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THE PRIMARY STRUCTURE

OF

BOVINE HEART CYTOCHROME $\underline{c}_1$

BY

SADAO WAKABAYASHI

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ABBREVIATIONS

Cm-	S-Carboxymethyl-
Cmc	S-Carboxymethylcysteine
CPase	Carboxypeptidase
C-terminal	Carboxyl terminal
Hse	Homoserine
Hsl	Homoserine lactone
Lys*	N^{ε}-Succinyllysine
NPSCl	p-Nitrophenylsulfenyl chloride
N-terminal	Amino terminal
Pe-	S-Pyridylethyl
PTH	Phenylthiohydantoin
SDS	Sodium dodecylsulfate
Suc-	Succinyl-
TLC	Thin layer chromatography
TPCK-trypsin	L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin

I SUMMARY

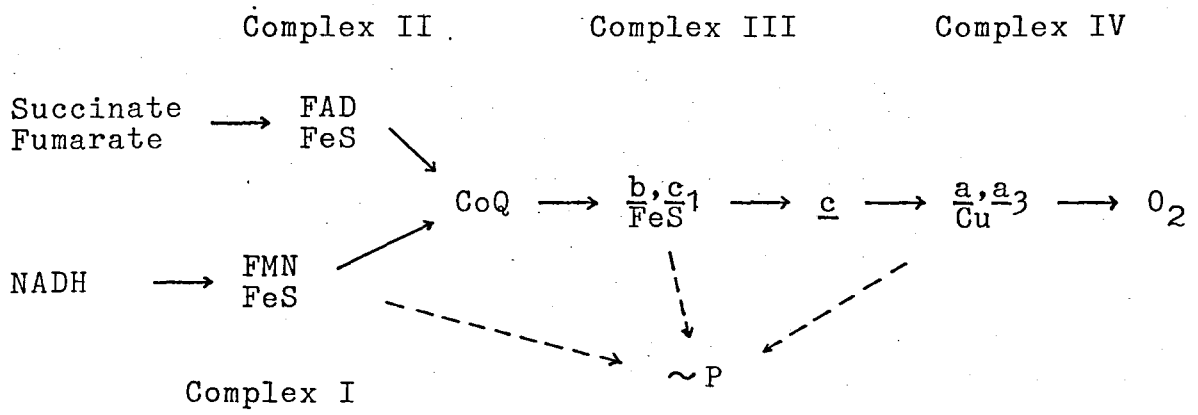
The complete amino acid sequence of bovine heart cytochrome $\underline{c}_1$ has been determined. It consists of a single polypeptide chain of 241 amino acid residues and heme $\underline{c}$. The molecular weight was calculated to be 27,256 without heme group and 27,874 with the heme.

Fourteen peptides were purified from tryptic digest of succinylated apoprotein which was prepared by differential modification of cysteine residues with iodoacetic acid and 4-vinylpyridine. The heme peptide and three tryptophanyl peptides were separately purified from the tryptic digest of carboxymethylated and succinylated cytochrome $\underline{c}_1$. Staphylococcal protease digestions were performed on carboxymethylated cytochrome $\underline{c}_1$ and carboxymethylated and succinylated cytochrome $\underline{c}_1$ and 13 peptides were purified. Chymotryptic digestion was also performed on carboxymethylated and maleylated cytochrome $\underline{c}_1$ and 20 peptides were obtained in pure form. The sequence analyses of these peptides enabled the construction of the primary structure of cytochrome $\underline{c}_1$. For the confirmation of this structure, cyanogen bromide fragmentation and hydroxylamine cleavage were also performed. The compositional and sequencial studies on these peptides firmly supported the proposed sequence.

The heme was located near the amino terminus and bound to the polypeptide chain through two cysteines-37 and -40 followed by histidine-41 which must chelate heme iron. Some notable structural features and prediction of secondary structure are also described.

II INTRODUCTION

Mitochondrial electron transfer chain can be divided into 4 enzyme complexes, Complexes I, II, III and IV (1) as shown below.



The middle segment, Complex III, is active in the reduction of cytochrome *c* by reduced coenzyme Q (ubiquinol) and corresponds to one of the energy coupling sites in the respiratory chain. It consists of 7-9 polypeptides including cytochromes *b* and c_1 and iron-sulfur protein (2,3), and therefore, the term "bc_1-complex" is used frequently.

One of the components of Complex III is cytochrome c_1 and it was first discovered in 1940 by Yakushiji and Okunuki (4). They showed that ox heart muscle suspension from which cytochrome *c* was washed out with buffer still had an absorption band at 552 nm and named cytochrome c_1. In 1949, Keilin and Hartree (5) observed a new band at 552 nm between cytochromes *b* and *c* with the aid of low temperature spectroscopy and designated it cytochrome *e*. Later it was realized that cytochromes c_1 and *e* were identical and Keilin and Hartree dropped the name "cytochrome *e*" (6). The

physiological role of cytochrome $\underline{c}_1$ was established by Ball and Cooper (7) and Estabrook (8) as the reductase for cytochrome $\underline{c}$.

Many attempts were made to isolate bovine heart cytochrome $\underline{c}_1$ but it was difficult to extract it from its association with cytochrome $\underline{b}$ and other components of mitochondrial succinate oxidase system without damaging it. The isolation methods in the earlier times involved heat treatments (9,11,12) or incubation with sodium dodecylsulfate (Duponol) (10,11) or guanidine-HCl (13). In 1972, Yu $\underline{et\ al}$. (14) established a reproducible method for isolation of cytochrome $\underline{c}_1$ utilizing a mild process. Trumpower and Katki (15) also succeeded to purify cytochrome $\underline{c}_1$. However, their preparations contained two polypeptides, heme containing polypeptide with a molecular weight of 29,000 and heme not containing polypeptide with molecular weight of 14,000, which had been considered to be one of the subunits of cytochrome $\underline{c}_1$. Recently, König $\underline{et\ al}$. (16) improved the method of Yu $\underline{et\ al}$. (14) and succeeded in a large scale preparation of highly purified and active cytochrome $\underline{c}_1$ which consisted of only one polypeptide. Independently, Robinson and Talbert (17) obtained cytochrome $\underline{c}_1$ as a single non-denatured subunit using gel filtration or high pressure liquid chromatography in deoxycholate.

Cytochrome $\underline{c}_1$ has heme $\underline{c}$ as the prosthetic group and exhibits an absorption spectrum similar to that of cytochrome $\underline{c}$. The reduced cytochrome $\underline{c}_1$ possessed maxima at 276 nm, 317 nm, 417 nm (Soret band), 512 nm, 522.5 nm, 530 nm (β bands) and 552.5 nm (α band)(14). Some characteristics of isolated cytochrome $\underline{c}_1$, its circular dichroism (18), fluorescence (19), autoxidation with alcohols (20), and photoreduction (21), and some other properties

(22) were studied. The kinetics and equilibrium of the electron transfer between isolated cytochromes *c* and c_1 (23) and complex formation of cytochrome c_1 with cytochrome *c* (19,24) and cytochrome oxidase (25) were also studied.

The location of cytochrome c_1 in mitochondrial membrane or Complex III was determined to be the C-side of mitochondrial inner membrane by various chemical modifications including cross-linking (26-28).

On the other hand, Trumpower's group (29-31) proposed the cyclic electron flow through Complex III, instead of the linear flow, named "protonmotive Q cycle", which was first brought by Mitchell (32,33), from the reconstitution experiment of succinate-cytochrome *c* reductase by iron-sulfur protein and the studies on effects of antimycin on the electron flow in this region. In this scheme cytochrome c_1 mediates the electron flow from iron-sulfur protein to cytochrome *c*.

In spite of these studies on cytochrome c_1, the exact mechanism of electron transport is not known yet. This fact is partly attributed to the lack of the information on the chemical structure of cytochrome c_1, while the very extensive studies have been performed on the structure including the tertiary structure of its redox partner, cytochrome *c*. Only a few attempts were performed for the elucidation of the structure of cytochrome c_1. The heme peptides of bovine cytochrome c_1 were obtained by digestions with chymotrypsin (34), trypsin (35) and pepsin (36), but those information are limited only to the amino acid compositions.

Cytochrome c_1 was purified from other organisms, yeast (37) and *Neurospora* , and only amino(N)-terminal sequence of

Neurospora cytochrome c_1 was reported (38).

Therefore, the determination of the amino acid sequence of cytochrome c_1 is very important and an acute desire to understand the structure-function relationship of this protein.

III MATERIALS & METHODS

Cytochrome c_1

Bovine heart cytochrome c_1 (so-called "heme subunit") was mainly supplied from Dr. King's laboratory (State University of New York at Albany). For cyanogen bromide cleavage a partially purified cytochrome c_1 was prepared as follows: Keilin-Hartree preparation (Green brei) was suspended in an equal volume of 0.2 M sodium phosphate buffer, pH 7.4, extracted by the addition of 40 % sodium cholate solution (25 ml per 288 ml of suspension), and fractionated by solid ammonium sulfate between 33 and 50 % saturation. The precipitate was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.5 % cholate in a 1/3 volume of Green brei suspension and cytochrome oxidase fraction was removed after dialysis against 40 mM sodium phosphate buffer, pH 7.4, for 90 min as described by Yoshikawa *et al.*(39). The supernatant was fractionated by ammonium sulfate between 25 and 45 % saturation and the precipitate was dissolved in 0.2 M sodium phosphate buffer, pH 7.4, in a 1/6 volume of the original suspension. To the cytochrome bc_1 rich fraction thus obtained a 1/10 volume of 20 % cholate and solid ammonium sulfate (114 g/l) were added and the solution was kept at 0°C overnight. After removal of cytochrome *b* by centrifugation the ammonium sulfate (59 g/l) was added and the precipitate was removed. The supernatant was diluted 7 times with water, reduced by dithionite and concentrated on a small DEAE-cellulose column. Cytochrome c_1 was eluted with 0.1 M sodium phosphate buffer, pH 7.4, containing 1 M NaCl and 0.5 % cholate. The eluate was dialyzed against 10 mM sodium phosphate buffer,

pH 7.4, containing 0.2 M NaCl, 0.5 mM EDTA, 0.01 % 2-mercaptoethanol and 0.1 % cholate and applied to a DEAE-Sephadex A-50 column equilibrated with the same buffer. Cytochrome $\underline{c}_1$ was eluted with a linear gradient from 0.2 M NaCl and 0.1 % cholate to 0.8 M NaCl and 0.5 % cholate. The fractions having the absorbance ratio A_{417}/A_{276} over 2.0 were collected, dialyzed against 10 mM NH_4HCO_3, and lyophilized. The preparation thus obtained contained three polypeptides with molecular weight of 29,000, 14,000 and 8,000.

Enzymes and Chemicals

Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), chymotrypsin and carboxypeptidases A and B were purchased from Worthington Biochemical Corp. Staphylococcal protease was a kind gift of Dr. R. P. Ambler (University of Edinburgh, U.K.). Carboxypeptidase Y was obtained from Oriental Yeast Co., Ltd. Bio-Gel P-6 and P-10 were obtained from Bio-Rad Laboratories. Sephadex G-50, DEAE-Sephadex A-25 and A-50, and SP-Sephadex C-25 were from Pharmacia Fine Chemicals. Whatman 3MM paper for paper electrophoresis and chromatography and DEAE-cellulose (DE-52) were from Whatman Biochemicals. p-Phenylene-diisothiocyanate, aminopropyl glass were obtained from LKB-Biochrome Ltd. Precoated silica gel plate 60-F254 was from Merck. Other reagents for sequence analyses were sequenal or analytical grade and obtained from Wako Pure Chemical Industries (Osaka, Japan) or Dojin chemical Laboratories (Kumamoto, Japan). p-Nitrophenylsulfenyl chloride (NPSCl), succinic anhydride, hydroxylamine and cyanogen bromide were purchased from Nakarai Chemicals Co. (Kyoto, Japan). 4-Vinylpyridine was the product of Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All the other reagents were

purchased from Nakarai Chemicals Co. or Wako Pure Chemical Industries.

Carboxymethylation (40)

About 100 mg of cytochrome $\underline{c}_1$ was dissolved in 10 ml of 0.6 M Tris-HCl buffer, pH 8.6, containing 6 M guanidine-HCl and 5 mM EDTA, and reduced with 0.15 ml of 2-mercaptoethanol under nitrogen at 40°C for 4 h. To this solution 400 mg of iodoacetic acid dissolved in 5 N NaOH and neutralized was added and the pH of the solution was maintained at about 8.5. After 15 min the reaction was stopped by the addition of 0.5 ml of 2-mercaptoethanol and the solution was dialyzed against deionized water and 10 mM NH_4HCO_3 successively and freeze-dried.

Heme Removal (41) and pyridylethylation (42)

Carboxymethyl(Cm)-cytochrome $\underline{c}_1$ (150 mg) was dissolved in 20 ml of 50 % acetic acid and 95 mg of NPSCl in 10 ml of acetic acid was added to remove heme moiety. After gentle stirring for 10 min, 60 ml of deionized water was added and heme was extracted with ethyl acetate three times using low speed centrifugation. The aqueous phase was dialyzed against deionized water and freeze-dried. These procedures were repeated once more. The lyophilized sample was dissolved in 10 ml of the buffer used for carboxymethylation and reduced with 0.1 ml of 2-mercaptoethanol to remove the p-nitrophenylsulfenyl groups. The solution was incubated at 40°C for 3 h under nitrogen and the generated cysteine residues were converted to pyridylethyl(Pe)-derivatives by reacting with 0.4 ml of 4-vinylpyridine under nitrogen at 40°C for 2 h. The

reaction was stopped by 0.25 ml of 2-mercaptoethanol and the solution was dialyzed against 10 mM NH_4HCO_3 and freeze-dried.

Succinylation (43) and Maleylation (44)

Cm-cytochrome $\underline{c}_1$ or Pe-Cm-cytochrome $\underline{c}_1$ was dissolved in 0.5 M $NaHCO_3$ containing 6 M guanidine-HCl at a concentration of 10 mg/ml. The powder of succinic anhydride (a 60-fold molar exess per amino group) was added and the pH of the solution was kept between 8.0 and 9.0 with 5 N NaOH. The solution was kept standing for 30 min and dialyzed against 10 mM NH_4HCO_3 followed by 0.5 M $NaHCO_3$. The dialysate was again succinylated with a 40-fold molar excess of succinic anhydride as above, dialyzed against 10 mM NH_4HCO_3, and lyophilized.

Maleylation was performed with an 80-fold molar excess of maleic anhydride in 0.5 M $NaHCO_3$ containing 6 M guanidine-HCl.

Tryptic Digestion

Succinyl(Suc)-Pe-Cm-cytochrome $\underline{c}_1$ (150 mg) was digested with TPCK-trypsin (3 mg) in 10 ml of 0.1 M NH_4HCO_3 at room temperature for 18 h.

Suc-Cm-cytochrome $\underline{c}_1$ (50 mg) was separately digested with 0.8 mg of TPCK-trypsin in 5 ml of 0.1 M NH_4HCO_3 at room temperature for 18 h to obtain the heme peptide and tryptophanyl peptides.

Staphylococcal Protease Digestion

Cm-cytochrome $\underline{c}_1$ (65 mg) was digested with 1.2 mg of staphylococcal protease in 5 ml of 0.1 M NH_4HCO_3 at room temperature for 18 h.

Suc-Cm-cytochrome $\underline{c}_1$ (70 mg) was separately digested with 1.2 mg of the enzyme in 5 ml of 0.1 M NH_4HCO_3 at 40°C for 4 h to isolate the peptides in carboxyl(C)-terminal region.

Chymotryptic Digestion

Maleyl-Cm-cytochrome $\underline{c}_1$ (65 mg) was digested with chymotrypsin (0.4 mg) in 3.8 ml of 0.1 M Tris-HCl buffer, pH 8.0, at 40°C for 2 h. Deblocking of maleyl groups was performed after purification of peptides in 30 % acetic acid at room temperature for 72 h.

Cyanogen Bromide Cleavage (45)

Partially purified cytochrome $\underline{c}_1$ was carboxymethylated, citraconylated (46) and chromatographed on a Sephacryl S-200 column in 0.2 M NH_4HCO_3-NH_4OH buffer, pH 9.0. Citraconyl-Cm-cytochrome $\underline{c}_1$ thus obtained (70 mg) was dissolved in 2 ml of 70 % formic acid and cyanogen bromide (320 mg) was added. After incubation at 0°C for 15 h the solution was diluted and lyophilized.

Hydroxylamine Cleavage (47)

Suc-Cm-cytochrome $\underline{c}_1$ (6 mg) was dissolved in 1 ml of 0.5 M $NaHCO_3$ containing 6 M guanidine-HCl and to this solution 1 ml of 4 M hydroxylamine-0.4 M K_2CO_3 solution, pH 9.5, was added. The precipitate formed at this step was solubilized by addition of solid guanidine-HCl. After incubation at 40°C for 3 h the solution was dialyzed against water, lyophilized, and directly applied to manual Edman degradation.

Peptide Separations

Each digest was applied to a Bio-Gel P-10 column (2 x 180 cm) equilibrated with 0.2 M NH_4HCO_3-NH_4OH buffer, pH 9.0. Peptides were monitored by absorbancies at 220 nm, 230 nm, 280 nm, and/or 410 nm. The peptide fractions were pooled, lyophilized, and further purified by ion exchange chromatography (DE-52, DEAE-Sephadex A-25 or SP-Sephadex C-25), paper electrophoresis at pH 3.6 (pyridine:acetic acid:water, 1:10:189, v/v) and/or 6.5 (pyridine:acetic acid:water, 10:0.4:180, v/v) and paper chromatography (BPAW; 1-butanol:pyridine:acetic acid:water, 60:40:12:48, v/v). The detailed conditions for ion exchange chromatographies are described in figures.

In the case of staphylococcal protease digest of Cm-cytochrome $\underline{c}_1$ the precipitate appeared during incubation and was removed by centrifugation. The supernatant was fractionated as mentioned above. The precipitate was dissolved in 0.2 M ammonium acetate buffer, pH 4.0, and chromatographed on a Sephadex G-50 column (2 x 180 cm) and further purified by an SP-Sephadex column.

Cyanogen bromide peptides were first fractionated on a Sephadex G-50 column (4 x 110 cm) equilibrated with 8 M urea and 10 % formic acid and the peptide fractions were desalted on a Sephadex G-10 column in 10 % formic acid, lyophilized, and further purified by column chromatography of SP-Sephadex and paper electrophoresis at pH 3.6.

Amino Acid Analysis

The protein and purified peptides were hydrolyzed with 6 N HCl containing a slight amounts of thioglycolic acid in evacuated, sealed tube at 110°C for 24 h. The hydrolysates were analyzed with an automated amino acid analyzer (Beckman Model 120B or Irica model A-3300, Irica Instruments Inc., Kyoto, Japan) essentially as described by Spackman et al. (48).

Sequence Determination

The N-terminal sequence was determined by manual Edman degradation procedure (49) using about 0.1 μmol of protein or peptide and phenylthiohydantoin (PTH) derivatives were identified by thin-layer chromatography (TLC) using solvent system V (n-heptane:ethylene dichloride:propionic acid, 29:12.5:8.5, v/v)(50) and II (methanol:chloroform, 5:45, v/v)(51). PTH-histidine and PTH-arginine were identified by iodine-azide reaction (52) after paper electrophoresis at pH 6.5. Some peptides were analyzed by automated Edman degradation on an LKB 4020 solid-phase sequencer. Peptides (0.1 to 0.2 μmol) were attached to aminopropyl glass through one of the following methods (53); conversion of arginine to ornithine with 50 % hydrazine followed by p-phenylenediisothiocyanate activation, water soluble carbodiimide activation, and homoserine lactone method. The anilinothiazolinone derivatives delivered from sequencer were converted to PTH-derivatives and identified by TLC.

PTH-N^{ε}-succinyllysine was semi-synthesized as follows: About 2 μmol of a peptide T-II, Val-Thr-Leu-Lys, isolated from Scenedesmus ferredoxin (54) was succinylated with 25 mg of succinic anhydride

in 0.1 M NH_4HCO_3. The succinylated peptide was digested with thermolysin and a peptide, Leu-Lys*, was purified by paper electrophoresis at pH 3.6. (Lys* means N^{ε}-succinyllysine.) The recovery of this peptide was about 50 % and PTH-N^{ε}-succinyllysine was obtained by Edman degradation after two steps on this peptide. The R_f values of PTH-N^{ε}-succinyllysine on TLC were 0.01 with solvent system V and 0.05 with solvent system II.

The C-terminal sequence was determined by digestion with carboxypeptidase (CPase) A, B or Y (55,56) and released amino acids were detected on an amino acid analyzer.

Peptide Nomenclature

T-, S-, C- and CB- refer to tryptic, staphylococcal protease, chymotryptic, and cyanogen bromide peptides, respectively. T'- and S'- refer to supplemental peptides derived by tryptic and staphylococcal protease digestions of Suc-Cm-cytochrome $\underline{c}_1$, respectively. The number of each peptide indicates its order in the sequence from the N-terminus.

IV RESULTS

Amino Acid Composition and Terminal Sequence Analysis

Amino acid composition of cytochrome $\underline{c}_1$ was determined on unmodified cytochrome $\underline{c}_1$ and Pe-Cm-cytochrome $\underline{c}_1$ as shown in Table I. The N-terminal sequence was determined by manual Edman degradation using 70 nmol of Cm-cytochorme $\underline{c}_1$. The established N-terminal sequence was as follows: Ser-Asp-Leu-Glu-Leu-His-Pro-Pro-Ser-Tyr-Pro-. The quantitative analysis was also performed by amino acid analyses of acid hydrolysates of PTH-derivatives with 6 N HCl containing 0.5 % $SnCl_2$ (57). The result of this analysis was as follows: 1st step, Ser (33 % as alanine) and Gly (19 %); 2nd step, Asp (32 %); 3rd step, Leu (20 %); 4th step, Glu (21 %). The C-terminal amino acid was analyzed by CPase A digestion (substrate/enzyme ratio, 1/50 (w/w)) of Cm-cytochrome $\underline{c}_1$ in 0.1 M borate buffer, pH 8.0. Lysine was detected in 63 % yield along with leucine (54 %), alanine (38 %) and serine (36 %) after 21 h digestion. These amino acids were found in almost the same ratio at any reaction time. These terminal sequence analyses suggested the presence of some impurities and an aliquot of Cm-cytochrome $\underline{c}_1$ was chromatographed on an SP-Sephadex column in the presence of 8 M urea as shown in Fig.1. The amino acid composition of the main fraction is also listed in Table I. It agrees well with that calculated from the sequence, but a quantitative N-terminal analysis showed that impurities were still present. The exact nature of the impurities could not be elucidated here but the following experiments definitely determined the terminal sequences of cytochrome $\underline{c}_1$ as discussed below.

Table I Amino acid composition of cytochrome $\underline{c}_1$

Amino acid	unmodified[a]	Pe-Cm	Cm[b]	sequence
Cm-cysteine		2.95	2.23	
Aspartic acid	19.0	19.4	18.8	19[c]
Threonine	7.86	7.52	6.50	7
Serine	14.8	13.6	12.9	16
Glutamic acid	22.1	22.5	20.8	21[d]
Proline	19.6	21.1	21.7	22
Glycine	16.8	16.4	17.8	17
Alanine	17.9	17.9	18.1	18
Half-cystine	2.98	1.85[e]	1.05	5
Valine	14.4	14.6	15.3	15
Methionine	9.40	5.87	10.0	10
Isoleucine	5.02	4.43	3.81	4
Leucine	22.0	22.7	24.5	25
Tyrosine	12.2	11.7	11.9	15
Phenylalanine	7.70	7.89	8.31	8
Lysine	13.8	13.5	11.5	12
Histidine	9.19	9.04	9.36	9
Arginine	14.5	14.7	14.3	15
Tryptophan	N.D.	N.D.	N.D.	3
Total residues				241

[a] Acid hydrolyses were performed on unmodified cytochrome $\underline{c}_1$ for 24 and 72 h. The values of threonine and serine were obtained by extrapolations to zero time of hydrolysis. Values of valine and isoleucine were of 72 h hydrolysate. Pe-Cm- and Cm-cytochrome $\underline{c}_1$ were hydrolyzed for only 24 h.

[b] Specifically purified Cm-cytochrome $\underline{c}_1$

[c] 13 aspartic acids and 6 asparagines

[d] 16 glutamic acids and 5 glutamines

[e] Determined as pyridylethyl-cysteine

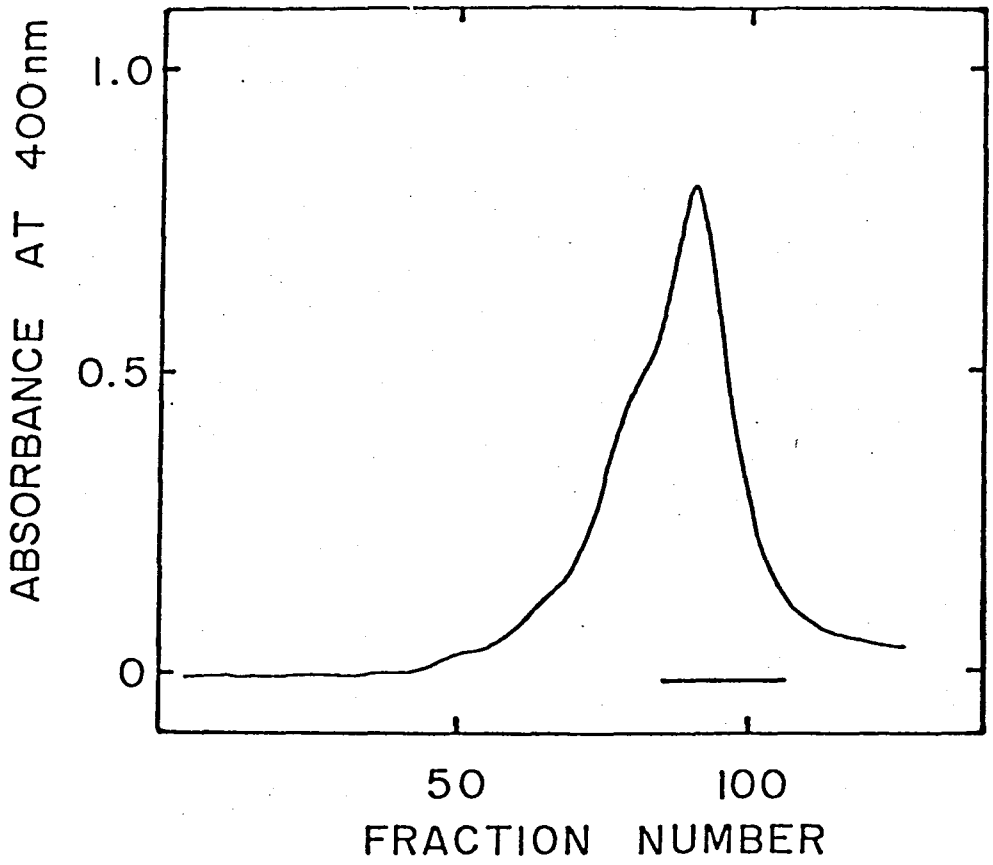


Fig.1. Purification of Cm-cytochrome $\underline{c}_1$. Cm-cytochrome $\underline{c}_1$ (30 mg) was dissolved in 2 % phosphoric acid containing 8 M urea and 50 mM NaCl, applied to an SP-Sephadex C-25 column (1.5 x 37 cm), and eluted with a linear gradient from 50 mM to 300 mM NaCl in 2 % phosphoric acid and 8 M urea.(300 ml each). The flow rate was 14 ml/h. The fractions (5 ml each) indicated by solid bar were pooled.

Tryptic Peptides

The tryptic peptides of Suc-Pe-Cm-cytochrome c_1 was divided in two parts and each was first fractionated on a Bio-Gel P-10 column as shown in Fig.2a. The pooled fractions I to V were further chromatographed on DE-52 columns and their elution profiles are shown in Fig.3. Other fractions were further separated by paper electrophoreses and paper chromatographies. Fifteen peptides and free arginine were purified and their amino acid compositions and some properties are listed in Table II, and their amino acid sequences were determined as summarized in Table III.

Peptide T-1 had a blocked N-terminus and was assumed to be the N-terminal peptide of the protein which had been succinylated. The manual Edman degradation following the staphylococcal protease digestion gave the sequence of Leu-His-Pro-Pro-Ser-Tyr-Pro-X-Ser-His. This sequence corresponded to the N-terminal sequence of the protein after the 5th residue and confirmed the assumption.

The amino acid sequences of Peptides T-2, T-4, T-5, T-8, T-12, T-13 and T-14 were completely determined by manual Edman degradations except for the 3rd residue of Peptide T-13 which was assumed to be tryptophan.

The largest peptide T-6 (1 μmol) was further digested with staphylococcal protease (0.1 mg) in 0.7 ml of 0.1 M Tris-HCl buffer, pH 8.0, at 40°C for 3 h, and resulted subfragments were purified by Bio-Gel P-4 column chromatography and paper electrophoreses. Their amino acid compositions are listed in Table IV. Sequence studies by Edman degradation on Peptide T-6 (22 steps) and subfragments T-6-S-3 (12 steps) and T-6-S-4 (10 steps) were performed. Tryptic peptides T-6a and T-6b were parts of Peptide

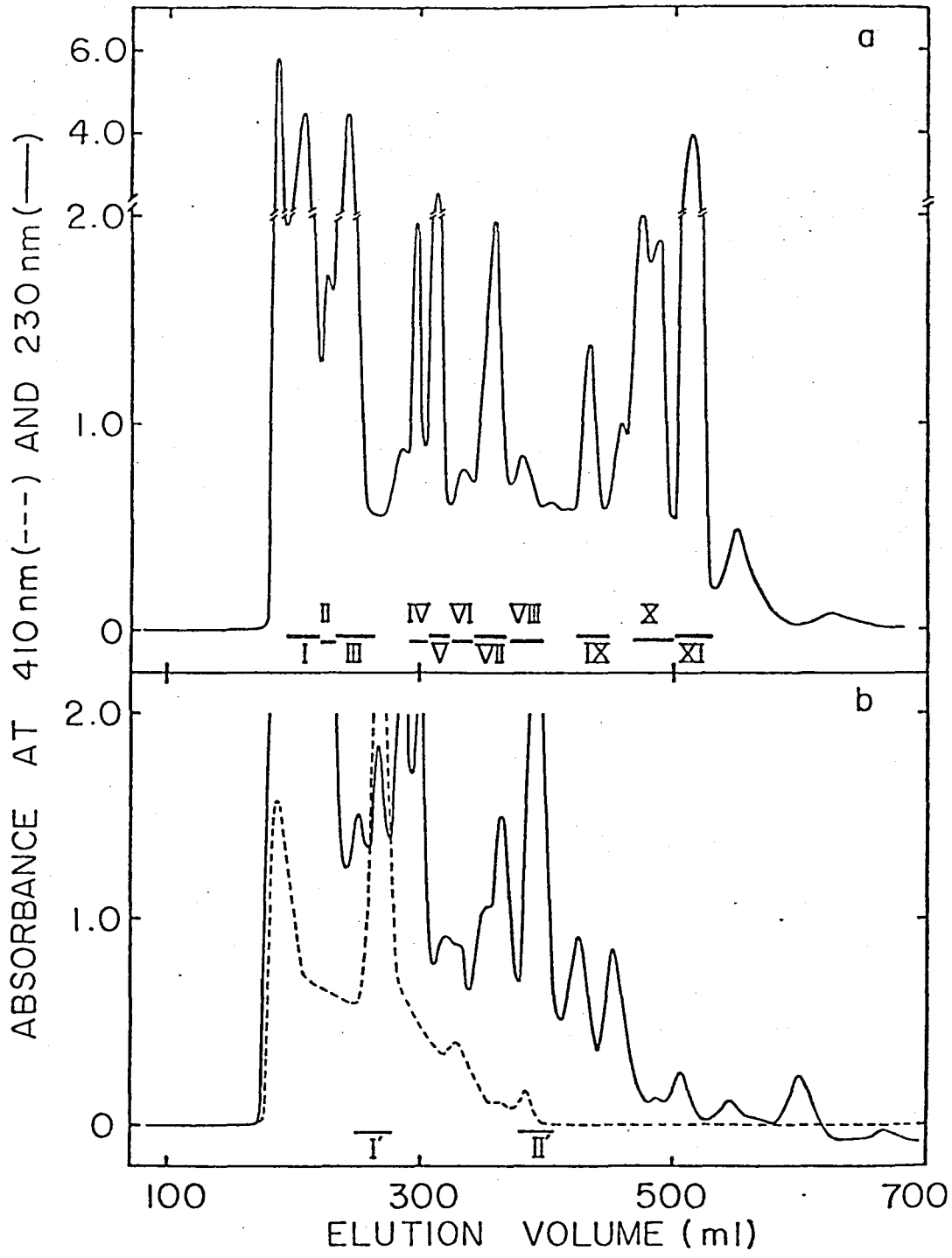


Fig.2. Elution patterns of tryptic digests.
a. Tryptic digest of Suc-Pe-Cm-cytochrome $\underline{c}_1$
b. Tryptic digest of Suc-Cm-cytochrome $\underline{c}_1$
Column: Bio-Gel P-10 (2 x 180 cm)
Buffer: ammonium bicarbonate, 0.2 M , pH 9.0
Flow rate: 10 ml/h

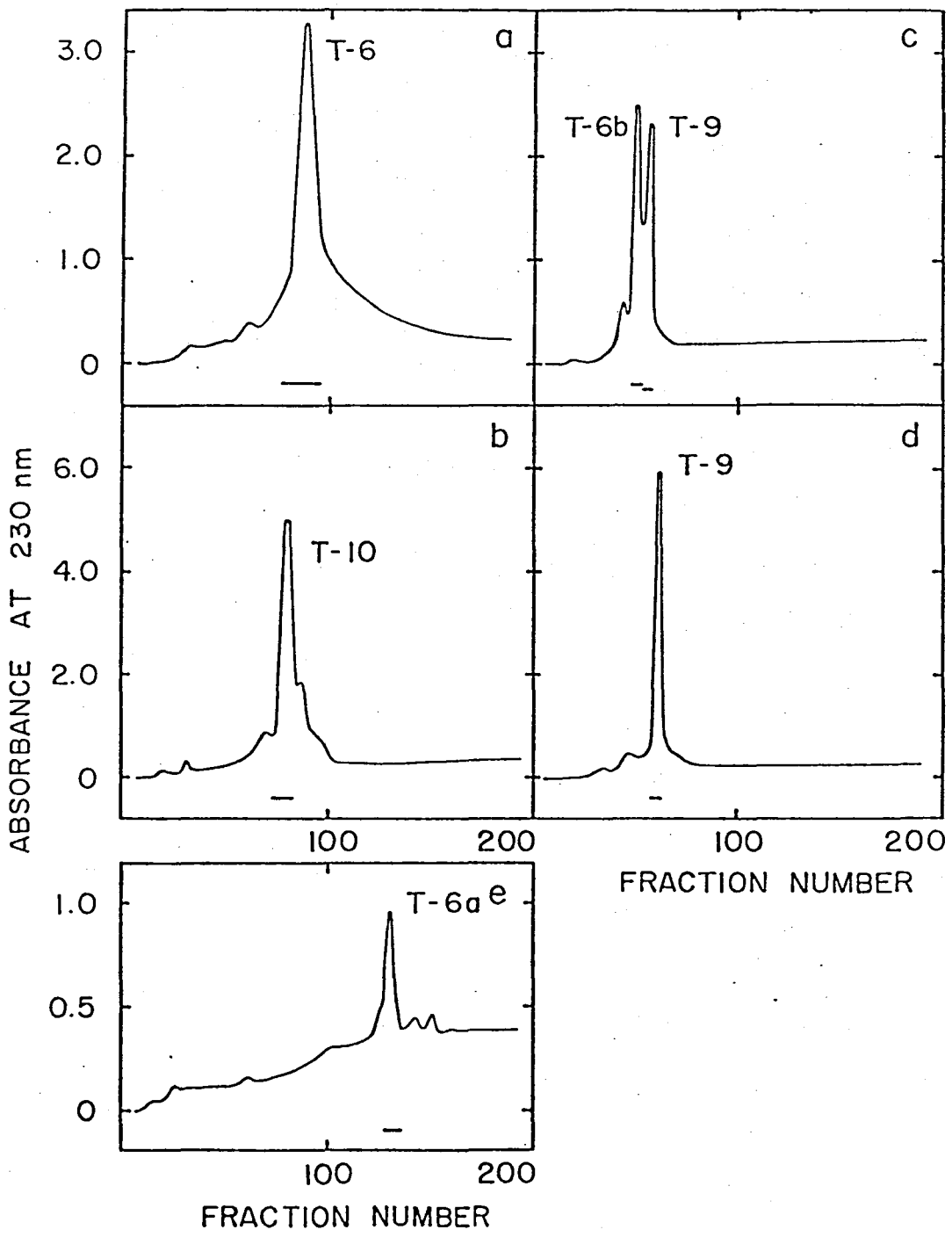


Fig.3. Chromatograms of pools I to V in Fig.2a on DE-52 columns
a: Pool I, b: Pool III, c: Pool IV, d: Pool V, e: Pool II
Column: a-e DEAE-cellulose (DE-52) (1.5 x 37 cm)
Elution: a-d, linear gradient from 50 mM to 1 M NH_4HCO_3
e, linear gradient from 50 mM to 0.75 M NH_4HCO_3 (300 ml each)
Flow rate: 20 ml/h Fraction: 3ml

Table II Amino acid compositions[a] and some properties of tryptic peptides

Peptide	T-1	T-2	T-3	T-4	T-5	T-6	T-6a	T-6b
Residue No.	1-15	16-27	28	29-33	42-49	50-102	50-83	84-102
Cmc						0.92(1)	0.95(1)	
Asp	0.97(1)	1.04(1)			0.98(1)	5.78(6)	4.02(4)	2.11(2)
Thr		0.99(1)				1.20(1)	1.02(1)	
Ser	2.73(3)	2.78(3)			0.96(1)	1.19(1)		0.93(1)
Glu	1.03(1)			1.01(1)		9.31(9)	7.76(8)	1.29(1)
Pro	2.93(3)					5.10(6)	1.25(1)	4.69(5)
Gly		0.98(1)		1.05(1)		3.55(4)	2.93(3)	1.13(1)
Ala					0.98(1)	4.93(5)	2.98(3)	2.07(2)
Val				0.93(1)	1.06(1)	3.54(4)	4.00(4)	
Met					0.94(1)	2.12(2)	1.87(2)	
Ile		0.96(1)						
Leu	2.00(2)	2.98(3)				3.23(3)	2.06(2)	1.03(1)
Tyr	0.92(1)			0.99(1)	1.93(2)	2.21(3)	0.99(1)	1.69(2)
Phe				0.99(1)		1.80(2)	1.07(1)	0.97(1)
Lys						2.80(3)	0.89(1)	1.89(2)
His	1.99(2)	0.95(1)				0.83(1)	0.92(1)	
Arg	1.14(1)	1.03(1)	1.00(1)		1.07(1)	2.21(2)	1.05(1)	0.93(1)
Trp	N.D.(1)							
Total	15	12	1	5	8	53	34	19
Yield(%)	14	38	42	7	8	34	12	16
Mobility[b]								
pH3.6	+5.2	+10.0	+18.0	+5.0	+6.4	N.D.	N.D.	N.D.
pH6.5	-1.5	+4.3	+15.0	+1.4	+1.4	N.D.	N.D.	N.D.
Rf(BPAW)	0.59	0.52	0.20	N.D.	N.D.	0.00	N.D.	N.D.
Nin.Col.	Negative	Yellow	Violet	Yellow	Yellow	Negative	N.D.	N.D.
Pool(Fig.2)	X	VII,VIII	XI	X	X	I	II	IV
Purification	6.5	6.5	3.6	6.5	6.5	DE-52	DE-52	DE-52
	3.6			3.6	3.6	BPAW		

[a] Amino acid analyses were performed on 24h hydrolysates. Values in parentheses are taken from sequence.

[b] Mobility is expressed in terms of cm (+) to the cathode at 43 V/cm for 1 h.

Table II (continued)

Peptide	T-7	T-8	T-9	T-10	T-11	T-12	T-13	T-14
Residue No.	103-118	119-120	121-144	145-191	192-201	202-203	225-233	234-241
Cmc			1.05(1)	0.79(1)				
Asp	2.93(3)		1.10(1)	4.93(5)	1.01(1)			
Thr			1.97(2)	2.79(3)				
Ser	0.96(1)		1.99(2)	1.12(1)			1.50(2)	
Glu			2.21(2)	4.99(5)	1.98(2)			
Pro	2.14(2)		1.97(2)	5.30(5)	1.12(1)			2.20(2)
Gly	1.05(1)		3.80(4)	3.82(4)				
Ala	2.88(3)	0.96(1)		4.05(4)	2.03(2)			0.94(1)
Val	0.75(1)		1.98(2)	2.75(3)			1.15(1)	
Met				1.94(2)				
Ile	0.75(1)			1.96(2)				
Leu	1.93(2)		2.93(3)	2.95(3)			0.91(1)	0.99(1)
Tyr	0.95(1)		1.95(2)	2.65(3)				0.92(1)
Phe			1.01(1)	3.68(4)				
Lys				0.98(1)		0.92(1)	1.72(2)	2.05(2)
His			0.86(1)		1.76(2)		0.99(1)	
Arg	1.08(1)	1.04(1)	1.02(1)	1.10(1)	1.03(1)	1.08(1)	1.11(1)	1.04(1)
Trp					N.D.(1)		N.D.(1)	
Total	16	2	24	47	10	2	9	8
Yield(%)	35	52	48	41	60	67	10	19
Mobility[b]								
pH3.6	+6.5[c]	+16.7	N.D.	N.D.	+10.0	+12.0	+7.0	+6.5
pH6.5	-1.5[c]	+11.3	N.D.	N.D.	+1.0	+2.0	-1.5	-1.5
Rf(BPAW)	0.39[c]	0.21	N.D.	N.D.	0.27	0.20	0.59	0.47
Nin.Col.	Violet	Violet	N.D.	N.D.	Violet	Violet	Violet	Violet
Pool(Fig.2)	VI,VII	X	IV,V	III	XI	IX	X	VII
Purification	6.5 BPAW	6.5	DE-52	DE-52	3.6	6.5	6.5 3.6	6.5 BPAW

[c]The minor peptide showed the mobilities of +2.0 (pH 3.6) and -4.0 (pH 6.5). The Rf value of this peptide was 0.36.

Table II (continued)

Peptide	T'-1	T'-2	T'-3	T'-4
Residue No.	1-15	29-49	192-201	225-233
Cmc		1.80(2)[d]		
Asp	1.08(1)	1.45(1)	1.04(1)	
Thr				
Ser	2.66(3)	2.84(3)		2.01(2)
Glu	1.08(1)	2.35(2)	2.04(2)	
Pro	2.97(3)		1.05(1)	
Gly		0.97(1)		
Ala		1.04(1)	2.04(2)	
Val		3.08(3)		1.19(1)
Met		0.77(1)		
Ile				
Leu	1.95(2)	0.35		1.00(1)
Tyr	0.96(1)	0.98(3)		
Phe		0.89(1)		
Lys		0.81(1)		1.81(2)
His	1.83(2)	1.30(1)	1.84(2)	0.85(1)
Arg	1.00(1)	0.82(1)	0.94(1)	1.04(1)
Trp[e]	N.D.(1)		N.D.(1)	N.D.(1)
Total	15	21	10	9
Yield(%)	19	9	62	21
Mobility[b]				
pH3.6	+5.0	N.D.	+9.0	+7.3
pH6.5	-1.8	N.D.	0	-1.8
Nin.Col.	Negative	N.D.	Violet	Violet
Pool(Fig.2)	II'	I'	II'	II'
Purification	DEAE 3.6	G-50	DEAE	DEAE 3.6

[d]This value was obtained after heme removal and carboxymethylation of purified peptide.
[e]Tryptophan was tested qualitatively by Ehrlich reaction

Table III. Summary of sequence studies on tryptic peptides[a]

Peptide	Residue No.	
T-1	1-15	(Ser,Asp,Leu,Glu)Leu-His-Pro-Pro-Ser-Tyr-Pro(Trp) Ser-His-Arg
T-2	16-27	Gly-Leu-Leu-Ser-Ser-Leu-Asp-His-Thr-Ser-Ile-Arg
T-4	29-33	Gly-Phe-Gln-Val-Tyr
T-5	42-49	Ser-Met-Asp-Tyr-Val-Ala-Tyr-Arg
T-6	50-102	His-Leu-Val-Gly-Val-Cmc-Tyr-Thr-Glu-Asp-Glu-Ala- ⊢——— S-1 ———⊣ Lys*-Ala-Leu-Ala-Glu-Glu-Val-Glu-Val-Gln-Asp-Gly- — S-2 ———⊣ Pro-Asn-Glu-Asp-Gly-Glu-Met-Phe-Met-Arg-Pro-Gly- — S-3 ———⊣ Lys*-Leu-Ser-Asp(Asx^1,Glx^1,Pro^4,Tyr^2,Phe^1,Lys^1)(Ala^2,Arg^1) —— S-4 ——⊣ ⊢ S-5 ⊣
T-6a	50-83	His-Leu-Val-Gly-Val-Cmc(Asx^4,Thr^1,Glx^8,Pro^1,Gly^2,Ala^3, Val^2,Met^2,Leu^1,Tyr^1,Phe^1,Lys^1,Arg^1)
T-6b	84-102	Pro-Gly-Lys*-Leu-Ser-Asp-Tyr-Phe-Pro-Lys*-Pro-Tyr- Pro-Asn-Pro-Glu-Ala(Ala^1,Arg^1)
T-7[b]	103-118	Ala-Ala-Asn-Asn-Gly-Ala-Leu-Pro-Pro-Asp-Leu-Ser- ⊢——— S-1 ———⊣ Tyr-Ile-Val-Arg — S-2 ———⊣

T-8 119-120 Ala-Arg

T-9 121-144 His-Gly-Gly-Glu-Asp-Tyr-Val-Phe-Ser-Leu-Leu-Thr-
Gly-Tyr-Cmc-Glu-Pro-Pro-Thr-Gly-Val-Ser-Leu-Arg

T-10 145-191 Glu-Gly-Leu-Tyr-Phe-Asn-Pro-Tyr-Phe-Pro-Gly-Gln-
S-1
Ala-Ile-Gly-Met-Ala-Pro-Pro-Ile-Tyr-Asn-Glu-Val-
S-1
Leu-Glu-Phe-Asp-Asp-Gly-Thr-Pro-Ala-Thr-Met-Ser-
S-2 S-3
Gln-Val-Ala-Lys*-Asp-Val-Cmc-Thr-Phe-Leu-Arg
S-4

T-11 192-201 (Trp)Ala-Ala-Glu-Pro-Glu-His-Asp-His-Arg
S-1 S-2

T-12 202-203 Lys*-Arg

T-13 225-233 His-Lys*(Trp)Ser-Val-Leu-Lys*-Ser-Arg

T-14 234-241 Lys*-Leu-Ala-Tyr-Arg-Pro-Pro-Lys*

T'-2 29-49 Gly-Phe-Gln-Val-Tyr-Lys*-Gln-Val-Cmc-Ser(Cmc^1, Asx^1,
Ser^2, Ala^1, Val^1, Met^1, Tyr^1, His^1)Tyr-Arg

T'-3 192-201 Trp-Ala(Asx^1, Glx^2, Pro^1, Ala^1, His^2, Arg^1)

[a] Cmc and Lys* refer to carboxymethylcysteine and N^{ε}-succinyllysine, respectively. Arrows (→) and (←) indicate that the residue above was identified by manual Edman degradation and carboxypeptidase, respectively. The solid lines indicate the subfragments.

[b] The amino acid sequence of T-7 was determined on minor peptide whose 4th residue was aspartic acid (See text in detail).

Table IV. Amino acid compositions of subfragments of tryptic peptides

	T-6					T-7	
	S-1	S-2	S-3	S-4	S-5	S-1	S-2
Cmc	0.83(1)						
Asp	1.17(1)		3.15(3)	2.05(2)		3.30(3)	
Thr	0.98(1)						
Ser				0.95(1)			1.12(1)
Glu	2.17(2)	2.03(2)	3.97(4)	1.13(1)			
Pro			0.97(1)	4.86(5)		1.86(2)	
Gly	1.07(1)		1.90(2)	1.05(1)		0.98(1)	
Ala		3.04(3)			1.99(2)	2.77(3)	
Val	2.02(2)		2.06(2)				0.80(1)
Met				1.72(2)			
Ile							0.70(1)
Leu	1.02(1)	0.97(1)		1.05(1)		1.07(1)	0.98(1)
Tyr	0.91(1)			1.64(2)			0.95(1)
Phe				1.87(2)			
Lys		0.90(1)		1.61(2)			
His	0.75(1)						
Arg				1.05(1)	1.01(1)		1.05(1)
Total	11	7	12	20	3	10	6
Yield(%)	39	54	39	19	72	12	12
Mobility							
pH 3.6	+3.7	+2.4	0	+4.0	+15.0	N.D.	N.D.
pH 6.5	-9.7	-11.3	N.D.	-5.0	+11.2	-6.7	+6.2

Table IV. (continued)

	T-10				T-11	
	S-1	S-2	S-3	S-4	S-1	S-2
Cmc				0.84(1)		
Asp	2.20(2)		2.95(3)			1.09(1)
Thr			1.85(2)	0.97(1)		
Ser			0.95(1)			
Glu	3.05(3)	1.04(1)	1.07(1)		1.73(2)	
Pro	3.69(4)		0.97(1)		1.20(1)	
Gly	3.01(3)		1.09(1)			
Ala	1.98(2)		1.91(2)		1.86(2)	
Val		1.01(1)	0.98(1)	0.95(1)		
Met	0.97(1)		0.97(1)			
Ile	1.90(2)					
Leu	1.00(1)	0.96(1)		1.01(1)		
Tyr	2.99(3)					
Phe	2.09(2)		1.01(1)	1.09(1)		
Lys			0.80(1)			
His						1.89(2)
Arg				0.96(1)		1.00(1)
Trp					N.D.(1)	
Total	23	3	15	6	6	4
Yield (%)	18	61	46	61	49	41
Mobility						
pH 3.6	+1.5	+4.8	0	+5.6	N.D.	N.D.
pH 6.5	-3.1	-6.2	-9.6	+1.5	-5.0	+7.0

T-6 which were derived by the partial cleavage of Arg-Pro bond. Sequence studies on these peptides, T-6a (6 steps) and T-6b (17 steps), together with those on T-6 revealed the amino acid sequence of Peptide T-6.

Manual Edman degradation on Peptide T-7 was not successful beyond the 3rd residue and the spots on thin-layer plate became very faint from asparagine at the 4th residue. The minor peptide, which showed the different electrophoretic mobility from Peptide T-7 and had the same amino acid composition, was also obtained in pure form. The 4th residue of this peptide was aspartic acid and the subsequent sequence could be determined up to the 13th residue. From these observations it was concluded that the 4th and 5th residues were asparagine and glycine, respectively, and the formation of β-aspartylglycine bond might cause the difficulty in sequence determination. The remaining part of Peptide T-7 was analyzed on subfragments derived from staphylococcal protease digestion. Since this protease cleaves Asp-X bond in phosphate buffer (58), Peptide T-7 (0.38 μmol) was digested with this enzyme (0.03 mg) in 0.3 ml of 50 mM sodium phosphate buffer, pH 7.8, at 40°C for 18 h. Although the yield was low (12 %), two subfragments were obtained by paper electrophoresis at pH 6.5. Their amino acid compositions are shown in Table IV and manual Edman degradation was carried out on T-7-S-2 up to the 5th residue. These studies established the amino acid sequence of Peptide T-7.

Manual Edman degradation revealed the sequence of Peptide T-9 up to the 22nd residue, and CPase B released arginine (99 %) and also leucine (90 %) in 0.1 M borate buffer, pH 8.0, at 40°C for 30 min.

After 30 steps of Edman degradation, Peptide T-10 (1.3 μmol) was digested with staphylococcal protease (0.1 mg) in 0.7 ml of 0.1 M Tris-HCl buffer, pH 8.0, at 40°C for 3 h and the digest was applied to a Bio-Gel P-4 column. Four subfragments were purified by paper electrophoreses and their amino acid compositions (Table IV) and amino acid sequences were studied.

Peptide T-11 (0.8 μmol) was digested with staphylococcal protease (0.015 mg) in 0.1 M Tris-HCl buffer, pH 8.0, at 40°C for 2 h and two subfragments were purified by paper electrophoresis at pH 6.5. Their amino acid compositions (Table IV) and amino acid sequences were determined. The first residue of Peptide T-11 was assumed to be tryptophan and it was confirmed as described below.

The peptide containing pyridylethylcysteine corresponding to the heme peptide could not be recovered. Tryptophan was modified by the reagent for heme removal, NPSCl, and formed yellow products. In this experiment three yellow peptides, T-1, T-11 and T-13, were obtained and they were considered to contain tryptophan residues. To confirm the location of tryptophan residues and recover heme peptide, Suc-Cm-cytochrome $\underline{c}_1$ was digested with trypsin and chromatographed on a Bio-Gel P-10 column (Fig.2b). The heme peptide fraction (pool I′ in Fig.2b) was further separated on a Sephadex G-50 (fine) column as shown in Fig.4. Tryptophan peptides were purified from fraction II′ by column chromatography on DEAE-Sephadex A-25 (Fig.5) and paper electrophoresis at pH 3.6. The amino acid compositions and some properties of the heme peptide T′-2, which was treated with NPSCl to remove heme group and carboxymethylated, and three tryptophanyl peptides, T′-1, T′-3 and T′-4 are

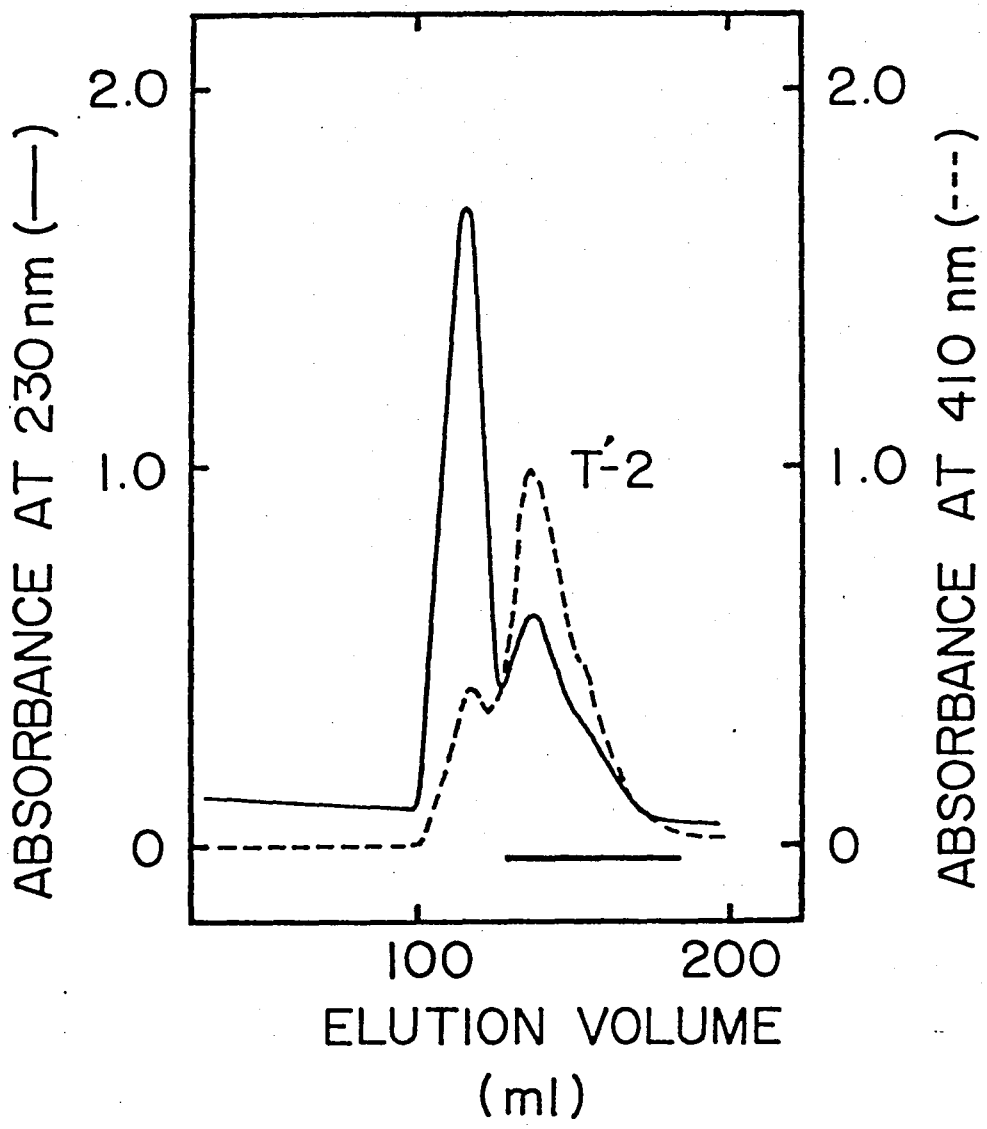


Fig.4. Elution pattern of pool I′ in Fig.2b.
Column: Sephadex G-50 (fine) (1.8 x 90 cm)
Buffer: 1 M NH_4OH
Flow rate: 4 ml/h

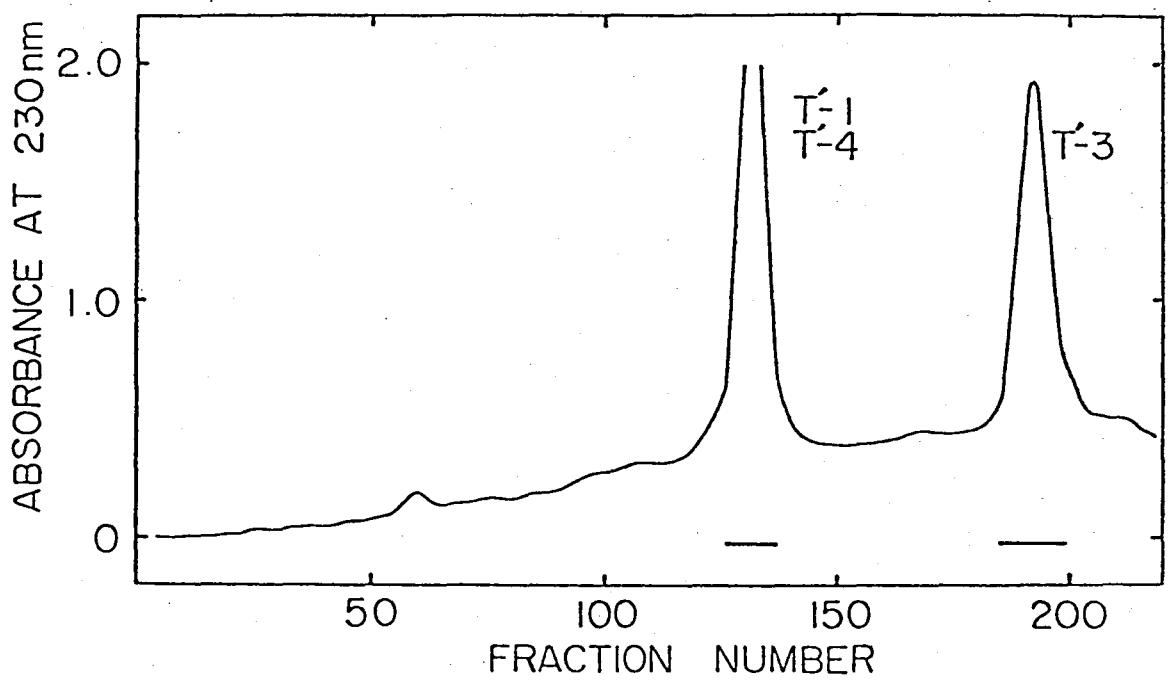


Fig.5. Chromatogram of pool II′ in Fig.2b on a DEAE-Sephadex column.
Column: DEAE-Sephadex A-25 (1.5 x 37 cm)
Elution: linear gradient from 0.1 M to 1 M NH_4HCO_3 (300 ml each)
Flow rate: 14 ml/h Fraction: 3 ml

also listed in Table II. The amino acid compositions of Peptides T'-1, T'-3 and T'-4 were the same as those of Peptides T-1, T-11 and T-13, respectively. Tryptophan was directly identified by manual Edman degradation of Peptide T'-3 as the first residue. Sequence determination was not attempted for the other two tryptophanyl peptides.

The heme peptide was analyzed after removal of heme group and carboxymethylation and the amino acid sequence up to the 10th residue was determined. CPase A and B released arginine (75 %) and tyrosine (72 %) along with a small amounts of alanine and valine at 40°C for 1 h in 0.1 M borate buffer, pH 8.0. Since the recovery of the heme peptide during purification procedures was low, further analysis could not be performed.

These studies on tryptic peptides revealed about 90 % of amino acid sequence of cytochrome $\underline{c}_1$. The peptide corresponding to residues 204 to 224 could not be obtained because of its low solubility. The leucine and methionine rich peptide was eluted when the Bio-Gel P-10 column was washed with the same buffer containing 30 % n-propanol, but further purification was unsuccessful.

Staphylococcal Protease Peptides

During the digestion of Cm-cytochrome c_1 with staphylococcal protease an insoluble material was produced. It was removed by centrifugation and the supernatant was fractionated on a Bio-Gel P-10 column as shown in Fig.6a. Pooled fractions I to VI were further purified by paper electrophoreses and chromatographies. The fractions eluted ahead of pool I contained heme peptide, but it was difficult to purify further. The precipitate was dissolved in 0.2 M ammonium acetate buffer, pH 4.0, and chromatographed on a Sephadex G-50 column as shown in Fig.7. The heme peptide, S-2, was purified from pool P-II by SP-Sephadex column chromatography (Fig.8). Several attempts were made to purify peptides from pool P-I, but none was obtained in pure form. Through these procedures 11 peptides were obtained and their amino acid compositions and properties are shown in Table V. Sequence studies of these peptides are summarized in Table VI. Manual Edman degradation was carried out on each peptide and amino acid sequence was determined as indicated.

Peptide S-8 (0.3 μmol) was further digested with chymotrypsin (0.02 mg) at room temperature for 4 h and subfragments S-8-C-1 and S-8-C-2 were obtained by paper electrophoresis at pH 6.5. Their amino acid compositions are listed in Table VII. Sequence studies were performed on S-8-C-1 (6 steps) and S-8-C-2 (9 steps).

To avoid the precipitation during incubation, Suc-Cm-cytochrome c_1 was digested with staphylococcal protease and chromatographed on a Bio-Gel P-10 column (Fig.6b). Almost all peptides obtained from the digest of Cm-cytochrome c_1 were also recovered from the fractions eluted after pool III', but the results on these

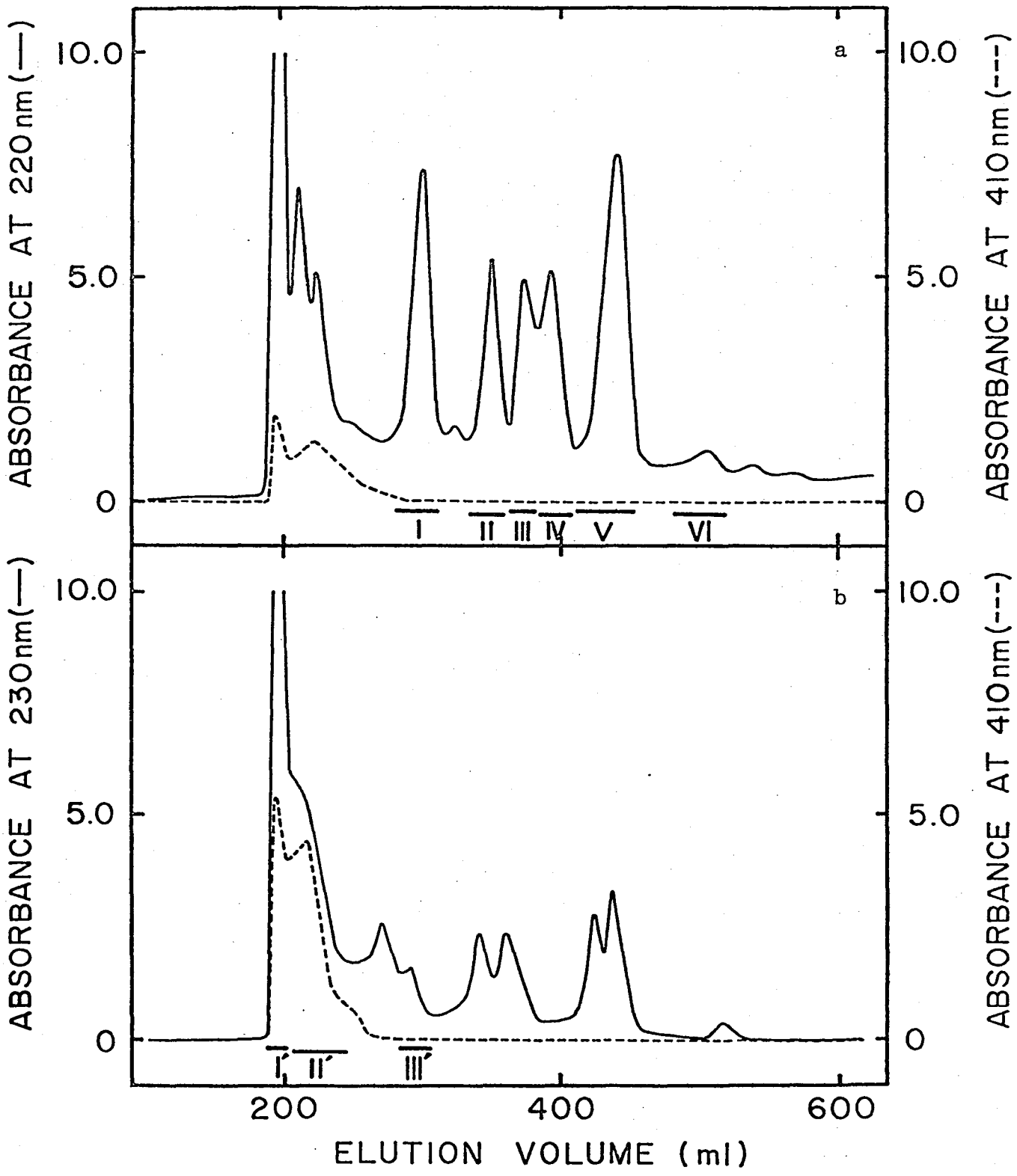


Fig.6. Elution patterns of staphylococcal protease digests.
a: Soluble part of staphylococcal protease digest of Cm-cytochrome $\underline{c}_1$
b: Staphylococcal protease digest of Suc-Cm-cytochrome $\underline{c}_1$
Column: Bio-Gel P-10 (2 x 180 cm)
Buffer: 0.2 M ammonium bicarbonate, pH 9.0
Flow rate: 10 ml/h

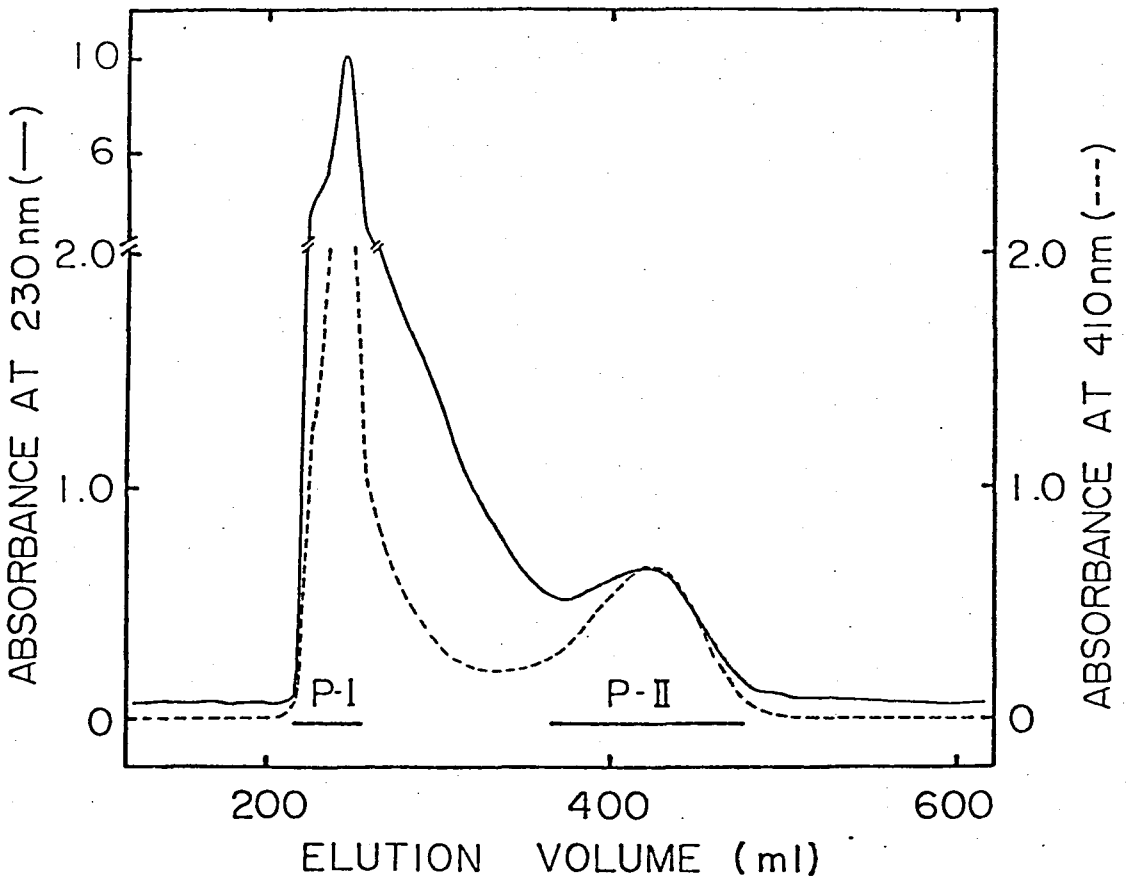


Fig.7. Elution pattern of insoluble part of staphylococcal protease digest of Cm-cytochrome $\underline{c}_1$
Column: Sephadex G-50 (fine) (2 x 180 cm)
Buffer: 0.2 M ammonium acetate, pH.4.0
Flow rate: 10 ml/h

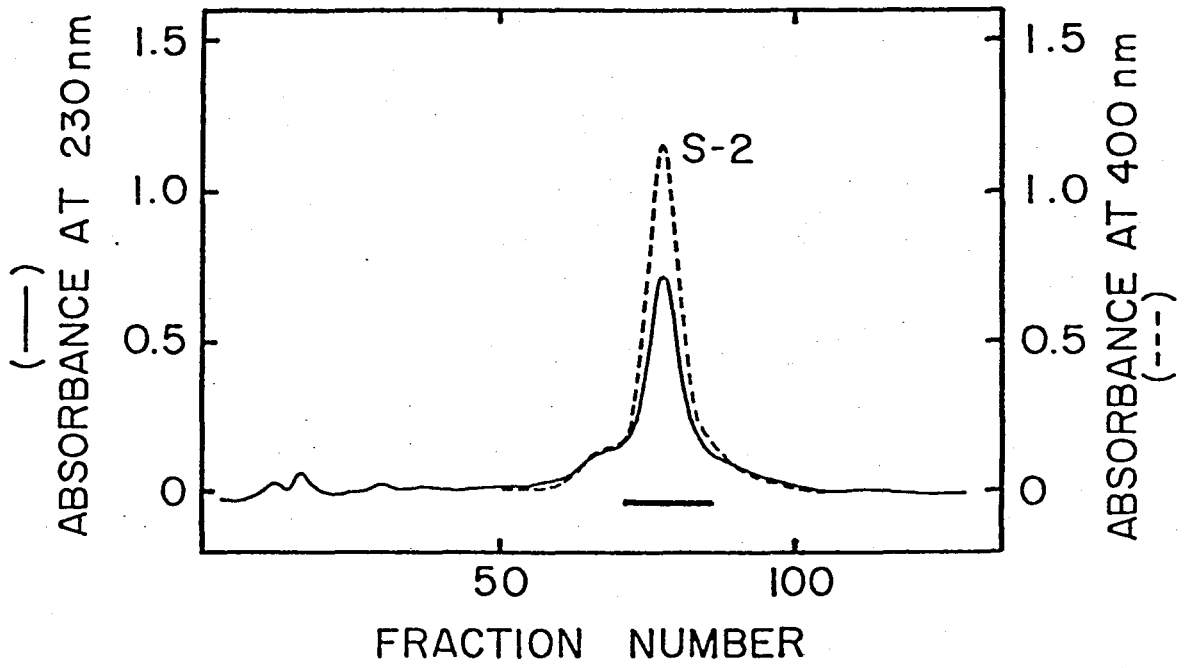


Fig.8. Chromatogram of pool P-II on an SP-Sephadex column.

Column: SP-Sephadex C-25 (1.3 x 30 cm)

Elution: linear gradient from 0.1 M to 0.55 M NaCl in 2 % phosphoric acid and 8 M urea (200 ml each)

Flow rate: 14 ml/h, Fraction: 3 ml

Table V Amino acid compositions[a] and some properties of staphylococcal protease peptides

Peptide Residue No.	S-1 1-4	S-2 5-60	S-3 61-67	S-4 68-69	S-5 70-79	S-6 80-89	S-7 90-99
Cmc		1.35(1)					
Asp	1.02(1)	3.27(3)			3.17(3)	1.07(1)	1.08(1)
Thr		1.93(2)					
Ser	0.90(1)	6.34(8)				0.97(1)	
Glu	1.08(1)	3.84(4)	2.01(2)	1.09(1)	3.19(3)		1.03(1)
Pro		3.01(3)			1.06(1)	0.97(1)	3.82(4)
Gly		3.36(3)			1.95(2)	1.00(1)	
Ala		1.35(1)	2.93(3)				
Val		4.69(5)		0.91(1)	0.84(1)		
Met		0.85(1)				1.45(2)	
Ile		1.07(1)					
Leu	0.89(1)	4.86(5)	1.03(1)			0.99(1)	
Tyr		2.83(5)					1.90(2)
Phe		1.18(1)				1.01(1)	0.99(1)
Lys		1.71(1)	0.98(1)			0.97(1)	1.00(1)
His		4.73(5)					
Arg		3.66(4)				1.01(1)	
Trp		N.D.(1)					
Total	4	56[b]	7	2	10	10	10
Yield(%)	57	16	45	14	15	13	42
Mobility[c]							
pH 3.6	+1.3	0~+4.0	+9.5	+4.0	N.D.	+12.0	+8.0
pH 6.5	-12.5	0.0	-3.3	N.D.	N.D.	+5.0	+1.0
Rf(BPAW)	0.36	N.D.	0.28	0.43	0.13	0.53[d]	0.58
Nin.Col.	Yellow	Negative	Violet	Violet	Violet	Violet	Violet
Pool(Fig.6)	III,IV	P-II	IV	V	I	VI	V
Purification	3.6	SP	3.6	3.6 BPAW	BPAW	3.6 BPAW	3.6

[a] Amino acid analyses were performed on 24h hydrolysates. Values in parentheses are taken from sequence.
[b] Including two cysteine residues linked to heme.
[c] Mobility is expressed in terms of cm (+) to the cathode at 43 V/cm for 1 h.
[d] Minor peptides (Rf=0.28 and 0.41) also gave the same amino acid composition.

Table V (continued)

Peptide Residue No.	S-8 100-124	S-9 125-145	S-10 146-167	S-11 168-170	S'-1 171-197	S'-2 198-241
Cmc		0.79(1)			0.90(1)	
Asp	3.15(3)	1.04(1)	2.20(2)		3.07(3)	1.27(1)
Thr		1.95(2)			2.73(3)	
Ser	1.03(1)	1.87(2)			0.95(1)	1.97(2)
Glu	1.10(1)	2.05(2)	2.03(2)	1.03(1)	3.08(3)	
Pro	1.82(2)	1.96(2)	3.57(4)		2.03(2)	2.62(3)
Gly	3.16(3)	1.93(2)	2.77(3)		1.10(1)	2.22(2)
Ala	5.84(6)		2.41(2)		3.80(4)	2.15(2)
Val	0.54(1)	2.14(2)		1.02(1)	1.88(2)	2.22(2)[e]
Met			0.56(1)		0.93(1)	5.13(5)
Ile	0.68(1)		1.52(2)			
Leu	1.91(2)	2.96(3)	1.11(1)	0.95(1)	1.00(1)	8.56(9)
Tyr	0.96(1)	1.87(2)	2.42(3)			1.90(2)
Phe		0.96(1)	1.82(2)		1.94(2)	
Lys					0.88(1)	6.63(7)[e]
His	0.75(1)					2.90(3)
Arg	2.99(3)	0.95(1)			0.90(1)	5.05(5)
Trp					N.D.(1)	0.33(1)
Total	25	21	22	3	27	44
Yield(%)	25	28	15	32	11	27
Mobility						
pH 3.6	+9.3	N.D.	+2.8	+4.7	0	N.D.
pH 6.5	+0.5	N.D.	-0.8	N.D.	-6.3	N.D.
Rf(BPAW)	0.18	0.64	N.D.	0.69	N.D.	N.D.
Nin.Col.	Violet	Violet	Yellow	Violet	Violet	N.D.
Pool(Fig.6)	II,III	I	II	V	III'	I'
Purification	6.5	BPAW	6.5	3.6 BPAW	DEAE 6.5	DEAE

[e]These values were obtained from 72h hydrolysate, since succinyllysine is rather resistant for 24h hydrolysis and co-elutes with valine in the single column system analyzer.

Table VI. Summary of sequence studies on staphylococcal protease peptides

Peptide	Residue No.	
S-1	1-4	Ser-Asp-Leu-Glu
S-2	5-60	Leu-His-Pro-Pro-Ser-Tyr(Cmc^{1}, Asx^{3}, Thr^{2}, Ser^{7}, Glx^{4}, Pro^{1}, Gly^{3}, Ala^{1}, Cys^{2}, Val^{5}, Met^{1}, Ile^{1}, Leu^{4}, Tyr^{4}, Phe^{1}, Lys^{1}, His^{4}, Arg^{4}, Trp^{1})
S-3	61-67	Ala-Lys-Ala-Leu-Ala-Glu-Glu
S-5	70-79	Val-Gln-Asp-Gly-Pro-Asn-Glu-Asp-Gly-Glu
S-6	80-89	Met-Phe-Met-Arg-Pro-Gly-Lys-Leu(Ser)Asp
S-8	100-124	Ala-Ala-Arg-Ala-Ala-Asn(Asx^{2}, Ser^{1}, Pro^{2}, Gly^{1}, Ala^{1}, Leu^{2}, Tyr^{1}) Ile-Val-Arg-Ala-Arg-His-Gly-Gly-Glu
S-9	125-145	Asp-Tyr-Val-Phe-Ser-Leu-Leu-Thr-Gly-Tyr-Cmc-Glu-Pro-Pro(Thr^{1}, Ser^{1}, Glx^{1}, Gly^{1}, Val^{1}, Leu^{1}, Arg^{1})
S-10	146-167	Gly-Leu-Tyr-Phe-Asn-Pro-Tyr-Phe-Pro-Gly-Gln-Ala-Ile-Gly-Met-Ala(Asx^{1}, Glx^{1}, Pro^{2}, Ile^{1}, Tyr^{1})

C-1

C-2

S-1 171-197 Phe-Asp-Asp-Gly-Thr-Pro-Ala-Thr-Met-Ser-Gln-Val-Ala-Lys*-Asp-Val(Cmc^{1}, Thr^{1}, Glx^{2}, Pro^{1}, Ala^{2}, Leu^{1}, Phe^{1}, Arg^{1}, Trp^{1})

S-2 198-241 His-Asp-His-Arg-Lys*-Arg-Met-Gly-Leu-Lys*-Met-Leu-Leu-Met-Met-Gly-Leu-Leu-Leu-Pro-Leu-Val-Tyr-Ala-Met-Lys*-Arg(Ser^{2}, Val^{1}, Leu^{1}, Lys^{2}, His^{1}, Arg^{1}, Trp^{1})(Pro^{2}, Ala^{1}, Leu^{1}, Tyr^{1}, Lys^{2}, Arg^{1})

T-1, T-2, T-3, T-4, T-5

The arrow (⟶) and (⟿) indicate that the residue above was identified by manual Edman degradation and automated Edman degradation on a solid-phase sequencer, respectively.

Table VII. Amino acid compositions of subfragments of staphylococcal protease peptides

	S-8		S-2				
	C-1	C-2	T-1	T-2	T-3	T-4	T-5
Asp	3.08(3)		1.05(1)				
Ser	0.98(1)					1.65(2)	
Glu		1.05(1)					
Pro	1.94(2)				1.07(1)		2.08(2)
Gly	1.29(1)	1.95(2)			2.08(2)		
Ala	4.94(5)	1.08(1)			1.02(1)		0.90(1)
Val		0.68(1)			1.33(1)	1.05(1)	
Met					4.70(5)		
Ile		0.62(1)					
Leu	2.05(2)				6.84(7)	0.95(1)	1.08(1)
Tyr	0.96(1)				0.98(1)		0.95(1)
Lys				1.03(1)	2.43(2)	1.75(2)	2.03(2)
His		0.90(1)	1.78(2)			1.23(1)	
Arg	1.02(1)	2.00(2)	1.06(1)	0.97(1)	1.42(1)	0.88(1)	0.96(1)
Trp						N.D.(1)	
Total	16	9	4	2	21	9	8
Yield(%)	44	31	43	33	24	26	50
Mobility							
pH 3.6	N.D.	N.D.	+15.0	+8.0	0	+5.3	+4.2
pH 6.5	-4.7	+7.5	N.D.	N.D.	N.D.	N.D.	N.D.

peptides will not be mentioned here. Only pools I′ to III′ are described from which the peptides in the C-terminal region and heme peptide were obtained. Pool I′ was further chromatographed on a DEAE-Sephadex column in the presence of 30 % n-propanol as shown in Fig.9, and very hydrophobic peptide, S′-2, could be purified. Pool II′ contained the heme peptide corresponding to Peptide S-2 and was directly digested with TPCK-trypsin and chromatographed on a Bio-Gel P-6 column (Fig.10), and 4 tryptic subfragments, S′-T-1 to S′-T-4 were obtained by paper electrophoreses. S′-T-3 was the heme peptide but impure. From pool III′, Peptide S′-1 was purified by paper electrophoresis at pH 6.5. The amino acid compositions of Peptides S′-1 and S′-2 are listed in Table V and those of Peptides S′-T-1 to S′-T-4 in Table VIII.

Manual Edman degradations were carried out on Peptides S′-1 (16 steps) and S′-2 (20 steps) as shown in Table VI. Peptide S′-2 (0.58 μmol) was further digested with TPCK-trypsin (0.06 mg) in 0.3 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 30 % n-propanol at 40°C for 2 h and 5 subfragments were purified by paper electrophoresis at pH 3.6. The amino acid compositions of these subfragments are listed in Table VII.

The subfragment S′-2-T-3 was analyzed on the solid-phase sequencer after treatment with 50 % hydrazine, giving two spots on TLC at each step except for the 1st, 2nd, 13th, and 15th steps. The identified residues were as follows: X-Gly-$\frac{\text{Met}}{\text{Leu}}$-$\frac{\text{Gly}}{\text{Lys}^*}$-$\frac{\text{Leu}}{\text{Met}}$-$\frac{\text{Lys}^*}{\text{Leu}}$-$\frac{\text{Met}}{\text{Leu}}$-$\frac{\text{Leu}}{\text{Met}}$-$\frac{\text{Leu}}{\text{Met}}$-$\frac{\text{Met}}{\text{Gly}}$-$\frac{\text{Met}}{\text{Leu}}$-$\frac{\text{Gly}}{\text{Leu}}$-Leu-$\frac{\text{Leu}}{\text{Pro}}$-Leu-$\frac{\text{Pro}}{\text{Val}}$-$\frac{\text{Leu}}{\text{Tyr}}$-$\frac{\text{Val}}{\text{Ala}}$-$\frac{\text{Tyr}}{\text{Met}}$-$\frac{\text{Ala}}{\text{Lys}^*}$-. This sequence was interpreted by two residues extension of succinyllysine and arginine at the N-terminus which was the result of incomplete cleavage of Arg-Met bond. This was supported by amino

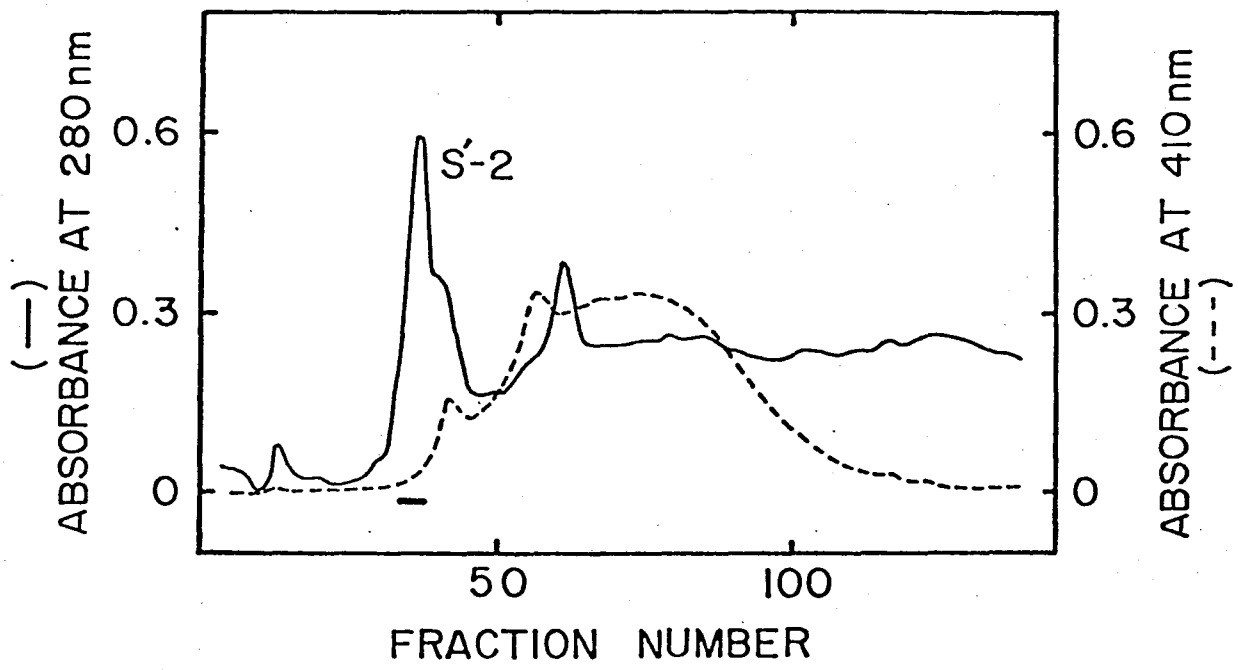


Fig.9. Chromatogram of pool I' in Fig.6b on a DEAE-Sephadex column.
Column: DEAE-Sephadex A-25 (1.3 x 30 cm)
Elution: linear gradient from 0.1 M to 1 M NH_4HCO_3 containing 30 % n-propanol (250 ml each)
Flow rate: 14 ml/h
Fraction: 3.5 ml

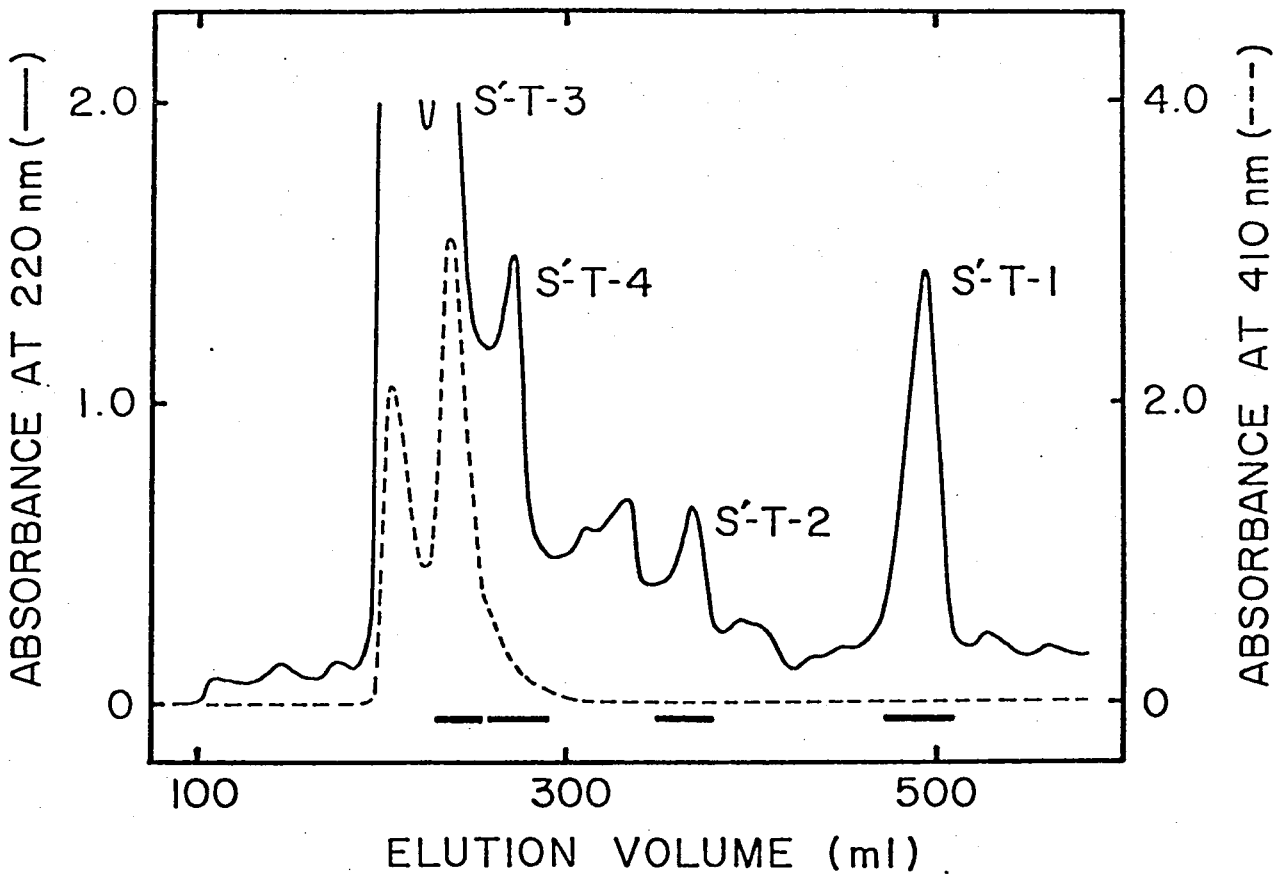


Fig.10. Elution pattern of tryptic digest of pool II′ in Fig.6b.
Column: Bio-Gel P-6 (2 x 180 cm)
Buffer: 0.2 M ammonium bicarbonate, pH 9.0
Flow rate: 10 ml/h

Table VIII. Amino acid compositions of Peptides S'-T-1 to S'-T-4

	S'-T-1	S'-T-2	S'-T-3	S'-T-4
Cmc			0.17	1.03(1)
Asp		1.09(1)	1.85(1)	1.26(1)
Thr		1.00(1)	0.58	1.01(1)
Ser	2.03(2)	2.78(3)	2.27(3)	
Glu			2.26(2)	2.07(2)
Pro	3.18(3)		0.89	
Gly		0.94(1)	1.49(1)	1.06(1)
Ala			1.38(1)	
½Cys			0.86(2)	
Val			2.78(3)	1.77(2)
Met			0.95(1)	
Ile		0.93(1)	0.31	
Leu	0.96(1)	2.93(3)	0.92	1.07(1)
Tyr	0.94(1)		1.49(3)	0.89(1)
Phe			1.02(1)	
Lys			1.03(1)	
His	2.04(2)	1.03(1)	1.03(1)	0.82(1)
Arg	1.01(1)	1.02(1)	0.91(1)	
Trp	N.D.(1)			
Total	11	12	21	11

acid composition of S-2-T-3. The heme containing subfragment S-T-3 was also analyzed on the solid-phase sequencer after hydrazine treatment and following sequence was revealed: X-Phe-Gln-Val-Tyr-Lys-Gln-Val-X-X-X-X-His-X-Met-Asp-Tyr-Val-Ala, where X was an unidentified residue and the last three steps were ambiguously identified.

Staphylococcal protease peptides thus obtained covered all the regions of cytochrome $\underline{c}_1$ and about 60 % of these peptides were sequenced.

Chymotryptic Peptides

Maleyl-Cm-cytochrome $\underline{c}_1$ was digested with chymotrypsin and fractionated on a Bio-Gel P-10 column as shown in Fig.11. Pool III was chromatographed on a Bio-Gel P-6 column as in Fig.12. Pool IIIa thus obtained was further separated on a small DEAE-Sephadex column (Fig.13). Pools I, II, IIIb, IV and V were separated on DEAE-Sephadex columns (Fig.14), and further purified by paper electrophoreses and chromatographies. Twenty peptides were purified and their amino acid compositions and properties are listed in Table IX. Several other peptides were also purified and found to be derived by heterogeneous cleavage, but they will not be described here. The amino acid sequences of these peptides were determined as summarized in Table X. Lysine-containing peptides and Peptide C-1 were demaleylated before sequence analyses.

Peptide C-3 was studied on the solid-phase sequencer after carbodiimide activation and its complete sequence was determined, but Peptide C-16 could not be attached to the glass support and sequence study could not be performed.

Peptide C-4 had a blocked N-terminus and was assumed that glutamine was the N-terminal residue which cyclized to form pyroglutamic acid.

Peptide C-5 was the heme peptide. It contained no carboxymethylcysteine residue before heme removal, but after heme removal and carboxymethylation of this peptide, over one residue of carboxymethylcysteine was recovered. Manual Edman degradation was carried out on modified peptide up to 11th residue and 8th and 11th steps were ambiguously identified. Tyrosine (81 %), alanine (33 %) and valine (27 %) were released by CPase for 1 h at 40°C in 0.1 M borate buffer, pH 8.0.

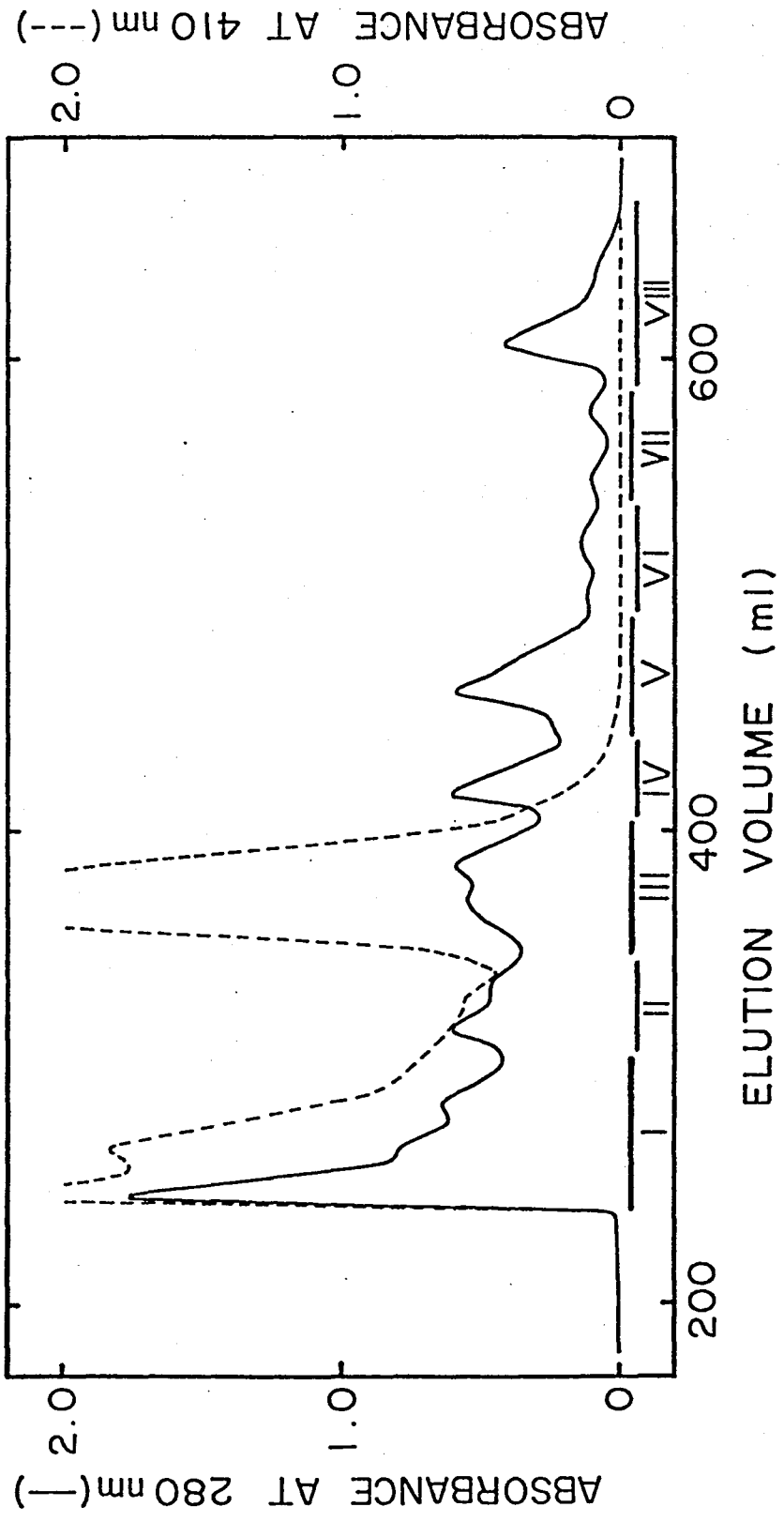


Fig.11. Elution pattern of chymotryptic digest of maleyl-Cm-cytochrome $\underline{c}_1$
Column: Bio-Gel P-10 (2 x 180 cm)
Buffer: 0.2 M ammonium bicarbonate, pH 9.0
Flow rate: 10 ml/h

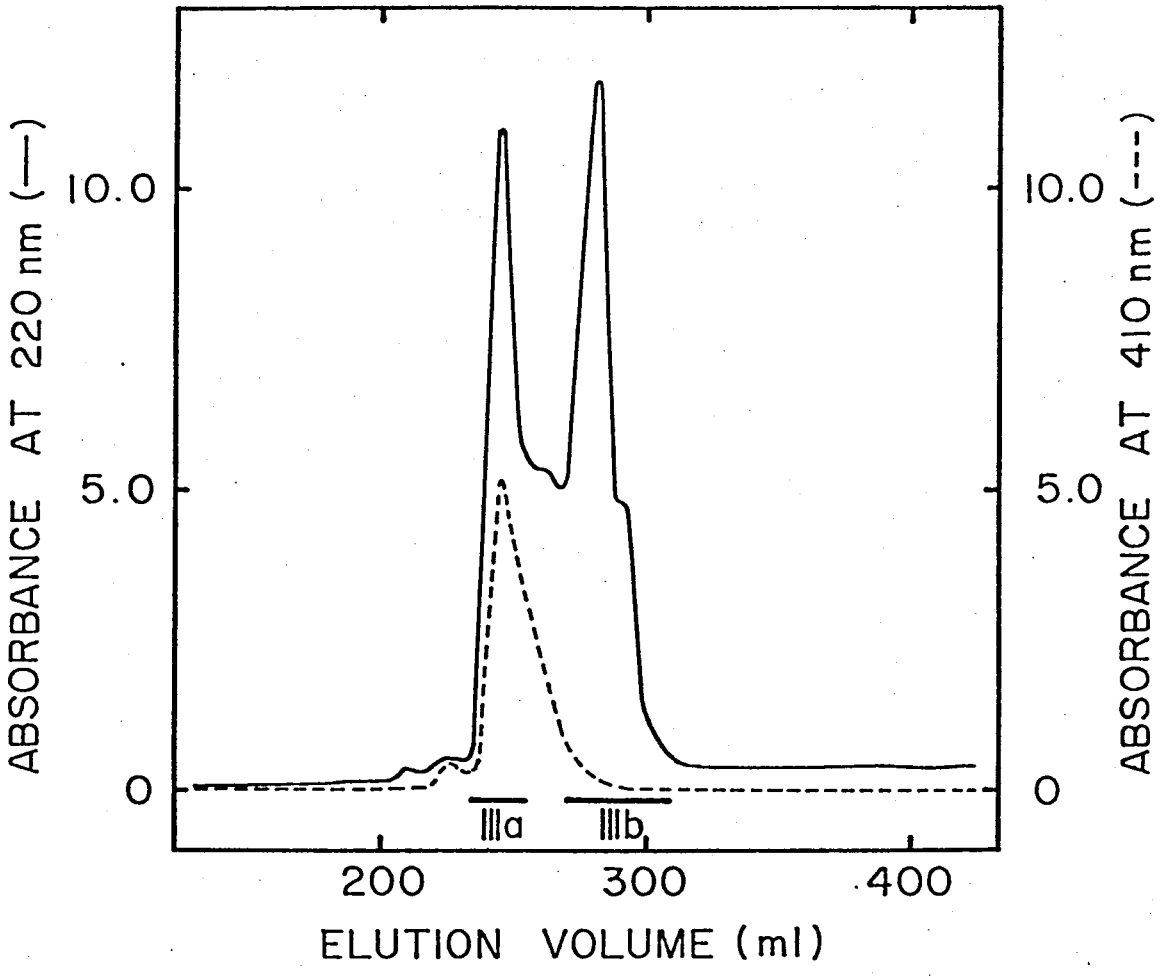


Fig.12. Elution pattern of pool III in Fig.11.
Column: Bio-Gel P-6 (2 x 180 cm)
Buffer: 0.2 M ammonium bicarbonate, pH 9.0
Flow rate: 10 ml/h

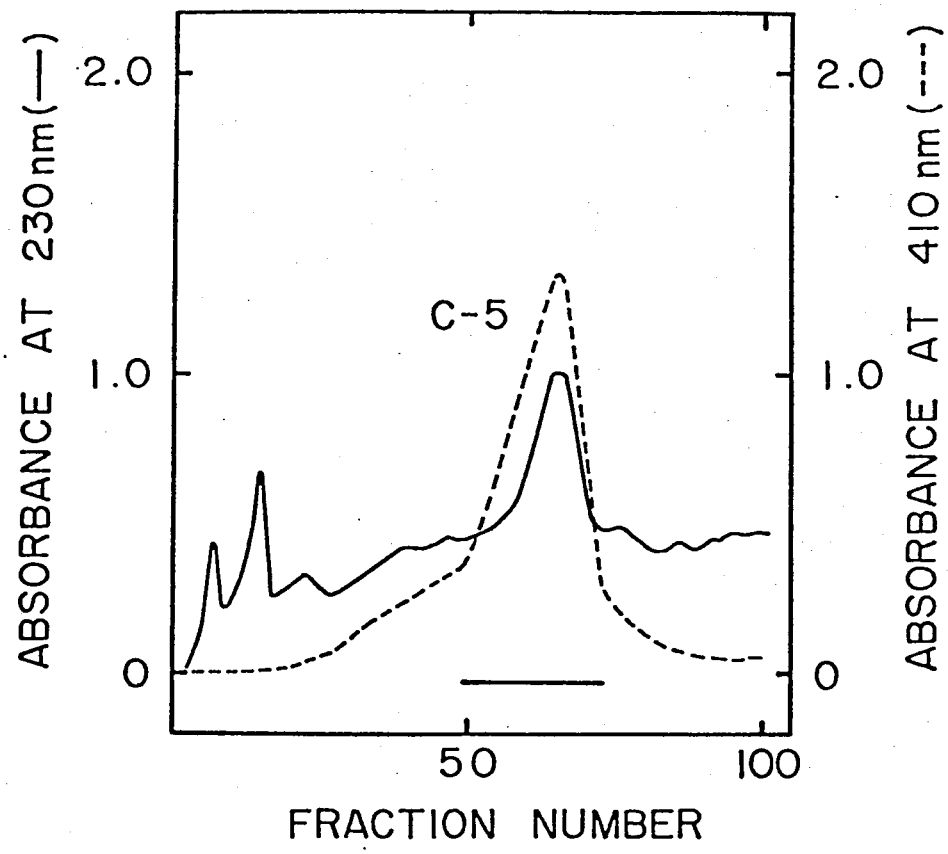


Fig.13. Chromatogram of pool IIIa on a DEAE-Sephadex column.
Column: DEAE-Sephadex A-25 (0.7 x 5 cm)
Elution: linear gradient from 0.2 M to 1 M NH_4HCO_3 (100 ml each)
Flow rate: 2.7 ml/h
Fraction: 2 ml

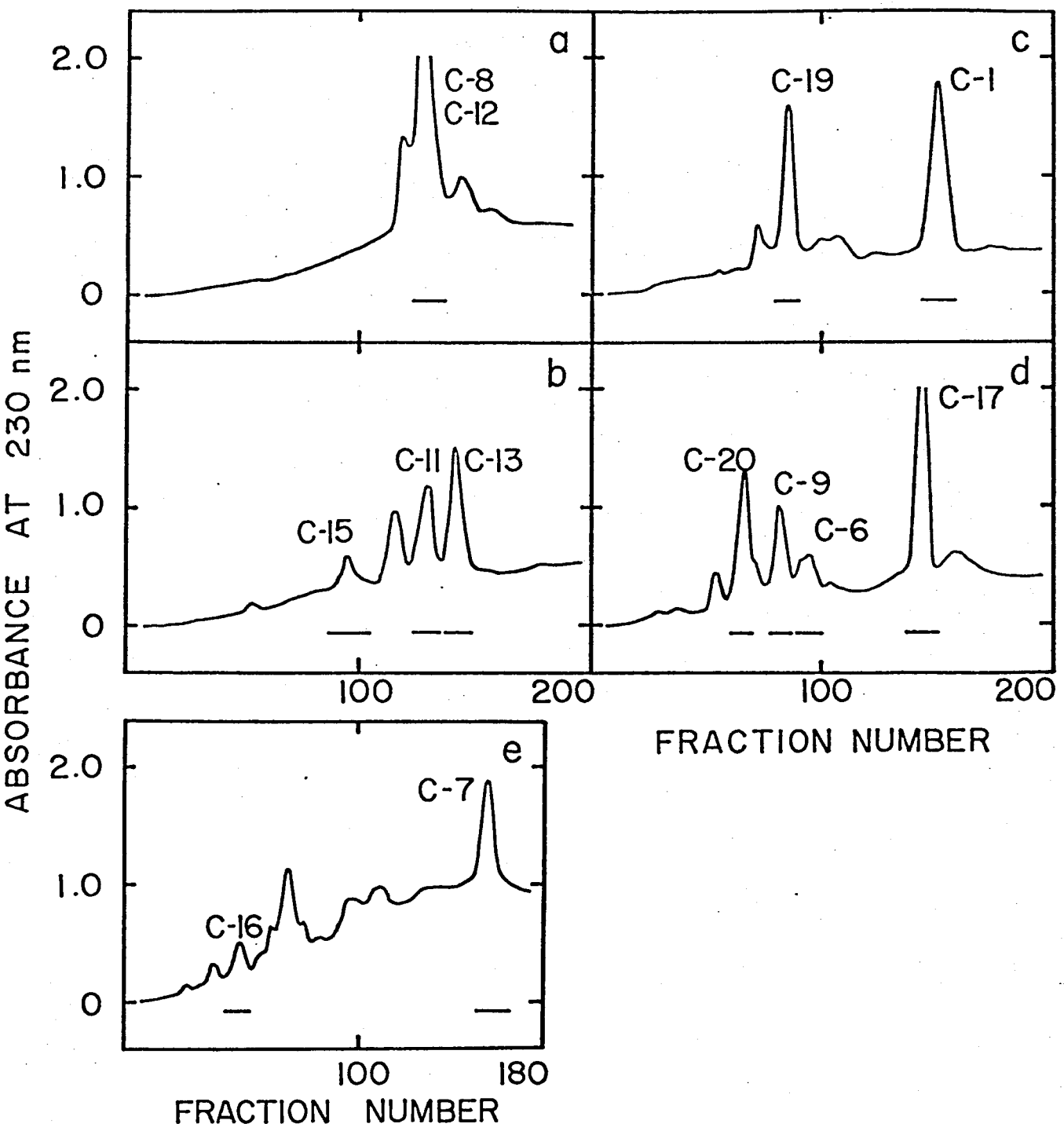


Fig.14. Chromatograms of pool I, II, IIIb, IV and V in Figs. 11 and 12.

a: Pool II, b: Pool IIIb, c: Pool IV, d: Pool V, e: Pool I

Column: a-d, DEAE-Sephadex A-25 (1.5 x 37 cm)
e, DEAE-Sephadex A-25 (1.3 x 27 cm)

Elution: a-d, linear gradient from 50 mM to 1 M NH_4HCO_3 (300 ml each)
e, linear gradient from 50 mM to 1 M NH_4HCO_3 (230 ml each)

Flow rate: 14 ml/h, Fraction: 2.7 ml

Table IX Amino acid compositions[a] and some properties of chymotryptic peptides

Peptide	C-1	C-2	C-3	C-4	C-5	C-6	C-7
Residue No.	1-12	13-18	19-30	31-33	34-48	49-56	57-81
Cmc					1.42(2)[b]	0.96(1)	
Asp	1.04(1)		0.99(1)		1.03(1)		4.03(4)
Thr			0.99(1)				0.86(1)
Ser	1.88(2)	0.93(1)	2.76(3)		2.58(3)		
Glu	1.04(1)			1.03(1)	1.28(1)		7.83(8)
Pro	2.95(3)						1.05(1)
Gly		1.01(1)	0.98(1)			1.05(1)	1.99(2)
Ala					0.94(1)		2.89(3)
Val				1.01(1)	1.98(2)	2.02(2)	2.10(2)
Met					0.83(1)		0.93(1)
Ile			0.95(1)				
Leu	1.98(2)	1.91(2)	1.10(1)			0.96(1)	1.04(1)
Tyr	0.87(1)			0.96(1)	0.99(2)	0.99(1)	
Phe			0.97(1)				0.92(1)
Lys					0.78(1)		1.06(1)
His	0.93(1)	0.99(1)	0.97(1)		0.94(1)	0.96(1)	
Arg		1.03(1)	2.10(2)			1.02(1)	
Trp	0.53(1)						
Total	12	6	12	3	15	8	25
Yield(%)	41	24	35	20	15	15	44
Mobility[c]							
pH 3.6	0.0	+13.1	+10.0	-1.9	N.D.	+7.2	0.0
pH 6.5	-6.8	+6.6	+5.2	-5.6	N.D.	+2.3	-12.3
Rf(BPAW)	N.D.	0.56	0.50	0.77	N.D.	N.D.	N.D.
Nin.Col.	Negative	Yellow	Yellow	Negative	N.D.	Violet	Yellow
Pool(Fig.11)	IV	VI	VI	VI,VII	III	V	I
Purification	DEAE	3.6	3.6	3.6	P-6 DEAE	DEAE 6.5	DEAE 6.5

[a] Amino acid analyses were performed on 24h hydrolysates. Values in parentheses are from sequence.
[b] This value was obtained after heme removal and carboxymethylation of purified peptide.
[c] Mobility is expressed in terms of cm (+) to the cathode at 43 V/cm for 1 h.

Table IX (continued)

Peptide Residue No.	C-8 82-115	C-9 116-128	C-10 129-131	C-11 132-148	C-12 149-179	C-13 180-189	C-14 190-192
Cmc				1.02(1)		0.81(1)	
Asp	4.68(5)	0.97(1)			3.75(4)	0.96(1)	
Thr				1.91(2)	2.09(2)	1.02(1)	
Ser	1.91(2)	0.23	0.92(1)	1.10(1)		0.98(1)	
Glu	1.11(1)	1.03(1)		1.99(2)	3.36(3)	1.06(1)	
Pro	6.98(7)			2.15(2)	5.25(5)		
Gly	2.00(2)	2.05(2)		2.61(3)	3.02(3)		
Ala	4.73(5)	0.99(1)			2.75(3)	1.01(1)	
Val		1.36(2)		1.20(1)	1.10(1)	2.05(2)	
Met	1.02(1)				2.09(2)		
Ile		0.46(1)			1.99(2)		
Leu	2.82(3)	0.44	2.00(2)	2.11(2)	1.06(1)		0.96(1)
Tyr	2.80(3)	1.06(1)		1.88(2)	1.72(2)		
Phe	0.89(1)	0.86(1)			2.78(3)	0.95(1)	
Lys	2.20(2)					1.01(1)	
His		0.99(1)					
Arg	2.25(2)	2.11(2)		1.06(1)			1.04(1)
Trp							0.44(1)
Total	34	13	3	17	31	10	3
Yield(%)	21	44	14	30	11	61	29
Mobility							
pH 3.6	+1.8	N.D.	+6.2	N.D.	0.0	N.D.	+7.0
pH 6.5	-4.9	N.D.	+0.7	N.D.	-4.9	N.D.	+3.4
Rf(BPAW)	N.D.	N.D.	0.79	N.D.	N.D.	N.D.	0.58
Nin.Col.	Violet	N.D.	Yellow	N.D.	Violet	N.D.	Violet
Pool(Fig.11)	II	V	VI	III	II	III	VIII
Purification	DEAE 3.6	DEAE	3.6 BPAW	P-6 DEAE	DEAE 3.6	P-6 DEAE	3.6

Table IX (continued)

Peptide Residue No.	C-15 193-206	C-16 211-220	C-17 221-227	C-18 228-230	C-19 231-235	C-20 236-241
Cmc						
Asp	1.07(1)					
Thr						
Ser				0.91(1)	0.89(1)	
Glu	2.21(2)					
Pro	1.17(1)	1.02(1)				2.04(2)
Gly	1.17(1)	1.19(1)	0.98(1)			
Ala	2.13(2)					1.01(1)
Val		1.00(1)		0.98(1)		
Met	0.91(1)	1.82(2)	1.00(1)			
Ile						
Leu	0.97(1)	4.33(4)		1.02(1)	1.05(1)	
Tyr		0.81(1)				0.97(1)
Phe						
Lys	1.00(1)		2.05(2)		1.87(2)	1.15(1)
His	1.80(2)		1.00(1)			
Arg	1.82(2)		1.01(1)		1.02(1)	0.87(1)
Trp			0.60(1)			
Total	14	10	7	3	5	6
Yield(%)	10	8	39	19	48	20
Mobility						
pH 3.6	+8.6	+5.6	N.D.	+6.2	N.D.	+6.6
pH 6.5	N.D.	+1.7	N.D.	+0.7	N.D.	+1.1
Rf(BPAW)	N.D.	N.D.	N.D.	0.72	N.D.	N.D.
Nin.Col.	Violet	Violet	N.D.	Yellow	N.D.	Violet
Pool(Fig.11)	III	I	V	VI	IV	V
Purification	P-6 DEAE 3.6	DEAE 3.6	DEAE	3.6 BPAW	DEAE	DEAE 3.6

Table X. Summary on sequence studies on chymotryptic peptides.

Peptide	Residue No.	
C-1	1-12	Ser-Asp-Leu-Glu-Leu-His-Pro-Pro(Ser)Tyr(Pro)Trp
C-2	13-18	Ser-His-Arg-Gly-Leu-Leu
C-3	19-30	Ser-Ser-Leu-Asp-His-Thr-Ser-Ile-Arg-Arg-Gly-Phe
C-5	34-48	Lys-Gln-Val-Cmc-Ser-Ser-Cmc-His-Ser-Met-Asx(Tyr) Val-Ala-Tyr
C-6	49-56	Arg-His-Leu-Val-Gly-Val-Cmc-Tyr
C-7	57-81	Thr-Glu-Asp-Glu-Ala-Lys-Ala-Leu-Ala-Glu-Glu(Asx^3, Glx^4, Pro^1, Gly^2, Val^2, Met^1)Phe
C-8	82-115	Met-Arg-Pro-Gly-Lys-Leu-Ser-Asp-Tyr-Phe-Pro-Lys-Pro-Tyr-Pro-Asn-Pro-Glu-Ala-Ala(Arg)Ala(Asx^3, Ser^1, Pro^2, Gly^1, Ala^2, Leu^2, Tyr^1)
C-9	116-128	Ile-Val-Arg-Ala-Arg-His-Gly-Gly-Glu-Asp(Val^1, Tyr^1, Phe^1)
C-11	132-148	Thr-Gly-Tyr-Cmc-Glu-Pro-Pro-Thr-Gly-Val-Ser-Leu-Arg-Glu-Gly(Leu^1, Tyr^1)

C-12 149-179 Phe-Asn-Pro-Tyr-Phe-Pro-Gly-Gln-Ala-Ile-Gly-Met-Ala-Pro-Pro-Ile-Tyr-Asn-Glu-Val-Leu-Glu(Asx^2, Thr^2, Pro^1, Gly^1, Ala^1, Met^1, Phe^1)

C-13 180-189 Ser-Gln-Val-Ala-Lys-Asp-Val-Cmc-Thr-Phe

C-14 190-192 Leu-Arg-Trp

C-15 193-206 Ala-Ala-Glu-Pro-Glu-His-Asp-His-Arg-Lys-Arg(Gly^1, Met^1, Leu^1)

C-17 221-227 Ala-Met-Lys-Arg-His-Lys-Trp

C-19 231-235 Lys-Ser-Arg(Lys)Leu

C-20 236-241 Ala-Tyr-Arg-Pro-Pro-Lys

CPase A released tryptophan from Peptide C-1 (50 %), C-14 (94 %) and C-17 (96 %) after 1 h digestion on 0.1 M borate buffer, pH 8.0. Peptide C-7 was incubated with CPase Y in 0.1 M pyridine-acetate buffer, pH 5.5, for 1 h and phenylalanine (57 %) was found on the analyzer.

Chymotryptic peptides covered about 99 % of the protein and about 75 % of them were sequenced.

Cyanogen Bromide Fragments

Although the complete amino acid sequence of cytochrome c_1 could be constructed from tryptic, staphylococcal protease and chymotryptic peptides, cyanogen bromide fragments were also isolated to confirm this sequence. The lyophilized cyanogen bromide peptide mixture was first fractionated on a Sephadex G-50 column as shown in Fig.15. Pools I to IV were further chromatographed on SP-Sephadex columns (Fig.16) and pools V to VII were subjected to paper electrophoreses at pH 3.6. Eight peptides were purified and their amino acid compositions are listed in Table XI. Manual Edman degradations were carried out on Peptides CB-1 (2 steps), CB-2 (12 steps), CB-3 (2 steps), CB-4 (2 steps), CB-5 (2 steps) and CB-8 (14 steps). Solid-phase sequencing was performed on Peptides CB-6 (4 steps) and CB-7 (10 steps) after attaching them to the glass through homoserine lactone method. These results are summarized in Table XII.

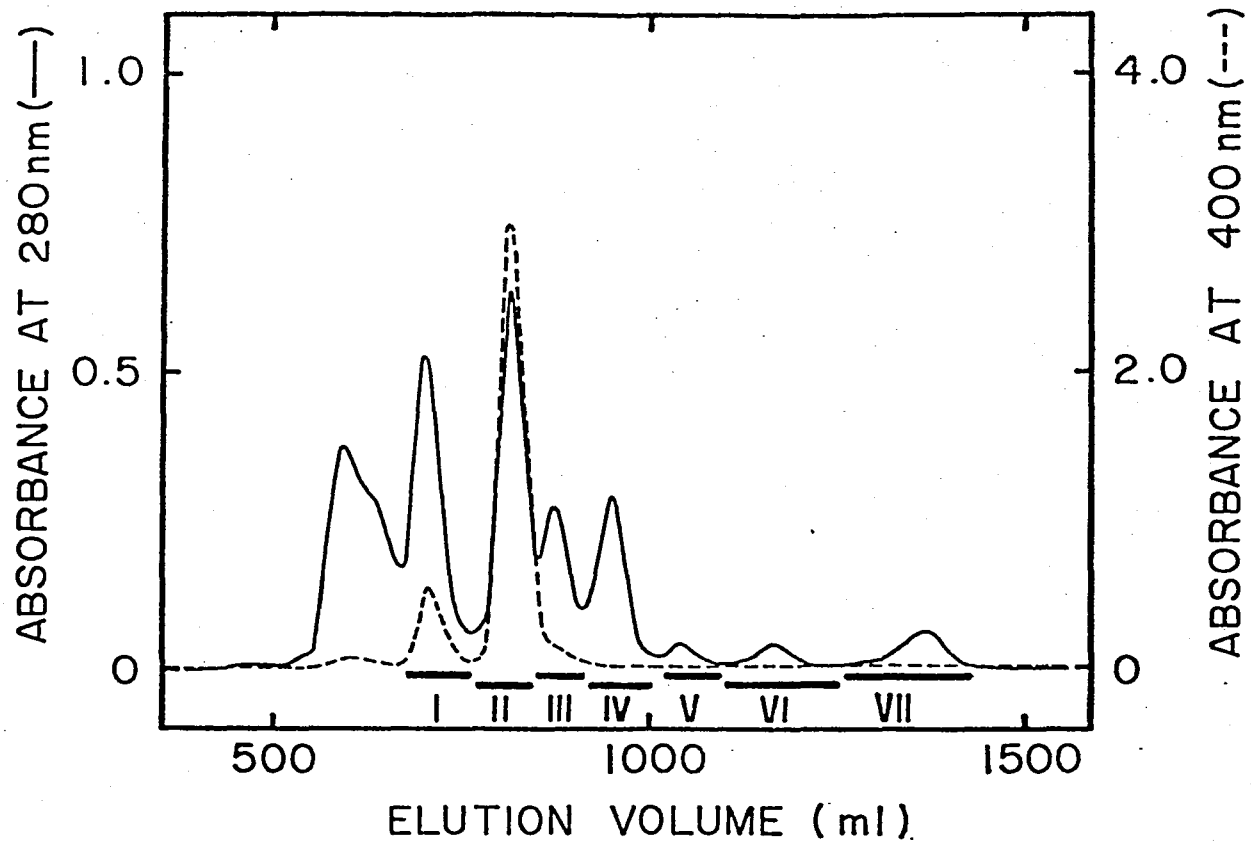


Fig.15. Elution pattern of cyanogen bromide peptide mixture.
Column: Sephadex G-50 (fine) (4 x 110 cm)
Buffer: 10 % formic acid containing 8 M urea
Flow rate: 20 ml/h

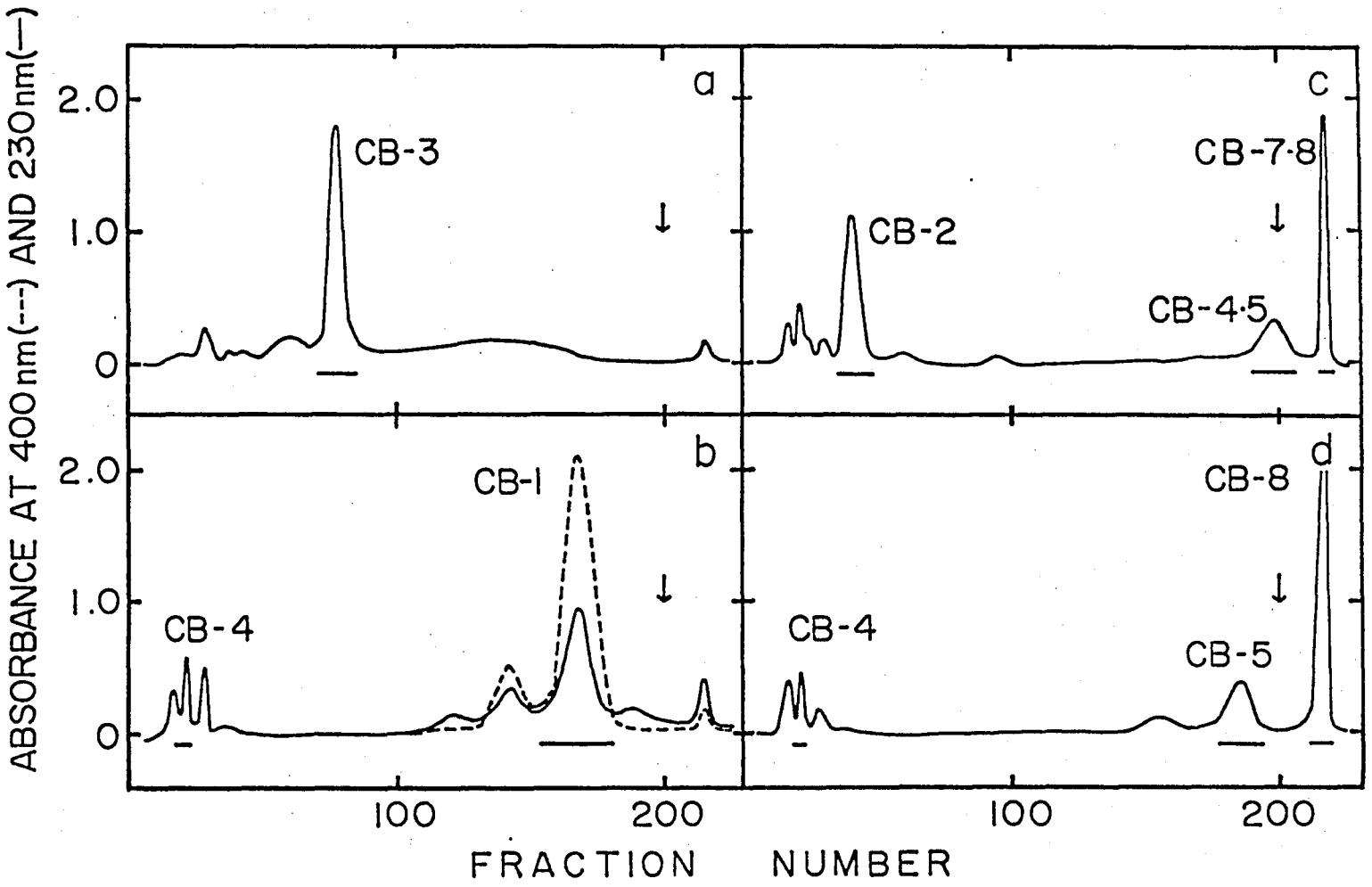


Fig.16. Chromatograms of pools I to IV in Fig.15 on SP-Sephadex columns
Column: SP-Sephadex C-25 (1.5 x 37 cm) a: I, b: II, c: III, d: IV
Elution: linear gradient from 50 mM to 350 mM NaCl in 2.5 % phosphoric acid and 8 M urea (300 ml each)
Flow rate: 20 ml/h, Fraction: 3 ml

Table XI. Amino acid compositions[a] of cyanogen bromide peptides

Peptide Residue No.	CB-1 1-43	CB-2 44-80	CB-3 83-160	CB-4 161-179	CB-5 180-204	CB-6 205-208	CB-7 213-222	CB-8 223-241
Cmc		0.91(1)	0.81(1)		0.92(1)			
Asp	2.09(2)	5.15(5)	7.08(7)	2.72(3)	1.99(2)			
Thr	1.00(1)	0.99(1)	2.03(2)	1.62(2)	0.95(1)			
Ser	6.85(9)		3.46(4)		0.93(1)			1.75(2)
Glu+Hse[b]	3.20(3)	8.33(8)	5.21(5)	2.61(2)	3.27(3)	0.27		
Pro	2.74(3)	1.05(1)	10.8(11)	2.81(3)	0.98(1)		1.03(1)	1.93(2)
Gly	2.11(2)	3.49(3)	9.20(9)	1.44(1)		0.99(1)	1.03(1)	
Ala	0.67	4.20(4)	6.79(7)	1.70(2)	2.92(3)		0.90(1)	0.97(1)
Val	2.16(2)	4.94(5)	3.21(3)	1.49(1)	2.27(2)		1.19(1)	1.16(1)
Ile	0.83(1)		1.69(2)	0.84(1)				
Leu	4.60(5)	2.05(2)	6.66(7)	0.93(1)	1.01(1)	0.97(1)	3.88(4)	1.98(2)
Tyr	0.73(2)	2.81(3)	6.24(7)	0.91(1)			0.88(1)	0.93(1)
Phe	1.04(1)		4.15(4)	0.97(1)	0.96(1)			
Lys	1.37(1)	0.97(1)	2.50(2)		2.16(2)	1.05(1)		5.06(5)
His	3.23(4)	1.07(1)	0.44(1)		2.74(2)			1.29(1)
Hsl[b]	0.61(1)	0.51(1)	0.53(1)	0.43(1)	0.45(1)	0.59(1)	0.49(1)	
Arg	2.83(3)	0.82(1)	4.51(5)		2.75(3)			2.94(3)
Trp	N.D.(1)				N.D.(1)			N.D.(1)
Total	43[c]	37	78	19	25	4	10	19
Yield (%)	25	29	23	24	15	10	16	42
Pool (Fig.15)	II	III	I	II,IV,V	IV	VII	VI	IV
Purification	SP	SP	SP	SP 3.6	SP	3.6	3.6	SP
Mobility[d] pH 3.6	N.D.	N.D.	N.D.	+2.6	N.D.	+17.3	+5.3	N.D.

[a] Amino acid analyses were performed on 24h-hydrolysates. Values in parentheses are taken from sequence.
[b] Hse and Hsl mean homoserine and homoserine lactone, respectively.
[c] Including two cysteine residues linked to heme.
[d] Mobility is expressed in terms of cm (+) to the cathode at 43 V/cm for 1 h.

Table XII. Summary of sequence studies on BrCN peptides.

Peptide	Residue No.	
CB-1	1-43	Ser→-Asp→-
CB-2	44-80	Asp→-Tyr→-Val→-Ala→-Tyr→-Arg→-His→-Leu→-Val→-Gly→-Val→-Cmc→-
CB-3	83-160	Arg→-Pro→-
CB-4	161-179	Ala→-Pro→-
CB-5	180-204	Ser→-Gln→-
CB-6	205-208	Gly⇉-Leu⇉-Lys⇉-Hse⇉
CB-7	213-222	Gly⇉-Leu⇉-Leu⇉-Leu⇉-Pro⇉-Leu⇉-Val⇉-Tyr⇉-Ala⇉-Hse⇉
CB-8	223-241	Lys→-Arg→-His→-Lys→-Trp→-Ser→-Val→-Leu→-Lys→-Ser→-Arg→-Lys→-Leu→-Ala→

Complete Amino Acid Sequence of Cytochrome $\underline{c}_1$

The complete amino acid sequence of cytochrome $\underline{c}_1$ is shown in Fig.17. Tryptic peptides, staphylococcal protease peptides, and chymotryptic peptides overlapped each other and the complete amino acid sequence was established. One-residue overlap of Peptides T-5 and T-6 by C-6 was supported by amino acid composition of Peptide S-2 and further confirmed by CB-2. Another one-residue overlap of Peptides T-10 and T-11 by C-14 was covered by amino acid compositions of S'-1 and CB-5. All the residues were identified on at least two different peptides or subfragments except for tryptophan-12, arginines-15 and -28, residues 39-41 and 107-112, and methionine-204. Histidine-41 was ambiguously identified on Peptide C-5, and further confirmed on Peptide S'-T-3 although this peptide contained small impurities. Residues 107 to 113 was confirmed by direct manual Edman degradation of Suc-Cm-cytochrome $\underline{c}_1$ after hydroxylamine treatment. It gave the sequence, Gly-Ala-Leu-Pro-Pro-Asp-Leu, and confirmed the residue 106 to be asparagine because hydroxylamine does not cleave Asp-Gly bond.

The total amino acid residues are 241 giving a molecular weight of 27,238 for apoprotein and 27,874 including heme $\underline{c}$.

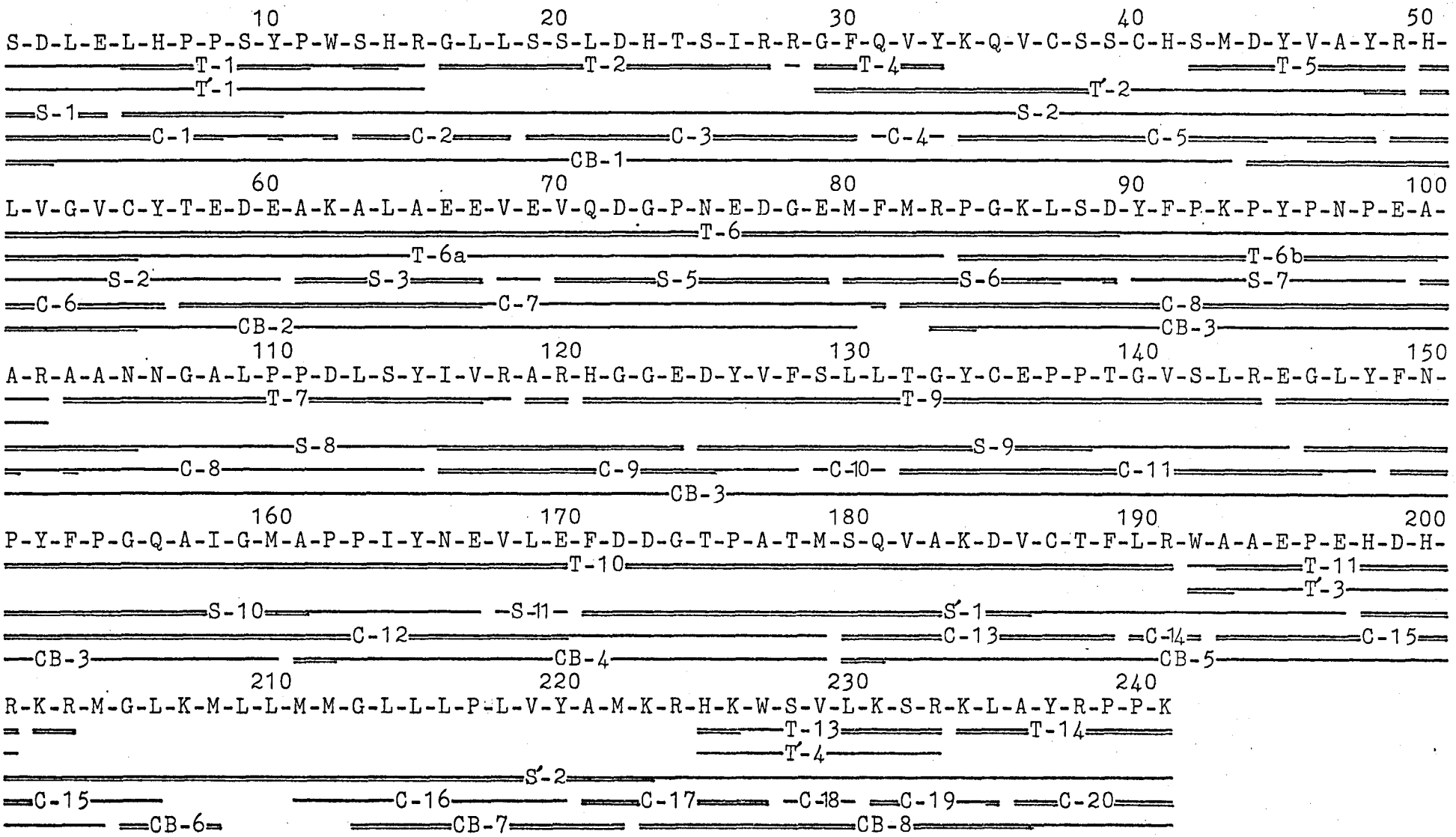


Fig.17. Complete amino acid sequence of bovine heart cytochrome c_1.
The sequenced region was indicated by double line.

V DISCUSSION

Reliability of Determined Amino Acid Sequence

The terminal analyses of Cm-cytochrome $\underline{c}_1$ threw some questions on N- and C-terminal regions. But recovery of the terminal peptides suggested that the proposed sequence must be correct. The composition of tryptic peptide T-1 or T'-1 whose N-terminus was blocked agreed well with the N-terminal sequence of the protein and deblocked chymotryptic peptide C-1 showed the same sequence as that of Cm-cytochrome $\underline{c}_1$. The tryptic peptides T-2 and T-4, staphylococcal protease peptide S-10 and cyanogen bromide peptides CB-6 and CB-7 had the N-terminal glycine, but it is evident that these peptides are not the N-terminal peptides of the protein. No other peptides which had glycine as the N-terminal residue was obtained. If glycine was the N-terminal residue of the protein, the N-terminal peptide containing the same amino acid sequence, even in partial, starting from glycine must have been obtained from different enzymic digests, but none of such a peptide was recovered. Therefore, it was concluded that glycine found in quantitative N-terminal analysis was not derived from cytochrome $\underline{c}_1$.

Lysine was concluded to be the C-terminal residue. The rationale to conclude such is as follows: The C-terminal residue of tryptic peptide T-14 was succinyllysine and that of chymotryptic peptide C-20 was maleyllysine. These residues did not fit the specificities of trypsin and chymotrypsin. Cyanogen bromide peptide CB-8 did not contain homoserine and its composition showed a good agreement with that deduced from the proposed sequence. The C-terminal sequence Pro-Pro-Lys might make it difficult to

determine C-terminal residue with CPase. CPase Y could not release lysine from Cm-cytochrome $\underline{c}_1$ even if SDS or 30 % propanol was present. However, the exact interpretation of the result of CPase digestion is not on our hands.

The overlapping of the peptides from three different enzymic digests and cyanogen bromide fragmentation was perfect and the complete amino acid sequence of cytochrome $\underline{c}_1$ was established.

The amino acid composition calculated from the established sequence is shown in Table I and shows a good agreement with those obtained for various original samples. The major difference between them exists on values of serine and tyrosine. As to serine, the recovery on amino acid analysis of each peptide showed a good agreement with the value from its sequence, although some destruction of serine were observed on a few large peptides during hydrolysis. The value of tyrosine in each peptide also agreed well except for the heme peptides (Tables II, V, IX and XI). The tyrosine recovery of heme peptides were always very low and the presence of tyrosine residues at residues 10, 33, 45, 48, and 56 became in question. However, each residue was recovered stoichiometrically and firmly identified by Edman degradation when it belonged to a peptide cleaved off from the heme peptide (T-1, T'-1, T-4, T-5, T-6, C-1, C-4, C-6, and CB-2). Therefore, the specific site of tyrosine was not modified and the tyrosine residues belonging to heme peptides might be modified or destroyed during purification or hydrolysis of heme peptides.

The amino acid compositions of cytochrome $\underline{c}_1$ reported by other workers (3,15-17,59) are shown in Table XIII. These values varied to some extent from one to another, but they showed a similar

Table XIII. Amino acid compositions of cytochrome c_1 reported by other workers

	present study	King	Trumpower & Katki	Bell & Capaldi	König[a] et al.	Robinson & Talbert G-200	Robinson & Talbert HPLC	Yeast	Neurospora[b]
Asp	19	21.9	23.2	23.3	21.2	22.0	23.6	27.4	30.2
Thr	7	8.6	9.2	10.2	8.4	9.9	10.1	12.7	16.8
Ser	16	14.5	15.8	17.3	13.3	16.8	15.9	14.7	16.8
Glu	21	23.5	27.9	28.3	34.5	32.6	31.7	28.7	27.4
Pro	22	25.8	24.8	24.9	16.9	20.0	19.3	24.9	21.0
Gly	17	17.8	19.4	21.7	13.9	17.7	20.2	25.0	22.7
Ala	18	18.6	21.9	22.8	15.9	18.4	19.6	30.0	31.4
Cys	5	7.5	4.3	N.D.	N.D.	4.3	6.0	N.D.	N.D.
Val	15	15.1	16.6	17.0	14.5	17.7	16.9	15.8	20.4
Met	10	8.8	8.5	7.3	7.2	7.7	6.0	5.0	2.5
Ile	4	4.5	5.0	7.9	4.3	4.8	5.2	9.1	7.8
Leu	25	28.8	28.3	28.3	28.0	28.3	28.3	20.2	23.8
Tyr	15	14.4	15.0	7.1	9.4	13.3	10.6	6.0	12.3
Phe	8	8.6	9.9	9.7	7.7	10.4	9.0	10.0	10.4
Lys	12	15.0	12.9	14.4	13.3	13.9	14.0	9.0	17.4
His	9	9.8	9.6	6.8	8.7	10.2	9.7	11.0	5.0
Arg	15	18.4	16.7	14.9	15.4	18.3	17.7	14.3	14.0
Trp	3	2.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Reference		(59)	(15)	(3)	(16)	(17)	(17)	(37)	(60)

[a] Amino acid composition was expressed in terms of mol% in the original report and these values were obtained by calculation assuming that 241 amino acid residues amount to 100 %.

[b] Amino acid composition was expressed in terms of mol% in the original report and these values were obtained by calculation assuming that 280 amino acid residues amount to 100 % for the molecular weight was reported to be 31,000.

distribution of amino acids, while considerable differences are noted between bovine cytochrome $\underline{c}_1$ and others such as yeast (37) and Neurospora (60) cytochrome $\underline{c}_1$. They also reported the molecular weight of cytochrome $\underline{c}_1$ ranging from 29,000 to 31,000. The molecular weight calculated from sequence was about 28,000 somewhat smaller than those reported. However, estimation of the molecular weight of membranous protein by SDS-polyacrylamide gel electrophoresis needs special cautions. Many membranous proteins do not migrate as the soluble standard proteins do and anomalous behavior of cytochrome $\underline{c}_1$ on SDS gel electrophoresis has also been reported (15). Moreover, the quantitative determination of protein by the Biuret reaction or Lowry's method is tending to overestimate. This results in an underestimation of heme content and accordingly in an overestimation of molecular weight. Recently Kubota and Tsugita (61) reported the molecular weight of 25,400 for Neurospora cytochrome $\underline{c}_1$ by a quantitative N-terminal analysis instead of 32,000 obtained by SDS gel electrophoresis.

Cytochrome $\underline{c}_1$ was reported to contain a small amount of carbohydrate, but no carbohydrate was found in the present sequence studies and they were probably contaminants.

Sequence Strategy and Specificity of Proteases

In the sequence studies of cytochrome $\underline{c}_1$ apocytochrome $\underline{c}_1$ was prepared, since the heme was considered to cause trouble during peptide purification. In fact the purification of the heme peptides was very difficult and the recoveries were always low. The enzymic or chemical cleavages were incomplete and heme peptides were separated into two fractions on gel filtration. Moreover, they often spread on the resin columns and a part of them was irreversibly adsorbed on ion exchange resins and was not recovered completely.

The heme group was effectively removed by a very simple procedure with NPSCl (41). However, partially deteriorated heme, for example the heme having a brown color in the fractions eluted from a Bio-Gel P-10 column at the void volume, wasn't effectively removed. In contrast, the second heme fraction eluted from this column which showed a red color was easily removed. Therefore, the intact nondeteriorated heme is necessary to be detached with NPSCl. Incidentally the prosthetic group of phycocyanin which attached to the protein through a thioether linkage like heme $\underline{c}$ was not removed by this reagent (Hase,T. personal communication).

To distinguish the cysteine residues linked to heme from other free cysteine residues, the formers were pyridylethylated after heme removal. Pyridylethylcysteine has a characteristic absorption band in the ultraviolet region and the peptide containing this residue is easily distinguished from other peptides. This differential modification of cysteine residues was applied as a model experiment to yeast cytochrome $\underline{c}$ which has one free cysteine residue and two linked to heme. Three different deriva-

tives of cytochrome c (Pe_3-, Cm_3- and Pe_2Cm_1-cytochorme c) were prepared and their tryptic digests were compared by finger printing method (62). The results showed that cysteine residues of heme peptide were specifically pyridylethylated in Pe-Cm-derivative.

The amino acid composition of Pe-Cm-cytochrome c_1 indicated that cysteine residues attached to the heme were specifically pyridylethylated. However, no pyridylethylcysteinyl peptide was recovered from tryptic digest. The major reason of no recovery of such derivatized peptide from Pe-Cm-cytochrome c_1 digest was the heterogeneous cleavage of the peptide bonds between tyrosine-33 and succinyllysine-34 and histidine-41 and serine-42, probably due to the chymotrypsin-like activity which contaminated in trypsin. The peptide corresponding to residues 29 to 49 was cleaved heterogeneously by trypsin and separated into 4 subspecies and it became very difficult to detect the pyridylethylcysteinyl peptide.

In general peptide bond involving proline, i.e. Arg-Pro or Lys-Pro, is resistant to trypsin, but Arg-Pro bond at residues 83 and 84 was partially cleaved by trypsin.

Staphylococcal protease showed a fairly strict specificity and only one peptide cleaved at aspartic acid residue was obtained. But to a small extent unspecific cleavages at serine-129 and glycine-159 were also observed.

Cytochrome c_1 has a very hydrophobic cluster near the C-terminus and the purification of the peptides in this region was rather difficult. Tryptic peptide could not be obtained as mentioned above and many cleaving sites for chymotrypsin resulted

in heterogeneous cleavages and low recovery of peptides. However, staphylococcal protease peptide could be purified on a column with the aid of n-propanol. The presence of n-propanol in buffer increases the solubility of some hydrophobic peptides and many proteases retain their activities under such conditions (38). The further digestion of Peptide S-2 with trypsin could be successfully achieved in the presence of 30 % n-propanol.

The sequencing of these peptides was performed on a solid-phase sequencer. It was a very useful tool for some small hydrophobic peptides, which were sequenced effectively. Cyanogen bromide peptides were also effectively sequenced by this method. But the sequence study on whole protein was not successful for the low efficiency of attachment to the support.

Some Characteristic Structural Features of Cytochrome $\underline{c}_1$

Cytochrome $\underline{c}_1$ contains heme $\underline{c}$ as a prosthetic group (9,14) and it is now revealed that the heme is attached to the protein through two cysteine residues at positions 37 and 40. Position 41 is occupied by histidine which is probably one of the ligands of heme iron as in cytochrome $\underline{c}$ (63). The apparent similarity is found between cytochromes $\underline{c}$ and $\underline{c}_1$ of bovine heart in the vicinity of heme binding site as shown in Fig.18 together with sequences of other cytochromes $\underline{c}$. Another ligand is not clear yet, but studies on the absorption band at 690 nm has suggested that the methionine is a possible ligand (22). Methionine-80 is the 6th rigand in cytochrome $\underline{c}$ (63) and the vicinity of this residue shows some similarity to that of methionine-208 in cytochrome $\underline{c}_1$ as shown in Fig.18. This fact may suggest that methionine-208 is the 6th ligand of heme iron in cytochrome $\underline{c}_1$. In spinach cytochrome $\underline{f}$, which is also a membrane-bound $\underline{C}$-type cytochrome and has a similar absorption spectrum to that of cytochrome $\underline{c}_1$, lysine was suggested to be one of the ligands by MCD and EPR studies (65). Therefore, further studies are necessary to confirm the present proposal.

Several workers have reported the isolation of heme peptides and it is possible to assign these peptides in the present sequence with the aid of amino acid compositions. The tryptic heme peptide (35) corresponds to residues 35 to 49 and the peptic heme peptide (36) to residues 34 to 44 with the N-terminus lysine, although minor disagreements exist. However, the chymotryptic heme peptide (34) was rather impure and cannot be assigned.

There are very interesting features on the distribution of

Fig.18. Sequence comparison between bovine cytochrome c_1 and various cytochromes c

| | | |

amino acids in cytochrome $\underline{c}_1$. Prolines are present in a row on 5 positions: residues 7-8, 110-111, 137-138, 162-163 and 239-240. Another proline cluster exists at residues 92 to 98 in which 4 prolines distribute alternately. Although the exact role of these sequences is not predictable at present, these are strong breakers for the peptide folding to make secondary structures, i.e., α-helix or β-sheet.

Leucine is the largest contributor of cytochrome $\underline{c}_1$ and accounts for over 10 % of the residues (25 out of 241), while only 4 isoleucine residues are present. This is a unique characteristic of bovine cytochrome $\underline{c}_1$ and the proportion of isoleucine to leucine is larger than a half in other mitochondrial components such as cytochrome oxidase (66) and cytochrome $\underline{b}$ (67).

The core of hydrophobic and basic amino acids are present near the C-terminal region and no acidic amino acids are present beyond aspartic acid-199. This distribution of amino acids must play an important role to bury cytochrome $\underline{c}_1$ into the inner membrane of the mitochondria.

There are two strongly acidic regions, from glutamic acid-58 to glutamic acid-79 including one exception of lysine-62 and from glutamic acid-167 to aspartic acid-173. These regions are probably exposed to the outer surface of the membrane to interact with cytochrome $\underline{c}$ (19,24).

Cytochrome $\underline{c}_1$ contains three cysteine residues besides the two linked to the heme and they are probably in free form, since some chemically titratable SH-groups are present (21). However, the possibility that two of them might be involved in the disulfide bridge formation in the mitochondrial membrane cannot be excluded,

because the reducing agent such as 2-mercaptoethanol was used for the purification of this cytochrome at rather high concentrations (14-16).

When the amino acid residues in cytochrome $\underline{c}_1$ are classified into hydrophobic, neutral, acidic and basic residues, each class amounts to 43.6, 29.5, 12.0 and 14.9 %, respectively. It should be noted that cytochrome $\underline{c}_1$ contains more basic residues than acidic residues. If the contribution of imidazole group of histidine is assumed to be half of lysine and arginine at neutral pH, the net charge of apocytochrome $\underline{c}_1$ is calculated to be +2.5. The isoelectric point of purified cytochrome $\underline{c}_1$ has not been reported, but it was reported to be 3.6 (11) or 5.5 (68) in complex form with some other protein components. Yet cytochrome $\underline{c}_1$ has been considered to be an acidic protein. In fact, cytochrome $\underline{c}_1$ is adsorbed on DEAE-cellulose even when 0.2 M NaCl is present at neutral pH. These facts mey reflect that the hydrophobic and basic core near the C-terminal region is buried inside of the protein complex or aggregates and the acidic regions are exposed to the outer surface.

The polarity defined by Capaldi and Vanderkooi (69) was calculated on cytochrome $\underline{c}_1$ to be 41.5 %. This value is slightly high among the membrane proteins and may reflect that cytochrome $\underline{c}_1$ is not deeply buried in the mambrane (28).

Cytochrome $\underline{c}_1$ has a common prosthetic group with cytochrome $\underline{c}$ and apparent similarities exist between these cytochromes only in the vicinity of heme binding site and a methionine region as described above, and no similarity is found in other parts. This was expected from the differences in properties between these

cytochromes such as molecular size and amino acid composition. Therefore, the similarities found may result from the functional requirement for heme binding, that is, these cytochromes evolved independently.

Among the many hemoproteins whose amino acid sequences were determined, only a few proteins are membrane-bound. They are cytochrome $\underline{b}_5$ (64,70-74) and several subunits of cytochrome $\underline{c}$ oxidase (66,75-84), and cytochrome $\underline{c}_1$ shows no apparent similarity to these proteins.

The terminal sequences of Neurospora cytochrome $\underline{c}_1$ (38) and spinach and algal cytochrome $\underline{f}$ (85) are also reported, but no homology is found between these sequences and present sequence of bovine cytochrome $\underline{c}_1$. More detailed comparison must be carried out after completion of sequence studies of these proteins.

Recently, the DNA sequence of cytochrome $\underline{b}$ gene of yeast mitochondria was determined and the amino acid sequence composed of 385 amino acid residues was deduced from the DNA sequence (86). This amino acid sequence was much different from that of bovine cytochrome $\underline{c}_1$.

Trumpower and Katki (15) performed a controlled digestion of cytochrome $\underline{c}_1$ with trypsin by which the cytochrome of molecular weight of 30,600 with blocked N-terminus was converted to a heme polypeptide of molecular weight of 29,000 with N-terminal lysine. They concluded that trypsin sensitive lysyllysine or arginyllysine bond was located in the N-terminal region. However, the present sequence study does not support their claim and the reason of the discrepancy is not clear without further study.

Prediction of the Secondary Structure

The secondary structure of cytochrome $\underline{c}_1$ was predicted according to the method of Chou and Fasman (87), since it is a relatively simple procedure and does not need any complex computer manipulations.

Of 241 amino acid residues 47 (20 %) was calculated to be α-helices and 82 (34 %) β-sheets, and these values are very close to the predicted values for subunit II of bovine cytochrome c oxidase, i.e., 17 % α-helices and 37 % β-sheets (66). Eight β-bends were also predicted. In some parts both α-helix and β-sheet could be assigned and the prediction bears some ambiguity. These results are illustrated in Fig.19. The hydrophobic cluster present near the C-terminal region are predicted as a long β-sheet although one strong breaker, proline, is present. But this region had a relatively high potentials for α-helix, too. At present, the studies on the tertiary structure of membrane proteins are limited, but they contained less β-sheets and had tendency to form α-helices (69). Taking this fact into account, the hydrophobic region ought to be predicted asan α-helix.

In any case, this prediction method is based on the conformational parameters derived from the studies on globular hydrophilic proteins, and therefore the special caution must be desired when it was extended to membranous protein.

Up to date, there are no report on the secondary structure of pure cytochrome $\underline{c}_1$. Yu *et al.*(18) reported the CD spectra of cytochrome $\underline{c}_1$ which contained one more polypeptide with the molecular weight of 14,000, and estimated that their preparation contained 20-25 % α-helices and 25 % β-sheets from CD spectrum at

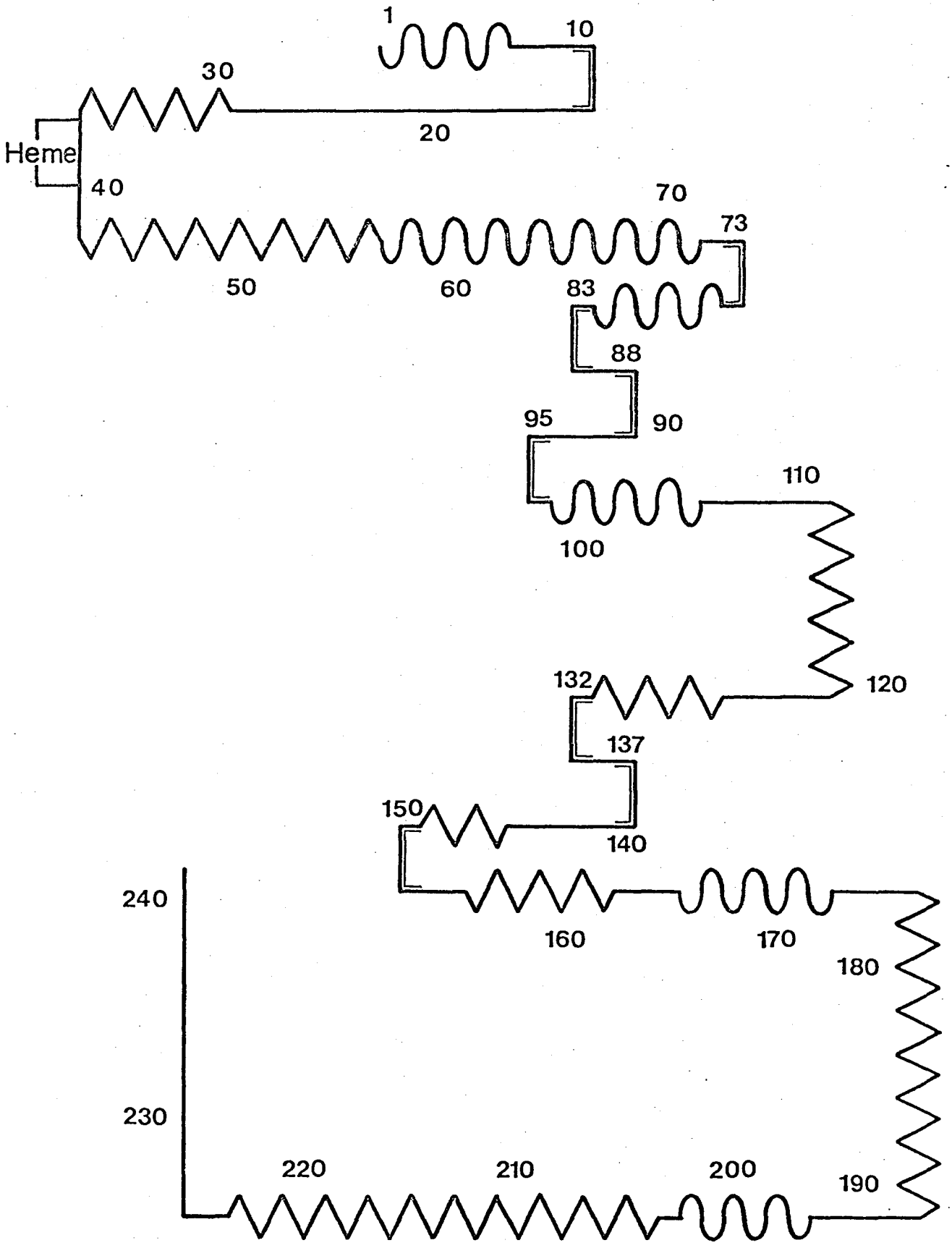


Fig.19. Prediction of secondary structure of cytochrome $\underline{c}_1$. α-helix, β-sheet and β-bend are expressed by (∿∿), (⩘) and (⟦), respectively.

far ultra violet region.

In near future, the crystallographic analysis will be attempted on mitochondrial electron carriers. The crystallization of cytochrome oxidase-cytochrome c complex (88,89) and cytochrome bc_1-cytochrome c complex (90) have been already accomplished. The present sequence will provide strong information to elucidate the tertiary structure of this cytochrome by X-ray diffraction analysis.

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