AMINO-ACID SEQUENCE OF CYANOGEN BROMIDE FRAGMENT CB5(Phe) OF HUMAN PLASMA ALBUMIN

L. MORÁVEK, V. KOSTKA, I. ROSENBERG and B. MELOUN

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received May 27th, 1975

Cyanogen bromide fragment CB5(Phe) representing the region of the polypeptide chain of human plasma albumin between methionine residues No IV and V was submitted to sequential studies. The data obtained by automatic sequential degradation of the fragment and by investigation of its enzymatic digests permitted the complete 117-residue amino.acid sequence of the fragment to be determined as follows: Phe-Leu-Tyr-Glu-Tyr-Ala-Arg-Arg-His-Pro-Asp-Tyr-Ser-Val-Val--Leu-Leu-Leu-Arg-Leu-Ala-Lys-Thr-Tyr-Glu-Thr-Thr-Leu-Glu-Lys-Cys-Cys-Ala-Ala-His-Asp--Pro-Tyr-Glu-Cys-Ala-Ala-Lys-Val-Phe-Asp-Glu-Phe-Lys-Pro-Leu-Val-Glu-Glu-Pro-Gln-Asn--Leu-Ile-Lys-Gln-Asn-Cys-Glu-Leu-Phe-Glu-Gln-Leu-Gly-Glu-Tyr-Lys-Phe-Gln-Asn-Ala-Leu--Leu-Val-Arg-Tyr-Thr-Lys-Lys-Val-Pro-Gln-Val-Ser-Thr-Pro-Thr-Leu-Val-Glu-Val-Ser-Arg--Asn-Leu-Gly-Lys-Val-Gly-Ser-Lys-Cys-Cys-Lys-His-Pro-Glu-Ala-Lys-Arg-Met.

The cyanogen bromide cleavage[1] of human plasma albumin with subsequent oxidation, leading to the interruption of the disulfide bonds between the fragments formed[2], gives rise to seven cyanogen bromide fragments isolated an characterized in our laboratory[3,4]. Studies on the amino-acid sequences around the six methionine residues of this protein permitted the order of these fragments in the polypeptide chain of human plasma albumin to be determined[5,6]. The determined order served as a basis of a new, rational nomenclature of the cyanogen bromide fragments[6] which replaced the earlier working symbols. In the course of the sequential studies on the whole human plasma albumin molecule, which have been pursued in our laboratory, the complete amino-acid sequences of four fragments[7-10], *i.e.* CB7(Asp), CB4(Pro), CB2(Ala), and CB1(Asp), and the partial amino acid sequences of fragments CB6(Pro) (ref.[11]) and CB5(Phe) (ref.[12]) were determined.

This paper reports on the determination of the complete amino-acid sequence of fragment CB5(Phe). This sequence is a result of the completion of the partial amino acid sequence[12] derived earlier from data obtained by automatic sequential degradation of oxidized fragment CB5(Phe), by the analysis of peptides from its tryptic and chymotryptic digest, and of tryptic peptides of the maleylated fragment.

EXPERIMENTAL

Material

Human plasma albumin was a product of the Institute for Sera and Vaccines, Prague, Czechoslovakia. Cyanogen bromide fragment CB5(Phe) was prepared in oxidized form as described elsewhere[3,4]. Chymotrypsin and TPCK-trypsin (trypsin treated with 1-chloro-4-phenyl-3-tosylamino--2-butanone[13]) were from Worthington Biochemical Corporation, Freehold, N. J., U.S.A. Soybean trypsin inhibitor was a B-grade preparation of Calbiochem, San Diego, California, U.S.A. Sephadex G-75, Sephadex G-25 fine, and QAE-Sephadex A-25 were purchased from Pharmacia, Uppsala, Sweden. Maleic anhydride pure was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. 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 used were of analytical purity grade. Silufol, thin-layer chromatography plates, were from Kavalier, Sázava, Czechoslovakia.

Methods.

The individual peptides were isolated from mixtures by techniques of paper chromatography and electrophoresis carried out on Whatman No 3 paper. Paper chromatography was effected in the system n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v). Descending electrophoresis[14] at pH 5·6 was carried out at 1400 V in the mixture water–pyridine–acetic acid (994 : 5 : 1, v/v), horizontal high-voltage electrophoresis[15] at pH 1·9 and 6000 V in the mixture acetic acid–-water–formic acid (3 : 16 : 1, v/v). The amino-acid analyses of 20-h and 70-h hydrolysates of the peptides were carried out by the method of Spackman and coworkers[16] on Model 6020 Amino Acid Analyzer, manufactured by the Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague. Since the starting material, *i.e.* fragment CB5(Phe) was in oxidized form, the half-cystine content was determined directly as cysteic acid. Homoserine lactone was converted into homoserine before the analysis[17].

The amino-acid sequences of the peptides isolated were determined by Edman degradation[18]; the phenylthiohydantoins of the amino acids were identified by thin-layer chromatography[18,19] on Silufol plates. Information on the presence of amides in peptides, provided by their sequential degradation, was compared with the net charge of the peptides determined electrophoretically at pH 5·6. In some cases the peptides were ascribed their C-terminal amino acids with respect to the specificity of the enzyme used for the digestion.

Automatic Sequential Degradation of Fragment CB5(Phe)

Fragment CB5(Phe) in oxidized form (11·0 mg, c. 0·9 μmol) was subjected to Edman degradation[18] in Beckman Model 890 C Sequencer (Beckman Instruments, Inc., Spinco Div., Palo Alto, California, U.S.A.). The degradation proceeded according to the "Fast Quadrol Program" recommended by the manufacturer. The phenylthiohydantoins obtained by the conversion of the thiazolinones were identified by gas chromatography in Beckman Model GC-65 Gas Chromatograph, either as such or after their silylation[20]. In several instances the phenylthiohydantoins were identified by thin-layer chromatography on silica gel as described above.

Preparation and Fractionation of Chymotryptic Digest of Fragment CB5(Phe)

A 600-mg portion of lyophilized fragment CB5(Phe) was dissolved in 38 ml of 0·1% ammonium carbonate. To this solution were added 6 mg of chymotrypsin in 1 ml of water, 0·12 mg of soy-

bean trypsin inhibitor in 0·5 ml of water, and phenol red as an indicator. The solution was incubated 2 h at 37°C. At the end of this period, the same quantity of the enzyme and the inhibitor was added and the incubation was continued for another 2-h period. The final weight ratio of enzyme to substrate was 1 : 50, of inhibitor to enzyme also 1 : 50. The color of the indicator did not change during the entire period of incubation. The reaction mixture (41 ml) was placed on top of a column of Sephadex G-25, (200 × 4·5 cm) equilibrated with 0·1% solution of ammonium carbonate, pH 8·5. The column was eluted by the same solution and 10-ml fractions were collected every 10 min. These fractions were evaluated by paper chromatography of aliquots containing 0·1% of the total quantity of the fraction. The contents of the tubes were pooled into six fractions with regard to the analysis of the aliquots. Individual peptides were isolated from the pooled fractions by paper chromatography and both electrophoretic procedures. The peptides isolated from this digest are marked by the letter "C".

Preparation of maleylated fragment CB5(Phe)

The procedure of Butler and coworkers[21], modified by Tang[22] was used for the maleylation. The material (835 mg) was dissolved in 83 ml of 5M guanidine hydrochloride and alternately treated with maleic anhydride (a total of 2·5 g) and solid sodium carbonate to keep the pH of the

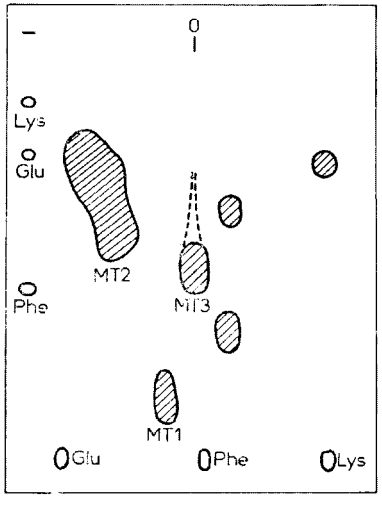

FIG. 1

Peptide Map of Tryptic Digest of Maleylated Fragment CB5(Phe)

First direction (horizontally), electrophoresis at 5·6, anode to the left. Second direction (vertically), paper chromatography. O origin, Lys, Glu, Phe reference mixture of amino acids. Peptides not marked by symbols were not isolated in this study.

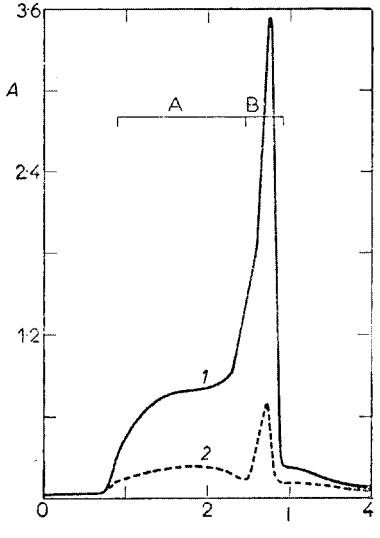

FIG. 2

Gel Filtration of Tryptic Digest of Maleylated Fragment CB5(Phe)

Column of Sephadex G-75 (195 × 4·5 cm), equilibrated with 5 mM ammonium bicarbonate, eluted by the same solution. Fractions 25 ml/15 min. *1* A_{250nm}; *2* A_{280nm}. A, B pooling of fractions.

solution at 8·5. The reaction was allowed to proceed 45 min at 22°C. The desalting was effected on a column of Sephadex G-25 (95 × 9·5 cm) equilibrated with 0·02M ammonium hydroxide. The course of the desalting was pursued by absorbance measurement at 280 nm. The protein-containing fraction (650 ml) was concentrated to 135 ml by partial freeze-drying.

Preparation and Fractionation of Tryptic Digest of Maleylated Fragment CB5(Phe)

The concentrated solution of the maleyl derivative (see above) was made up to 150 ml with 1M ammonium bicarbonate, pH 8·3. The solution was incubated with 8·5 mg of TPCK-trypsin, 2 h at 37°C. The same addition of trypsin was made afterwards and the incubation was continued for another 4-h period. The volume of the digest was reduced to 70 ml by partial lyophilization. Aliquots (containing 3 mg of material) of this solution were withdrawn for the preparation of peptide maps (Fig. 1). The main portion of the digest was subjected to gel filtration on a column of Sephadex G-75 (195 × 4·5 cm), equilibrated with 5mM ammonium bicarbonate. The course of the fractionation was monitored by absorbance measurement of the effluent at 250 and 280 nm. Fractions (25 ml) were collected at 15-min intervals. The elution profile is shown in Fig. 2. Pooled fractions A and B were obtained; individual peptides were isolated by paper chromatography and electrophoresis at pH 5·6. The peptides obtained from this digest were marked MT.

TABLE I

Amino-Acid Composition of Fragment CB5(Phe)

The values are not corrected. Methionine and tryptophan are not present in fragment CB5(Phe)

Amino acid	Hydrolysis		Number of residues found in sequence	Analysis (ref.[4])[a]
	20 h	70 h		
Lysine	13·1	[b]	12	12·6
Histidine	2·8	[b]	3	3·3
Arginine	6·5	[b]	6	6·0
Cysteic acid	6·0	[b]	6	6·2
Aspartic acid	7·8	7·4	7	8·5
Threonine	6·1	5·1	6	6·5
Serine	4·3	3·1	4	3·9
Glutamic acid	17·3	16·9	17	16·5
Proline	7·4	7·1	7	6·1
Glycine	3·6	3·3	3	2·4
Alanine	8·4	8·1	8	8·9
Valine	9·8	10·0	10	9·7
Isoleucine	1·4	1·2	1	1·1
Leucine	14·0	14·0	14	14·0
Tyrosine	6·2	4·9	7	6·2
Phenylalanine	5·3	4·8	5	5·1
Homoserine	1·2	1·2	1	1·1
TOTAL			117	

[a] Recalculated analysis based on the content of 14·0 residues of leucine; [b] undetermined.

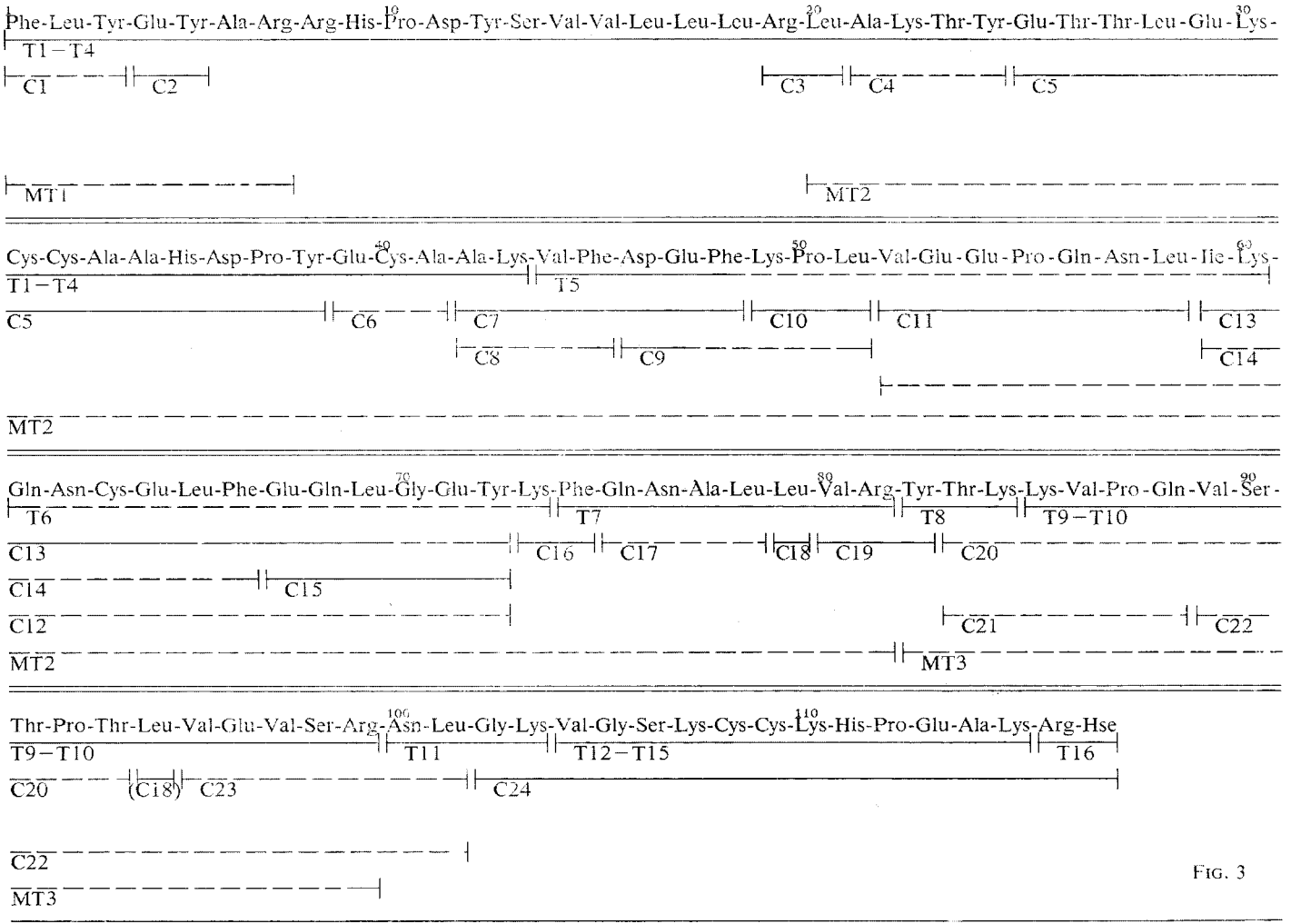
Phe-Leu-Tyr-Glu-Tyr-Ala-Arg-Arg-His-Pro-Asp-Tyr-Ser-Val-Val-Leu-Leu-Leu-Arg-Leu-Ala-Lys-Thr-Tyr-Glu-Thr-Thr-Leu-Glu-Lys-
T1–T4
C1
C2
C3
C4
C5
MT1
MT2
Cys-Cys-Ala-Ala-His-Asp-Pro-Tyr-Glu-Cys-Ala-Ala-Lys-Val-Phe-Asp-Glu-Phe-Lys-Pro-Leu-Val-Glu-Glu-Pro-Gln-Asn-Leu-Ile-Lys-
T1–T4
T5
C5
C6
C7
C10
C11
C13
C8
C9
C14
MT2
Gln-Asn-Cys-Glu-Leu-Phe-Glu-Gln-Leu-Gly-Glu-Tyr-Lys-Phe-Gln-Asn-Ala-Leu-Leu-Val-Arg-Tyr-Thr-Lys-Lys-Val-Pro-Gln-Val-Ser-
T6
T7
T8
T9–T10
C13
C16
C17
C18
C19
C20
C14
C15
C12
C21
C22
MT2
MT3
Thr-Pro-Thr-Leu-Val-Glu-Val-Ser-Arg-Asn-Leu-Gly-Lys-Val-Gly-Ser-Lys-Cys-Cys-Lys-His-Pro-Glu-Ala-Lys-Arg-Hse
T9–T10
T11
T12–T15
T16
C20
(C18)
C23
C24
C22
MT3

FIG. 3

Fraction A contained one main component only, peptide MT 2; this peptide was obtained in a yield of 237 mg by paper chromatography (once repeated development). Fraction B afforded after the same procedure four main bands which were subjected to final purification by electrophoresis at pH 5·6. Peptides MT1 (6 mg) and MT3 (30 mg) were obtained from two of these bands, the purification of the remaining components was discontinued. The chromatographic and electrophoretic characteristics of all peptides isolated are obvious from Fig. 1.

RESULTS AND DISCUSSION

Fragment CB5(Phe), isolated in our laboratory under the earlier working symbol IV-Phe, was characterized by amino-acid analysis and determination of its N- and C-terminal sequence[4]. For the sequential studies reported here, a new preparation of oxidized fragment CB5(Phe) was obtained in a large-scale experiment, carried out as described earlier[3,4]. The amino-acid composition of this preparation is given in Table I. For the calculation of the number of residues of the individual amino acids, the value of leucine was taken to represent 14·0 residues. The analysis reported earlier[4] is also given in the Table for reasons of comparison.

The automatic sequential degradation of forty residues from the amino terminus of fragment CB5(Phe) and the results of the investigation of its tryptic peptides permitted a partial amino-acid sequence of the fragment to be derived[12]. This information is summarized in Fig. 3 in the form of sequential regions marked "T". To obtain data necessary for the completion of the amino-acid sequence of the fragment, two digests were prepared, *i.e.* the chymotryptic digest and the tryptic digest of the maleylated fragment in which the lysine residues are protected against the action of trypsin[21].

A number of 24 peptides were isolated from the chymotryptic digest; their amino acid analyses are given in Table II, their partial or complete amino-acid sequences in Table III. The sequential data thus obtained are aligned with other information in Fig. 3. The chymotryptic peptides isolated cover the entire polypeptide chain of the fragment, except for residues No 8–18. We were not able to purify a fraction of the chymotryptic digest, rich in valine and leucine and most likely containing material derived from this site. The chymotryptic digestion proceeded according to the specificity of this enzyme and bonds involving lysine and arginine residues remained intact

FIG. 3

Amino-Acid Sequence of Cyanogen Bromide Fragment CB5(Phe)
Sequential regions determined earlier[12] are marked T. Region T1–T4 includes the result of 40 degradation steps of the fragment. Peptides isolated in this study from the chymotryptic digest are marked C, from the tryptic digest of the maleyl derivative MT. Peptides and sequential regions are symbolized by horizontal bars (full line — determined sequence, broken line — undetermined sequence).

TABLE II

Amino-Acid Analyses of Peptides Isolated from Chymotryptic Digest of Fragment CB5(Phe) and from Tryptic Digest of Maleylated Fragment CB5(Phe)

The analyses were performed on 20-h hydrolysates of the peptides. The values are not corrected. None of the peptides isolated contained methionine or tryptophan.

Amino acid	Number of residues in peptide											
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
Lysine				1·1	1·2		1·0	1·0	0·9	0·9		0·9
Histidine					0·7							
Arginine			0·9									
Cysteic acid					2·1	1·0						1·1
Aspartic acid					1·0		1·0		1·0		1·0	2·0
Threonine				1·0	1·4							
Serine												
Glutamic acid		1·1			2·3	0·8	1·0		1·1		2·8	8·3
Proline					0·9				1·0	0·7	1·0	1·0
Glycine												1·2
Alanine				1·0	2·1	0·9	1·0	0·8				
Valine							1·0	1·0			1·0	1·0
Isoleucine												0·8
Leucine	1·1		1·0		1·0				1·0	1·0	1·0	3·1
Tyrosine	0·8	1·0		0·5	0·6							0·7
Phenylalanine	0·8						1·9	0·7	1·1			1·1
Homoserine												

because of the presence of the soy-bean trypsin inhibitor. However, the cleavage of the bond -Ala-Ala- (res. No 41, 42) and -Thr-Leu- (res. No 93, 94), also observed to occur during the chymotryptic digestion of peptide T9 (ref.[12]), is unusual. To verify the sequential data on the region involving residues No 8–18, the automatic sequential degradation of 25 residues from the N-terminal end was repeated and the earlier data were fully confirmed[12].

The peptides from the tryptic digest of the maleylated fragment were isolated in maleyl form by gel filtration, paper chromatography and electrophoresis at pH 5·6; these peptides are marked "MT". The amino acid analyses of these peptides are given in Table II, their partial amino acid sequences in Table III. Peptides MT1–MT3 were isolated from this digest. Peptide MT1 covers the N-terminal region of the entire fragment. The large 62-residue peptide MT2 represents the extensive middle part of the chain and is directly followed in sequence by peptide MT3 (Fig. 3). All three peptides resulted from specific tryptic cleavage at the carboxyl side of the arginine residues. Since the summary of all the sequential data obtained was sufficient for

TABLE II
(*continued*)

Number of residues in peptide														
C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	MT1	MT2	MT3
1·0	0·9		1·0				1·9	2·1			3·9		6·1	1·8
													1·1	
						1·0			1·0	0·9	0·8	0·9	0·8	1·0
1·1	1·1										2·3		4·1	
1·3	1·0			1·0					1·1	1·2			4·7	
							2·6	0·7	2·0				3·1	2·9
							1·0		2·0	1·1	1·0			2·0
5·3	2·0	3·0		0·8			1·0	0·8	1·2	1·2	1·1	1·0	13·0	2·1
							2·1	1·0	1·0		0·7		3·2	2·1
		0·9									1·9		1·2	
				1·0							1·1	1·0	6·0	
						0·9	1·7	0·8	3·0	2·0	1·0		3·0	4·2
0·9	0·8												1·2	
2·0	1·0	1·2		1·0	1·0				2·0	1·0		1·0	7·6	1·3
0·8		0·6				0·8						1·5	2·4	0·8
1·0	1·0		0·8									1·0	3·4	
											1·0			

unambiguous determination of the complete amino-acid sequence of fragment CB5(Phe), the isolation of the remaining peptides from this digest was not completed. Interest deserves the effect of maleylation on the chromatographic and electrophoretic properties of peptides which made it possible to isolate the unexpectedly large peptide MT2 of 62 residues on paper.

The data obtained by duplicate sequential degradation of fragment CB5(Phe) (40 and 25 degradation steps), by the analyses of tryptic and chymotryptic peptides, and by the analysis of the tryptic digest of the maleylated fragment, are summarized in Fig. 3. These data enable us to determine unambiguously the complete amino acid sequence of fragment CB5(Phe), involving 117 residues. The amino acid composition of the fragment (Table I) is in good agreement with the number of the individual residues found in its sequence. The analytical value for lysine is higher, the sequential overlaps obtained, however, do not allow for any other lysine residue to be incorporated into the fragment.

In their recent communication Behrens and coworkers[23] have published an amino

acid sequence of human plasma albumin, including the region studied in this paper. However, the missing sequence information concerning res. No 1–19 and 23–24 of our fragment as well as information on the acid/amide form of res. 53, 61, 62 and 68 (Fig. 3), undetermined by the authors[23], were obtained in our Laboratory. The alignment of the amino-acid sequence of fragment CB5(Phe) with the sequence proposed by these authors reveals several differences. In their sequence, the position of Thr (23) (Fig. 3) is occupied by a glutamic acid residue, the position of Glu(64) by a glutamine, and for res. 35–41, sequence -Asp-Gln-Pro-His-Ala-Cys-Tyr-, different from our results, is proposed.

In his study on the specific reaction of certain amino acids of enzymes and other

TABLE III

Amino-Acid Sequences of Peptides Isolated

Designation of peptide	Sequence of peptide
CI	(Phe,Leu,Tyr)
C2	Glu-Tyr
C3	Arg-Leu
C4	(Ala,Lys,Thr)Tyr
C5	Glu-Thr-Thr-Leu-Glu-Lys-Cys-Cys-Ala-Ala-His-Asp-Pro-Tyr
C6	(Glx,Cys,Ala)
C7	Ala-Lys-Val-Phe-Asp-Glu-Phe
C8	(Ala,Lys,Val)Phe
C9	Asp-Glu(Phe,Lys,Pro,Leu)
C10	Lys-Pro-Leu
C11	Val-Glu-Glu-Pro-Gln-Asn-Leu
C12	(Lys,Cys,Asx_2,Glx_8,Pro,Gly,Val,Ile,Leu_3,Tyr,Phe)
C13	Ile-Lys-Gln-Asn-Cys-Glu-Leu-Phe-Glu-Gln-Leu(Gly,Glx)Tyr
C14	(Ile,Lys,Glx,Asx,Cys,Glx,Leu)Phe
C15	Glu-Gln-Leu-Gly-Glu-Tyr
C16	Lys-Phe
C17	(Gln,Asn,Ala,Leu)
C18	Leu
C19	Val-Arg-Tyr
C20	(Thr,Lys,Lys,Val,Pro,Glx,Val,Ser,Thr,Pro,Thr)
C21	(Thr,Lys,Lys,Val,Pro,Glx)
C22	(Val,Ser,Thr,Pro,Thr,Leu,Val,Glx,Val,Ser,Arg,Asx,Leu)
C23	(Val,Glx,Val,Ser,Arg,Asx,Leu)
C24	Gly-Lys-Val-Gly-Ser-Lys-Cys-Cys-Lys-His-Pro-Glu-Ala-Lys-Arg-Hse
MT1	(Phe,Leu,Tyr,Glu,Tyr,Ala)Arg
MT2	(Lys_6,His,Cys_4,Asx_5,Thr_3,Glx_{13},Pro_3,Gly,Ala_6,Val_3,Ile,Leu_8,Tyr_3,Phe_4)Arg
MT3	(Lys_2,Thr_3,Ser_2,Glx_2,Pro_2,Val_4,Leu,Tyr)Arg

biological preparations with radioactive diisopropyl fluorophosphate, Shaw[24] observed that a single tyrosine residue of rabbit and human serum albumin is labelled. This residue is contained in the sequence A-B-C-D-Arg-Tyr-Thr-Lys, where symbols A through D stand for neutral amino acids. Our results permit us to assign this sequence to positions 77–84 of fragment CB5(Phe) (Fig. 3, sequence -Ala-Leu-Leu--Val-Arg-Tyr-Thr-Lys-).

Sequential studies on the remaining cyanogen bromide fragment of human plasma albumin, CB3(Cys), representing the region of the chain between methionine residues No II and III of the protein, are in progress in our laboratory at present.

We acknowledge gratefully the skillful technical assistance of Mrs J. Viková, Mrs E. Bulantová, and Mrs A. Kulhánková. We are indebted to Mr K. Grüner and Miss V. Špindlerová for the Edman degradation experiments. We thank Mr J. Zbrožek, Miss V. Himrová, and Mrs E. Dršková for the amino-acid analyses of the peptides.

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Translated by the author (V. K.).