SEQUENCE OF THE CYSTEINE PEPTIDE FROM THE COPPER-SUBUNIT OF BOVINE CARDIAC CYTOCHROME OXIDASE

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SUMMARY: It has been proposed that the subunit of molecular weight about 22,000 is the copper-containing subunit of bovine cardiac cytochrome oxidase. It has been suggested that the copper in this subunit may bind to the cysteine residues. A 36 amino acid residue tryptic peptide has been isolated from this subunit and the amino acid sequence of this peptide has been determined. Construction of molecular models show that the peptide has potential copper binding sites of the type suggested by Hemmerich.

The nature and the number of subunits of cytochrome oxidase have been under investigation in several laboratories (1-6). Ten years ago, MacLennan and Tzagoloff (1) obtained from succinylated cytochrome oxidase, a Cu-protein which contained 2 g atoms of copper per mole of oxidase. The molecular weight of the subunit was reported to be about 25,000. Results from subsequent investigations (2-6), mainly from polyacrylamide gel electrophoresis of samples of the highly purified oxidase in dissociating media, suggest a more probable molecular weight of about 21-22,000. A number of hypotheses (6-13) on the role of copper and the mode of copper binding in the oxidase but no conclusive evidence, espeically from the structural point of view is available. In the present investigation, the cysteine containing peptide obtained by tryptic cleavage of the S-carboxymethylcysteine derivative of the Cu-subunit has been isolated and the sequence determined. The methods used for the isolation of the subunit and the tryptic peptide, sequence determination of the peptide and the possible binding of copper to the peptide are discussed.

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MATERIALS AND METHODS

Preparation of Cu-protein from Cytochrome Oxidase: Phospholipid depleted (less than 0.1% lipid) cytochrome oxidase was prepared according to the method developed recently in this laboratory (Yu et al. (14)). For the large scale preparation of the Cu-subunit, the fractionation method was done in a modular manner. Each modular unit-containing two hundred mg protein was incubated with 1 gm SDS and 0.2 ml of β-ME , in 20 ml of 50 mM phosphate buffer, pH 7.4. The incubation time was extended to 8 hours at 37°. A large column (5.0 x 80 cm) of Sephadex G-150, "superfine" grade, was used. The column was equilibrated and eluted with 50 mM phosphate buffer, pH 7.4 in the presence of 0.1% of β-ME-0.1% of SDS mixture. A better resolution was observed (cf. Fig. 1). The second peak of Fig. 1, which was found to contain mainly 21,000 protein subunit (naturally, Cu was no longer detected under these conditions), was pooled, concentrated and combined with the same portions obtained from a number of columns and rechromatographed. The pure 21,000 subunit thus obtained was cooled to 0° for the removal of the SDS and then concentrated by ammonium sulfate precipitation. About 20 mg of the purified protein subunit was obtained from 200 mg phospholipid-depleted oxidase. A similar experiment without β-ME lead to the isolation of the subunit with about 2 g atoms of Cu present per 21,000 g of protein.

Carboxymethyl Derivative of Cu-Protein. About 8.5 ml of the copper-subunit preparation (9 mg per ml) has converted the carboxymethylcysteine derivative by the procedure of Crestfield et al. (15)

Trypsin Digestion of Carboxymethyl Derivative of the Copper Subunit and Chromatography of the Tryptic Peptides. About 1 μmole (22.4 mg) of carboxymethylated derivative was suspended in 1.1 ml of deionized water and

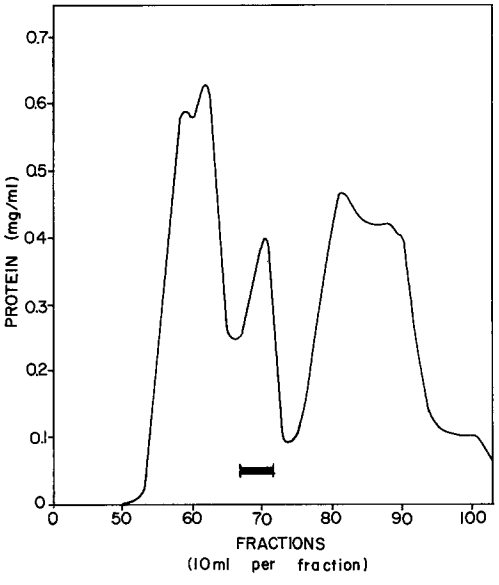

Figure 1. Elution pattern of cytochrome oxidase in the presence of β-mercaptoethanol. 200 mg of pure protein were incubated with 1 gram of sodium dodecylsulfate and 0.2 ml of β-mercaptoethanol, in phosphate buffer. A Sephadex G-150 column (5.0 x 80 cm) was used. See "Materials" for experimental details.

pH was adjusted to 8 with N-ethylmorpholine. Then, 1.0 mg of TPCK-trypsin was added, and pH was again adjusted to 8 with N-ethylmorpholine. After a period of 6 hours, 1.0 mg of TPCK-treated trypsin was again added and the pH was adjusted to 8. After reaction for 24 hours, the tryptic mixture was taken to dryness, dissolved in 0.1 M NH_4OH and applied to a Sephadex G-50 column (1.9 x 55 cm). The elution buffer was 0.1 M NH_4OH and the flow rate was 55 ml per hour. The fractions of 3.80 ml were collected and were detected from the absorbance of the samples at 280 nm. The elution pattern is shown in Fig. 2.

Isolation and Purification of the Tryptic Peptide. The fractions from peak 2 marked by a bar in Fig. 2 were pooled and rechromatographed on the same Sephadex G-50 column. The peptide was finally purified by paper chromatography in the solvent which contained pyridine/isoamyl alcohol/0.1 N NH_4OH (60:30:50, v/v).

Thermolysin Hydrolysis of Tryptic Peptide and Dowex 1-X2 Column Chromatography of Thermolytic Peptides. The tryptic peptide (0.70 µmole) was dissovled in 0.5 ml of deionized water and pH was adjusted to 8 with N-ethylmorpholine. Thermolysin (enzyme to substrate was 1:20, w/w) was added and the reaction time was 20 hours at 40°C. The sample dissolved in 0.1 M pyridine was applied to a Dowex 1-X2 column (0.7 x 20 cm). Linear gradient elution was carried out by mixing 100 ml of 0.1 M pyridine in mixing chamber and 100 ml of 6.0 M acetic acid in reservoir. The flow rate was 58 ml per hour and the fractions of 2.3 ml were collected. An aliquot of each fraction was assayed by ninhydrin reaction after alkaline hydrolysis (16). The elution pattern is shown in Fig. 3.

Amino Acid Composition and Sequence Analyses. The amino acid compositions of the protein and peptides were determined on acid hydrolysates in a Beckman-

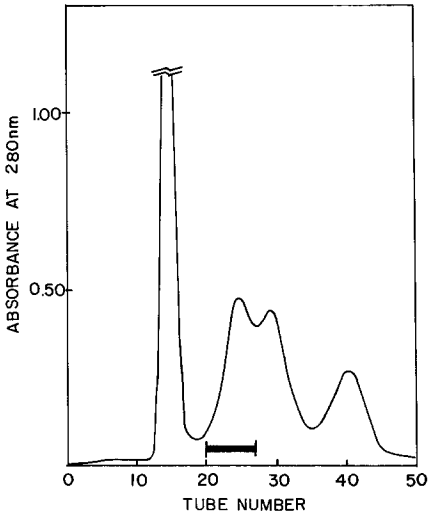

Figure 2. Sephadex G-50 column chromatography of tryptic peptides of carboxymethylated copper subunit protein of bovine cardiac cytochrome oxidase. About 1 µmole of carboxymethylated copper protein was digested with trypsin and the tryptic peptides were chromatographed on a Sephadex G-50 column (1.9 x 55 cm). See "Methods" for details. Fractions which contained the cysteine-containing peptide are shown by a solid bar.

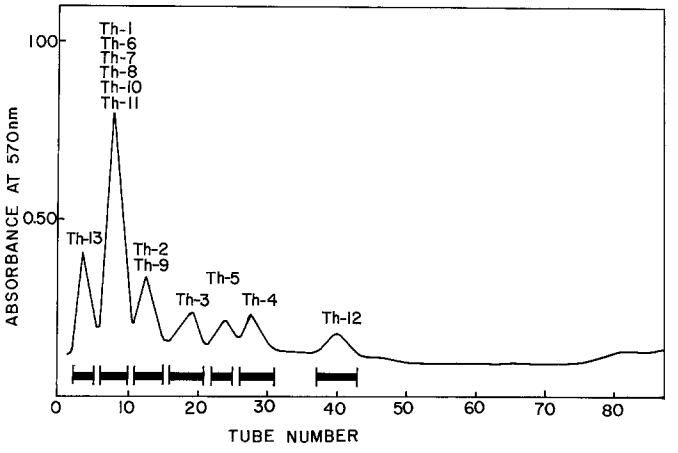

Figure 3. Dowex 1-X2 column chromatography of the thermolytic peptides of cysteine-containing peptide. Approximately 0.70 μmole of cysteine-containing tryptic peptide was hydrolyzed with thermolysin and the digestion mixture was applied on a Dowex 1-X2 column (0.7 x 20 cm). See "Methods" for details of experimental conditions. Fractions under each peak which were pooled are indicated by solid bars.

Spinco Model 120 C automatic amino acid analyzer as described by Spackman et al. (17). The instrument was equipped with high sensitivity cuvettes and a 4 to 5 mV full scale range card. The methods used for the amino-terminal sequence determinations have been described previously in the publications from our laboratory (18,19). The sequence analyses of cysteine-containing tryptic peptide were carried out on both the 4-sulfophenylthiocarbamyl derivative of the peptide and the underivatized peptide (20). The NH_2-terminal sequences of all the thermolytic peptides were determined by the usual manual Edman procedure (21). Peptide samples used for sequence analyses ranged from 50 to 200 nmoles. The phenylthiohydantoins of the amino acids were identified by gas chromatography (22), by thin layer chromatography (23), or by amino acid analyses of the 6N HCl hydrolysates of the amino acid phenylthiohydantoins (24). The COOH-terminal amino acids were determined by the use of carboxypeptidase B followed by A(25). Hydrazinolysis was performed on the peptide as described by Bradbury (26).

RESULTS

Amino Acid Composition of the Copper Subunit of Bovine Cardiac Cytochrome Oxidase. The amino acid composition of the purified copper subunit from bovine heart cytochrome oxidase was obtained from a 24-hr hydrolysate of the carboxymethyl derivative. The results are shown in Table 1 along with the amino acid composition data of cytochrome oxidase (4). The preliminary amino acid composition data indicate that the copper subunit of cytochrome oxidase contains two moles of cysteine residues per mole of protein.

TABLE I

Amino Acid Composition of Bovine Cardiac Cytochrome Oxidase and Its Copper Subunit Protein.

Amino acid	Cytochrome oxidase[a] Preparation 1	Preparation 2	Copper subunit protein[b]
Aspartate	52	52	13.40 (13)
Threonine	57	51	16.55 (17)
Serine	53	53	13.90 (15)
Glutamate	51	52	17.42 (17)
Proline	47	48	9.71 (10)
Glycine	54	53	9.00 (9)
Alanine	57	55	8.39 (9)
Cysteine	7	7	2.04 (2)
Valine	47	45	9.81 (10)
Methionine	15	13	10.50 (11)
Isoleucine	37	40	10.05 (10)
Leucine	76	79	27.70 (28)
Tyrosine	29	29	9.02 (9)
Phenylalanine	42	43	7.35 (7)
Tryptophan[c]	28	27	(6)
Lysine	27	28	4.87 (5)
Histidine	18	20	6.21 (6)
Arginine	19	21	5.05 (5)
Total residues	716	716	189
Molecular weight[d]	79,757	80,054	21,659

[a] Values of Kuboyama et al. (4). A recent report by Rubin and Tzagoloff (6) indicate the molecular weight of cytochrome oxidase is about 226,000 and the number of residues must be corrected once the molecular weight has been definitely established.

Amino Acid Composition and Some Properties of the Tryptic Peptide Isolated from the Copper Subunit. The fractions from the second peak contained chiefly the cysteine-containing peptide (Fig. 2). Therefore, the second peak fractions were pooled, concentrated to dryness, and rechromatographed on Sephadex G-50. Finally, the cysteine-containing peptide was purified by paper chromatography in the solvent system, pyridine/isoamyl alcohol/0.1 N NH_4OH (60:30:50, v/v). The pure cysteine-containing tryptic peptide was obtained in 50% yield from the carboxymethyl derivative of the copper subunit. Amino acid analyses were carried out on the 24- and 48-hour hydrolysates of the peptide and the amino acid composition was found as follows: aspartic acid: 2.87 (3), threonine: 0.96 (1), serine: 3.76 (4), glutamic acid: 4.01 (4), proline: 2.78 (3), glycine: 2.95 (3), carboxymethylcysteine: 1.80 (2) , valine: 2.11 (2), methionine: 0.84 (1), isoleucine: 1.96 (2), leucine: 4.84 (5), tyrosine: 2.98 (3), phenylalanine: 1.00 (1), lysine: 0.99 (1), and histidine: 0.93 (1); where the numbers in parentheses represent the assumed stoichiometric number of residues per mole of the pure peptide. The total number of amino acid residues in this peptide was 36.

Amino Acid Composition and Some Properties of Thermolytic Peptide From the Cysteine-containing Peptide. As shown in Fig. 3,a total of seven peaks were detected by the ninhydrin assay after alkaline hydrolysis. The methods used for the purification of the peptides from the pooled samples, the amino acid compositions and properties of the various thermolytic peptides are summarized in Table II.

Sequence Determination of the Cysteine-containing Tryptic Peptide.

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[b]Acid hydrolysis was performed on the carboxymethyl derivative of copper subunit protein for 24 hr at 110° with 6 N HCl. The amino acid residues were calculated on the basis of a glycine content of 9.00 mol/mol of protein. Amide determination and extrapolation for the threonine and serine were not made in this experiment. Values in parentheses indicate values rounded off to nearest whole number.

[c]Determined by the spectrophotometric procedure of Goodwin and Morton (27).

[d]Based on amino acid composition. The prosthetic groups have not been included.

TABLE II

Amino Acid Composition[a] and Properties of the Thermolytic Peptides of Cysteine-Containing Peptide in Bovine Cardiac Cytochrome Oxidase

Amino acid	Th-1	Th-2 + Th-9[c]	Th-3	Th-4	Th-5	Th-6	Th-7	Th-8 + Th-11[d]	Th-10	Th-12	Th-13	Total residues[b]
Carboxymethylcysteine				0.94(1)	0.95(1)	0.96(1)						2
Aspartic acid						1.00(1)	1.00(1)			1.88(2)		3
Threonine										0.96(1)		1
Serine				0.93(1)	0.92(1)	1.85(2)	1.83(2)	0.91(1)				4
Glutamic acid		1.00(1)		2.09(2)	1.99(2)					1.00(1)		4
Proline	0.90(1)							1.02(1)	0.98(1)			3
Glycine	1.00(1)			1.00(1)	1.00(1)	1.02(1)						3
Valine		0.98(1)							1.00(1)			2
Methionine								0.82(1)				1
Isoleucine		0.97(1)				0.98(1)						2
Leucine		1.60(2)	1.00(1)					1.00(1)	1.00(1)		1.08(1)	5
Tyrosine		0.56(1)	1.85(2)	0.98(1)						0.92(1)		3
Phenylalanine								0.94(1)				1
Lysine											1.00(1)	1
Histidine						0.97(1)	0.91(1)					1
Total residues	2	6	3	6	5	7	4	5	3	5	2	36
Recovery (%)	53	38, 63	15	24	32	47	20	67, 67	78	47	50	
R_f[e]	0.38	0.80	0.90	0.30	0.15	0.26	0.18	0.57	0.84	0.26	0.43	
Color reaction with ninhydrin	Purple	Purple	Purple	Violet	Yellow to Violet	Violet	Violet	Purple	Purple	Purple	Purple	
Pauly reaction	-	+	+	+	-	+	+	-	-	+	-	
Purification method[f]	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	

TABLE II (Con't.)

[a] Results from 6N HCl hydrolyses for 24 and 48 hr. The numbers in parentheses refer to the assumed stoichiometric number of residues per molecule of pure peptide.

[b] Sum of the peptides, Th-1, Th-2, Th-4, Th-6, Th-8, Th-9, Th-10, Th-11, Th-12, and Th-13.

[c] Could not be separated by paper chromatography with BPAW, but manual Edman degradation results showed to be a mixture of two peptides, Leu-Tyr (Th-2, 38% recovery) and Ile-Val-Leu-Glu (Th-9, 63% recovery).

[d] Mixture of equal molar ratio of two peptides, Phe-Met-Pro (Th-8) and Leu-Ser (Th-11).

[e] Paper chromatography with 1-butanol/pyridine/acetic acid/water (60:40:12:48, v/v).

[f] BPAW is the abbreviation of paper chromatography in the solvent system, 1-butanol/pyridine/acetic acid/water (60:40:12:48, v/v).

Two steps of the manual Edman degradation results showed that the NH_2-terminal sequence of the cysteine-containing tryptic peptide was Pro-Gly- . Manual Edman degradation of the 4-sulfophenylthiocarbamyl derivative of the same peptide established the sequence of the first 30 residues from the amino-terminal end of the peptide. The average repetitive yield on this peptide was 91%. In order to establish the complete sequence of the cysteine-containing tryptic peptide, manual Edman degradation was performed on all the thermolytic peptides (Th-1 to Th-13). Hydrazinolysis of peptide Th-6 showed that serine was the C-terminal amino acid. Carboxypeptidase B followed by A digestion showed that the COOH-terminal sequence of the cysteine-peptide was -Leu-Lys. All of the sequence experiments which were performed on the tryptic peptide are summarized in Fig. 4.

DISCUSSION

Throughout this present report, the subunit with a molecular weight of about 22,000 has been referred to as the copper containing subunit of cytochrome oxidase. There is a need to confirm that this subunit indeed is the copper-containing subunit. Tzagoloff and MacLennan (10) have shown the ESR spectrum of the Cu-subunit obtained from succinylated cytochrome oxidase, although it contained 2 g atoms of copper per mole of protein, showed a different ESR spectrum from the Cu-subunit in the native cytochrome oxidase. The reason for this behavior is not known at present. If it is assumed that the subunit of molecular weight 22,000 is indeed the Cu-binding subunit in cytochrome oxidase, Hemmerich (11,12) has postulated that the copper containing active site may consist of a RSSR-2 RS^- couple. Therefore, in the present investigation, an attempt was made to isolate the cysteine (cystine) containing peptide.

By tryptic digestion of the S-β-carboxymethylcysteinyl-derivative of the subunit, a 36 residue peptide fragment was isolated. The peptide contained the two cysteine residues present in the subunit. The sequence of the peptide is summarized in Fig. 4. The two cysteine residues were located at positions

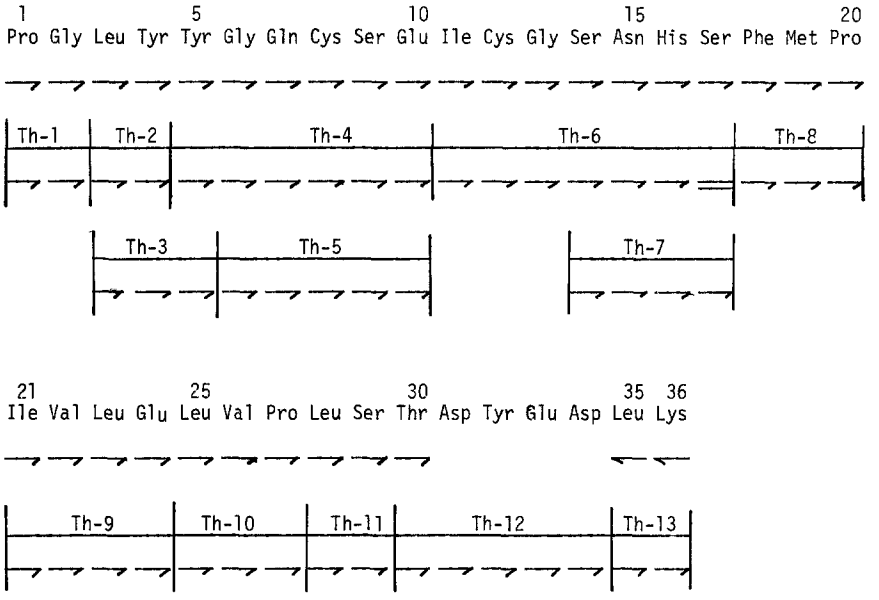

Figure 4. Reconstruction of the complete sequence of a cysteine-containing tryptic peptide obtained from the copper subunit protein of bovine heart cytochrome oxidase and all of the sequence data which were performed on peptide fragments. In the figure, the symbols →, ←, and ⇉ represent sequences determined by use of the direct manual Edman degradation, carboxypeptidase B followed by A, and hydrazinolysis experiments, respectively.

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8 and 11 and were three residues apart. Construction of a molecular model of the peptide showed the peptide could bind copper and function in the manner proposed by Hemmerich.

Studies are in progress to determine the complete sequence of the subunit as well as to determine whether the subunit of molecular weight 22,000 is the Cu-containing subunit of cytochrome oxidase.

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