed.

Specific precipitation in gel. The double-diffusion technique of Ouchterlony (1949) was used.

Quantitative specific precipitation. Specific precipitation was done by the technique of Heidelberger & Kendall (1938); the amounts of precipitates were measured by the biuret reagent as described by McDuffie & Kabat (1956).

Inhibition of the specific precipitation. Inhibition experiments were carried out by adding increasing amounts of fragments A, B and C to constant amounts of fragment D and rabbit anti-(human serum albumin) sera corresponding to the equivalence point. Inhibiting fragments were incubated with the serum for 30 min at 37°C before the addition of fragment D. Inhibition by fragment A of the specific precipitation of human serum albumin was performed under the same conditions. Controls were made by incubating the same amounts of fragments A, B and C with rabbit sera but without the addition of fragment D or human serum albumin. The slight amount of precipitate obtained was subtracted from the amount of precipitate obtained after the addition of fragment D or human serum albumin.

Results

Preparation of fragments A, B, C and D

The three fragments A, B and C were obtained from human serum albumin in a 50% yield. Fragment D was obtained in a smaller amount: 20 mg/500 mg of human serum albumin. These results were roughly the same whatever the source of the albumin (Mann, Squibb or mercaptalbumin). The amino acid compositions of fragments A, B and C were essentially the same as those of McMenamy *et al.* (1971).

Ultracentrifugation of fragment D

By ultracentrifugation, fragment D was homogeneous and had a sedimentation coefficient of 2.49 S. Human serum albumin under the same conditions had a sedimentation coefficient of 4.04 S. The molecular weight of fragment D measured by the Yphantis method (1960) was 33 900.

Immunochemical properties of fragment D

(a) Fig. 4 shows the specific precipitation in gel of fragment D and human serum albumin with an anti-(human serum albumin) rabbit serum. Fragment D forms one line of precipitation and gives a cross reaction with human serum albumin.

(b) Fig. 5 shows the precipitation curves obtained with human serum albumin or fragment D reacting with the same anti-(human serum albumin) rabbit serum. The amount of antibodies precipitated at the equivalence point is 8 mg/ml for human serum albumin and 3.1 mg/ml for fragment D.

(c) Table 1 shows the maximum inhibition obtained with fragments A, B and C of the specific precipitation of fragment D with rabbit anti-(human serum albumin) serum. Fragments B and C each partially inhibit fragment D precipitation and when mixed together completely inhibit the precipitation. Fragment A does not inhibit the precipitation of fragment

Fig. 4. Specific precipitation in gel of human serum albumin (HSA) and fragment D with a rabbit anti-(human serum albumin) serum

Fragment D and human serum albumin were used at a concentration of 1 mg/ml. Rabbit serum was used undiluted. See the text for further details.

Fig. 5. Specific precipitation curves of human serum albumin (○) and fragment D (●) with a rabbit anti-(human serum albumin) serum

For details see the text.

D, but partially inhibits the precipitation of human serum albumin with the same rabbit anti-(human serum albumin) serum.

Table 1. Inhibition of the specific precipitation of fragment D and human serum albumin

The results correspond to the maximum values of inhibition obtained and are expressed in percentages.

Inhibiting fragments	Inhibition of the precipitation of:	
	Fragment D	Human serum albumin
A	0	52
B	56	
C	28	
B+C	91	

Amino acid composition of fragment D

Table 2 shows the amino acid composition of fragment D. It is close to the sum of fragments B and C. The unique tryptophan residue of human serum albumin which is located in fragment C is present in fragment D. It should be noted that there are two homoserine residues in fragment D whereas fragments B and C have three homoserine residues. No methionine was found in fragment D. No methionine sulphone was found in fragment D oxidized with performic acid.

CNBr treatment of fragment D

Fig. 6 shows the electrophoretic pattern in polyacrylamide-agarose gel of fragments B, C and D and fragment D treated with CNBr. Fragment D consists of one component having an electrophoretic mobility intermediate between those of fragments B and C. CNBr treatment of fragment D does not change its electrophoretic mobility and does not give rise to fragments B and C. CNBr also failed to cleave fragment D after it had been reduced as described by Adelstein & Kuehl (1970).

Isolation and amino acid composition of the chains of fragment D

Kusnir & Meloun (1973) have shown that carboxymethylated and maleylated chains of reduced fragment N can be separated on Sephadex G-100. Three chains are eluted: III-Cys, I-Asp and II-Ala corresponding respectively to the C-Cys, B-Asp II and B-Ala fragments of McMenamy *et al.* (1971).

When fragment D was processed in the same manner, two chains only were eluted (Fig. 7). Their amino acid compositions are given in Table 2. Chain I has the same composition as C-Cys+B-Ala fragments; however, it should be noted that it has only one homoserine residue and no methionine residue. Chain II has the same composition as B-Asp II fragment. For both chains, there is no cysteine but only *S*-carboxymethyl cysteine, indicating that all disulphide

Table 2. *Amino acids analysis*

Results are expressed as mol of amino acid/mol of peptide, except for composition of fragments B+C, which is given as nearest integers.

	Fragment D*	Fragments B+C†	Chain I from fragment D‡	B-Ala+C-Cys fragments†	Chain II from fragment D‡	B-Asp II fragment†
Lys	25.2	25	19.3	18.6	6.7	6.8
His	7.7	9	5.7	5.3	3.4	3.7
Arg	11.6	13	11.0	11.1	2.3	2.1
CmCys§			12.6	13.6	3.2	3.8
Asp	26.6	28	21.0	20.1	8.5	9.2
Thr	11.5	11	6.0	5.0	4.6	5.4
Ser	11.6	11	8.3	7.4	2.9	3.0
Glu	40.2	44	26.3	28.7	11.6	13.1
Pro	9.9	10	8.6	8.8	1.8	1.5
Gly	7.7	6	3.4	3.0	2.7	3.0
Ala	28.8	32	23.4	25.2	7.8	8.8
Val	14.7	15	8.0	7.7	5.8	6.6
Cys	13.6	18	0	0	0	0
Met	0	0	0	0	0	0
Ileu	4.3	5	3.6	3.6	0.9	0.9
Leu	27.4	29	20.6	21.0	7.5	9.2
Tyr	7.0	7	5.2	5.4	1.8	1.8
Phe	14.4	15	11.0	11.1	5.0	5.6
Hse	2.2	3	0.7	1.6	0.6	0.7
Trp	0.4	1	—	0.6	—	0

* Mean of five analyses on five different preparations.

† Results from McMenamy *et al.* (1971).

‡ Results of one analysis.

§ S-Carboxymethyl cysteine.

bonds have been reduced and all thiol groups alkylated.

N-Terminal analysis of fragment D and its chains I and II

For fragment D, two terminal amino acids have been found: aspartic acid and alanine (respectively 0.40 and 0.44 mol/mol of fragment D). For chain I, two *N*-terminal amino acids were found: alanine (0.27 mol/mol of chain I) and aspartic acid in trace amounts indicating a slight contamination by chain II. For chain II, one *N*-terminal amino acid was found aspartic acid (0.31 mol/mol of chain II).

Discussion

The component described by McMenamy *et al.* (1971) as unsplit human serum albumin, and designated fragment D in the present work, was always contaminated by fragment A. This contamination was eliminated by passage through CM-cellulose at pH 6.55 which completely retains fragment A. Fragment D, which is not absorbed on CM-cellulose under these conditions, is still contaminated by traces of human serum albumin detectable by rabbit anti-(human serum albumin) serum. They are eliminated by chromatography on CM-cellulose at pH 3.25.

The yield of fragments A, B and C is lower than that obtained by McMenamy *et al.* (1971); this may be due to the use of commercial albumin preparations as starting material.

The fact that fragment D is a portion of an albumin molecule is demonstrated by ultracentrifugation. Ultracentrifugation analysis was done at an acidic pH (3.25) to avoid aggregate formation which occurs at more alkaline pH. Under this condition, the sedimentation coefficient of human serum albumin was unchanged and the sedimentation coefficient of fragment D was much lower than that of albumin. Its molecular weight (33 900) explains why it is eluted from Sephadex G-100 with fragment A, the molecular weight of which was estimated to be of the order of 35 000 (McMenamy *et al.*, 1971).

Immunochemical properties of fragment D agree with the fact that it corresponds to a portion of an albumin molecule since it gives a cross-reaction with human serum albumin in gel, and precipitates only a portion of the antibodies of a rabbit anti-(human serum albumin) serum in liquid medium.

Fragment D must consist of fragments B and C since its specific precipitation with anti-(human serum albumin) serum is not inhibited by fragment A and is inhibited almost completely by fragments B plus C. The absence of the inhibiting ability of fragment A is not due to an alteration of its structure

Fig. 6. Electrophoresis in polyacrylamide-agarose gels of fragments B, C and D and fragment D treated with CNBr

Duration of electrophoresis was 3 h at 10V/cm at pH 8.7. For further details see the text.

during its preparation since it is able to inhibit at 50% the specific precipitation of human serum albumin.

Physical and chemical properties of fragment D confirm that it is made up of fragments B plus C. Its molecular weight is similar, within experimental errors, to the sum (33 500) of the molecular weights of fragments B and C, calculated from their amino acid compositions. The amino acid composition of fragment D is close to that of fragments B plus C. It contains the only tryptophan residue of human serum albumin which belongs to fragment C. It has two *N*-terminal amino acids: alanine, which is one of the *N*-terminal residues of fragment B, and aspartic acid, which is one of the *N*-terminal residues of fragment A but also of fragment B (McMenamy *et al.*, 1971).

Reduction of fragment D gives rise to two chains which have been separated on Sephadex G-100: a small one, chain II, which is the same as the subfragment B-Asp II and a large one, chain I, which has alanine as *N*-terminus and is made up of subfragments B-Ala+C-Cys. The B-Ala fragment is linked to the C-Cys fragment by a peptide bond since chain I has one *N*-terminal residue and one homoserine residue as C-terminal. As the *N*-terminal is alanine, the B-Ala

Fig. 7. Chromatography of components of reduced fragment D

A column (1 cm × 250 cm) of Sephadex G-100 was eluted with 0.1M-NH₄HCO₃ as described by Kusnir & Meloun (1973).

fragment must precede the C-Cys fragment in the sequence of human serum albumin, which agrees with the structure established for human serum albumin by Behrens *et al.* (1975).

The existence of fragment D means that the peptide bond, which is cleaved by CNBr in most of the molecules of human serum albumin giving rise to fragments B and C, is not cleaved in the molecules of human serum albumin from which fragment D comes from. This cannot be due to an incomplete cleavage of human serum albumin molecules by CNBr since CNBr-treated fragment D does not give rise to fragments B and C. Neither can it be due to the fact that the methionine residue situated at the C-terminus of the B-Ala fragment would be in methionine sulfoxide form, which is uncleavable by CNBr, since no cleavage was observed when reduced fragment D was treated with CNBr. The absence of methionine from fragment D and from its chain I, as well as the absence of methionine sulphone from fragment D oxidized with performic acid, shows that this methionine is missing from the molecule of human serum albumin giving rise to fragment D. This residue is number 123 according to Behrens *et al.* (1975). The absence of this methionine residue could be the result of either an amino acid deletion or substitution.

The yield of fragment D allows an estimation of the proportion of human serum albumin molecules lacking this methionine residue. The fact that 20mg of fragment D can be prepared from 500mg of human serum albumin means that 8% of human serum albumin molecules give rise to fragment D. This number is a minimum since losses may occur during the preparation. If a correction is made for this loss, assuming that it is the same (approx. 50%) as for the other CNBr-cleaved fragments, 16% of human serum albumin molecules give rise to fragment D.

This heterogeneity of human serum albumin molecules has no relationship with the heterogeneity responsible for the existence of mercaptalbumin and non-mercaptalbumin molecules (Hughes, 1974). When mercaptalbumin is submitted to CNBr cleavage, fragments B, C and D are produced in amounts roughly similar to those produced from human serum albumin. If the lack of methionine was peculiar to mercaptalbumin molecules, fragment D only would have been obtained and if the lack of a methionine residue was found solely in non-mercaptalbumin molecules, no fragment D would have been obtained.

Fragment D has been isolated from commercial albumin samples, prepared from a pool of different human sera, and some might have been sera of bisalbuminaemia. This would not explain our results for the following reasons: for bisalbuminaemia, the variants have abnormal electrophoretic mobilities, which was not found in the present albumin samples; bisalbuminaemia is a very rare trait observed once in several thousand sera (Melartin, 1967) and so cannot explain the ratio of human serum albumin molecules lacking a methionine residue; in the case where the structural modifications responsible for albumin variants has been elucidated, it was due to an amino acid change, glutamic acid to lysine, located in the A fragment (Winter *et al.*, 1972).

We thank Mr. S. Mangalo for performing the ultracentrifugations. The expert technical assistance of Mrs. J. Cavillon, Mrs. D. Leduc and Miss A. Robbe is gratefully acknowledged.

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