AMINO ACID SEQUENCE OF CYANOGEN BROMIDE FRAGMENT CB5 OF HUMAN HEMOPEXIN

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Cyanogen bromide fragment CB5 represents the region of the polypeptide chain of hemopexin between the fourth and fifth methionine residue (residues 232—352). It contains 120 amino acid residues in the following sequence: Arg-Cys-Ser-Pro-His-Leu-Val-Leu-Ser-Ala-Leu-Thr-Ser--Asp-Asn-His-Gly-Ala-Thr-Tyr-Ala-Phe-Ser-Gly-Thr-His-Tyr-Trp-Arg-Leu-Asp-Thr-Ser-Arg--Asp-Gly-Trp-His-Ser-Trp-Pro-Ile-Ala-His-Gln-Trp-Pro-Gln-Gly-Pro-Ser-Ala-Val-Asp-Ala-Ala--Phe-Ser-Trp-Glu-Glu-Lys-Leu-Tyr-Leu-Val-Gln-Gly-Thr-Gln-Val-Tyr-Val-Phe-Leu-Thr-Lys--Gly-Gly-Tyr-Thr-Leu-Val-Ser-Gly-Tyr-Pro-Lys-Arg-Leu-Glu-Lys-Glu-Val-Gly-Thr-Pro-His--Gly-Ile-Ile-Leu-Asp-Ser-Val-Asp-Ala-Ala-Phe-Ile-Cys-Pro-Gly-Ser-Ser-Arg-Leu-His-Ile-Met. This sequence was derived from the data on peptides prepared by cleavage of fragment CB5 by mild acid hydrolysis, by trypsin and chymotrypsin.

Hemopexin is a serum beta-glycoprotein consisting of a single polypeptide chain. It binds strongly the heme during catabolic degradation of hemoglobin thus participating in the mechanism maintaining the iron balance in the organism. The determination of the complete amino acid sequence of this important blood protein was carried out independently in this Institute and at Indiana University, Bloomington, in U.S.A. In our preceding study[1] we reported as the first ones an extended sequence of the N-terminal part of the chain of human hemopexin comprising cyanogen bromide fragments CB1—CB4 and corresponding to the 232-residue N-terminal region. This study was followed soon thereafter by a paper by Takahashi and coworkers[2] reporting on the complete amino acid sequence of this carrier protein.

The present paper describes an independent determination of the amino acid sequence of fragment CB5 corresponding to the central part of the peptide chain of hemopexin (Fig. 1).

EXPERIMENTAL

Chemicals

Cohn fraction IV of human plasma was a product of Imuna, Šarišské Michal'any. Bio-gel P-100 (50—150 mesh) was from Bio-Rad, Richmond, U.S.A. Rivanol (2-ethoxy-6,9-diaminoacridine

lactate) was supplied by Zdravotnické zásobování, Prague. Spheron P 300 (63—100 μm) was supplied by Lachema, Brno, Czechoslovakia. 1,2-Diaminoethane (98%) was from BDH Chemicals, G. B. 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide. HCl and trypsin from bovine pancreas (DCC-treated, 37·6 U/mg) were from Serva, Heidelberg, F.R.G. Sephadex G-25 Coarse (100—300 μm) was purchased from Pharmacia, Uppsala, Sweden. Ultrapore RPSC (4·6 mm × × 75 mm) and Ultrasphere ODS (4·6 mm × 250 mm) chromatographic columns were supplied by Beckman Instruments, Fullerton, CA, U.S.A. Acetonitrile LiChrosolv was from Merck, Darmstadt, F.R.G. The chemicals used for the sequence degradation were purchased from Applied Biosystems, Foster City, CA, U.S.A. Hemin was prepared from bovine blood as described elsewhere[3]. The remaining chemicals were of analytical purity.

Preparation of Affinity Support

Bio-gel P-100 was aminoethylated by 1,2-diaminoethane as described elsewhere[4]. The aminoethylated gel was washed with water and 30% dimethylformamide. The gel thus prepared (100 ml) was suspended in 30% dimethylformamide (100 ml) and subsequently were added with gentle stirring 50 mg of hemin in 5 ml of pyridine and 1 g of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide. HCl dissolved in 5 ml of 50% dimethylformamide. The reaction was allowed to proceed 18 h at room temperature. The gel slurry was then filtered off and was washed with 50% pyridine until the eluate was free of the tinge of hemin. The gel slurry was stabilized in 0·12M-Tris–HCl buffer, pH 7·0, containing 0·2M-NaCl.

Preparation of hemopexin from Cohn fraction IV. Cohn fraction IV of human plasma (10 g) was suspended in 100 ml of water and the pH of the suspension was adjusted to pH 7·0 by 0·1M-NaOH. The serum albumin present was removed from the solution by precipitation with rivanol[5]. The excess of rivanol was salted out by sodium chloride. The last traces of rivanol were removed by adsorption to Spheron. The resulting solution was made 0·1M in Tris-HCl and 0·2M in NaCl; its pH was 7·0. This solution was then applied to the affinity column (20 mm × × 250 mm). The column was washed with 0·2M Tris-HCl buffer, pH 7·0, until the effluent showed zero absorbance at 280 nm. The hemopexin which was retained in the column was displaced by elution with 0·1M sodium citrate, pH 2·4, containing 0·2M-NaCl. After completion of the elution the pH of the effluent was adjusted to pH 7·0 by 1M-NaOH and the effluent was desalted on a column of Sephadex G-25 equilibrated with distilled water. Desalted hemopexin was lyophilized. The affinity column was washed with 0·1M Tris-HCl, pH 7·0, containing 0·2M-NaCl and was ready for repeated use.

Preparation of carboxymethyl-hemopexin. Hemopexin (50 mg) was dissolved in 10 ml of 6M guanidine hydrochloride. 2-Mercaptoethanol (50 μl) was added and the pH of the solution was adjusted to pH 8·6 by 1M-NaOH. The reduction of the disulfide bonds was completed after 2 h,

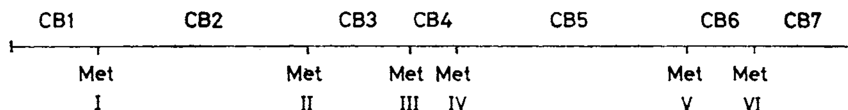

FIG. 1
Distribution of cyanogen bromide fragments in the polypeptide chain of hemopexin

at 40°C in the atmosphere of nitrogen. After the reaction mixture had been cooled down to room temperature 140 mg of monoiodoacetic acid was added in several portions. During this process the reaction mixture was stirred vigorously and the pH was maintained at pH 8·6 by additions of 30% NaOH. The carboxymethylation was terminated in 30 min. The reaction mixture was diluted with the same volume of water and was dialyzed against water whose pH had been adjusted to pH 8—9 by ammonia. The clear or only slightly opalescent dialyzed solution was lyophilized.

Digestion of carboxymethyl-hemopexin with cyanogen bromide. A 50-mg portion of carboxymethyl-hemopexin was dissolved in 5 ml of 50% trifluoroacetic acid. Subsequently 50 mg of cyanogen bromide was added and the digestion was allowed to proceed 16 h at room temperature. The reaction mixture was then diluted with 300 ml of water and was lyophilized.

Preparation of cyanogen bromide fragment CB5. A 1-mg portion of the cyanogen bromide digest of carboxymethyl-hemopexin was dissolved in 100 μl of 0·1% trifluoroacetic acid. The solution was placed onto a Beckman Ultrapore reversed phase column. Elution of the column was effected by a gradient of acetonitrile containing 0·1% of trifluoroacetic acid. One mg of the cyanogen bromide digest afforded 100 μg of fragment CB5 (Fig. 2).

Acid hydrolysis of fragment CB5. The fragment (2 mg) was dissolved in 1 ml of 1 mM-HCl and the solution was heated in a sealed-off tube and in the atmosphere of nitrogen for 90 min at 110°C. The reaction mixture was taken to dryness in a desiccator, was dissolved in 0·1% trifluoroacetic acid and placed onto a Beckman Ultrapore column. The conditions of elution of the column were the same as those described for the preparation of fragment CB5.

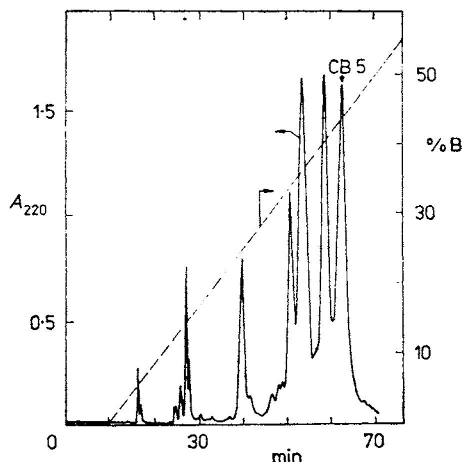

FIG. 2

Separation of cyanogen bromide digest of carboxymethyl-hemopexin. The CNBr digest of carboxymethyl-hemopexin (1 mg) was resolved on an Ultrapore RPSC column (5 μ dimethylsilica packed into a 4·6 mm × 75 mm column. Elution was effected by a linear gradient of acetonitrile with 0·1% trifluoroacetic acid as the aqueous component. Concentration of acetonitrile (in % B) is shown by the dashed line. Elution profile of peptidic material measured as absorbance at 200 nm (A_{220}) is shown by the solid line. Flow rate 60 ml/h

Enzymatic hydrolyses. The tryptic hydrolysis of D2 was effected by DCC-treated trypsin at an enzyme to substrate ratio of 1 : 100 at pH 8·0, 2 h at 10°C. The tryptic digest was separated by HPLC under the conditions described for the cyanogen bromide digest (Beckman Ultrasphere ODS column). The chymotryptic hydrolysis of fragment D1 was effected by chymotrypsin at an enzyme to substrate ratio of 1 : 100. Fifty nmol of the peptide was cleaved at pH 8·0, 6 h at room temperature. The chymotryptic digest was resolved by thin-layer chromatography on a thin layer of Polygram Cel 300.

Amino acid analyses. The peptides (2—10 nmol) were hydrolyzed in 5·6M-HCl, 20 h at 110°C. An aliquot of the digest was analyzed in Durrum D-500 Amino Acid Analyzer.

Sequence analyses. Fragment CB5 (50 nmol) was sequenced in Beckman Model 890 C Amino Acid Sequenator using the Protein Quadrol Program with dual cleavage. The peptides from either acid or enzymatic hydrolysates were sequenced (5—10 μmol) in Applied Biosystems Model 470A Protein Sequencer using program 03RPTH provided by the sequencer manufacturer. The phenylthiohydantoins were analyzed by HPLC on a Beckman Ultrasphere ODS column (ref.[6]). The amino acid sequence of peptide C1 was determined by the 4-N,N'-dimethylaminoazobenezene-4-isothiocyanate/phenylisothiocyanate double coupling method[7].

RESULTS AND DISCUSSION

The isolation of hemopexin from Cohn fraction IV of human plasma by affinity chromatography is advantageous because it is simple and because the resulting product is of a good purity. The preparation of the affinity support based on Bio-gel P-100 is easier than the procedure described earlier. The bond between hemin and the gel is so strong that hemin is not released into the effluent. We have confirmed a finding reported earlier[8] that the presence of a spacer between the hemin molecule and the support is unnecessary. A certain disadvantage of the adsorbent is its dependence on the ionic strength of the eluant. For this reason all eluants contained 0·2M-NaCl. This high ionic strength was used to suppress simultaneously the ion-exchange function of the affinity adsorbent. The removal of serum albumin before the affinity chromatography is necessary since it is otherwise adsorbed together with hemopexin. The homogeneity of our preparation of hemopexin was confirmed by discontinuous electrophoresis in 7·5% polyacrylamide gel at pH 8·3 in Tris-glycine buffer and by N-terminal sequence analysis of 16 amino acid residues.

The preparation of fragment CB5 was difficult because the fragment has a tendency to form aggregates. It was probably for this reason why efforts to prepare the fragment by classical methods of gel permeation chromatography were unsuccessful even when chaotropic reagents were used. The isolation of the fragment by reversed phase chromatography was on the contrary easier because of hydrophobic interactions between the fragment and dimethylpropylsilica gel. Fragment CB5 is eluted as the last one of all cyanogen bromide fragments of hemopexin at a high concentration of acetonitrile. The purity of the fragment was demonstrated by sequence degradation of 37 amino acid residues from the N-terminus of its molecule.

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                                     10                                      20
Arg-Cys-Ser-Pro-His-Leu-Val-Leu-Ser-Ala-Leu-Thr-Ser-Asp-Asn-His-Gly-Ala-Thr-Tyr-Ala-Phe-Ser-Gly-Thr-
                                          fragment CB5

                   30                                      40                                      50
-His-Tyr-Trp-Arg-Leu-Asp-Thr-Ser-Arg-Asp-Gly-Trp-His-Ser-Trp-Pro-Ile-Ala-His-Gln-Trp-Pro-Gln-Gly-Pro-
              fragment CB5                                          D1
                                                                                           C1

                                     60                                      70
-Ser-Ala-Val-Asp-Ala-Ala-Phe-Ser-Trp-Glu-Glu-Lys-Leu-Tyr-Leu-Val-Gln-Gly-Thr-Gln-Val-Tyr-Val-Phe-Leu-
----D1-------                                                D2
         C1                                                           D4

                   80                                      90                                      100
-Thr-Lys-Gly-Gly-Tyr-Thr-Leu-Val-Ser-Gly-Tyr-Pro-Lys-Arg-Leu-Glu-Lys-Glu-Val-Gly-Thr-Pro-His-Gly-Ile-
                                                    D2                     ------------------
                                                                   D4      ----------------
                                                                                 T1

                                     110                                     120
-Ile-Leu-Asp-Ser-Val-Asp-Ala-Ala-Phe-Ile-Cys-Pro-Gly-Ser-Ser-Arg-Leu-His-Ile-Met
-------------------------------- D2 -------------------------------------------
---D4---                                            D3
                     T1          ------------------------
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FIG. 3

Deduction of the amino acid sequence of fragment CB5 of human hemopexin. D1—D4 are peptides obtained from the acid hydrolysate of fragment CB5, T1 is a tryptic fragment of peptide D2, C1 is a peptide obtained from the chymotryptic digest of peptide D1. → → designates parts of peptides whose amino acid sequence was established by either automatic or manual sequencing, ----- designates parts of peptides not sequenced

TABLE I

Numbers of amino acid residues found in sequenced peptides and compared with amino acid analysis of intact fragment CB5

Amino acid	Number of residues in:				CB5	
	N-terminal sequence (1–35)	fragment D1 (36–54)	fragment D2 (55–120)	total (1–120)	analysis[a]	nearest integer
Asp	4	1	2	7	6·5	7
Thr	4		4	8	7·6	8
Ser	5	2	5	12	10·5	11
Glu		2	6	8	8·2	8
Pro	1	3	3	7	6·7	7
Gly	2	2	7	11	11·5	11
Ala	3	2	4	9	8·7	9
Cys/2	1		1	2	1·8[b]	2
Val	1	1	6	8	7·9	8
Met			1	1	0·9[c]	1
Ile		1	4	5	4·5	5
Leu	4		7	11	10·6	11
Tyr	2		4	6	5·4	5
Phe	1		3	4	3·9	4
His	3	2	2	7	6·5	7
Lys			4	4	4·4	4
Arg	3		2	5	4·7	5
Trp	1	3	1	5	—[d]	—
Total	35	19	66	120		119

[a] mol of amino acid per mol of peptide; [b] present as carboxymethyl-cysteine; [c] determined as homoserine; [d] not determined.

The methods of deeper cleavage of fragment CB5 were chosen to yield subfragments suitable for automatic sequencing. Acid hydrolysis by hydrochloric acid[9] was more advantageous than enzymic hydrolyses. From the acid hydrolysate peptides D1, D2, D3 and D4 were isolated and were subsequently characterized by amino acid analyses and by the determination of either complete or partial amino acid sequences (Fig. 3).

Peptide D1 (residues 36 – 54). Automatic degradation afforded the sequence of 15 amino acid residues (residues 36 – 50). The sequence of the C-terminal portion was established by manual sequencing of peptide C1 (residues 47 – 54), obtained by chymotrpytic cleavage of peptide D1.

Peptide D2 (residues 55 – 120). The N-terminal sequence of 40 amino acid residues (residues 55 – 94) was obtained by automatic degradation. The middle portion of the molecule (residues 93 – 109) was elucidated by automatic degradation of peptide T1 (residues 93 – 116) isolated from the tryptic digest of peptide D2.

Peptide D3 (residues 107 – 120). The complete amino acid sequence of the peptide was determined by its automatic degradation.

Peptide D4 (residues 55 – 103). The 41-residue N-terminal amino acid sequence of the peptide (residues 55 – 95) was obtained by its automatic sequencing.

Deduction of the amino acid sequence of fragment CB5 (Fig. 3). Peptides D2 and D3 were aligned with the C-terminal part of fragment CB5 because they contain homoserine lactone. Peptide D4 overlaps the N-terminal sequence of peptide D2 and tryptic peptide T1. The N-terminal sequence of fragment CB5 (residues 1 – 37) was obtained by automatic sequencing of fragment CB5 and by the determination of the sequence of peptide D1 (residues 36 – 54). The existence of the bond between amino acid residues 54 and 55, linking together peptides D1 and D2 (or, alternatively, D4), was not proved experimentally. The existence of this link, however, follows from the total number of amino acid residues present in the N-terminal 35-residue sequence of fragment CB5, determined by its automatic degradation, in peptide D1 (residues 36 – 54) and in peptide D2 (residues 55 – 120); this number is in accordance with the amino acid composition of fragment CB5 (Table I). The result of American authors[2] obtained independently is in agreement with the sequence determined by us.

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