Chemical Structure of Two Fragments of Human Serum Albumin and their Location in the Albumin Molecule

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(Received 2 December 1974)

1. 'Inhibitor fragment' isolated from human serum albumin degraded by rabbit cathepsin D is composed of one peptide chain with two intrachain disulphide bonds. There are two kinds of inhibitor molecules having different *N*-terminal amino acids: one is threonine and the other glutamine. 2. Fragment F1, isolated from inhibitor degraded by trypsin, is composed of two chains linked by a disulphide bond. There are three kinds of fragment F1. All have one α chain in common, which has an intrachain disulphide bond. They differ by the nature of the second chain, which is linked to the α chain by a disulphide bond. The ϵ chain is present in trace amounts. The two other chains, β and γ , differ by their *C*-terminal amino acid, which is respectively arginine and lysine. 3. Inhibitor is composed of the last 92 or 89 residues of the human albumin molecule and fragment F1 is composed of two parts of this *C*-terminal portion of the albumin molecule.

A component was isolated from human serum albumin degraded by rabbit spleen extract which did not precipitate with most rabbit anti-albumin sera but partially inhibited the specific precipitation of these antisera by albumin (Lapresle & Webb, 1960). This product, designated 'inhibitor', has a molecular weight of 11 000 (Lapresle & Webb, 1964).

Further degradation of inhibitor by trypsin gave rise to a smaller immunologically active fragment designated 'F1': its molecular weight as measured by the Yphantis (1960) method in the ultracentrifuge, is 6600 (Lapresle & Webb, 1965).

The chemical structure of inhibitor and fragment F1 and their location in the albumin molecule are described in this paper.

Experimental

Materials

Human serum albumin (Squibb fraction V) prepared by ethanol fractionation, was kindly provided by the American Red Cross. Inhibitor fragment was prepared from human serum albumin degraded by rabbit cathepsin D as described by Webb & Lapresle (1964) but without addition of cysteine. Fragment F1 was prepared from inhibitor degraded by trypsin as described by Lapresle & Goldstein (1969).

Trypsin was once-crystallized Novo-Trypsin from Martinet (222 Bal Pereine, Paris 17^e, France), treated as described by Kostka & Carpenter (1964) with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (Calbiochem, San Diego, Calif., U.S.A.). Di-isopropyl phosphorofluoridate was purchased from Schuchardt (München, W. Germany). Carboxy-

peptidase A was a suspension of 35 units/mg obtained from Merck (Darmstadt, W. Germany). Carboxypeptidase B was a solution of 135 units/mg treated with di-isopropyl phosphorofluoridate and obtained from Sigma (St. Louis, Mo., U.S.A.). 2-Mercaptoethanol was from Eastman (Rochester, N.Y., U.S.A.).

Iodo[2-¹⁴C]acetamide, obtained from The Radiochemical Centre (Amersham, Bucks., U.K.), had a specific radioactivity of 5 mCi/mmol.

Ethyleneimine monomer was from Fluka (Buchs, Switzerland); it was redistilled and kept in the presence of KOH. Anhydrous hydrazine was prepared from hydrated hydrazine (95%, w/v) by the method of Braun & Schroeder (1967). *p*-Chloromercuribenzoate was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). Succinic anhydride was from Sigma Chemical Co.

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.

Methods

Gel filtration. Peptides were desalted by filtration on Sephadex G-25 equilibrated with 0.02 M-NH₄HCO₃ adjusted to pH 8.7 with 2 M-NH₃. Eluates were read at 220 nm.

Electrophoresis in polyacrylamide-agarose plates. Electrophoresis was performed in Tris-glycine buffer, pH 8.7, and the peptides were detected by staining with Coomassie Blue, as described by Uriel (1966). Preparative electrophoresis was performed by the method of Bellon (1973) with the following modifications: the peptides were not extracted from the gel

by a second electrophoresis in agarose but by grinding the slices of the gel containing the peptides at 0°C in a Potter apparatus. The ground gel was suspended in 0.02M-NH₄HCO₃, pH8.7, and kept at 4°C for 12h before being centrifuged. The precipitate was resuspended in the same buffer. After this had been done three times, the supernatants were pooled, freeze-dried and filtered on Sephadex G-25. The fractions containing the peptides which emerged 10ml ahead from salts and glycine were collected and freeze-dried.

In some instances, electrophoresis was performed on reduced but not alkylated peptides. The polyacrylamide-agarose plates were then rehydrated in the same Tris-glycine buffer but containing 0.08M-2-mercaptoethanol.

Ultracentrifugation. Inhibitor (10mg/ml) and inhibitor reduced and alkylated with ethyleneimine (8.75mg/ml) were dissolved in acetic acid-formic acid-water (2:8:90, by vol.). These solutions were centrifuged at 20°C at 29470g in a Spinco model E ultracentrifuge. The molecular weights were calculated by the method of Yphantis (1960).

Reduction and alkylation of the fragments. Reduction of the fragments was performed as described by Raftery & Cole (1966). The freeze-dried fragment (1μmol) was dissolved in 8M-urea (1ml). To this solution was added 10μl of 2-mercaptoethanol, 50μl of a solution of EDTA (2mg of the disodium salt/ml) and 300μl of 3M-Tris-HCl buffer, pH8.6. The reaction mixture was left under N₂ for 2h at room temperature.

Alkylation with iodoacetamide was performed by the method of Kolb *et al.* (1974). Iodoacetamide was added to the peptide to give a 1.1 molar excess of alkylating agent relative to thiol concentration. The reaction mixture was stirred at 0°C in the dark for 1h and filtered in the dark on Sephadex G-25. The eluates containing peptide were collected and freeze-dried.

For ultracentrifugation experiments, alkylation was performed with ethyleneimine as described by Raftery & Cole (1966). Reduced inhibitor was treated with an eightfold molar excess of redistilled ethyleneimine in three equal portions at intervals of 10min. The nitroprusside test for thiol (Toennies & Kolb, 1951) was then negative. The reaction mixture was immediately filtered on Sephadex G-25 and the eluates containing peptide were collected and freeze-dried.

Determination of radioactivity of ¹⁴C-labelled peptides. Aqueous samples of peptide were dissolved in scintillation fluid consisting of 60g of naphthalene, 4g of PPO (2,5-diphenyloxazole), 0.2g of dimethyl POPOP [1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene], 100ml of distilled methanol, 20ml of ethylene glycol and dioxan to 1 litre. The radioactivity was

counted on an Intertechnique liquid scintillation counter.

Determination of thiol groups. Free SH groups were assayed at pH4.6 with *p*-chloromercuribenzoate as described by Boyer (1954). The assay was performed by the addition of 5μl samples of 10mM-*p*-chloromercuribenzoate solution to 2.5ml of fragment solution (0.04μmol/ml).

Tryptic digestion of succinylated inhibitor. Succinylation of inhibitor (10mg/ml) was performed by the method of Slobin & Singer (1968). Trypsin treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (2mg/ml in 0.02M-CaCl₂) was added to 10mg of succinylated inhibitor dissolved in 1ml of 0.1M-sodium phosphate buffer, pH8. Two final concentrations of trypsin were used, 0.005 and 0.05%. The reaction mixture was kept for 2h at 37°C and the degradation was stopped by the addition of 2 drops of a solution of 0.1M-di-isopropyl phosphorofluoridate in propanol-2-ol.

Amino acid analysis. Fragments and peptides were hydrolysed at a concentration of 0.5mg/ml with 5.7M-HCl at 110°C for 18h. Amino acid analysis was performed on a Technicon automatic analyser.

N-Terminal analysis. N-Terminal amino acids of fragments and peptides were determined by the fluorodinitrobenzene method of Sanger & Thompson (1953) under the conditions described by Kasper *et al.* (1965). Hydrolysis of the dinitrophenylated peptides was performed during different time-periods from 10 to 72h. 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 1ml of 1M-NaOH at 60°C for 30min. After centrifugation, the absorbance of the supernatants was determined at 360nm. 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.

C-Terminal analysis. C-Terminal amino acids were determined with carboxypeptidase as described by Guidotti *et al.* (1962). A suspension of carboxypeptidase A (0.1ml) was dissolved by raising the pH to 10.5 with 0.1ml of Na₂CO₃ (7.5mg/ml). The concentration of carboxypeptidase A was determined at 280nm, and 0.02μmol (25units) was added to 1μmol of peptide dissolved in 1.5ml of 0.2M-sodium phosphate buffer, pH7.8. For peptides having lysine or arginine as C-terminal residues, 35μl (25units) of carboxypeptidase B was also added. The reaction mixtures were incubated at 25°C for various periods

of time. The reaction was stopped by lowering the pH to 3 with 1 M-HCl and samples were kept frozen until analysis in the amino acid analyser. Control solutions containing only substrate or enzyme were also incubated and analysed.

C-Terminal analysis of fragments was also performed by hydrazinolysis as described by Niu & Fraenkel-Conrat (1955). First 1 μ mol of freeze-dried fragments were dried *in vacuo* over P₂O₅ and then 1 ml of anhydrous hydrazine was added and the mixture was heated at 70°C for 16h. Excess of hydrazine was removed by evaporation over H₂SO₄. The dry residue was suspended in 2 ml of water and shaken for 15 min with 2 ml of freshly distilled benzaldehyde. This process was repeated twice. The aqueous phase was extracted with 4 $\times$ 1 ml of diethyl ether and evaporated. The dry residue was dissolved in 0.05 M-HCl and analysed in the amino acid analyser.

Sequential analysis. Sequential analysis was performed on 2 μ mol of peptide with a Sequenceur de Protéines 'Socosi', by the degradation method of Edman & Begg (1967).

Results

Amino acid composition of inhibitor

The amino acid composition of inhibitor is given in Table 1. The molecular weight calculated from this composition is 11 500. There are four half-cystine residues. Determination of free SH with *p*-chloro-mercuribenzoate gave negative results, indicating that these residues form two S-S bonds.

Molecular weight of inhibitor before and after reduction

Before reduction, the molecular weight of inhibitor measured by ultracentrifugation was 10500. After reduction and alkylation with ethyleneimine the molecular weight was 10320, i.e. unchanged within experimental error.

N-Terminal analysis of inhibitor

N-Terminal determination of inhibitor was done on 1 μ mol of Dnp-inhibitor hydrolysed for various periods of time from 10 to 72h. The best result was

Table 1. Amino acid analysis

Results are expressed as mol of amino acid/mol of peptide. No correction was made for destruction during hydrolysis. Total is of the best integer values. For component II, the best integer chosen for Gly was 1 because the sum of Gly of component I and II must be 3 and component II can be contaminated with Gly during its isolation by electrophoresis.

Amino acids	Inhibitor	Components obtained after tryptic degradation and reduction of succinylated inhibitor			Fragment F1	Chains obtained after reduction of F1		
		I	II	III		α	β	γ
Lys	13.1*	9.0	2.1	2.0	4.7*	3.9*	1.0*	1.1*
His	2.2	1.0	1.1	1.1	1.1	0	1.0	1.0
Arg	1.2	0.2	1.0	1.0	1.0	0	1.0	0.1
Asp	6.0	3.9	2.6	3.0	6.0	3.8	3.1	3.0
Thr	6.8	3.0	4.0	4.0	4.1	1.0	2.9	3.2
Ser	2.1	1.1	1.4	1.2	2.0	0	1.2	1.2
Glu	15.1	10.0	5.3	4.1	5.8	3.9	4.1	3.8
Pro	2.0	0.9	1.3	1.0	0	0	0	0
Gly	2.9	2.1	1.8	1.1	1.8	0.8	1.0	0.9
Ala	13.6	10.0	3.2	2.5	6.9	4.9	3.2	2.9
Val	5.9	6.2	2.0	1.2	3.0	1.8	0.3	0.2
Cys	4.1	3.0	1.0	1.0	3.7	2.7	1.0	0.7
Met	0.9	0.9	0	0	1.0	1.1	0	0
Ile	1.9	1.0	1.1	1.0	2.2	0	1.0	1.2
Leu	6.7	5.2	1.9	1.2	2.1	0	1.2	1.2
Tyr	0.7	0.1	1.0	0.9	0	0	0	0
Phe	5.6	2.9	3.1	2.7	5.2	2.7	3.8	3.1
Trp†	0	—	—	—	0	—	—	—
Total	92	60	32	29	51	28	25	23

* Means of three analyses on three different preparations.

† No tryptophan has been found in previous analyses of inhibitor (Lapresle & Webb, 1964) and fragment F1 (Lapresle & Webb, 1965).

obtained with a hydrolysis time of 40h. In this case, after correction for losses, 0.5 mol of threonine and 0.23 mol of glutamic acid or glutamine were detected per mol of inhibitor. With other samples of inhibitor, the same two *N*-terminal residues were found, but the relative amount of each varied with the sample analysed.

C-Terminal analysis of inhibitor

By hydrazinolysis, one *C*-terminal residue, leucine, was found for inhibitor with a yield of 75%. Degradation with carboxypeptidases A and B released 1 mol of leucine/mol of inhibitor and only traces of other amino acids.

Tryptic digestion of succinylated inhibitor

Inhibitor treated with succinic anhydride was hydrolysed by trypsin to split the molecule at the unique arginine residue. The hydrolysate was reduced and submitted to electrophoresis in polyacrylamide-agarose gel in the presence of 2-mercaptoethanol. Four components were observed (Plate 1). They were isolated by electrophoresis and their amino acid composition determined.

The slowest component, which has the same mobility as succinylated inhibitor, has the same composition as inhibitor. It corresponds to a portion of undegraded inhibitor and disappears almost completely when the highest concentration of trypsin is used. The three other components have been named I, II and III in the order of increasing mobility. Table 1 shows their amino acid compositions.

Components II and III each contain 1 mol of arginine/mol of peptide and have a similar amino acid composition. The sum of the amino acids of component I and component II or III is very close to the amino acid composition of inhibitor. Component I corresponds therefore to the *C*-terminal portion and components II and III to the *N*-terminal portion of inhibitor with respect to the arginine residue.

N-Terminal amino acids of components II and III were determined. For component II, there was 86% glutamic acid or glutamine and 14% threonine. For component III, there was 75% threonine and 25% glutamic acid or glutamine. These results indicate that components II and III each have one of the two *N*-terminal residues of inhibitor. The presence of the other residue in lower amount is explained by the fact that components II and III, which have very similar mobilities, were isolated by electrophoresis and were slightly contaminated one by the other.

Electrophoretic analysis of fragment F1

Plate 2 shows the electrophoretic pattern of fragment F1 in polyacrylamide-agarose plates. Fragment F1 has three components, which have

been named fragments F1a, F1b and F1c in order of increasing mobility. As judged from the intensity of the staining, the relative amount of these components varied from one preparation to another. However, fragment F1a was always a minor component and fragment F1b was the major one. The three components of fragment F1 were isolated by preparative electrophoresis, but there was always a slight contamination of each one by the neighbouring components, as can be seen in Plate 2.

Fragment F1 and its three isolated components were reduced and alkylated. Plate 2 shows the peptide chains obtained from each one. Fragment F1 gave rise to four chains named ϵ , α , β and γ in order of increasing mobility. Fragments F1a, F1b and F1c, gave rise to two chains: the α chain which is common to the three components and another chain which differs from one component to another. The ϵ chain which comes from fragment F1a was present in trace amounts and was not studied further. Chains β and γ came respectively from fragments F1b and F1c. The three chains α , β and γ were isolated in a pure state by preparative electrophoresis.

Amino acid composition of fragment F1 and chains α , β and γ

The amino acid composition of fragment F1 and the α , β and γ chains is given in Table 1. The molecular weights calculated from these compositions is 6067 for fragment F1, 3110 for the α chain, 2876 for the β chain and 2724 for the γ chain.

The composition of fragment F1 is close to the sum of the α chain and the β or γ chains. The slight discrepancies observed can be explained by the fact that the proportion of β and γ chains in fragment F1 is not known, nor the contribution to fragment F1 of the ϵ chain.

There are four half-cystine residues in fragment F1, three in the α chain, one in the β or γ chain. This result is confirmed by the determination of the radioactivity of fragment F1 and the three chains reduced and alkylated with iodo[^{14}C]acetamide. The results in d.p.m./ μmol of peptide were: 273250 for fragment F1; 210000 for the α chain; 62500 for the β chain and 64000 for the γ chain. These activities are for fragment F1, α , β and γ chains respectively in the proportion 4:3:1:1.

Determination of free SH-groups of fragment F1 with *p*-chloromercuribenzoate gave negative results, indicating that the four half-cystine residues form two S-S bonds.

N-Terminal amino acids of fragment F1 and α , β and γ chains

N-Terminal determination of fragment F1 and α , β and γ chains was made on 1 μmol of Dnp-

EXPLANATION OF PLATE I

Electrophoresis in polyacrylamide-agarose gel of inhibitor succinylated, reduced and hydrolysed by trypsin

From left to right: the concentration of trypsin was 0, 0.005 and 0.05%. Duration of electrophoresis was 3 h at 10V/cm at pH8.7. Inh: Inhibitor. For details of components I, II and III see the text.

EXPLANATION OF PLATE 2

Electrophoresis in polyacrylamide–agarose gel of fragment F1 and its components

Duration of electrophoresis was 2 h at 10 V/cm at pH 8.7. For details of α , β , γ and ϵ chains and of the various components of fragment F1 see the text.

Table 2. Time-dependent release of amino acids from chains β and γ hydrolysed by carboxypeptidase A+B

Results are expressed as mol of amino acid released/mol of hydrolysed peptide.

β chain					
Time (min)	Arg	Phe	Lys	Thr	Ala
30	0.95	0.74	0.45		
60	1	0.84	0.51	0.39	
120	0.97	1.10	0.51	0.60	0.53
240	0.94	1.55	0.62	0.92	1

γ chain					
Time (min)	Lys	Phe	Thr	Ala	Ser
15	0.47				
60	0.89	0.33	0.27		
240	1.03	0.90	0.71	0.58	0.53

peptides hydrolysed for 16h. Two *N*-terminal amino acids were found for fragment F1, alanine and glutamic acid or glutamine, in equimolar amounts. The α chain has alanine and the β and γ chains have glutamic acid or glutamine as *N*-terminal residue.

C-Terminal amino acids of fragment F1 and α , β and γ chains

Study of the *C*-terminal residues of fragment F1 by hydrazinolysis demonstrated the existence of two *C*-terminal residues, arginine and lysine. This agrees with the fact that fragment F1 is obtained from inhibitor by tryptic degradation.

Arginine, being absent from α and γ chains, must be the *C*-terminal amino acid of the β chain. Lysine, on the other hand, must be the *C*-terminal amino acid of α and γ chains.

This structure was confirmed by hydrolysis of the α , β and γ chains with carboxypeptidase A+B.

For α chain, there was a release of 1 mol of lysine/mol of α chain and only trace amounts of other amino acids.

For the β or γ chains, several amino acids were released; Table 2 shows the kinetics of the hydrolysis reaction.

These results suggest that the *C*-terminal sequence of the β chain is Arg $\rightarrow$ Phe $\rightarrow$ (Lys,Phe,Thr,Ala) and that the *C*-terminal sequence of the γ chain is Lys $\rightarrow$ Phe $\rightarrow$ (Thr,Ala,Ser).

Sequence of the *N*-terminal region of the α chain

We have shown that immunological activity of fragment F1 was due mainly to the α chain (F. Bellon & C. Lapresle, unpublished work); the sequence of this chain was investigated. Fig. 1 shows the sequence

Fig. 1. Sequence of the α chain and of the *C*-terminal region of human serum albumin
Chain V-Pro is as described by Kusnir & Meloun (1973) and chain VII-Asp as described by Meloun & Kusnir (1972). Sequence of chain α is the result of the single run in an automatic sequencer.

of the first 20 amino acid residues. This sequence includes the only methionine residue of fragment F1.

Discussion

Peptides were isolated by electrophoresis in polyacrylamide-agarose gel plates. Extraction from the gel could be performed by letting the products diffuse after grinding the gel and not by a second electrophoresis as described by Bellon (1973). This method is very efficient (75%), which is probably due to the low molecular weight of the products analysed. However, the buffer used for electrophoresis contains a large amount of glycine which could not always be eliminated completely. This may have disturbed the determination of this residue.

The molecular weight of inhibitor and fragment F1 calculated from their amino acid composition agrees with those previously determined by ultracentrifugation (Lapresle & Webb, 1964, 1965). For determination of the molecular weight of reduced inhibitor, alkylation was performed with ethylenimine rather than with iodoacetamide because the product so obtained is more soluble.

Inhibitor molecule is composed of one chain, as is shown by two observations: the molecular weight is the same before and after reduction, and there is only one C-terminal amino acid, leucine, in the proportion of 1 mol/mol of inhibitor. The two S-S bonds of inhibitor must therefore be intrachain bonds.

Two kinds of inhibitor molecules must exist since inhibitor has two N-terminal amino acids, threonine and glutamic acid or glutamine, the sum of which is lower than 1 mol/mol of inhibitor. These two kinds of inhibitor molecule have not been separated so far. However, their existence was confirmed by cleavage of succinylated inhibitor with trypsin on the carboxyl side of its arginine residue. This cleavage followed by reduction gives rise to three components: one that does not contain arginine corresponds to the C-terminal portion, and the two containing arginine that correspond to the N-terminal portion of inhibitor. These last two components could be separated by electrophoresis and each had one of the two N-terminal amino acids of inhibitor.

Component II has a lower electrophoretic mobility than component III and therefore is more basic. As it differs from component III by having one more residue of leucine, valine and glutamic acid or glutamine, this suggests that the glutamic residue is in the amide form.

On the other hand, the number of amino acids (60) of the first component permits the location of the arginine residue in the inhibitor chain. It is the 61st residue starting from the C-terminus.

Fig. 2 shows the schematic representation of the two molecules of inhibitor and of the components obtained after cleavage at the arginine residue.

Electrophoresis of fragment F1 in the polyacrylamide-agarose gel containing 9% (w/v) acrylamide showed that component F1 was composed of two kinds of molecules (Lapresle & Goldstein, 1969). The demonstration in this work of three forms of fragment F1 can be ascribed to the use of a gel containing a lower amount (5.5%) of acrylamide.

Each form of fragment F1 is composed of two chains linked by disulphide bonds since they could be separated after reduction. This interchain linkage is formed between the unique half-cystine residue of the β or γ chain and one of the three half-cystine residues of the α chain. The two other half-cystine residues of the α chain form therefore an intrachain bond.

Fig. 2 gives the schematic representation of the structure of fragments F1.

Homology between the sequence of the N-terminal portion of the α chain from fragment F1 and certain portions of human serum albumin permits the location of fragment F1 and inhibitor in the albumin molecule. Cleavage at the six methionine residues of human serum albumin followed by disruption of the disulphide bonds produces seven peptide chains (McMenamy *et al.*, 1971; Kusnir & Meloun, 1973). They have been named differently and we shall use the nomenclature of the latter authors. Chains I-ASP and VII-ASP have been identified as the N-terminus and C-terminus of the molecule (McMenamy *et al.*, 1971; Kusnir & Meloun, 1973) and chain VII-ASP has been completely sequenced by Meloun & Kusnir (1972).

The sequence of the α chain includes a methionine residue which is at the third position. The 17 sequenced amino acids that follow methionine are identical with the first 17 residues of chain VII-ASP (Fig. 1). The first two amino acids which precede methionine have been identified with the C-terminal portion of chain V-Pro. These results indicate that chain V-Pro precedes chain VII-ASP in the albumin structure (Lapresle & Bellon, 1973). As inhibitor has no other methionine residue and is composed of one chain, no other CNBr fragments than V-Pro and VII-ASP can participate in its structure as well as in that of fragment F1.

The eight last residues of the α chain, which has not been sequenced, are the same as the eight residues (18-25) sequenced by Kusnir & Meloun (1973). It is most probable that the order of these amino acids is the same as the corresponding part of chain VII-ASP (Fig. 1). In this case, the C-terminal lysine of the α chain is preceded by glycine, which agrees with the observation that with carboxypeptidase degradation, 1 mol of lysine/mol of the α chain, and no other amino acid, was released.

The two kinds of inhibitor have the same C-terminal leucine residues. The fact that no other residue is released by carboxypeptidase degradation suggests

Fig. 2. Schematic representation of the structures of inhibitor and fragment F1

One of the two possible forms of disulphide linkage of the contiguous half-cystine residues to the non-contiguous half-cystine residues has been arbitrarily represented.

that the penultimate or the antepenultimate residue is resistant to carboxypeptidase attack (Ambler, 1967). Among the leucine residues of the remaining part of chain VII-Asp, not common to the α chain, only the one situated at the C-terminal end, which is preceded by a glycine residue, agrees with this requirement. Therefore the two kinds of inhibitor (92 or 89 residues) must have the same C-terminal amino acid as the albumin molecule. They are composed of the 37 residues of chain VII-Asp and of 55 or 52 residues of the C-terminal portion of chain V-Pro.

Component I, obtained by cleavage of the succinylated inhibitor at the arginine residue, and which does not contain arginine, is the C-terminal portion of inhibitor with respect to this residue; therefore it is composed of the 60 C-terminal residues

of the human albumin molecule. By subtracting from the amino acid composition of this component the last 40 amino acids of albumin beginning at the N-terminal alanine residue of the α chain, we can deduce the nature of the 20 amino acids of the portion of inhibitor molecule situated between the N-terminal alanine of the α chain and the arginine residue. This composition is (Lys₄, His₁, Thr₂, Glu₅, Pro₁, Ala₁, Val₃, Ile₁, Leu₂).

The unique arginine residue of inhibitor and fragment F1 is the C-terminal amino acid of component II (32 residues), component III (29 residues) and the β chain (25 residues). Carboxypeptidase degradation of the β and γ chains shows that the sequence of the inhibitor region starting from the arginine residue must be Arg $\rightarrow$ Phe $\rightarrow$ Lys $\rightarrow$ Phe. This result agrees with the fact that the β chain has

one more arginine and phenylalanine residue than the γ chain. However, there is a discrepancy in the phenylalanine content of components II and III which has not been explained. These components, which must include all the amino acids of the β chain, should have four and not three phenylalanine residues.

The amino acids present in fragment III and not in the β chain (Thr, Tyr, Val, Pro, Lys) are located beyond the N -terminus of the β chain. The amino acids present in fragment II and not in fragment III (Gln, Val, Leu) are located beyond the N -terminus of fragment III. These residues are from the N -terminal portion of inhibitor which must have the following order: Gln-(Val, Leu)-Thr-(Tyr, Val, Pro)-Lys. Glutamine and threonine can be located because they are the N -terminal residues of components II and III and lysine can be located because inhibitor is cleaved at this level by trypsin to give fragment F1.

Among the four half-cystine residues of inhibitor and fragment F1, two are contiguous. For this reason, the two S-S bonds of these molecules must have the following location. One linkage is formed between one of the two contiguous half-cystine and the last half-cystine residue of the α chain. The second linkage is formed between the other contiguous half-cystine residue and the fourth half-cystine residue of inhibitor; this last residue is located in the human serum albumin chain between the N -terminal glutamic acid or glutamine of fragment F1 and the unique arginine residue of inhibitor. These two S-S bonds give rise to two loops: a small one composed of at most eight residues, and a larger one composed of at least 36 residues. This structure is most probably that of the C -terminal part of the albumin molecule. Inhibitor is obtained by degradation of human serum albumin at an acidic pH (3.5) where rearrangement of disulphide bonds cannot occur. The structure of albumin is well preserved in the inhibitor and fragment F1, as shown by their capacity to react with anti-(human-serum-albumin) antibodies (Lapresle & Webb, 1960; Lapresle & Goldstein, 1969). It should be noted that a similar structure has been found for bovine serum albumin molecule (Brown, 1974).

We are grateful to Mrs. M. J. Escribano for helpful discussions. We thank Mr. S. Mangalo for performing the ultracentrifugation and Mr. Santis and Mrs. Nebenzahl

from Socosi for performing sequential analysis. The expert technical assistance of Mrs. P. Daillan and Mrs. J. Cavillon is gratefully acknowledged. F. B. is Attaché de Recherche au C.N.R.S.

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