SEQUENTIAL ANALYSIS OF FRAGMENT CB3(Cys) OF HUMAN PLASMA ALBUMIN. N-TERMINAL SEQUENCE AND TRYPTIC PEPTIDES

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At the concluding stage of sequential studies on human plasma albumin the remaining so far unsequenced fragment CB3(Cys) was investigated which represents an approximately 175-residue region of the chain between methionine residues No II and III. The following partial amino acid sequence of 171 residues of the fragment was determined by automatic Edman degradation of the fragment and by the analysis of peptides from the tryptic digest of the S-sulfo derivative of the fragment: Cys-Thr-Ala-Phe-His-Asp-Asn-Gln-Glu-Thr-Phe-Leu-Lys-Lys-Tyr-Leu-Tyr-Glu--Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe-Tyr-Ala-Pro-Glu-Leu-Leu-Phe-Phe-Ala-Lys-Arg-Tyr-Lys--Ala/Ala-Ala-Phe-Thr-Glu-Cys-Cys-Glu-Ala-Ala-Asp-Lys/Ala-Ala-Cys(Leu,Leu,Pro)Lys/Leu--Asp-Glu-Leu-Arg-Asp-Glu-Gly-Lys/Ala-Ser-Ser-Ala-Lys/Leu-Lys/Cys-Ala-Ser-Leu-Gln-Lys/ /Phe-Gly-Glu-Arg/Ala-Phe-Lys/Ala-Trp-Ala-Val-Ala-Arg/Leu-Ser-Gln-Arg/Ala-Glu-Phe-Ala--Glu-Val-Ser-Lys/Leu-Val-Thr-Asp-Leu-Thr-Lys/Val-His-Thr-Glu-Cys-Cys-His-Gly-Asp-Leu--Leu-Glu-Cys-Ala-Asp-Asp-Arg/Ala-Asp-Leu-Ala-Lys/Tyr-Ile-Cys-Glu-Asn-Gln-Asp-Ser-Ile--Ser-Ser-Lys/Leu-Lys-Glu-Cys-Cys-Glu-Lys-Pro-Leu-Leu-Glu-Lys/(Ser,His,Cys,Ile,Ala,Glx,Val, Glx,Asx,Asx,Glx)Hse

Cyanogen bromide cleavage[1] of human plasma albumin with intact disulfide bonds gives rise to three products isolated and characterized in an earlier study[2,3] from this Laboratory and designated by symbols "N", "M", and "C". The cyanogen bromide fragments in products "N" and "C" remain linked together by disulfide bonds whereas product "M" comprises the region of one cyanogen bromide fragment only. After cleavage of these disulfide bonds seven cyanogen bromide fragments were isolated and characterized[4] in this Laboratory; these fragments account for the entire polypeptide chain of human plasma albumin. Studies on amino acid sequences around methionine residues permitted us to determine[5,6] the order of these fragments in the albumin molecule and to derive a rational nomenclature of the fragments. The complete amino acid sequences of fragments CB1(Asp) (ref.[3]), CB2(Ala) (ref.[7]), CB4(Pro) (ref.[8]), CB5(Phe) (ref.[9,10]), CB6(Pro) (ref.[11,12]), and CB7(Asp) (ref.[13]) have been determined in our studies. This paper reports on the results of sequential work on the remaining fragment CB3(Cys). Sequential data on this region of the polypeptide chain of human plasma albumin have been published by Gambhir and McMenamy[14]

and later also by Behrens and coworkers[15]. The partial amino acid sequence of fragment CB3(Cys) was derived in this Laboratory from data obtained by automatic sequential degradation of the fragment and by the analysis of peptides from the tryptic digest of the S-sulfo derivative of the fragment.

EXPERIMENTAL

Material

Human plasma albumin was a product of the Institute of Sera and Vaccines, Prague, Czechoslovakia. Fragment CB3(Cys) with intact disulfide bonds (identical with product "M" obtained by fractionation of the cyanogen bromide digest was prepared as described earlier[3]. TPCK-Trypsin (trypsin treated with 1-chloro-4-phenyl-3-tosylamino-2-butanone[16]) was from Worthington Biochemical Corporation, Freehold, N. J., U.S.A. Sephadex G-100, G-25 fine, and SE-Sephadex C-25 were products of Pharmacia, Uppsala, Sweden. Dowex 1-X2, 200–400 mesh, was from Fluka, Buchs, Switzerland. The chemicals used for the automatic sequential degradation were supplied by the manufacturer of the instrument (Beckman Instruments, Spinco Div., Palo Alto, California, U.S.A.) All the remaining chemicals were of analytical purity. Silufol, aluminum sheets coated with a thin layer of silica gel, were from Kavalier, Sázava, Czechoslovakia.

Methods

Paper chromatography of peptides was carried out in the system n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v). Descending electrophoresis[17] at pH 5·6 was performed at 1 400 V in the system water–pyridine–acetic acid (994 : 5 : 1, v/v).In both cases Whatman No 3 paper was used. Amino acid analyses of 20- and 70-h hydrolysates of peptides were effected by the method of Spackman and coworkers[18] in Model 6020 Amino Acid Analyzer manufactured by the Instrument Development Workshops of the Czechoslovak Academy of Sciences, Prague. Half-cystine was determined as cysteic acid in samples oxidized by performic acid[19]. Homoserine lactone was converted into homoserine[20] before the analysis.

Amino acid sequences of peptides were determined by Edman degradation[21]; the phenylthiohydantoins of amino acids were determined by thin-layer chromatography[21,22] on "Silufol" layers. The information on the presence of amides in peptides as provided by the sequential degradation was complemented in some instances by data on the net charge of peptides determined electrophoretically. In some cases the peptides were ascribed C-terminal amino acids with respect to the specificity of trypsin.

Automatic sequential degradation of fragment CB3(Cys). Fragment CB3(Cys) (15·0 mg, c. 0·8 μmol) with intact disulfide bonds was subjected to Edman degradation[21] in Beckman Model 890C Sequencer (Beckman Instruments, Inc., Spinco Div., Palo Alto, California, U.S.A.). The "Fast Quadrol Program" as recommended by the manufacturer was used. The phenylthiohydantoins were identified as such or after their silylation[23] by gas chromatography in Beckman Model GC-65 Gas Chromatograph, in some cases also by thin layer chromatography as described for the manual procedure.

Preparation of S-sulfo derivative of fragment CB3(Cys). Fragment "M" (ref.[3]) (5·5 g) was dissolved in 420 ml of 1mM-HCl containing 8M urea; the pH of the solution was adjusted to 3·0 by conc. HCl. The solution was treated with 110 ml of 1·5M-Na_2SO_3 and by a mixture of 22·3 ml of conc. NH_4OH and 22·3 ml of 2M-$Cu(NO_3)_2$. The pH of the reaction mixture was ajdusted

TABLE I

Amino Acid Analyses of Peptides Isolated

The values are not corrected; half-cystine residues were determined as cysteic acid. None of the peptides analyzed contained methionine.

Designation of peptide	Lys	His	Arg	Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	Hse[a]	Trp
T1	1·1	0·8		0·6	2·0	1·8		2·0			1·0			1·1		1·9		
T2	1·0																	
T3			1·0					1·0			1·1		0·9	1·2	1·6			
T4		1·0	1·0						0·9						0·7			
T5								1·0	0·9		1·0			2·0	0·8	1·1		
T6	1·0										1·0					2·0		
T7			1·0															
T8	1·0														0·6			
T9	1·1			1·2	1·1	1·0		2·0			4·0					1·0		
T10	1·0			0·8					0·9		2·0			2·1				
T11	1·1		1·0		2·0			2·1		0·9				2·0				
T12			1·0		1·0			1·0						1·9				

Tl3	1·0				1·0			1·0		1·0									
T14	1·2						1·8				2·0								
T15	1·1													0·9					
T16	1·1			0·8			0·8	1·0			1·0			1·0					
T17			1·1					1·0		1·0						1·0			
T18	1·2										0·8					1·0			
T19			1·1								2·9	1·0							+ [b]
T20			1·1				0·9	1·1						1·0					
T21	1·0						0·9	2·0			1·9	1·0				1·0			
T22	1·3				0·9	1·7						0·9		1·8					
T23		2·0	1·1	3·0	3·0	0·9		2·0		0·9	1·0	0·8		2·0					
T24	1·1				1·0						2·0			1·1					
T25	1·0			1·0	2·1		2·6	2·0					1·8		0·6				
T26	2·8			1·9				3·2	1·0					2·9					
T27		0·9		1·0	2·3		0·8	3·0			1·3	0·9	0·8				1·0		

[a] Homoserine; [b] the presence of tryptophan determined qualitatively.

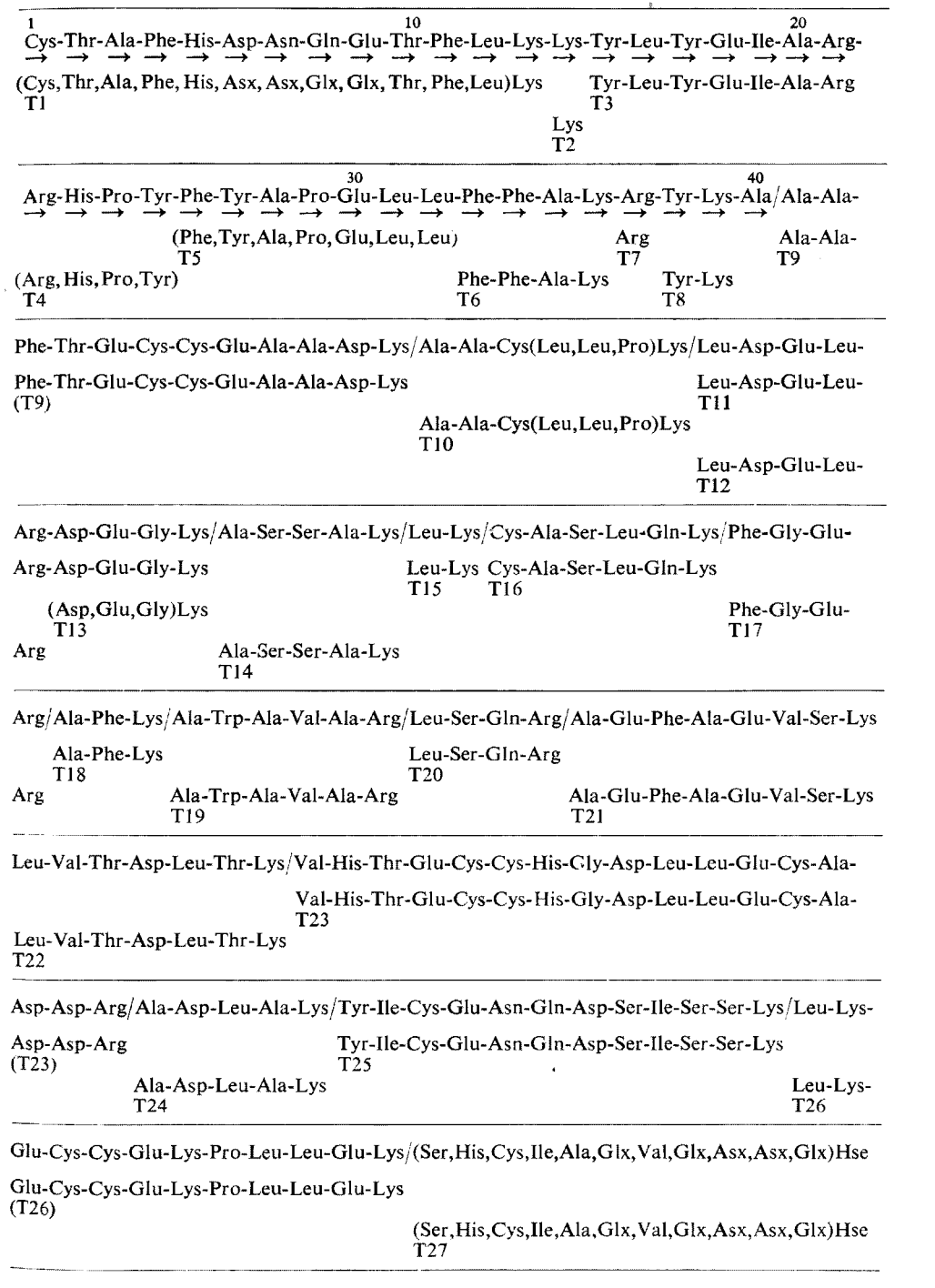
to 10·2 by conc. NH_4OH. The mixture was set aside for 1 h at room temperature and then subjected to gel filtration on a column of Sephadex G-25 (90 × 10 cm) equilibrated with 0·2M ammonium carbonate. The protein-containing fraction was concentrated to 350 ml by ultrafiltration in an Amicon Ultrafiltration Cell (Amicon, N.V., The Hague, Holland), provided with a UM-10 filter.

Preparation and fractionation of tryptic digest of fragment CB3(Cys). TPCK-Trypsin (40 mg) was added to 270 ml of the solution concentrated by ultrafiltration and containing c. 4·2 g of fragment CB3(Cys) in 0·2M ammonium carbonate. The mixture was incubated 2 h at 37°C. Another addition of 40 mg of TPCK-trypsin was made after this period and the mixture was incubated two more hours at 37°C. The volume was made up to 400 ml with water and the tryptic digest was fractionated on a column of Sephadex G-25 (200 × 4·5 cm) equilibrated with 0·01M ammonium carbonate. The same solution was used for elution and fractions were collected at a rate of 50 ml/30 min. The course of the separation was examined by electrophoresis of aliquots representing 1/250 of each fraction. According to the results obtained the column effluent was pooled to fraction "A" (fractions 26—38), "B" (fractions 39—47), and "C" (fractions 49—79). The peptide material contained in these pooled fractions was dried and freed of ammonium carbonate by lyophilization. The yield was 1 200 mg of fraction A, 1 700 mg of fraction B, and 1 200 mg of fraction C. Subsequently these fractions were separated in individual experiments by ion-exchange chromatography following a slight modification of the procedure described by Guest and coworkers[24]. The peptide material dissolved in 20 ml of 1% *sym*-collidine (the pH of the solution was adjusted to 10·0 by conc. NH_4OH) was applied onto a column of Dowex 1-X2 (66 × 1·8 cm) equilibrated with 1% solution of *sym*-collidine (pH 9·6). The column was eluted by 100 ml of the same collidine solution mixed stepwise with 500-ml volumes of acetic acid of increasing concentration (0·1M, 0·3M, 1·0M, 5·0M, and glacial acetic acid) in a closed mixing device. Glacial acetic acid (300 ml) was used for the final washing of the column. Fractions were collected at a rate of 20 ml/10 min and examined by paper chromatography of aliquots corresponding to 1/200 of the volume of each tube. With respect to the chromatographic results the effluent was pooled to fractions which were subsequently dried by lyophilization or in a rotary evaporator. Individual peptides were purified by paper chromatography and electrophoresis if necessary. The amino acid composition of the peptides isolated is given in Table I.

RESULTS AND DISCUSSION

As determined in our earlier study[4], the N-terminal amino acid of cyanogen bromide fragment CB3(Cys) of human plasma albumin is a half-cystine residue. Since this half-cystine residue is bound by a disulfide bond no product was detected in the first step of sequential degradation of the fragment with intact disulfides. This peculiar feature of the material analyzed, however, did not hinder the subsequent degradation which yielded a 40-residue N-terminal sequence as marked by arrows in Fig. 1.

Before tryptic digestion of fragment CB3(Cys) its disulfide bonds were interrupted by conversion of the fragment into its S-sulfo derivative. The digest was group-separated into fractions A, B, and C by gel filtration on Sephadex G-25; these fractions were subjected to ion-exchange chromatography on Dowex 1. The final purification of the peptides was effected by paper chromatography and electrophoresis. A total of 27 peptides were obtained whose amino acid composition is given in Table I. Selected peptides were submitted to sequential examination; the latter was omitted with


1 10 20
Cys-Thr-Ala-Phe-His-Asp-Asn-Gln-Glu-Thr-Phe-Leu-Lys-Lys-Tyr-Leu-Tyr-Glu-Ile-Ala-Arg-
(Cys,Thr,Ala, Phe, His, Asx, Asx,Glx, Glx, Thr, Phe,Leu)Lys
T1
Tyr-Leu-Tyr-Glu-Ile-Ala-Arg
T3
Lys
T2
30 40
Arg-His-Pro-Tyr-Phe-Tyr-Ala-Pro-Glu-Leu-Leu-Phe-Phe-Ala-Lys-Arg-Tyr-Lys-Ala/Ala-Ala-
(Phe,Tyr,Ala, Pro, Glu, Leu, Leu)
T5
Arg
T7
Ala-Ala-
T9
(Arg, His, Pro,Tyr)
T4
Phe-Phe-Ala-Lys
T6
Tyr-Lys
T8
Phe-Thr-Glu-Cys-Cys-Glu-Ala-Ala-Asp-Lys/Ala-Ala-Cys(Leu,Leu,Pro)Lys/Leu-Asp-Glu-Leu-
Phe-Thr-Glu-Cys-Cys-Glu-Ala-Ala-Asp-Lys
(T9)
Leu-Asp-Glu-Leu-
T11
Ala-Ala-Cys(Leu,Leu,Pro)Lys
T10
Leu-Asp-Glu-Leu-
T12
Arg-Asp-Glu-Gly-Lys/Ala-Ser-Ser-Ala-Lys/Leu-Lys/Cys-Ala-Ser-Leu-Gln-Lys/Phe-Gly-Glu-
Arg-Asp-Glu-Gly-Lys
Leu-Lys
T15
Cys-Ala-Ser-Leu-Gln-Lys
T16
(Asp,Glu,Gly)Lys
T13
Phe-Gly-Glu-
T17
Arg
Ala-Ser-Ser-Ala-Lys
T14
Arg/Ala-Phe-Lys/Ala-Trp-Ala-Val-Ala-Arg/Leu-Ser-Gln-Arg/Ala-Glu-Phe-Ala-Glu-Val-Ser-Lys
Ala-Phe-Lys
T18
Leu-Ser-Gln-Arg
T20
Arg
Ala-Trp-Ala-Val-Ala-Arg
T19
Ala-Glu-Phe-Ala-Glu-Val-Ser-Lys
T21
Leu-Val-Thr-Asp-Leu-Thr-Lys/Val-His-Thr-Glu-Cys-Cys-His-Gly-Asp-Leu-Leu-Glu-Cys-Ala-
Val-His-Thr-Glu-Cys-Cys-His-Gly-Asp-Leu-Leu-Glu-Cys-Ala-
T23
Leu-Val-Thr-Asp-Leu-Thr-Lys
T22
Asp-Asp-Arg/Ala-Asp-Leu-Ala-Lys/Tyr-Ile-Cys-Glu-Asn-Gln-Asp-Ser-Ile-Ser-Ser-Lys/Leu-Lys-
Asp-Asp-Arg
(T23)
Tyr-Ile-Cys-Glu-Asn-Gln-Asp-Ser-Ile-Ser-Ser-Lys
T25
Ala-Asp-Leu-Ala-Lys
T24
Leu-Lys-
T26
Glu-Cys-Cys-Glu-Lys-Pro-Leu-Leu-Glu-Lys/(Ser,His,Cys,Ile,Ala,Glx,Val,Glx,Asx,Asx,Glx)Hse
Glu-Cys-Cys-Glu-Lys-Pro-Leu-Leu-Glu-Lys
(T26)
(Ser,His,Cys,Ile,Ala,Glx,Val,Glx,Asx,Asx,Glx)Hse
T27

peptides derived from the N-terminal region, which had been determined by sequential degradation of fragment CB3(Cys) and in cases where sequential data were available from analyses of other material[25]. The sequential data on the individual peptides are summarized in Fig. 1 in the form of a partial structure of fragment CB3(Cys). The peptides are arranged with regard to the N-terminal sequence determined and to some preliminary results[25] of studies on a chymotryptic digest of the same substrate and on a tryptic digest of maleylated fragment CB3(Cys) which follow the present work.

The partial structure proposed contains 171 amino acid residues of the total number[25] of approximately 175 residues of fragment CB3(Cys); the most probable overlap of Ala(40) and the N-terminal alanine of peptide T9 decreases this number to 170. If the sequential data obtained by us are compared with the results of other authors[14,15] several differences emerge. Thus, *e.g.* residues No 7 and 8, which we and also other authors[14] have determined as -Asn-Gln-, Behrens and coworkers[15] report in the acid form, *i.e.* as -Asp-Glu. Similarly, Behrens and coworkers propose a different sequence, -Phe-Ala-Thr-Lys-Tyr- for the region between residues No 33–38 which has been determined by us and also by Gambhir and McMenamy[14] as -Phe-Phe-Ala-Lys-Arg--Tyr-. Likewise, a comparison of individual peptides which follow after the fully determined sequence of residues No 1–40 reveals several differences to be discussed with the knowledge of the complete amino acid sequence of fragment CB3(Cys) which will complete the sequential studies on human plasma albumin in our Laboratory.

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FIG. 1

Partial Amino Acid Sequence of Cyanogen Bromide Fragment CB3(Cys) of Human Plasma Albumin

The results of the sequential degradation of the fragment (marked by arrows →) and of the analyses of tryptic peptides T1–T27 (bottom line) are summarized in the top line. The N-terminal half-cystine was characterized in our earlier study[4]. Slanting lines are used to separate individual sequential regions which are interchangeable.

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