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Amino Acid Sequence of a Myoglobin Isolated from Map Turtle, *Graptemys geographica**

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Nobuyo Maeda and Walter M. Fitch

From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706

Myoglobin was isolated from the skeletal muscle of map turtle, *Graptemys geographica*, and purified by Sephadex G-75 and DEAE-cellulose column chromatography. The myoglobin was cleaved by cyanogen bromide treatment, by peptic digestion, and by tryptic digestion of myoglobin whose lysine residues had been modified with citraconic anhydride or 1,2,4-benzenetricarboxylic anhydride. From the amino acid sequences of these fragments, the complete amino acid sequence was deduced.

The use of the amino acid sequences of orthologous proteins for the study of the evolutionary relationships was first suggested by Crick (1958), given some conceptional flesh by Zuckerkandl and Pauling (1962), and first applied systematically by Eck and Dayhoff (1966) and by Fitch and Margoliash (1967). Since then a great variety of proteins has been examined in this way and the general value clearly demonstrated. If one is to believe protein results, however, it is important to assess quantitatively the degree to which the phylogenetic trees based on different proteins agree with each other and/or with the tree as presently conceived on the basis of other biological data, obtained from the study of current representatives and the analysis of fossils.

To this end we need to compare several protein sets different in function but covering the same organisms as far as possible. There has previously been little effort to sequence proteins from species of taxonomic interest or which round out a set of proteins from a single taxon. In order to study tetrapod and amniote origins, as well as to fill the gaps in the available data of lower vertebrate proteins, the myoglobin of map turtle, *Graptemys geographica*, was studied. This paper describes the isolation and amino acid sequence of turtle myoglobin.

MATERIALS AND METHODS

Proteinases—Trypsin (treated with L-tosylamide-2-phenylethyl chloromethyl ketone) and α-chymotrypsin (from bovine pancreas) were obtained from Worthington. Pepsin (from hog stomach mucosa, twice crystallized) was purchased from Sigma.

Reagents and Chromatographic Media—Phenylisothiocyanate, trifluoroacetic acid, triethylamine, and 3 N mercaptoethanesulfonic acid were from Pierce. Citraconic anhydride was from Eastman and 1,2,4-benzenetricarboxylic anhydride was from Aldrich.

DEAE-cellulose, DE52, was obtained from Whatman, phosphocellulose (0.9 meq/g) was from Sigma, and Sephadex gels were from Pharmacia. Precoated Silica Gel $60F_{254}$ aluminum sheets and cellulose flexible sheets were from E. Merck and Eastman, respectively.

Isolation of Myoglobin—Skeletal muscle of map turtle, *G. geographica* (342 g), was minced and homogenized with distilled water containing approximately 10 mM KCN (500 ml). The homogenate was centrifuged at 9000 × *g* for 30 min. Solid ammonium sulfate was then slowly added to the supernatant liquid under stirring to 55% saturation and the stirring continued for another hour at 4 °C. Precipitate was removed by centrifugation at 9000 × *g* for 30 min. The supernatant was dialyzed against 1 mM KCN and concentrated to 40 ml using a Diaflo apparatus with PM10 membrane.

The concentrated solution (8 ml) was applied on a Sephadex G-75 column (1.8 × 60 cm) and eluted with 0.05 M Tris/HCl buffer, pH 8.5, containing 1 mM KCN. Chromatography was repeated four times more and the myoglobin-containing fractions were combined and concentrated to 50 ml, and the buffer was changed to 0.02 M Tris/HCl buffer, pH 8.5, containing 1 mM KCN, using a UM-2 membrane (final volume, 98 ml). Half of the solution (49 ml) was applied on a DEAE-cellulose, DE52, column (1.8 cm × 26 ml) equilibrated with 0.02 M Tris/HCl buffer, pH 8.5, containing 1 mM KCN and eluted with the same buffer, increasing the NaCl concentration in the buffer to 0.1 M linearly. Chromatography was repeated. The myoglobin containing fractions were collected, concentrated, and dialyzed against water.

To remove the heme group, the solution (21 ml) was added dropwise to 1.5% (v/v) of HCl/acetone, kept at −15 °C, stirring vigorously. The precipitate was collected by centrifugation at 8000 × *g* for 15 min, washed with HCl/acetone (1.5%, v/v) twice and with acetone three times, and dried under nitrogen to give 361 mg dry weight of purified dehemed myoglobin.

Amino Acid Analysis—Protein or peptides (10–50 nmol) were hydrolyzed with 0.3 ml of 6 M HCl in sealed tubes *in vacuo* at 110 °C, usually for 24 h. Amino acid analyses were performed as described by Niece (1975) with a Technicon TSM amino acid analyzer. Values of valine and isoleucine were obtained from the analyses of 72-h hydrolysates when necessary. Tryptophan contents were determined after hydrolyzing with 3 N mercaptoethanesulfonic acid for 22 h (Penke *et al.*, 1973). Cysteine was analyzed as the *S*-pyridylethylated derivative (Hermodson *et al.*, 1973) or as cysteic acid (Moore, 1963).

Blocking of ε-Amino Groups—To block with benzene tricarboxylate, we first dissolved 110 mg of apomyoglobin in 4 ml of 0.5 M acetic acid. The stirred solution became turbid upon addition of 2 ml of 1 M pyridine. The pH of the mixture was adjusted to 8.5 with 1 M NaOH. Solid 1,2,4-benzenetricarboxylic anhydride (850 mg) was gradually added to the mixture at 4 °C, keeping the pH between 8 and 9 with 1 M NaOH (final volume, 10 ml). As the reaction proceeded, the mixture clarified. The reaction mixture was stirred for 2 h more at room temperature and then chromatographed on a Sephadex G-25 column (coarse grade, 3.0 × 110 cm) in ammonium acetate buffer, pH 8.0. The protein fraction was freeze-dried and 120 mg of modified protein was obtained.

To block with citraconate, we dissolved 90 mg of apomyoglobin in 1 M acetic acid (3 ml) and the solution was adjusted to pH 7.5 with *N*-methylmorpholine under stirring. The precipitate produced was dissolved again by the dropwise addition of 1.0 M citraconic anhydride in dioxane (1.2 ml). The pH of the reaction mixture was kept between 8 and 9 with 2 N NaOH under continuous stirring at 4 °C. After 1.5 h at room temperature, the mixture was applied on a Sephadex G-50 column (fine grade, 1.4 × 160 cm) and eluted in 0.05 M ammonium bicarbonate, and the protein fraction was freeze-dried.

Fragmentation of the Peptide Chain—Cyanogen bromide cleavage was performed on 26.5 mg of apomyoglobin in 70% formic acid (2.5 ml) with CNBr (120 mg) for 16 h at 25 °C. The digest was freeze-dried, dissolved in 0.2 M acetic acid (2.0 ml) and separated into its components by Sephadex G-50 column (fine grade, 1.4 × 167 cm) chromatography in 0.2 M acetic acid.

Tryptic digestions of apomyoglobin modified with 1,2,4-benzene-

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tricarboxylic anhydride or with citraconic anhydride were carried out in 0.05 M Tris/HCl buffer, pH 7.8, at 37 °C for 20 and 8 h, respectively, at a substrate concentration of approximately 20 mg/ml. The enzyme/substrate ratios were 1:200 (w/w) and 1:100 (w/w), respectively. Three peptic digestions of apomyoglobin, at enzyme/substrate ratios of 1:1000, 1:700, and 1:100 (w/w), respectively, were performed at 25 °C for 1 h in 0.01 M HCl. Apomyoglobin concentration was approximately 10 mg/ml in each experiment. α-Chymotryptic digestion was performed in 1% (w/v) NH_4HCO_3 at 37 °C for 18 h at an enzyme/substrate ratio of 1:30 (w/w).

Separation of Peptides—Peptides were separated from each other by gel filtration on Sephadex G-25 or G-50 columns in 0.5 M acetic acid or in 0.05 M ammonium acetate buffer, pH 7.8, by DEAE-cellulose column chromatography with increasing concentration of ammonium bicarbonate, and by phosphocellulose column chromatography in 0.025 M phosphoric acid with increasing KCl concentration in the eluant. The beginning and ending concentrations of ions varied from one experiment to another and are provided in the miniprint supplement.[1] Thin layer electrophoresis on cellulose plates was performed at pH 4.8 using pyridine/acetic acid/water (8:5.5:986.5, v/v) at 20 V/cm for 60–75 min in a Desaga migration chamber. Thin layer chromatography on cellulose plates was carried out in butane-1-ol/acetic acid/water (4:1:2, v/v).

The amino acid compositions of the resulting peptides are presented in the miniprint supplement.

Sequence Determination—Automatic sequence analysis of apomyoglobin (0.3 μmol) was carried out by Beckman Sequencer 890C using the Quadrol program. Polybrene (0.2 mg) was added to the cup with protein.

The amino acid sequence of each peptide (50–100 nmol) was determined by manual Edman degradation as described by Tarr (1975). As a routine procedure, 30% (v/v) triethylamine, 50% (v/v) pyridine was used as coupling buffer. The reaction mixture was washed twice with heptane/ethyl acetate (2:1, v/v, followed by washing with benzene twice. Trifluoroacetic acid was used for cleavage and the thiazolinones were extracted twice with benzene/ethylacetate (1:1, v/v).

Each phenylthiohydantoin derivative was identified by thin layer chromatography using precoated silica gel plates which were developed by the method of Tarr (1975). After the spots were detected under a UV lamp, the same plate was redeveloped with solvent system V (Jeppsson and Sjoquist, 1967). The colors of the spots produced by heating the developed plates helped the differentiation of some derivatives with close mobilities, such as: glutamic acid methyl ester, alanine, and tryptophan; glycine and lysine; and glutamine and asparagine. Phenylthiohydantoin-derivatives were also hydrolyzed with alkali or acid (Smithies *et al.*, 1971) and the products were subjected to amino acid analysis.

RESULTS

The Sephadex G-75 and DEAE-cellulose column chromatographies of turtle myoglobin are shown in Figs. 1 and 2, respectively. The main fraction of heme protein gave only a very faint band of contaminants on the disc gel electrophoresis at pH 7.5 (Davis, 1964) and was therefore used for sequence analysis without further purification. The amino acid composition of myoglobin is given in Table I. The values were obtained from analyses of eight separate hydrolysates. The several minor heme proteins, shown in Fig. 2, were not analyzed further.

Apomyoglobin is not soluble in buffers near neutral pH, but can be dissolved in acids (0.5 M acetic acid, 0.01 M HCl, or 70% (v/v) formic acid). To change the nature of apomyoglobin so that the protein can be dissolved in neutral or slightly alkaline buffer, the lysine ε-amino groups were modified prior to tryptic digestion. 1,2,4-Benzenetricarboxylic anhydride specifically blocks ε-amino groups and the modification is irreversible under mild conditions.[2] The advantage of this modification, in addition to the increase in solubility of the peptides in alkaline buffer, is the increase of absorbance at 280 nm of modified protein or peptides. This makes it easier to follow the elution of peptides on column chromatography. Tryptic digestion of the derivative gave the expected cleavage at the carboxyl side of arginine residues, although the one at position 56 (Fig. 3) seemed to be only partially cleaved. The peptide terminating at residue 56 expected from that cleavage was found but the peptide beginning at residue 57 was not obtained in pure form. Possibly the blocking reagent attacked the serine residue next to this arginine slowing the rate of tryptic digestion. The disadvantage of this blocking reaction is the introduction of two carboxyl groups in place of the previous amino group, making the net charge of protein or peptides too negative. Thus, the derivative was much less soluble in acidic conditions

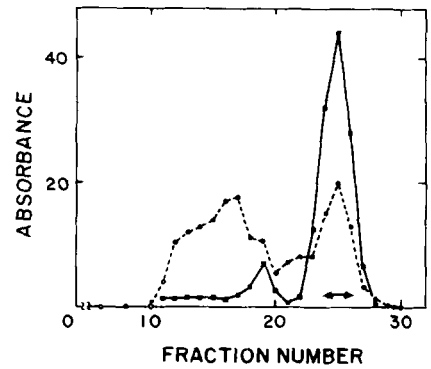

FIG. 1. **Gel filtration of muscle extract in Sephadex G-75.** The protein solution (8 ml, see "Materials and Methods") was applied on a Sephadex G-75 column (1.8 × 60 cm) and eluted with 0.05 M Tris/HCl buffer, pH 8.5, containing 1 mM KCN. Each 4.0-ml fraction was collected at a rate of 12 ml/h. Elution was monitored by measuring the absorbance at 280 nm (○- - -○) and 410 nm (■——■). Fractions marked by ↔ were pooled.

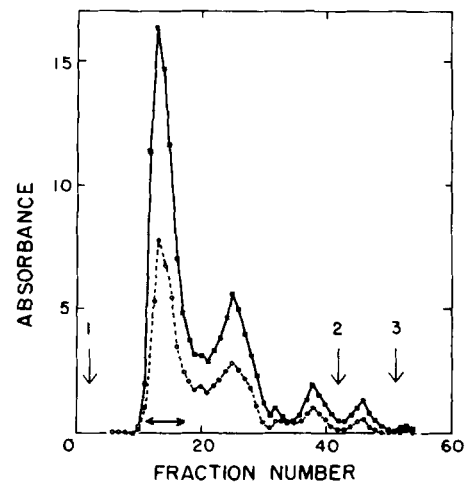

FIG. 2. **Purification of turtle myoglobin on DEAE-cellulose column (1.8 × 26 cm).** At the *arrow 1*, a linear gradient elution with NaCl concentration in 0.02 M Tris/HCl buffer, pH 8.5, containing 1 mM KCN was applied with 400 ml of the buffer in the mixing chamber and 400 ml of the same KCN buffer but also containing 0.1 M NaCl in the reservoir. At *arrows 2* and *3*, the NaCl concentration of the buffer entering the column was abruptly stepped up to 0.1 and 1 M, respectively. Each 11.4-ml fraction was collected at a flow rate of 84 ml/h. *Symbols* are as defined in the legend to Fig. 1.

[1] Portions of this paper (including part of "Materials and Methods," Figs. 1 to 20, and Tables I to XII) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80M-1602, cite author(s), and include a check or money order for $14.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] G. E. Tarr, personal communication.

TABLE I

Amino acid composition of map turtle myoglobin

The results are expressed in molar proportions of the amino acids, assuming that the most accurately determinable residues, Asp, Ala, Leu, and His total 47 in number. Values in parentheses give the numbers obtained from sequence analysis.

Amino acid	Molar proportion
Aspartic acid	10.96 (11)
Threonine	5.62 (6)
Serine	7.85 (8)
Glutamic acid	20.04 (21)
Proline	5.68 (6)
Glycine	8.35 (8)
Alanine	12.16 (12)
Valine[a]	8.14 (9)
Methionine	1.79 (2)
Isoleucine[a]	8.27 (10)
Leucine	14.82 (15)
Tyrosine	1.75 (2)
Phenylalanine	8.17 (8)
Lysine	16.84 (17)
Histidine	9.09 (9)
Arginine	6.03 (6)
Cysteine[b]	1.06 (1)
Tryptophan[c]	1.89 (2)
Total	153

[a] The value obtained from 72-h hydrolysates are given.
[b] Determined as pyridylethylcysteine.
[c] Tryptophan was determined by the analysis of the hydrolysates with 3 N mercaptoethenesulfonic acid for 22 h.

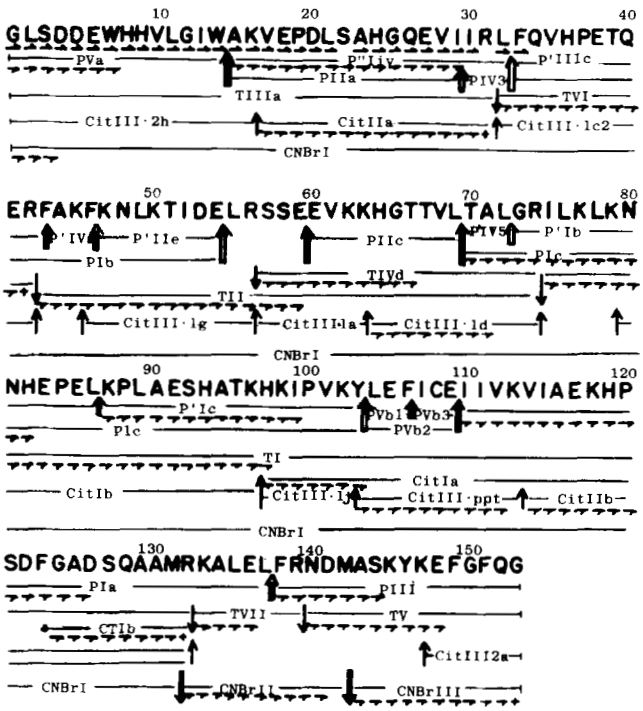

FIG. 3. **Sequence of map turtle, *G. geographica*, myoglobin.** → and →, residue determined by manual and automatic Edman degradation; +, residue determined by amino acid analysis of the remainder after several cycles of Edman degradation. Sites of chemical and enzymatic cleavage are indicated by: ⇓, cyanogen bromide; ⇑, pepsin; ↓, trypsin, after blocking lysines with 1,2,4-benzenetricarboxylic anhydride; ↑, trypsin, after blocking lysines with citraconic anhydride; ●, α-chymotrypsin.

and purification of the peptides was difficult.

Citraconylation, followed by tryptic digestion, gave more peptides than expected, indicating incomplete blocking of amino groups. The isolation of sets of peptides partially digested by trypsin was, however, helpful in determining the sequence of peptides.

Cleavage with CNBr gave one large fragment of 131 residues, CNBrI, and two smaller ones of 11 residues each, CNBrII and -III. The sequence of the NH_2-terminal three residues of peptide CNBrI was identical to that of whole apomyoglobin. Alignment of CNBrII and CNBrIII was confirmed by peptic peptide PIII and by tryptic peptides TVII and -TV.

Fig. 3 shows the peptides used to establish the sequence of turtle myoglobin.

DISCUSSION

The amino acid sequence of map turtle myoglobin was compared with those of other animals available (40 mammals, 2 birds, 3 fish, and alligator). Map turtle myoglobin is composed of 153 amino acid residues, of which 29 residues are common to all without any exception. The number of common residues goes up to 55 if myoglobins of the 3 fish are excluded.

Interesting features of residue replacements found in the sequence of turtle myoglobin are as follows.

Asp-5—The negative charge of the side chain at this position is unique. All the known myoglobins of mammals have glycine while birds and alligator (Dene *et al.*, 1980) have glutamine at this position. Fish myoglobin chains start from this position with *N*-acetylated residues (histidine in carp,[3] alanine in tuna (Brown *et al.*, 1979), and threonine in shark (Fisher and Thompson, 1979)).

His-8—This is common to harbour seal. All other mammalian and bird myoglobins have glutamine. Lysine occurs in alligator, glutamic acid occurs in carp and shark, and aspartic acid occurs in tuna.

His-9—This is common to alligator and shark. Most others have leucine with the occurrence of variations of serine, alanine, and glutamine.

Ile-29—This is common to alligator. All others have leucine at this position.

Gln-40—All mammals plus chicken (Deconinck *et al.*, 1971) have leucine (methionine in penguin, Peifer, 1973), while alligator, carp, and tuna myoglobins have glutamine (lysine in shark).

Arg-42—This is common to birds and alligator. Lysine occurs in all the mammals and leucine in fish.

Ala-44—Aspartic acid occurs here in all the mammals and birds, glutamic acid in alligator and proline in fish. It may be noted that, from the proline present in the lower vertebrates to the dicarboxylic acids present in the higher vertebrates, two base changes are required. The alanine found here may well be the intermediate form, one base change away from the codons of both the higher and lower (than turtle) vertebrate forms.

Ile-52—This hydrophobic residue at this position is unique, although shark myoglobulin has valine. Most others have glutamic acid with a few having proline, alanine, serine, or glutamine.

Leu-55—All known mammal, bird, and alligator myoglobins have methionine at this position, while the rest have leucine (isoleucine in tuna).

Val-61—This is a unique replacement. Methionine occurs in penguin and alligator. All others have leucine at this position.

Arg-74—This positively charged residue at this position is unique.

Leu-78—This is a unique replacement. All the mammal and bird myoglobins have lysine. Glutamine is present here in

[3] A. E. Romero-Herrera, personal communication.

alligator and shark; alanine is present in tuna and carp.

Asn-80—This is a unique replacement. All others have glycine.

Pro-84—This is unique. Glutamic acid occurs here in alligator, threonine occurs in shark, and alanine occurs in all others.

Cys-108—This is unique, too. Threonine occurs here in carp and shark and serine occurs in all others.

In the absence of functional data for map turtle myoglobin, the effect of these replacements on the function of the molecule is uncertain. Most of the replacements can be explained by single base changes in the triplet code. The evolutionary significance of the map turtle myoglobin will be discussed further in the accompanying paper (Maeda and Fitch, 1981).

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Isolation and Amino Acid Sequence of Myoglobin from Map Turtle, Graptemys geographica

Nobuyo Maeda and Walter M. Fitch

CNBr Cleavage of Turtle Apomyoglobin

Apomyoglobin (26.5 mg) was dissolved in 70% (v/v) formic acid and cleaved with CNBr (120 mg) for 16 h at 25°C. Water (25 ml) was added and the material was freeze-dried. Freeze-drying was repeated twice after addition of 0.2 M-acetic acid (15 ml). The cleaved product was subjected to gel filtration on a Sephadex G-50 column (fine grade, 1.4 cm x 167 cm), which gave the elution pattern shown in Fig. 1.

Amino acid composition of the first two fractions, CNBr-I and CNBr-II was the same. Rechromatography of each peak gave the same pattern of two peaks. Also the N-terminal four residues of each peak's protein were the same as those of apomyoglobin. CNBr-IV and CNBr-V were pure peptides of eleven amino acids. The amino acid composition of these peptides is given in Table 1.

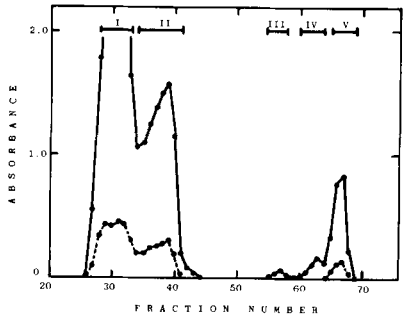

Fig. 1. Gel Filtration of Cyanogen Bromide Cleaved Fragments

CNBr cleaved products were freeze-dried, dissolved in 0.2M-acetic acid (2.0 ml), applied on a column (1.4 cm x 167 cm) of Sephadex G-50, fine grade, and eluted with 0.2 M-acetic acid. The elution was monitored at 280 nm (●–●) and 230 nm (○—○). Each 3.6 ml fraction was collected at a flow rate of 12 ml/h.

Table 1. Amino acid compositions of cyanogen bromide cleavage peptides of apomyoglobin. Results are expressed as molar ratio of amino acids. Numbers in parentheses are from the subsequent sequence studies.

	CNBr I-II		CNBr IV		CNBr V	
Asp	9.1	(9)			2.00**	(2)
Thr	5.6	(6)				
Ser	6.9	(7)	0.99**	(1)		
Glu	17.0	(18)	2.00	(2)	1.72	(1)
Pro	6.7	(6)				
Gly	5.9	(6)	1.91	(2)		
Ala	10.1	(10)	1.03	(1)	1.02	(1)
Val	7.8	(9)				
Met*		(1)				(1)
Ile	7.1	(10)				
Leu	12.0	(13)			2.13	(2)
Tyr	0.9	(1)	0.37	(1)		
Phe	5.0	(5)	1.94	(2)	0.90	(1)
Lys	12.3	(14)	2.31	(2)	1.19	(1)
His	7.6	(9)				
Arg	4.9	(6)			2.06	(2)
Trp		(2)				
Cys		(1)				
Total		131		11		11
Yield		59%		40%		41%
Residue		1-131		143-153		132-142

*Homoserine and homoserine lactone could not be separated from glutamic acid and lysine respectively.

**The amount of these amino acids are taken as standards.

Blocking of Lysine Residues by 1,2,4-Benzenetricarboxylic Anhydride

Apomyoglobin (110 mg) was completely dissolved in 4 ml of 0.5 M-acetic acid. The solution became turbid upon addition of 2 ml of 1 M-pyridine under stirring. The pH of the mixture was adjusted to between 8 and 9 with 1 M-NaOH. Solid benzenetricarboxylic anhydride (850 mg) was added to the mixture maintaining the pH of the reaction mixture between 8 and 9 with 1 M-NaOH. As the reaction proceeded the reaction mixture clarified. The final volume was approximately 10 ml. The reaction was completed by stirring at room temperature for 2 h and the mixture was desalted by gel filtration on Sephadex G-25 column (coarse grade, 3.0 cm x 40 cm) in 0.02 M-ammonium acetate buffer, pH 8.0. The protein fraction was freeze-dried.

The derivative (45 mg) was dissolved in 2 ml of 0.1 M-tris/HCl buffer pH 7.8, and digested with trypsin at 37°C for 20 h. The enzyme/protein ratio was 1:200 (w/w). The digest was applied to one of two Sephadex G-50 columns (1.4 cm x 150 cm and 1.5 cm x 85 cm) connected in series and eluted with 0.02 M-ammonium acetate buffer, pH 8.0 (Fig. 2). Fractions shown were collected. The amino acid composition of the first peak (unlabelled) was the same as that of second peak, T-I. T-II contains two arginine residues. The N-terminal amino acid sequence of T-II indicated that the peptide was the result of incomplete digestion at an arginine. The reason for the incomplete digestion under this condition was not clear but it is possible that the serine residue next to arginine was modified by 1,2,4-benzenetricarboxylic anhydride in a manner which made the arginine residue resistant to digestion by trypsin.

Fraction T-III was mainly the N-terminal peptide, containing two tryptophans, although the amino acid composition of T-III indicates some contamination (Table 2). DE-52 column chromatography did not remove the contamination from fraction T-III.

Fraction T-IV was applied on a DE-52 column (1.5 cm x 8 cm) equilibrated with 0.02 M-ammonium bicarbonate and chromatographed as shown in Fig. 3. Fraction T-IVa had a high absorbance at 230 nm, but the fraction contained no amino acids. Fraction T-IVd was a pure peptide and the C-terminal half of peptide T-II.

Peptides T-V, T-VI and T-VII were pure. Their amino acid compositions are given in Table 2.

Fraction T-I (5 mg) was dissolved in 1% (w/v) ammonium bicarbonate (0.6 ml) and digested with 0.18 mg of α-chymotrypsin at 37°C for 18 h. The digest was chromatographed on a DE-52 column (1.5 cm x 8 cm, Fig. 4). Peptide fractions a, b and c were pure but fractions d, e and f needed further purification.

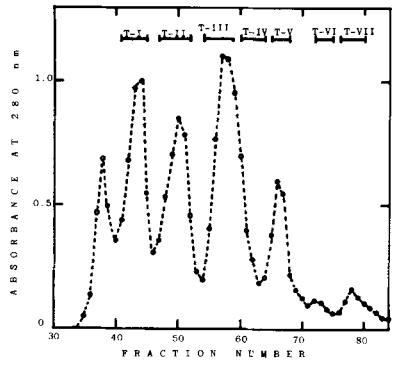

Fig. 2. Gel Filtration of Tryptic Digest of 1,2,4-Benzenetricarboxylic Apomyoglobin

The digest was applied on a column (1.4 cm x 150 cm) of Sephadex G-50, fine grade, which was connected to a second column (1.5 cm x 85 cm) and eluted with 0.02M-ammonium acetate buffer, pH 8.0. Each 5 ml fraction was collected at a flow rate of 10 ml/h.

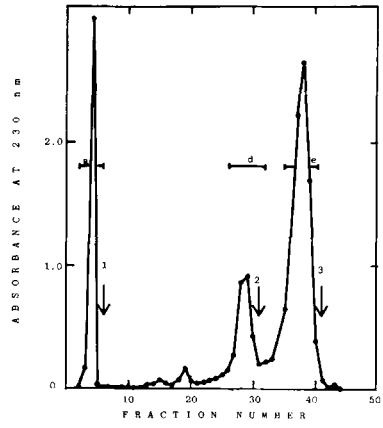

Fig. 3. Chromatography of Tryptic Fraction TIV on DE-52

Fraction TIV (Fig. 2) was freeze-dried, dissolved in 0.02 M-ammonium bicarbonate (2 ml) and applied on a column (15 cm x 8 cm) of DE-52. At arrow 1 linear gradient elution was begun with 0.02M-ammonium bicarbonate in the mixing chamber and 0.5M-ammonium bicarbonate in the reservoir (100 ml each). At arrows 2 and 3, the concentration of ammonium bicarbonate was changed stepwise to 0.5M and 1.0M respectively. Each 4.8 ml fraction was collected at a flow rate of 40 ml/h.

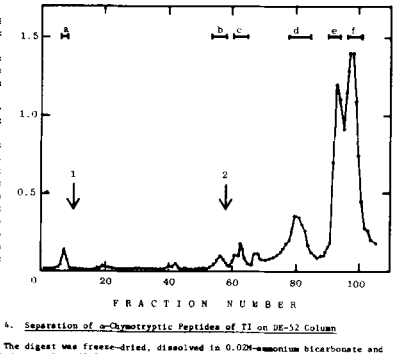

Fig. 4. Separation of α-Chymotryptic Peptides of TI on DE-52 Column

The digest was freeze-dried, dissolved in 0.02M-ammonium bicarbonate and applied on column (1.5 cm x 8 cm) of DE-52. At arrow 1, a linear gradient elution was begun with 150 ml each of 0.02M and 0.2M ammonium bicarbonate in the mixing chamber and reservoir respectively. Similarly at arrow 2, a second gradient was begun with 150 ml of 0.2M and 0.5M ammonium bicarbonate in the respective chambers. Each 4.4 ml fraction was collected at a flow rate of 60 ml/h.

Citraconylation

Turtle apomyoglobin (90 mg) was dissolved in 1 M-acetic acid (3 ml) and the solution adjusted to pH 7.5 with N-methylmorpholine under stirring. The precipitate produced was dissolved again by the dropwise addition of 1.2 ml of 1.0 M-methylmaleic anhydride in dioxane. The pH of the reaction mixture was kept between 8 and 9 with 2 M-NaOH under continuous stirring at 4°C. After 1.5 h at room temperature, the mixture was applied on a Sephadex G-50 column (fine grade, 1.4 cm x 160 cm) and eluted in 0.05 M-ammonium bicarbonate. The protein fraction was freeze-dried, dissolved in 0.05 M-tris/HCl buffer, pH 7.8, and digested with 1 mg of trypsin at 37°C for 8 h. The digest was chromatographed on Sephadex G-50 column (fine grade, 1.4 cm x 160 cm) with 0.05 M-ammonium bicarbonate (Fig. 5). The eluates were separated into four fractions, cit I-IV, freeze-dried, incubated with 60% (v/v) formic acid for 20 h to remove citraconyl groups, and freeze-dried separately.

Fraction cit-I was dissolved in 1 M-acetic acid (1 ml). To this solution added 2 ml of 0.2 M-tris/HCl buffer, pH 8.5, and the solution adjusted to pH 8 with 2 M-NaOH under stirring. Tryptic digestion was carried out by adding 80 μg of trypsin and incubating the mixture at 37°C for 20 h. The digest was applied on Sephadex G-25 column (fine grade, 1.5 cm x 110 cm) and eluted with 0.05 M-ammonium bicarbonate (Fig. 6). The amino acid composition of the peptides isolated is given in Table 3.

Fraction cit-II was dissolved in 0.025 M-phosphoric acid and applied on a DEAE cellulose, DE-52, column (1.5 cm x 8.8 cm) equilibrated with the same solution. The elution pattern is shown in Fig. 7. Two steps of linear gradient with KCl concentration were applied. Four pure peptides, cit-IIa, -IIb, -IIc and -IId were obtained. The amino acid composition of these peptides is given in Table 4.

Fraction cit III was dissolved in 0.2 M-acetic acid (2 ml) and the insoluble material was collected by centrifugation, and washed with 0.2 M-acetic acid (2 ml). The precipitate was a pure peptide of eleven amino acids (Table 5). Although the performic acid oxidation of the peptide gave only a trace (0.1mole/mole) of cysteic acid, other evidence, such as peptic peptides, demonstrated that the blank at the sixth cycle of Edman degradation was a cysteine residue. The supernatant was applied on a Sephadex G-25 column (fine grade, 1.5 cm x 97 cm), eluted with 0.2 M-acetic acid and pooled into three fractions (Fig. 8). Fraction cit-III-1 was freeze-dried, dissolved in 0.025 M-phosphoric acid and chromatographed on phosphocellulose (Fig. 9). Peak c was further separated into two peptides by thin layer electrophoresis at pH 4.8. Amino acid compositions of the resulting peptides are given in Table 5. Fraction Cit-III-2 was separated on phosphocellulose by the same method (Fig. 10). Only Cit-III-2a and Cit-III-2b were pure peptides. The remaining fractions containing several peptides, were not purified further. The amino acid compositions are given in table 6. Fraction Cit-III-3 was separated into two components by thin layer chromatography. One was the same as cit-III-2a and the other was lysine.

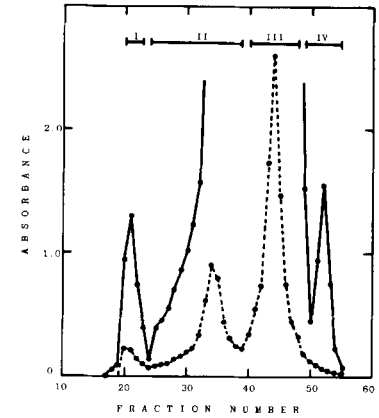

Fig. 5. Gel Filtration of Tryptic Digest of Citraconylated Apomyoglobin

The digest was applied on a Sephadex G-50 column (1.4 cm x 160 cm, fine grade) and eluted with 0.05M-ammonium bicarbonate. Elution was monitored at 280 nm (●–●) and 230 (○—○). Fractions of 5.4 ml were collected at a flow rate of 11 ml/h.

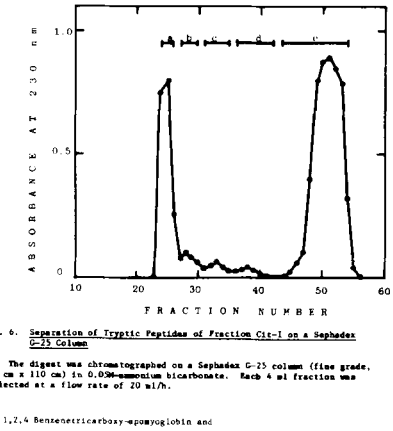

Fig. 6. Separation of Tryptic Peptides of Fraction Cit-I on a Sephadex G-25 Column

The digest was chromatographed on a Sephadex G-25 column (fine grade, 1.5 cm x 110 cm) in 0.05M-ammonium bicarbonate. Each 4 ml fraction was collected at a flow rate of 20 ml/h.

Table 2. Amino acid compositions of tryptic peptides of 1,2,4 Benzenetricarboxy-apomyoglobin and α-chymotryptic peptides of TI. Parenthetical values are from the subsequent sequence studies.

	TI	TII	TIII	TIVd	TV	TVI	TVII	CTIa	CTIb	CTIc
D	4.03 (4)	2.16 (2)	2.84 (3)		2.00* (2)				0.96 (1)	
T	0.95 (1)	2.52 (4)	0.60	1.91 (3)		0.99 (1)				
S	2.53 (3)	1.84 (2)	1.90 (2)	1.01 (2)	0.95 (1)				0.84 (1)	
E	6.74 (7)	3.00 (3)	4.00 (4)	2.00 (2)	2.03 (2)	4.00* (4)	1.34 (1)		1.07 (1)	
P	2.35 (4)	0.55	0.65 (1)			(1)				
G	1.12 (1)	1.82 (2)	2.91 (3)	1.90 (2)	1.64 (2)				1.00* (1)	
A	5.79 (6)	1.75 (2)	2.03 (2)	1.00 (1)	1.01 (1)		1.00* (1)	1.19 (1)	2.57 (3)	
V	2.13 (3)	1.75 (2)	2.52 (3)	2.05 (2)		1.54 (1)				
M	0.74 (1)				0.60 (1)			0.40 (1)	0.74 (1)	
I	3.60 (6)	0.85 (1)	1.84 (3)							0.90* (1)
L	4.72 (5)	3.00 (4)	3.16 (3)	1.92 (2)		0.88 (1)	1.84 (2)			2.00 (2)
Y	0.70 (1)				0.65 (1)					
F	1.95 (2)	1.86 (2)			2.00 (2)	1.24 (1)	1.15 (1)			
K	7.14 (8)	4.18 (5)	1.62 (1)	1.86 (2)	2.33 (2)		1.06 (1)			1.70 (1)
H	3.78 (4)	1.01 (1)	2.30 (3)	0.94 (1)		1.00* (1)				
R**	1.00 (1)	1.35 (2)	1.21 (1)	0.91 (1)		0.78 (1)	0.98 (1)	1.00* (1)	0.94 (1)	
W***			1.57 (2)							
C	0.50 (1)									
Total	58	32	31	18	14	11	7	3	9	4
Yield (%)****	67	40		9	48	30	53	13	21	7

*The amounts of these residues were taken as standards.

**Tryptophan content was measured by 3N-mercaptoethene sulfonic acid hydrolysis.

***Value was obtained as cysteic acid after performic oxidation of peptide.

****Yield was given as molar percent.

Table 3. Amino acid composition of tryptic peptide from Cit I

	CitIa	Ib	Ic-1	Ic-3	Id-1	Id-2	Id-4
D	2.42 (2)	1.82 (2)		2.00* (2)			
T		0.91 (1)					
S	2.17 (2)	0.91* (1)		1.85 (2)			
E	3.16 (4)	3.00 (3)		1.80 (1)			1.0* (1)
P	2.29 (2)	2.05 (2)	1.10 (1)	0.80 (1)			
G	1.28* (1)			1.44 (1)			
A	4.00* (4)	1.74 (2)		2.77 (3)			0.8 (1)
V	1.60 (3)		1.38 (1)	1.24			1.2 (1)
M	0.91 (1)			1.04 (1)			
I	2.10 (5)		0.89 (1)				0.4 (1)
L	2.28 (1)	1.96 (2)				0.6 (1)	
Y	1.09 (1)						
F	1.92 (2)			0.92 (1)			
K	3.52 (4)	2.08 (2)	1.92* (2)		1.00* (1)	1.0* (1)	0.8 (1)
H	2.02 (2)	1.92 (2)	1.00* (1)	1.02 (1)	0.84 (1)		
R	1.10 (1)			0.83 (1)			
Cys**	(1)						
Total	36	17	6	14	2	2	5
TLE (Arg)***	0	0.41	0.86	0.25	1.12	0.79	0.33
Residues	97-132	80-96	97-102	119-132	97-98	78-79	114-118

*The amount of these amino acid residues were taken as standards.

**Not determined.

***The mobilities of peptides on thin layer electrophoresis are given as ratio to that of arginine (1.00). Under this condition mobilities of glycine and glutamic acid were 0.24 and -0.20 respectively.

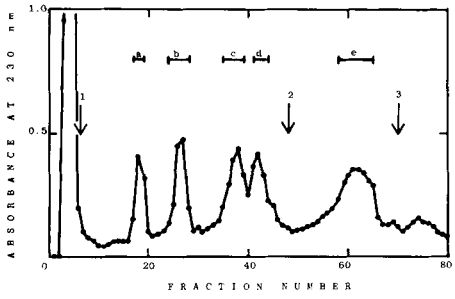

Fig. 7. Separation of Peptide Fraction Cit-II on P-cellulose

Fraction cit-II was chromatographed on a phospho-cellulose column (1.5 cm x 6.8 cm). At arrow 1, a linear gradient with increasing KCl concentration in 0.025M-phosphoric acid was begun with 125 ml of phosphoric acid containing 0.3M-KCl in the reservoir. At arrow 2, a second gradient was begun with 100 ml of phosphoric acid containing 0.3M-KCl in the mixing chamber and 100 ml of acid containing 0.6M-KCl in the reservoir. Each 5.6 ml fraction was collected at a flow rate of 40 ml/h.

Table 4. Amino acid composition of peptides CitIIa - CitIId and CitIIppt. Results are expressed as a molar ratio of amino acids.

	CitIIa	CitIIb	CitIIc	CitIId	CitIIppt
D	1.13 (1)	2.00* (2)	2.00* (2)	2.00* (2)	
T			1.13 (1)	0.96 (1)	
S	0.92 (1)	1.67 (2)	0.99 (1)	0.89 (1)	
E	2.81 (3)	2.01 (2)	2.99 (3)	2.90 (3)	2.00* (2)
P	1.13 (1)	1.25 (1)	1.81 (2)	1.84 (2)	
G	1.07 (1)	1.08 (1)			
A	1.02 (1)	3.47 (4)	1.88 (2)	1.86 (2)	
V	1.66 (2)	1.03 (1)			0.97 (1)
M		1.04 (1)			
I	1.42* (2)	0.58 (1)			1.54 (3)
L	1.00* (1)		2.10 (2)	2.84 (3)	1.20 (1)
Y					0.88 (1)
F		1.27 (1)			0.95 (1)
K		1.13 (1)	2.17 (2)	3.11 (3)	1.14 (1)
H	0.92 (1)	0.98 (1)	2.01 (2)	2.10 (2)	
R	0.98 (1)	0.97 (1)			
Cys.					(1)
Total	15	19	17	19	11
Yield (µmole)	0.70	0.76	0.56	0.52	
Residues	17-31	114-132	80-96	78-96	103-113

*The amounts of these amino acids were taken as standards.

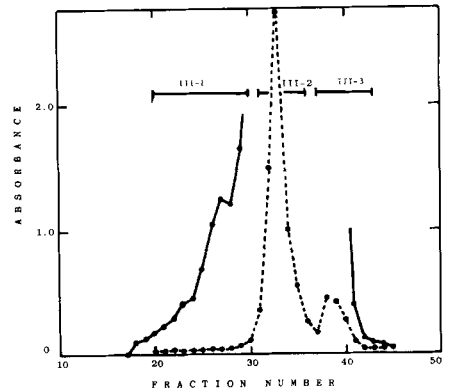

Fig. 8. Separation of Fraction Cit-III (supernatant) on a Sephadex G-25 Column

Fraction cit-III was suspended in 2.0 ml of 0.2M-acetic acid and centrifuged. The precipitates was re-suspended in 2.0 ml of 0.2M-acetic acid and centrifuged. The supernatants were combined and applied to a Sephadex G-25 column (fine grade, 1.5 cm x 97 cm) and eluted with 0.2M-acetic acid. Each 4 ml fraction was collected at a flow rate of 22 ml/h. ●--●; E_{280}, ●—●; E_{230}.

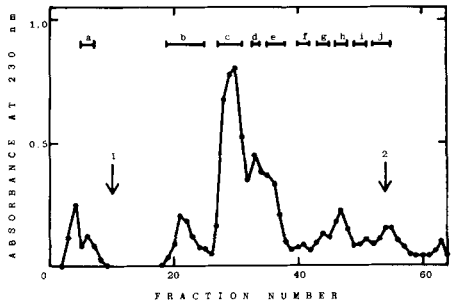

Fig. 9. Purifications of Fraction Cit-III-1 on a P-cellulose Column

Fraction CitIII-1 (Fig. 8) was chromatographed on a phospho-cellulose column (1.5 cm x 68 cm). At arrow 1, a linear gradient with KCl concentration in 0.025M-phosphoric acid was begun with 0.025M acid in the mixing chamber and 0.025M acid containing 0.3M-KCl in the reservoir (125 ml each). At arrow 2, KCl concentration in the eluent was changed stepwise to 1M. Each 5 ml fraction was collected at a flow rate of 46 ml/h.

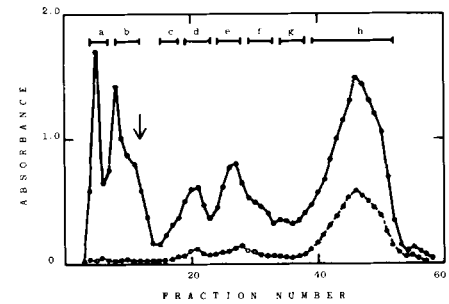

Fig. 10. Chromatography of Fraction Cit-III-2 on P-cellulose

Cit-III-2 was evaporated to dryness, dissolved in 0.025M-phosphoric acid and applied on a phosphocellulose column (1.5 cm x 6.8 cm). At the arrow a linear gradient with KCl concentration in the eluent was begun with 0.025M-phosphoric acid in the mixing chamber and 0.025M acid containing 0.3M-KCl in the reservoir (125 ml each). Each 5 ml fraction was collected at a flow rate of 36 ml/h. ●--●; E_{280}, ●—●; E_{230}.

Peptic Digestion of Apomyoglobin

Apomyoglobin (24 mg) was dissolved in 2 ml of 0.01 M-HCl and digested with pepsin at 25°C for 1 h at an enzyme/substrate ratio of 1:700 (w/w). The digest was applied on a Sephadex G-25 column (fine grade, 1.5 cm x 110 cm) and eluted with 0.2 M-acetic acid (Fig. 11).

Fractions P-I, P-II and P-III were freeze-dried, redissolved in 0.025 M-phosphoric acid and independently chromatographed on phosphocellulose (Figs 12, 13 and 14). Amino acid compositions of resultant peptides are given in Table 7.

Fraction P-V was evaporated to dryness, suspended in 1.0 ml of 1 M-pyridine and centrifuged. The precipitate, washed with 0.5 ml of 1 M-pyridine, was a pure peptide, P-Va. The supernatants were combined, evaporated to dryness, redissolved in 0.2 M-acetic acid and subjected to thin layer chromatography. Three peptides, P-Vb-1, P-Vb-2 and P-Vb-3 were obtained. Amino acid analysis of P-Vb-3 gave 0.51 moles of half cystine residues per mole of molecule (Table 8). Performic oxidation of peptide produced 0.80 mole of cysteic acid per mole of P-Vb-3.

A second peptic digestion of 29 mg. of apomyoglobin was identical except that the enzyme/substrate ratio was 1:100 (w/w). The digest was separated into five fractions by gel filtration on a Sephadex G-25 column, (fine grade, 1.5 x 110 cm, Fig. 15). Fractions P'-I, P'-II and P'-III were individually chromatographed on phosphocellulose (Figs. 16, 17 and 18). Fraction P'-IV was electrophoresed on thin layer plates at pH 4.8. Amino acid compositions of the resulting peptides are given in Table 9 and 10.

The third peptide digestion was performed on apomyoglobin (10.5 mg) at the enzyme/substrate ratio of 1:1000 (w/w) at 23°C for 1 h. The digest was applied on a Sephadex G-25 column (fine grade, 1.5 cm x 110 cm) and eluted in 0.2 M-acetic acid (Fig. 19). Fraction I was freeze-dried, redissolved in 0.2 M-acetic acid and chromatographed on a Sephadex G-50 column (fine grade, 1.4 cm x 167 cm, Fig. 20). Peptides P-II, P-III and P-V were further purified by thin layer electrophoresis and by thin layer chromatography. Amino acid compositions of peptides are given in Table 11.

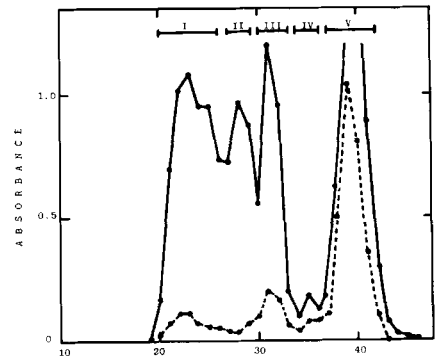

Fig. 11. Gel Filtration of Peptic Digest of Apomyoglobin on a Sephadex G-25 Column

The digest was chromatographed on a Sephadex G-25 column (fine grade, 1.6 cm x 110 cm) in 0.2M-acetic acid. Each 5 ml fraction was collected at a flow rate of 20 ml/h. ●--●; E_{280}, ●—●; E_{230}.

Table 5. The amino acid compositions of peptides from CitIII-1.

	IIIIa	IIIIc-1	IIIc-2	IIIId	IIIIe	IIIIf	IIIIg	IIIIh	IIIII	IIIIj
D		1.00* (1)			1.93 (2)		1.81 (2)		2.00* (2)	
T			0.95 (1)	2.63 (3)		1.85 (2)	1.38 (1)	2.38 (3)	1.01 (1)	
S	1.8 (2)	1.03 (1)	3.69 (4)		2.27 (2)				0.93 (1)	
E	1.8 (2)	2.89 (3)**	-- (1)**		2.70 (2)		1.39 (1)		3.08 (3)	
P		-- (1)			1.44 (1)				1.15 (2)	1.04 (1)
G		1.25 (1)		2.00* (2)	1.97 (1)	1.05 (1)		1.89 (2)		
A		1.19 (1)		1.16 (1)	3.06 (4)			1.16 (1)	2.05 (2)	
V	1.5 (1)	2.04 (2)	0.98 (1)	1.15 (1)	1.08 (1)	0.95 (1)		1.42 (1)		1.09 (1)
M					1.02 (1)**					
I		1.40 (2)			-- (1)		0.81 (1)			0.95 (1)
L		1.36 (1)	1.00* (1)	1.97 (2)		1.00* (1)	2.06 (2)	2.00* (2)	2.09 (2)	
Y										
F			0.90 (1)		1.29 (1)		0.79 (1)			
K	1.0* (1)				1.07 (1)	1.48 (1)	2.19 (2)	1.34 (1)	2.01 (2)	2.00* (2)
H		1.04 (1)	0.78 (1)	1.18 (1)	1.15 (1)	1.47 (1)		1.22 (1)	1.83 (2)	1.14 (1)
R		1.07 (1)	0.90 (1)	1.01 (1)	1.08 (1)		1.00* (1)	1.09 (1)		
C										
W										
Total	6	15	11	11	19	7	11	12	17	6
Residues	57-62	17-31	32-42	64-74	114-132	63-69	46-56	63-74	80-96	97-102
Yield (µmole)	0.40	0.29	0.49	1.0	0.57	0.08	0.16	0.64	0.17	0.80

*The amount of these amino acids were taken as standards.

**--indicate the trace amount.

Table 6. Amino acid composition of peptides from CitIII2 and CitIII3

	(III3-1) III2-a	III2-b	III3-5
D		2.00* (2)	
T			
S		0.94 (1)	
E	1.86 (2)	0.97 (1)	
P			
G	2.06 (2)	1.94 (2)	
A		1.14 (1)	
V		1.08 (1)	
M			
I		0.81 (1)	
L		1.94 (2)	
Y			
F	2.00* (2)		
K		1.53 (1)	1.00*
H		1.97 (2)	
R			
C			
W		(2)**	
Total	6	16	1
Residue	148-153	1-16	
Yield (µmole)	3.0	1.44	0.18

*The amount of these amino acids were taken as standards.

**Tryptophan was detected by spot test with Ehrlich reagent.

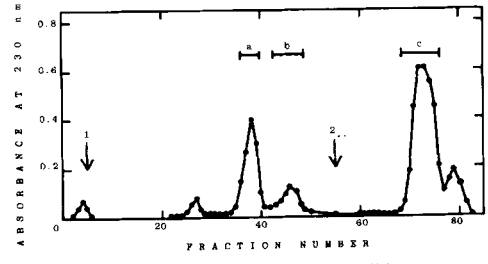

Fig. 12. Chromatography of Peptic Digest Fraction PI on P-cellulose

Fraction PI (Fig. 11) was freeze-dried, dissolved in 0.025M-phosphoric acid and chromatographed on a phosphocellulose column (1.5 cm x 6.8 cm) under the same condition as in Fig. 7. Each 4.7 ml fraction was collected at a flow rate of 40 ml/h.

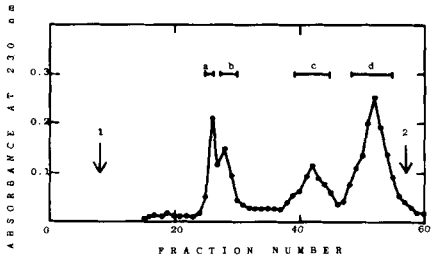

Fig. 13. Chromatography of Peptic Digest Fraction PII on P-cellulose

Fraction PII (Fig. 11) was freeze-dried, dissolved in 0.025M-phosphoric acid and chromatographed on a Phospho-cellulose column (1.5 cm x 68 cm). At arrow 1, a linear gradient elution was begun with 125 ml of 0.025M-phosphoric acid in the mixing chamber and 125 ml of acid containing 0.3M KCl in the reservoir. At arrow 2, the concentration of KCl in the eluant was changed stepwise to 0.6M. Each 4.5 ml fraction was collected at a flow rate of 40 ml/h.

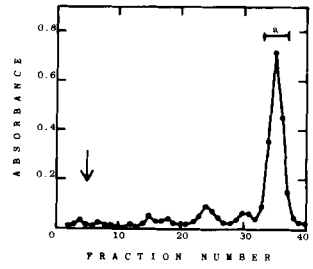

Fig. 14. Chromatography of Peptic Digest Fraction PIII on P-cellulose

Fraction PIII (Fig. 11) was freeze-dried, dissolved in 0.025M-phosphoric acid and chromatographed on a phospho-cellulose column (1.5 cm x 6.8 cm). At the arrow, a linear gradient elution was begun with 100 ml of 0.025M-phosphoric acid in the mixing chamber and 100 ml of same acid containing 0.3 M KCl in the reservoir. Each 5 ml fraction was collected at a flow rate of 40 ml/h.

Table 7. Amino acid compositions of peptic peptides.

Results are expressed as molar ratio of amino acids.

	pIa	pIb	pIc	pIIa	pIIb	pIIc	pIIIa
Asp	2.00*(2)	2.00*(2)	2.00*(2)	1.00*(1)	1.00*(1)		2.00*(2)
Thr		1.72 (2)	1.75 (2)			1.08 (2)	
Ser	1.77 (2)		0.90 (1)	0.78 (1)	0.84 (1)		0.97 (1)
Glu	2.77 (3)	4.36 (5)	3.42 (3)	2.97 (3)	2.84 (3)	1.20 (1)	2.02 (2)
Pro	1.01 (1)	1.07 (1)	3.07 (3)	0.90 (1)	0.91 (1)		
Gly	1.13 (1)		1.05 (1)	1.10 (1)	1.12 (1)	0.86 (1)	1.81 (2)
Ala	4.50 (5)	1.06 (1)	2.87 (3)	2.02 (2)	2.13 (2)		1.04 (1)
Val	2.05 (2)	1.40 (1)	1.50 (1)	1.91 (2)	1.49 (1)	2.05 (2)	
Met	0.91 (1)						1.06 (1)
Ile	1.61 (3)	0.93 (1)	1.68 (2)	0.73 (1)			
Leu	1.95 (2)	1.09 (1)	4.82 (5)	1.04 (1)	1.06 (1)	1.00*(1)	
Tyr			(1)				0.87 (1)
Phe	0.91 (1)	2.83 (3)					3.24 (3)
Lys	3.04 (3)	3.17 (3)	6.07 (6)	1.25 (1)	1.12 (1)	1.93 (2)	
His	1.10 (1)	1.03 (1)	3.23 (3)	1.17 (1)	0.99 (1)	0.93 (1)	2.10 (2)
Arg	1.04 (1)	1.05 (1)	0.92 (1)				0.90 (1)
Cys							
Trp							
Total	28	22	34	15	13	10	16
Yield (μmole)	0.55	0.15	0.57	0.16	0.34	0.29	0.49
Residues	110-137	33-54	70-103	15-29	15-27	60-69	138-153

*The amounts of these amino acids were taken as standards.

Table 8. Amino acid compositions of peptic peptides.
Results are expressed as molar ratio of amino acids.

	PVa	PVb-1	PVb-2	PVb-3
D	1.86 (2)			
T				
S	1.05 (1)			
E	1.21 (1)	1.00*(1)	2.00*(2)	1.00*(1)
P				
G	2.00*(2)			
A				
V	0.94 (1)			
M				
I	0.94 (1)		0.96 (1)	0.94 (1)
L	1.92 (2)	1.00 (1)	0.74 (1)	
Y				
F		1.01 (1)	1.28 (1)	
K				
H	1.62 (2)			
R				
C			0.66 (1)**	0.51 (1)**
W	(2)***			
Total	14	3	6	3
Residue	1-14	104-106	104-109	107-109
Yield (μmole)	0.70	0.10	0.03	0.46
TLC (Rf)	0.43	0.83	0.61	0.43

*These residues were taken as the reference amount.

**Recovered as 1/2 cystine on the analysis.

***Tryptophan was detected by spot test with Ehrlich reagent.

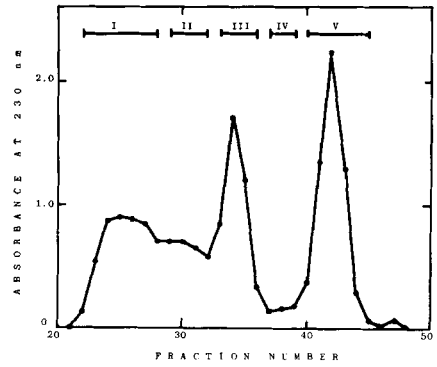

Fig. 15. Gel Filtration of Peptic Digest on Sephadex G-25 (P')

The digest was chromatographed on a Sephadex G-25 column (fine grade, 1.5 cm x 110 cm) in 0.2M-acetic acid. Each 5 ml fraction was collected at a flow rate of 40 ml/h.

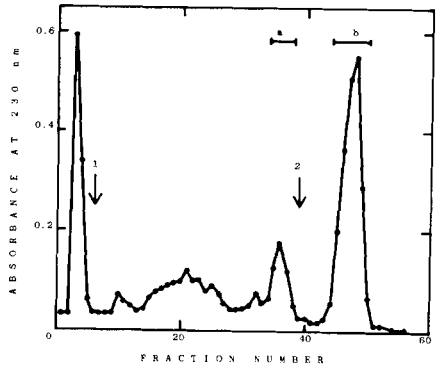

Fig. 16. Chromatography of Peptic Fraction P'I on P-cellulose

Fraction P'I (Fig. 15) was chromatographed on a phospho-cellulose column (1.5 cm x 6.8 cm) under the same condition as in Fig. 7. Each 7 ml fraction was collected at a flow rate of 36 ml/h.

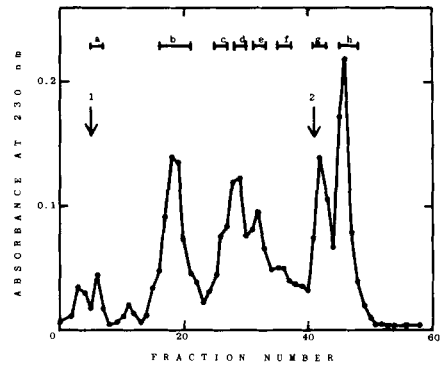

Fig. 17. Chromatography of Peptic Fraction P'II on P-cellulose

Fraction P'II (Fig. 15) was chromatographed on a phospho-cellulose column (1.5 cm x 6.8 cm) under the same condition as in Fig. 7, except that at the arrow 2, the KCl concentration in the eluant was changed stepwise to 0.5M. Each 5.6 ml fraction was collected at a flow rate of 36 ml/h.

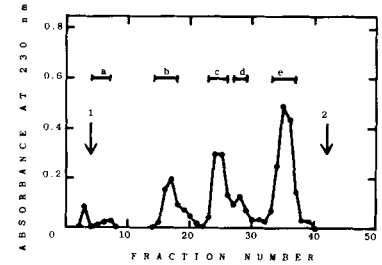

Fig. 18. Chromatography of Peptic Fraction P'III on P-cellulose

Fraction P'III (Fig. 15) was chromatographed on a phospho-cellulose column (1.5 cm x 6.8) under the same condition as in Fig. 14. Each 5.1 ml fraction was collected at a flow rate of 36 ml/h.

Table 9. Amino Acid Composition of Peptic Peptides.

	P'Ib	P'Ic	P'IIa	P'IIb	P'IIc	P'IIe	P'IIf	P'IIg	P'IIh
D	1.68 (2)		1.00 (1)	1.23 (1)	1.00*(1)	1.75 (2)	2.00 (2)		1.00
T		0.80 (1)	(0.94)			0.85 (1)		1.92 (2)	2.33
S		0.84 (1)	0.67 (1)	1.12*(1)	0.84 (1)				0.88
E	2.00*(2)	1.09 (1)	1.10 (1)	3.00*(3)	1.67 (2)	1.00*(1)	2.09 (2)	1.00*(1)	1.70
P	1.00 (1)	1.67 (2)		1.58 (1)	0.68 (1)		0.78 (1)		
G	1.05 (1)		0.73 (1)	0.64 (1)	0.91 (1)			0.93 (1)	1.42
A		1.72 (2)	2.68 (3)	1.94 (2)	1.99 (2)		0.87 (1)		
V		1.04 (1)		1.68 (2)	0.71 (1)			1.47 (2)	1.92
M			0.66 (1)						
I	0.82 (1)	0.92 (1)		0.67 (1)		1.08 (1)			
L	2.57 (3)	1.08 (1)	(0.91)	1.41 (1)	0.85 (1)	1.15 (1)	2.34 (2)	0.88 (1)	2.23
Y		0.99 (1)							
F									
K	2.07 (2)	3.49 (4)		1.56 (1)	1.00 (1)	2.00 (2)	2.76 (2)	2.14 (2)	3.50
H	1.11 (1)	2.00 (2)		0.75 (1)	0.77 (1)		0.90 (1)	0.87 (1)	1.39
R	1.08 (1)						0.83 (1)		0.90
C									
W									
Total	14	17	8	15	12	8	12	10	
Residue	73-86	87-103	124-131	15-29	15-26	47-54	73-86	60-69	
Yield (μmole)	0.4	1.16	0.24	0.51	0.22	0.39	1.10	0.48	

*The amount of these amino acids were taken as standards.

Table 10. Amino Acid Composition of Peptic Peptides.

	P'IIIa	P'IIIb	P'IIIc	P'IIId	P'IIIe	P'IV1	P'IV3	P'IV4	P'IV5	P'IV6	P'IV7	P'IV8
D	1.16 (1)				2.00*(2)						1.89	1.00 (1)
T	0.91 (1)		0.91 (1)						0.79 (1)			
S	0.86*(1)				1.00 (1)							
E	2.00 (2)	1.24 (1)	3.44 (4)	0.95 (1)	1.14 (1)					1.38 (1)	1.92	1.17 (1)
P			1.04 (1)									
G	2.27 (2)				0.50							
A	2.59 (2)			1.00*(1)	1.02 (1)	1.00 (1)			1.12 (1)			
V		1.45 (1)	1.23 (1)									
M					1.00 (1)							
I	1.04*(2)						1.00 (1)	1.21 (2)		1.00 (1)	1.74	0.96 (1)
L	1.10 (1)	1.00 (1)		0.78 (1)			1.00 (1)	0.81 (1)	1.00 (1)	0.46	1.00	
Y					0.83 (1)							
F	1.66 (2)		1.94 (2)		1.58 (2)	1.42 (1)				0.52		
K				1.76 (2)	1.98 (2)	1.10 (1)						
H			1.00*(1)									
R		0.71 (1)	0.98 (1)	0.97 (1)	1.05 (1)		1.15 (1)	0.64 (1)				
C										0.83 (1)**		
W												
	6	11	6	12	3	1	4	3	3		3	
Yield (μmole)	0.8	1.03	0.34	0.48	0.08	0.30	0.04	0.29	0.05	0.04	0.02	
Residue	27-32	33-43	132-137	138-149	44-46	30-32	29-32	70-72				
(Rarg)***					0.58	0.50	0.30	0.22	0.03	-0.11	-0.26	

*The amount of these amino acids were taken as standards.

**Detected as 1/2-Cystine residues.

***Mobility of each peptide on thin layer electrophoresis is given as a ratio to that of arginine (1.00).

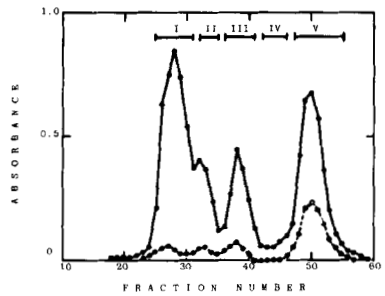

Fig. 19. Gel Filtration of Peptic Digest of Apomyoglobin on Sephadex G-25 (F'')

The digest was applied on a Sephadex G-25 column (fine grade, 1.5 cm x 110 cm) and eluted in 0.2M-acetic acid. Each 3.8 ml fraction was collected at a flow rate of 22.5 ml/h.

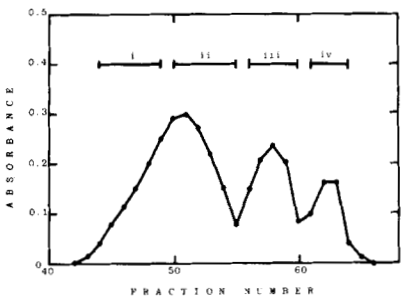

Fig. 20. Further Separation of F''I on Sephadex G-50

Fraction F''I (Fig. 19) was freeze-dried, redissolved in 0.2M-acetic acid and chromatographed on a Sephadex G-50 column (fine grade, 1.4 x 107 cm) in 0.2M-acetic acid. Each 3.6 ml fraction was collected at a flow rate of 11 ml/h.

Table 11. Amino Acid Composition of Peptic Peptides

	F''III	F''IIII	F''IIV	F''II	F''IIC	F''Va	F''Vb
D	1.97 (2)	2.0 (2)	1.18 (1)	1.0 (1)	1.9 (2)	1.99 (2)	
T	1.98 (2)	0.5	0.70				
S	1.15$_a$(1)	1.9$_a$(2)	1.15$_a$(1)	0.8 (1)	0.77 (1)	0.88$_a$(1)	
E	4.00 (4)	3.0 (3)	3.00 (3)	2.5 (3)	2.04 (2)	1.00 (1)	1.00*(1)
P	2.17 (3)	0.8 (1)	0.47 (1)	1.6 (1)			
G	1.09 (1)	1.1 (1)	0.92 (1)	1.0 (1)	1.59$_a$(2)	2.20 (2)	
A	2.81 (3)	3.5 (5)	1.51 (2)	1.6 (2)	1.00 (1)		
V	1.25 (1)	1.4 (2)	1.62 (2)	1.3 (1)		1.21 (1)	
M		0.6 (1)			0.34 (1)		
I	1.72 (2)	1.2 (3)	0.96 (2)			0.82 (1)	0.7$_a$ (1)
L	4.71 (6)	1.8 (2)	1.51 (2)	1.0 (1)		1.83 (2)	
Y	0.89 (1)				0.57 (1)		
F	1.13 (1)	0.9 (1)	0.60		2.58 (3)		
K	5.07 (5)	2.6 (3)	1.69 (2)	1.0 (1)	1.98 (2)		
H	2.73 (3)	0.7 (1)	1.01 (1)	0.8 (1)		1.92 (2)	
R	1.08 (1)	0.8 (1)	0.84 (1)		1.08 (1)		
C							**0.56 (1)
W						(2)	
Total	37	28	18	13	16	14	3
Yield (µmole)	0.17	0.14	0.4	-	-	0.20	0.16
Residue	70-106	110-137	15-32	15-27	138-153	1-14	107-109
TLC (Rf)				0.65	0.64	0.77	0.77
TLE (Rarg)				0.20	0.36	0	0.24

*These residues were taken as the reference amount.

**Tryptophan was detected by spot test with Ehrlich reagent.

Table 12. Automatic sequence analysis.

	Residue	Yield 0.2 N NaOH Hydrolysis nmole	TLC
1	Gly	151	G
2	Leu	140	L
3	Ser	-	S
4	Asp	134	D
5	Asp	126	D
6	Glu	63	E
7	(Trp)	-	W
8	His	46	H
9	His	79	H
10	Val	98	V
11	Leu	106	L
12	Gly	109	G
13	Ile	77.2	I
14	(Trp)	-	W
15	Ala	60.7	A
16	Lys	48.3	
17	Val	50.2	
18.	Glu	26.6	
19	Pro	26.0	
20	Asp	27.1	
21	Leu	40.8	
22	(Ser)	-	
23	Ala	25.7	
24	His	13.9	
25	Gly	17.9	
26	Gln	25.1	
27	Glu	30.3	
28	Val	19.9	
29	Ile	17.8	
30	Ile	21.3	
31	(Arg)	-	
32	Leu	16.2	
33	Phe	10.7	

Polybrene (0.2 mg) was added in the cup with apomyoglobin (0.3 µmole)

Initial yield 50.3%

repetitive yield 93% (Rleu)

Sequence Analysis

Apomyoglobin (0.3 µmole) was subjected to 33 cycles of automatic sequence analysis using a Beckman sequencer 890C with the Quadrol program. Table 12 gives the yield of amino acid at each cycle after hydrolysis with 0.2 M-NaOH. Carry over begins at the eleventh cycle of degradation and after 15th cycle, the background in the amino acid analysis became significant. Nanomoles of each amino acid recovered was calculated as the difference between the nanomoles analyzed on a given cycle and the nanomoles for that particular amino acid on the previous cycle. The residues ser-3, Trp-7 and Trp-14 were identified by thin layer chromatography. The whole sequence of turtle myoglobin was determined as shown in Fig. 3 in the main paper.