DISULFIDE BONDS IN HUMAN SERUM ALBUMIN

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The cyanogen bromide fragments of human serum albumin with intact disulfide bonds were stepwise digested with pepsin and chymotrypsin or with pepsin and trypsin. From the resulting digests cystine peptides were isolated permitting all 35 half-cystine residues of albumin to be characterized in the form of nine disulfide groupings D1 through D9. Two nonadjacent half-cystine residues are always linked to the central -Cys-Cys- pair through two disulfide bonds in groupings D2 through D9. The presence of a disulfide bond between the two adjacent half-cystines was experimentally eliminated in all cases. The bonds between these central half-cystines and the remaining two half-cystine residues have not been exactly defined as yet (as designated by the residue numbers in brackets). Grouping D1 is not complete and involves three half-cystine residues with one disulfide bond only. The individual groupings consist of the following half-cystine residues: D1: 34 (53—62); D2: 75 (90, 91) 101; D3: 124 (168, 169) 177; D4: 200 (245, 246) 253; D5: 265 (278, 279) 289; D6: 316 (360, 361) 369; D7: 392 (437, 438) 448; D8: 461 (476, 477) 487; D9: 514 (558, 559) 567. The regularities in the distribution of disulfide bonds and homologies in the amino acid sequence of the protein point to the existence of three homologous domains in the molecule (groupings D1—D3, D4—D6, and D7—D9) suggesting that the albumin molecule originated in smaller units.

The amino acid sequence[1] of human serum albumin involves 585 amino acid residues and is in good agreement with the amino acid composition[2,3] of the protein. Its molecule consists of a single polypeptide chain; the three-dimesional structure of albumin is maintained by 17 disulfide bonds. The remaining half-cystine residue of the total number of 35 is present as a cysteine. This study was aimed at the determination of disulfide bonds in the human serum albumin molecule which had not been carried out so far. The disulfide bonds appearing in the schematical presentation of the structure of the serum albumin molecule, as published by Behrens and coworkers[4], are based on analogy with bovine albumin[5] only.

A specific problem in the investigation of disulfide bonds in serum albumin is the determination of disulfide bonds in which half-cystine residues participate on the sequence -Cys-Cys-; such sequence repeats eight times in human serum

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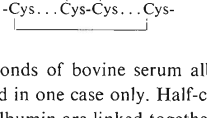
albumin[1]. From the theoretical viewpoint, two dosilfide bonds involving the sequence -Cys-Cys- can exist in three different forms:



In studies on the disulfide bonds of bovine serum albumin[5] the form of the disulfide bonds has been identified in one case only. Half-cystine residues No 511, 555, 556, and 564 of bovine serum albumin are linked together to a grouping correspondind to form (*a*). In this study the presence of form (*c*) has been eliminated in all cases by experimental results; no effort, however, was made to distinguish between forms (*a*) and (*b*).

In their sequential studies King and Spencer[6] carried out the initial fragmentation of native bovine serum albumin by cyanogen bromide cleavage. This procedure gave equally good results when applied to human serum albumin[7,8], containing six methionine residues. Three large fragments are formed in which the products of cyanogen bromide cleavage are held together by intact disulfide bonds. These fragments, whose nomenclature has been presented in one of the earlier papers[9], account for the entire molecule of albumin. Fragments N (residues 1–123), M (res. 124–298), and C (res. 299–585) were found to be a suitable starting material for the isolation of cystine peptides from the enzymatic digests of the fragments thus the individual parts of the albumin chain could be investigated separately. Since the complete amino acid sequence of human serum albumin is known and the distribution of amino acids around the individual half-cystine residues is different, the individual cystine peptides could be identified according to their amino acid composition.

EXPERIMENTAL

Material

Human serum albumin was a product of the Institute for Sera and Vaccines, Prague. Cyanogen bromide fragments N, M, and C were prepared as described elsewhere[10]. Pepsin chymotrypsin, and trypsin were from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. Sephadex G-10, G-25, and G-50, and SE-Sephadex C-25 were purchased from Pharmacia, Uppsala, Sweden. Dowex 1-X2 (200–400 mesh) was from Fluka, Switzerland. Silufol, aluminum sheets with a thin lager of silica gel, were a product of Kavalier, Sázava, Czechoslovakia. Polyamide layer sheets were purchased from BDH Chemical Ltd., Poole, U.K.

Methods

Separation of peptide mixtures. Peptide mixtures were separated by paper chromatography in system S1: n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v) and S2: n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 16 v/v). The electrophoretic separation of peptide mixtures on paper was effected in system E1: pyridine–acetic acid–water (5 : 1 : 494 v/v), pH 5·6, at 30 V/cm in the descending arrangement[11]. Peptide maps were prepared by a combination of electrophoresis (1st direction) and chromatography in system S1 (2nd direction). These separation procedures were carried out on Whatman No 3 paper.

Detection and elution of peptides. Peptides were detected by 0·2% solution of ninhydrin in acetone. Cystine peptides were specifically detected essentially according to Toennis and Kolb[12] as follows. Solution I: Methanol (90 ml) and 20 ml of 28% NH_4OH were added to 3 g of sodium nitropruside dissolved in 10 ml of 2N-H_2SO_4. Solution II: Sodium cyanide (4 g) was dissolved in 10 ml of water and the solution was diluted with 100 ml of methanol. Equal volumes of both solutions were mixed before use and the mixture was filtered. The chromatograms were dipped in this reagent and allowed to dry in a fume hood. Cystine peptides were stained pink. The peptides were eluted from preparative chromatograms by solution E1 as used for the electrophoretic separation.

Enzymatic digestion of peptides. The cystine peptides studied here were isolated from enzymatic digests of cyanogen bromide fragments N, M, and C as described. The analysis of the disulfide peptides as well as of their oxidation products are given in Table I. Some of the peptides were cleaved further by trypsin or chymotrypsin. The peptide (1 μmol) was dissolved in 250 μl of 0·1M-NH_4HCO_3, pH 8·0, and 0·01 μmol of the enzyme dissolved in 250 μl of water was added to this solution. The reaction mixture was incubated 2 h at 37°C. Another 0·01-μmol portion of the enzyme in 250 μl of water was added afterwards and the incubation of the reaction mixture was continued for 3 h at 37°C.

Oxidation of cystine peptides. The oxidation by performic acid was effected by the method of Hirs[13]. A solution of 1·5 μmol of peptide in 0·5 ml of 99% formic acid and 0·1 ml of methanol, and 1 ml of performic acid were cooled down to −5°C for 30 min. Both solutions were mixed afterwards and the reaction was allowed to proceed at the same temperature for 150 min. The reaction mixture was diluted with 40 ml of water afterwards and freeze-dried.

Amino acid analysis. The amino acid analyses were carried out by the method of Spackman and coworkers[14] in Model 6020 Amino Acid Analyzer, manufacturad by the Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague. The peptides were hydrolyzed in 6M-HCl, 20 h at 110°C. The half-cystine content was determined as cysteic acid in samples oxidized by performic acid[15].

Determination of N-terminal amino acids. The N-terminal end groups were characterized as a rule by one Edman degradation step[16]. The amino acid phenylthiohydantoins were identified by chromatography on Silufol thin lager steets[17]. The technique of Gray and Smith was used in some cases. The dansyl (1-dimethylaminonaphthalene-5-sulfonyl) derivatives of amino acids were identified chromatographically[19] on 37 × 37 mm polyamide layer sheets.

TABLE I

Aminocid Composition of Disulfide Peptides (D) and Products Obtained after their Oxidation (ox) or Cleavage with Trypsin (T)

The values for the numbers of the amino acid residues are not corrected.

Peptides isolated from fragment N

Amino Acid	D1	D1-ox1	D2	D2-ox1	D2-ox2	D2-ox3
Lysine	1·1	0·9	1·8	1·0	1·0	
Histidine						
Arginine						
Cysteic acid	1·8	1·9	4·2	1·1	1·8	0·8
Aspartic acid	3·1	3·0	3·0	1·0	1·1	1·0
Threonine	1·0	0·9	0·7	0·9		
Serine	0·9	0·9				
Glutamic acid	2·1	1·9	1·2			1·0
Proline						
Glycine			1·0	0·9		
Alanine	2·0	1·9	2·1		2·0	
Valine	1·0	0·9				
Isoleucine						
Leucine			0·8	1·0		
Tyrosine						
Phenylalanine			0·6			1·0
Homoserine						

Peptides isolated from fragment M

Amino Acid	D3	D3-ox1	D3-ox2	D4	D4-ox1	D4-ox2	D4-ox3	D5	D5-ox1	D5-ox2
Lysine	1·0		1·0	2·2	1·0	1·0		5·2	2·0	1·9
Histidine				1·6		1·6		1·2		1·0
Arginine				1·0	0·8					
Cysteic acid	4·0	0·7	3·0	4·0	1·1	2·1	1·0	3·7	1·0	1·0
Aspartic acid	1·4		1·0	2·6		1·0	2·0	2·2	2·0	
Threonine	1·9	0·9	0·9	1·5		1·8				
Serine				1·1	1·0			3·6	2·5	0·8
Glutamic acid	2·2		2·1	2·5		1·0	1·1	6·0	2·3	2·0
Proline								1·1		0·9
Glycine				1·0		1·0				
Alanine	5·2	1·1	3·8	2·0	1·1		1·0	2·1	1·0	0·8
Valine				0·6		0·8				
Isoleucine								2·7	1·9	0·6
Leucine	1·1		1·0	3·8	2·0	1·0	1·0	3·2	1·2	2·2
Tyrosine								0·5	0·7	
Phenylalanine										
Homoserine										

Peptides isolated from fragment C

Amino Acid	D6	D6-ox1	D6-ox2	D6-ox3	D7	D7-ox1	D7-ox2	D7-ox3	D8	D8-Ta	D8T-ox1	D8T-ox2	D9	D9T-ox1	D9T-ox2	D9T-ox3
Lysine	3·2	1·9	1·0		4·6	0·8	4 0			2.1			3·3		1·0	0·8
Histidine	1·0		0·7		0·9		0·3		0·5				0·8	0·7		
Arginine					1·3		1·2		2·8	0·7		1·1				
Cysteic acid	3·7	1·2	1·9	1·0	3·6	1·2	1 8	1 1	3·9		2·0	1·0	3·7	1·0	1·9	1·3
Aspartic acid	2·9	2·1	0·9		2·1	1·2		1·0	3·5	1·0			3·0	1·0		1·8
Threonine									3·3	0·9	0·9		1·7	0·9		0·9
Serine	0·8	1·0			1·1		1·0		1·7	1·0	0·9					
Glutamic acid	3·8	2·2	1·0	0·9	4·0	2·3	1·0	1·1	3·3		0·9		2·0			0·8
Proline	0·9		0·8		1·7		0·9	0·9	2·0	1·0		1·1				
Glycine					1·6		2·2									
Alanine	4·0	1·2	1·9	1·0	1·8		1·0	0·9					2·0	1·0		1·0
Valine	1·8	2·1			1·4		1·1		3·4	1·0			1·0			
Isoleucine					0·8	0·8							0·9	0·9		
Leucine					1·7	1·0			2·9		1·0		0·9	0·9		
Tyrosine	1·6	0·4	0·2		0·3			0·4								
Phenylalanine					0·9	1·0			1·0			0·9	1·0			0·8
Homoserine					0·9		0·8									

RESULTS

Preparation and Analysis of Cystine Peptides of Fragment N

A solution of 730 mg of fragment N in 35 ml of 0·1M-HCOOH was treated with 24 mg of pepsin, dissolved in 1 ml of water, 4 h at 37°C. The digest was placed onto a column of Sephadex G-50 equilibrated with 0·1M-HCOOH. The course of the separation was examined by paper electrophoresis of 0·1 ml and 0·2 ml aliquots taken from each fraction and by measuring the absorbance of the fractions at 260 nm (Fig. 1). Electropherograms obtained with 0·1 ml aliquots were stained with ninhydrin, electropherograms of 0·2 ml aliquots with the reagent for disulfides. The presence of cystine peptides was detected in fractions No 25—33. This material was digested further with trypsin. The fractions were pooled and the pH of the solution was adjusted to 6·5. After the addition of 8 mg of trypsin the cleavage was allowed to proceed 16 h at 25°C. The digest was lyophilized, the lyophilysate was dissolved in 10 ml of 0·05M-HCOOH and fractionated on a column of Sephadex G-50 stabilized with 0·05M-HCOOH. The fractionation of the sample was monitored by absorbance measurement of the fractions at 260 nm (Fig. 2). The disulfides were detected as described above; fractions No 38—44 gave a positive reaction. These fractions were pooled, the material was lyophilized, the residue dissolved and desalted on a 1·6 × 50 cm column of Sephadex G-10 equilibrated with 0·05M-HCOOH. An additional fractionation was

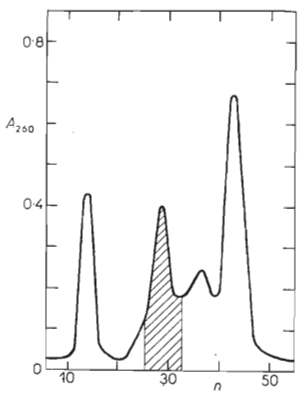


FIG. 1

Fractionation of Peptic Digest of Fragment N on Sephadex G-50

The column (145 . 2·8 cm) was eluted by 0·1M-HCOOH. Flow rate: 18 ml (one fraction) per 30 min. The disulfides were detected in fractions marked by hatching.

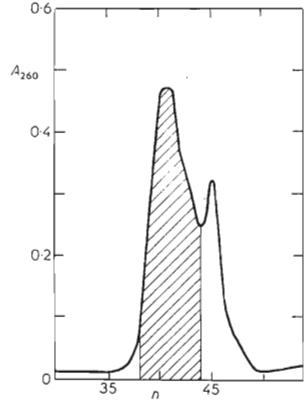


FIG. 2

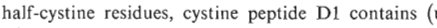
Fractionation of Tryptic Digest of Disulfide Fraction of Fragment N (Fig. 1) on Sephadex G-50

The column (145 . 2·8 cm) was eluted by 0·05M-HCOOH. Flow rate: 18 ml (one fraction) per 30 min. The disulfides were detected in fractions marked by hatching.

effected by paper electrophoresis. Cystine peptides were contained in the neutral and acidic zone only, none of the basic components gave a positive test for disulfides.

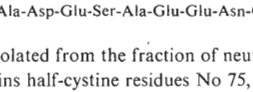
From the fraction of acidic peptides (30 mg), peptide D1 (Table I) was isolated by chromatography in system S1; its N-terminal amino acid was threonine. Peptide D1 afforded after oxidation one component only, D1ox1. Having regard to the presence of two half-cystine residues, cystine peptide D1 contains (unlike all the remaining peptides) one disulfide bond only. An inspection of the amino acid sequences around half-cystine residues in fragment N permits us to align unambiguously this peptide with the region between residues No 52 and 64 (numbered with respect to their positions in the amino acid sequence of albumin[1]); the disulfide bond thus links together half-cystines No 53 and 62:

Peptide D1

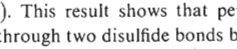


Peptide D2, which was isolated from the fraction of neutral peptides by chromatography in system S1, contains half-cystine residues No 75, 90, 91 and 101, as follows from an analogous comparison with the amino acid sequence of albumin. This finding can be schematically presented as follows

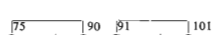
Peptide D2



Peptide D2 gave one spot on chromatography and electrophoresis. Three N-terminal amino acids were revealed in the first Edman degradation step: glycine, alanine, and aspartic acid (the use of the dansyl technique excluded the possibility of the determination of asparagine). This result shows that peptide D2 consists of three components joined together through two disulfide bonds between its four half-cystine residues. After oxidation of the peptide, three components, D2ox1, D2ox2, and D2ox3, were obtained. The N-terminal amino acids of these components as well as their summary amino acid composition are in agreement with the amino acid composition of original peptide D2. These results lead us to conclude that two disulfide bonds present in this grouping link together the half-cystine residues either according to variant (*a*)



or according to variant (*b*)



The result excludes variant (*c*)

since in this case cystine grouping D2 would yield two separable components.

Preparation and Analysis of Cystine Peptides of Fragment M

Fragment M (2·5 g) was dissolved in 250 ml of 0·01M-HCl and to the solution was added 50 mg of pepsin dissolved in 1 ml of 0·01M-HCl. The pH of the mixture was adjusted to pH 2·2 by 1M-HCl and the mixture was incubated 18 h at 23°C. After this period the pH of the mixture was raised to 6·5 by the addition of 1M-NH_4OH, chymotrypsin (50 mg in 1 ml of water) was added, and the solution was incubated 4 h at 37°C. The product was freeze-dried. The fractionation of the combined peptic — chymotryptic digest of fragment M was effected on a column of Sephadex G-25 (Fig. 3). Aliquots (0·1 ml) of each fraction were separated by paper electrophoresis; the electropherograms were stained for the presence of disulfides. According to the result of the detection the effluent was pooled to two fractions which were lyophilized. The yield of fraction M1 containing larger peptides was 529 mg, fraction M2 containing low molecular weight material weighed 585 mg.

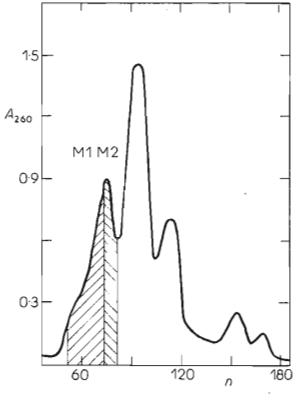


FIG. 3

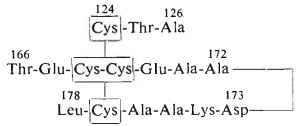
Fractionation of Combined Peptic-Chymotryptic Digest of Fragment M on Sephadex G-25

The column (55 × 6·6 cm) was eluted by 0·05M-HCOOH. Flow rate: 12·5 ml (one fraction) per 1·5 min. The disulfides were detected in fractions M1 and M2.

The peptide material of fraction M1 was dissolved in 5 ml of 1% collidine acetate (pH 6·5) and applied onto a column of Dowex 1 (32 × 1·4 cm) equilibrated with the same collidine acetate solution. The eluting solution was pumped to the column from a mixing chamber of constant volume of 250 ml, connected to an open reservoir. Both vessels were filled up with 1% collidine acetate, pH 6·5. After 150 ml of this buffer had been pumped to the column, the reservoir was filled up stepwise with 150 ml of 0·1, 1·0, and 5·0M-CH_3COOH. Fractions 8 ml in volume were collected at 5-min intervals. A 0·1 ml aliquot of each fraction was separated by electrophoresis; according to the results of this analysis, the effluent was pooled to fraction M11 containing cystine peptides. The pooled fraction was lyophilized. Fraction M2 was chromatographed on Dowex 1 by an analogous procedure and effluents containing cystine peptides were pooled to fraction M21

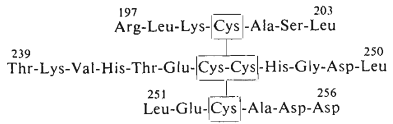
Fraction M21 was purified further by electrophoresis and yielded cystine peptides D3 and D4; fraction M11 yielded peptide D5.

Peptide D3



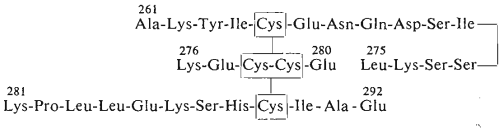
involves half-cystine residues No 124, 168, 169, and 177. Threonine was the only N-terminal amino acid found in the first Edman degradation step (half-cystine is bound by a disulfide bond); this finding indicates that no peptide bond between residues No 166 and 178 was cleaved by enzymatic hydrolysis. This conclusion was evidenced by the isolation of peptides D3ox1 and D3ox2. Peptide D3 consists of two components linked together by two disulfide bonds. This peptide was subsequently cleaved by trypsin. In addition to threonine, alanine appeared as a new N-terminal amino acid; the products of tryptic cleavage, however, remained held together by a disulfide bond and behaved as one component when subjected to different separation procedures. This result eliminates variant (*c*) with one disulfide bond between half-cystines No 124 and 177 and the other disulfide bond between half-cystines No 168 and 169.

Peptide D4



involves half-cystine residues No 200, 245, 246, and 253. Three N-terminal amino acids, arginine, threonine, and leucine, were obtained in the first Edman degradation step. Hence peptide D4 consists of three components held together by two disulfide bonds. Peptides D4ox1, D4ox2, and D4ox3 were isolated after oxidation of peptide D4.

Peptide D5



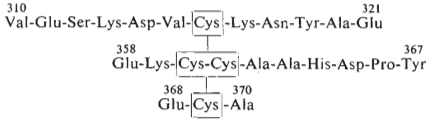
involves half-cystine residues No 265, 278, 279, and 289. Two N-terminal amino acids, alanine and lysine, were found by the dansyl-Edman procedure. Peptides D5ox1, D5ox2, and D5ox3 were isolated after oxidation; their N-terminal amino acids were identical with the N-terminal end groups of parent peptide D5.

Preparation and Analysis of Cystine Peptides of Fragment C

Fragment C (4 g) was dissolved in 400 ml of 0·01M-HCl and to the solution was added 80 mg of pepsin in 2 ml of 0·01M-HCl. The pH of the solution was adjusted to pH 2·2 by 1M-HCl and incubated 16 h at 23°C. The pH was raised to 6·5 after this period, 80 mg of chymotrypsin in 3 ml of water was added, and the digestion was allowed to proceed 16 h at 23°C. The combined peptic-chymotryptic digest of fragment C was lyophilized. The digest was chromatographed on a Sephadex G-25 column equilibrated with 0·001M-HCl (Fig. 4). The yield of pooled fractions No 59—80 containing disulfides was 2·31 g. A part of this material (1·8 g) was dissolved in 15 ml of 1% collidine and the solution was applied to a 30 × 2·5 cm column of Dowex 1 equilibrated with 1% collidine acetate buffer at pH 6·5. The column was eluted by 250 ml of this buffer and subsequently by an elution gradient developed by mixing 800 ml of 1% collidine acetate, pH 6·5, with 800 ml of 1% collidine acetate, pH 3·5, placed in open vessels of equal diameter. Fractions were collected at a rate of 15 ml/10 min. Two 1 ml aliquots of each fraction were taken for the ninhydrin and nitroprusside test after electrophoresis. Cystine peptides were found in the basic portion of the material (fractions No 3—6, 880 mg) and also among predominantly neutral peptides in fractions No 63—64 (216 mg). This neutral fraction was subjected to separation electrophoretically and chromatographically in systems S1 and S2 and yielded cystine peptides D6 and D9. The basic fraction was chromatographed on a column of SE-Sephadex (60·7 × × 2·5 cm). Elution was effected by a gradient developed by mixing 1 500 ml of 0·1M pyridine acetate buffer, pH 3·1, with 1 500 ml of 2M pyridine acetate buffer, pH 5·0, in open vessels of equal diameter. Fractions were collected at a rate of 19 ml/20 min and 1 ml of each fraction was taken for the detection of disulfides. The subsequent separation of the disulfide fractions was carried out electrophoretically and chromatographically in system S1 and S2; the chromatographic technique of repeated development was used.

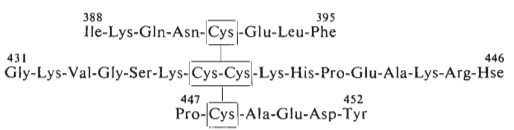
Cystine peptide D7 was isolated from fractions No 112–140 and cystine peptide D8 from fractions No 68–91 in this manner.

Peptide D6



contains half-cystines residue No 316, 360, 361, and 369. This peptide gave after oxidation peptides D6ox1, D6ox2, and D6ox3 with N-terminal valine and N-terminal glutamic acid in the remaining cases.

Peptide D7



contains half-cystine residues No 392, 437, 438, and 448. Isoleucine, glycine, and proline were found in the first Edman degradation step. Lysine only was found in the

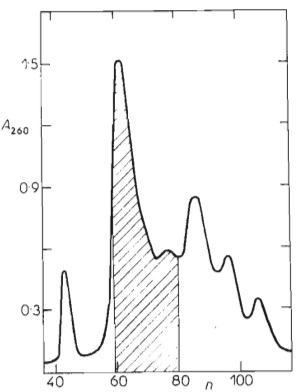


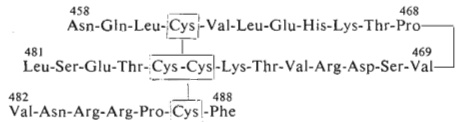
FIG. 4

Fractionation of Combined Peptic-Chymotryptic Digest of Fragment C on Sephadex G-25

The column (70 . 6·2 cm) was eluted by 0·001M-HCl. Flow rate: 20 ml (one fraction) per 3 min. The disulfides were detected in fractions marked by hatching.

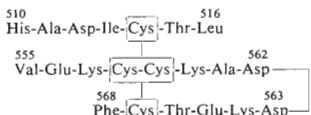
second step (half-cystine No 448 remains attached by a disulfide bond). Peptide D7 was oxidized and peptides D7ox1, D7ox2, and D7ox3 permitted the structure of peptide D7 to be derived.

Peptide D8



contains half-cystine residues No 461, 476, 477, and 487. Peptide D8 represents the region of the polypeptide chain of albumin between residues No 458 and 488 which has been cleaved by chymotrypsin at the carboxyl side of residue No 481. In subsequent experiments peptide D8 was digested with trypsin, the chain was thus cleaved and peptide D8Ta, Thr-Pro-Val-Ser-Asp-Arg was liberated. The remaining disulfide peptide D8T was uniform when subjected to electrophoresis and to chromatography in system S1. After oxidation of peptide D8T only two peptides containing cysteic acid were isolated: peptide D8Tox1, involving residues No 476–481, and peptide D8Tox2 (residues No 485–488). The remaining peptide (res. No 458–466) was not isolated. The results provide evidence that the disulfide bonds of peptide D8 are arranged as shown.

Peptide D9



includes half-cystine residues No 514, 558, 559, and 567; histidine and valine are its N-terminal amino acids. Peptide D9 was digested with trypsin to one single cystine-linked peptide D9T which yielded peptides D9Tox1 (res. No 510–516), D9Tox2 (res. No 558–560), and D9Tox3 (res. No 561–568) after oxidation. Tryptic digestion resulted in the cleavage of the peptide bond between residues No 560 and 561 and in the liberation of peptide Val-Glu-Lys. As in the preceding cases, the results obtained eliminate the possibility that both disulfide bonds are arranged according to variant (*c*), *i.e.* that the adjacent half-cystines No 558 and 559 are joined to one another by a disultide bond.

DISCUSSION

One half of the total number of 35 half-cystine residues in the molecule of human serum albumin occurs in the sequence -Cys-Cys-, repeating 8-times in the albumin molecule[1]. The sequence -Cys-Cys- participating on disulfide bonds occurs in a great number of proteins and plays an important role in the stabilization of their three--dimensional structure. This sequence has been found to exist, *e.g.* in insulin[20] and proinsulin[21], alpha-keratin[22], cobrotoxin[23-25], cardiotoxin[26], neurotoxin[27], interstitial stimulating hormone[28], ovine luteinizing hormone[29], neurophysin[30], rubredoxin[31], and phospholipase A2 (ref.[32]). As stated in the introduction, the sequence -Cys-Cys- can participate on the disulfide bonds in the form of three different groupings. Grouping -Cys---Cys-Cys---Cys- has been found in proinsulin[21] and also in bovine serum albumin[5] as the only disulfide grouping in albumin completely determined so far. Grouping -Cys---Cys-Cys---Cys- has been likewise found in proteins, namely in cardiotoxins[26] and neurotoxins[27]. As yet there is no evidence of a disulfide bond between adjacent half-cystine residues in proteins; however, a number of peptides with such a bond have been synthetized[33]. Hence, from the theoretical viewpoint, the presence of any of these disulfide groupings in proteins cannot be excluded beforehand.

The results obtained by the experimental procedures used in this study permit us to conclude that none of the disulfide groupings existing in human serum albumin involves a disulfide bond between adjacent half-cystine residues. It is to be solved powever, which of the two remaining variants is present in the molecule. A systematical approach to this problem would require a method for cleavage of the bond between the two adjacent half-cystines, a method which would leave intact the disulfide bond and at least a part of the peptide necessary for the identification of the half-cystine residue. Partial acid hydrolysis might provide the desired effect in some cases; yet this type of random cleavage obviously is not a general solution of the problem.

The study of the disulfide bonds of human serum albumin was easier after the isolation of the three cyanogen bromide fragments marked N, M, and C. These fragments were enzymatically digested and disulfide peptides were isolated from these digests. The amino acid composition of the disulfide peptides is given in Table I, together with the composition of products of thir oxidation. In their study, in addition to the main cystine peptides, minor quantities of other cystine peptides which were by several amino acid residues longer or shorter than the main peptides were also isolated. Only those peptides which were characterized further, including N-terminal end group analysis, are listed in this paper. Final results were obtained with cystine peptides corresponding to structures of three chains interlinked through two di-

sulfide bonds. In such cases the existence of the -Cys-Cys- disulfide bond could directly be eliminated. In other cases it was necessary to cleave the peptides by other enzymes; three-chain structures were always obtained. The amino acid sequence of the cystine peptides was derived from the knowledge of their N-terminal amino acid, their total amino acid composition, from the composition of their oxidation products, and from a comparison with the known amino acid sequence of the albumin molecule. The manner in which the cystine peptides interlink the molecule of human serum albumin is shown in Fig. 5. Regions corresponding to cyanogen bromide fragments N, M, and C are marked by horizontal bars.

Fragment N consists of 123 amino acid residues which involve the only unpaired cysteine residue whose position (No 34) has been determined in earlier work. The peptide chain of fragment N is covalently interlinked through 3 cystines. This fragment was digested stepwise with pepsin and trypsin and disulfide peptides D1 and D2 were isolated from this digest. Half-cystine residues No 53 and 62, which are linked through one disulfide bond, were identified in peptide D1. The remaining four half-cystine residues of fragment N are grouped together in disulfide peptide D2; the latter contains two disulfide bonds in the arrangement 75 (90, 91) 101, where numbers designate the positions of the half-cystine residues. According to this designation residue No 75 can be linked through a disulfide bond either to residue number 90 or 91; similarly, residue No 101 can be joined to residue No 90 or 91. Groupings of such pairs of disulfide bonds were found in all the remaining peptides (D3–D9). The data on disulfide bonds of fragment N have been presented in the thesis by one of the authors[34].

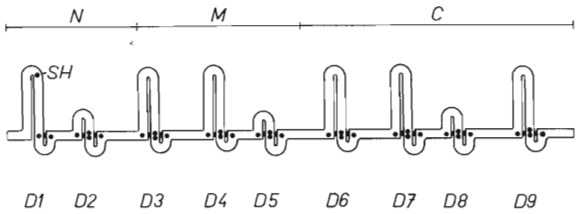


FIG. 5

Arrangement of Disulfide Bonds in Molecule of Human Serum Albumin

Regions N, M, and C denote the cyanogen bromide fragments of native albumin. ● half-cystine residues, SH unpaired cysteine residue (No 34). Groupings of two disulfide bonds (D1–D9) involve the following half-cystine residues. D1: 34 (53, 62); D2: 75 (90, 91) 101; D3: 124 (168, 169) 177; D4: 200 (245, 246) 253; D5: 265 (278, 279) 289; D6: 316 (360, 361) 369; D7: 392 (437, 438) 448; D8: 461 (476, 477) 487; D9: 514 (558, 559) 567. The meaning of this system of numerical symbols as well as the anomalous situation observed with grouping D1 are described in the text.

Fragment M comprises 175 amino acid residues of their number 12 half-cystines interlinked through six disulfide bonds. Fragment M was cleaved stepwise with pepsin and chymotrypsin. Disulfide peptides D3, D4, and D5 were isolated from the combined digest. Disulfide grouping D5 contains two disulfide bonds joining together half-cystines 265 (278, 279) 289 and therefore belongs to the same type of cystine groupings as the remaining ones. From the data of Behrens and coworkers[4] the half--cystine residues corresponding to our residues No 278 and 279 have not been sequentially characterized as being adjacent (res. No 277 and 281, *cf.* ref.[4]); this finding represents an exception not conforming to the regularities in the distribution of half--cystine residues along the entire molecule of human serum albumin.

Fragment C is the largest of the products of cyanogen bromide cleavage of the albumin molecule and contains 287 amino acid residues, of their number 16 half-cystines. It was treated in a manner analogous to that used with fragment M. Disulfide peptides designated D6, D7, D8, and D9 were isolated. Disulfide grouping D9 is obviously involved in the fragment of human serum albumin described by Bellon and Lapresle[35].

In this study peptides were experimentally characterized which account for all the disulfide bonds of human serum albumin; these bonds were arranged to form disulfide groupings D1 – D9. Eight of the latter (D2 – D9) contain a pair of disulfide bonds with the sequence -Cys-Cys- in its center. A fact deserving interest is the homology in the distribution of these groupings in the albumin molecule. The disulfide bonds link together several regions of equal size (Fig. 5). Most striking are long loops which include 43 – 44 amino acid residues in groupings D1, D3, D4, D6, D7, and D9. Another category represent medium-sized loops of 12 – 14 residues in groupings D2, D5, and D8, and finally short loops connecting 6 – 9 residues. The loops are joined to one another through horizontal regions, also homologous in size. Grouping D1 involves half-cystines No 34, 53, and 62 which do not comply with the regularity observed to govern the distribution of the remaining half-cystine residues. The arrangement observed within the large loop in grouping D1 is proper from the viewpoint of sequential homology emerging from a comparison with grouping D4 and, to a certain degree, also with grouping D7.

A characteristic feature of the albumin molecule is the presence of three regions built in an analogous manner, D1 – D3, D4 – D6, and D7 – D9. These domains include, as observed also by other authors[4], the same number of loops and connecting regions; this suggest that the albumin molecule has its origin in three large subunits. This assumption is also evidenced by a homology in its amino acid sequence (highest degree between loops D3 and D6). The disulfide bonds successively interlink the half-cystine residues along the albumin chain in such a manner that either large or small loops only are fixed. There are no disulfide bonds joining together distant parts of the chain. The whole molecule is so folded that the connecting regions

may allow for its expansion. Such an arrangement offers an explanation of the flexibility of the molecule and also of the causes of extensive conformational changes[36] which the molecule undergoes as a result of changes in its environment. The biological function and the characteristic structure of its molecule makes serum albumin the fundamental representant of one protein group in their classification.

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