Euglena gracilis CYTOCHROME bc_1 COMPLEX:

STRUCTURE OF CYTOCHROME c_1

WITH AN ATYPICAL HEME-BINDING

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CONTENTS

ABBREVIATIONS

PMSF	phenylmethylsulphonyl fluoride
DBMIB	2,5-dibromo-3-methyl-6-isopropylbenzoquinone or dibromothymoquinone
K_m	Michaelis constant
SDS	Na dodecylsulfate
PAGE	polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
CM	carboxymethyl
PTH	phenylthiohydantoin
HPLC	high performance liquid chromatography

PART I

Purification and characterization of complex III from *Euglena gracilis* mitochondria

SUMMARY

Complex III was purified from submitochondrial particles prepared from Euglena gracilis by a two-step extraction with Triton X-100 and a single ion exchange chromatography of the extract. The purified complex showed an atypical difference absorption spectrum for cytochrome c_1 with its α-band maximum at 561 nm. Cytochrome(s) b had its α-band maximum at 563 nm on a difference absorption spectrum, and the ratio to cytochrome c_1 is approximately one. The Euglena complex III consisted of 10 subunits. On the basis of their relative electrophoretic mobilities on a sodium dodecylsulfate-urea polyacrylamide gel, these have molecular masses of 50, 42, 37, 32.5, 32, 30, 18, 9.6, 8.8, and 7.2 kDa. The 37- and 32.5-kDa subunits have been identified as cytochrome b and c_1, respectively. Rieske iron-sulfur protein was not identified and is probably present from content of acid-labile sulfur of the complex.

Ubiquinol-cytochrome c oxidoreductase activity of freshly prepared Euglena mitochondria was inhibited potently by antimycin and partially by myxothiazol. During storage of Euglena cells and purification of complex III, however, the inhibitor-sensitivity to both antibiotics was lost. The isolated complex III had ubiquinol-2-horse heart cytochrome c oxidoreductase activity with a maximal turnover of 5.9 sec^{-1}. The activity was inhibited by dibromothymoquinone, suggesting the presence of Rieske iron-sulfur protein. When Euglena cytochrome c(c-558) was used as electron acceptor, turnover number increased about two-fold. According to the electron transfer mechanism of the Q-cycle

or its related schemes, the results of inhibitor-sensitivity of the activity of the isolated complex III suggests that electron transfer from ubiquinol through iron-sulfur protein and cytochrome $\underline{c}_1$ to cytochrome $\underline{c}$ is normal, but functional electron transfer route through cytochrome(s) $\underline{b}$ is lacked in the isolated Euglena complex III.

INTRODUCTION

The ubiquinol-cytochrome $\underline{c}$ reductase (cytochrome $\underline{bc}_1$ complex, complex III; EC 1.10.2.2.) is an oligomeric enzyme in the respiratory chain located in the mitochondrial inner membranes and some bacterial cell membranes (1,2). The $\underline{bc}_1$ complex, involving cytochrome $\underline{b}$, Rieske iron-sulfur protein and cytochrome $\underline{c}_1$ as electron carrier subunits, catalyzes the oxidation of ubiquinol and reduction of cytochrome $\underline{c}$ coupled with formation of an electrochemical potential of proton across the membrane. The mechanism is explained by the Q-cycle or other schemes (3,4).

The enzyme isolated from the mitochondria of bovine heart(5-9), yeast (8-12), Neurospora crassa (13,14), or sweet potato (15) consists of about 10 subunits including in addition to the three redox carrier subunits two large subunits, designated as core proteins, and several small subunits. On the other hand, the complexes isolated from bacteria (Rhodobacter capsulatus (9), Rhodobacter sphaeroides (8,9,16,17) and Paracoccus denitrificans (9,18,19)) were devoid of the core proteins and most of the small subunits. To date there has been no report for cytochrome $\underline{bc}_1$ complex of any protozoan mitochondria.

The protozoan, Euglena gracilis, occupies a unique position

in evolution and its mitochondria have several distinct properties. The ultrastructure of the mitochondria has been shown to be a network of mitochondrial reticulum(20). The pyruvate dehydrogenase complex and the 2-oxoglutarate dehydrogenase complex were replaced by pyruvate-$NADP^+$ oxidoreductase (21), and a 2-oxoglutarate decarboxylase (22) in conjunction with succinate-semialdehyde dehydrogenase(23), respectively.

The respiratory chain of the Euglena mitochondrion seems to consist of typical complexes I to IV(24). As to complex III, antimycin-sensitivity was shown for both activities of NADH oxidase and succinate oxidase, although the ubiquinol-cytochrome c reductase activity of complex III has not yet been established. In addition, Euglena cells can grow in the presence of antimycin, since an alternative succinate oxidase is induced(25). Moreover, the cytochrome c has an α-band maximum at 558 nm in contrast to cytochromes c from other sources which have an α-band maximum at 550 nm, and its heme is linked via a single thioether bond instead double to a cysteine (26-28).

This part describes the purification of Euglena complex III and reports some unique properties of the complex including the unusual spectrum for cytochrome c_1, the subunit composition, and the inhibitor-sensitivity of its ubiquinol-cytochrome c oxidoreductase activity.

MATERIALS AND METHODS

Materials

Euglena gracilis SM-ZK, a stable chloroplast-lacking mutant,

was used. Cells were cultured aerobically in the dark in Oda medium (29) containing 50 mg/l streptomycin, and kindly supplied by Toyo Jozo Co., Ltd.(Shizuoka, Japan). The cells were harvested at early stationary phase and stored at -20 °C. Crude trypsin(type II), phenylmethylsulphonyl fluoride(PMSF), horse heart cytochrome c(type VI), crude phospholipid(phosphatidylcholine, type IV-S), cardiolipin and dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropylbenzoquinone, DBMIB) were all purchased from Sigma. Antimycin A and myxothiazol were from Boehlinger. Triton X-100 was from Nakarai Chemicals (Kyoto). DEAE-Toyopearl 650M and Toyopearl HW-65F and HW-55F were from Toyosoda (Tokyo). Sephadex G-25 was from Pharmacia. Ubiquinone-2 was a gift from Eizai (Tokyo). 3,3',5,5'-tetramethylbenzidine was a product of Dojin (Kumamoto, Japan).

Preparation of submitochondrial particles

To prepare mitochondrial membranes, a method including limited protease digestion (30) was modified as follows. The frozen cell paste(1 kg) was thawed and suspended in 1 l of 50 mM K-phosphate, pH 7.5, containing 0.35 M sucrose and 0.5 mM Na-EDTA (buffer I). Subsequent steps were carried out on ice. The cell suspension was filtered through two layers of gauze and was centrifuged at 2000 x g for 5 min in a swinging- bucket rotor. The cells were resuspended in 1.08 l of buffer I, mixed with 1.2 g of crude trypsin dissolved in 120 ml of buffer I, and incubated for 1 h with gentle shaking. PMSF was added to the suspension at 1 mM to inhibit protease activity. The trypsin-treated cells were collected as before and resuspended in 50 mM

Tris-Cl, pH 7.5, containing 0.25 M sucrose and 0.5 mM Na-EDTA (buffer II). The suspension was homogenized with a Polytron homogenizer at medium speed for 1 min in the presence of 1 mM PMSF, and was centrifuged as before. The supernatant was decanted carefully to avoid disturbing the pellet, which consisted of a white layer of storage polysaccharide, paramylum, and a soft upper layer of unbroken cells and debris. This upper layer was resuspended by gentle swirling in 300 ml of buffer II containing 1 mM PMSF and homogenized and centrifuged as before. The combined supernatants were centrifuged again at 2000 x g for 5 min in an angle rotor to remove debris before centrifugation at 17,000 x g for 15 min to pellet the mitochondrial membranes. The yellowish brown pellet was suspended in 200 ml of buffer II containing 0.5 mM PMSF using a 50-ml glass-Teflon homogenizer. The crude mitochondrial membranes were sonicated for 1 min at three equal bursts in a Sonicator model W225 (Heat Systems-Ultrasonics, Inc.) at a power setting of 8. The sonicated membranes were centrifuged at 100,000 x g for 30 min. The precipitate which consisted of submitochondrial particles was resuspended in buffer II containing 0.5 mM PMSF at a protein concentration of 20 mg/ml and stored at -80 °C.

Purification of ubiquinol-cytochrome c reductase

For the first extraction of the submitochondrial particles, the suspension (130 ml) was thawed and mixed with an equal volume of a solution containing 1.8 % Triton X-100 and 0.2 M NaCl. The final concentrations were as follows: 10 mg protein/ml, 25 mM

Tris-Cl, pH 7.5, 0.125 M sucrose, 0.25 mM Na-EDTA, 0.25 mM PMSF, 0.1 M NaCl, and 0.9 % Triton X-100. The mixture was stirred for 5 min and centrifuged for 30 min at 100,000 x g. The yellowish orange supernatant was discarded and the brown pellet was resuspended in buffer II giving the original volume of the submitochondrial particle preparation. For solubilization of ubiquinol-cytochrome c reductase, the suspension was mixed with an equal volume of a solution of 1.4 % Triton X-100 and 0.2 M NaCl. The concentrations of protein and Triton X-100 were approximately 3 mg/ml and 0.7 %, respectively. The mixture was stirred and centrifuged as before.

The reddish orange supernatant was applied directly to a 3.6 x 14-cm DEAE-Toyopearl 650M column equilibrated with 50 mM Tris-Cl, pH 7.5, containing 0.1 % Triton X-100, 0.1 M NaCl and 0.5 mM Na-EDTA (buffer III). The column was washed with 150 ml of buffer III and the enzyme was eluted with 600 ml of a linear gradient of 0.1 to 0.3 M NaCl in buffer III. The red fractions were combined and stored at -80 °C.

Isolation of 10 Subunits from Euglena Complex III

Isolation of cytochrome c_1 (subunit IV) was described in part II (51). Other subunits were isolated by gel-filtration chromatography and preparative SDS-PAGE. Purified Euglena complex III (40 mg) was denatured with 4 % SDS and 4 % 2-mercaptoethanol, and the subunits were separated by combination of a Toyopearl HW-55 column and a Sephadex G-150 column using a buffer containing 0.2 M Na-phosphate, pH 7.0, and 0.1 % SDS as described in part II. A fraction containing subunit VII was obtained from

carboxymethyl (CM-) complex III from which CM-cytochrome $\underline{c}_1$ was isolated in the part II. Purification of subunit I, II, V, VI, VII, VIII, IX, and X was further performed on preparative SDS-gels. The gels were stained with Coomassie blue for 2-3 min and excised. The gel pieces were homogenized in a buffer containing 25 mM Tris-Cl, pH 7.5, and 0.1 % SDS, and centrifuged. The supernatant was concentrated using ultrafiltration membranes, and desalted on a spun column of Sephadex G-25F equilibrated with 0.1 % triethylamine and 0.1 % SDS. The subunit III was treated with methanol containing 1 N HCl (58) before sequencing.

Analytical procedures

Protein was determined by the Lowry procedure (31) using bovine serum albumin as a standard. Phospholipid content of the isolated complex III was determined by extracting lipids (32) and assaying for inorganic phosphate (33). Contents of acid-labile sulfur (34), and of Triton X-100 and ubiquinone bound to the reductase were determined by published methods (35 and 36, respectively). Amino acid composition and amino-terminal amino acid sequence were analyzed as described in part II.

Ubiquinol-cytochrome $\underline{c}$ oxidoreductase activity was assayed with ubiquinol-2 and horse heart cytochrome $\underline{c}$. Ubiquinone-2 was reduced by the method of Rieske(37) and stored in ethanol containing 6 mM HCl at -20 °C. The concentration of ubiquinol was determined spectrophotometrically using an absorption coefficient of 4.14 $mM^{-1}cm^{-1}$ at 288 nm for the reduced form (38). Horse heart cytochrome $\underline{c}$ was oxidized by a few grains of solid K-

ferricyanide, passed through a Sephadex G-25 column equilibrated with 50 mM Na-phosphate, pH 7.5, containing 0.5 mM Na-EDTA, and stored at -20 °C. Ubiquinol-cytochrome *c* reductase activity was measured in a buffer containing 50 mM Na-phosphate, pH 7.5, 0.5 mM Na-EDTA, 20 mM Na-malonate, 0.2 mM KCN, 37.5 µM ubiquinol-2, and 10 µM oxidized horse heart cytochrome *c*. Reduction of cytochrome *c* was determined spectrophotometrically using a difference absorption coefficient of 21.1 $mM^{-1}cm^{-1}$ at 550 nm for reduced minus oxidized form (39). Observed rates were corrected for any non-enzymic reduction of cytochrome *c*. One unit of reductase activity is defined as one µmol cytochrome *c* reduced per minute. *Euglena gracilis* mitochondrial cytochrome *c*(*c*-558) was prepared essentially following the method reported by Pettigrew *et al*. (26).

SDS-PAGE and SDS-urea-PAGE were performed on 15 % acrylamide slab gels after the method of Laemmli (40) and on 18 % acrylamide slab gels containing 6 M urea after the method of Kadenbach *et al*. (41), respectively. Samples analyzed on SDS- or SDS-urea-gels were incubated at 37 °C for 15 min in SDS-sample buffer containing 125 mM Tris-Cl, pH 6.8, 5 % glycerol, 4 % SDS and 2 % 2-mercaptoethanol. After electrophoresis, gels were stained for protein with Coomassie Brilliant Blue R-250 or for peroxidase activity using 3,3',5,5'-tetramethylbenzidine/ H_2O_2 to detect heme (42). In some cases the peroxidase stain was removed by soaking gels in a fixing solution containing 70 mM Na-sulfite, and the gels were stained for protein. Relative molecular mass of the subunits was determined against molecular mass standards (phosphorylase b, 94 kDa; bovine serum albumin, 67

kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and lysozyme, 14.4 kDa) and 11 subunits of bovine complex III (7) isolated by a reported method (5).

Difference absorption spectra were recorded at 22°C with a Hitachi 220A or U3200 spectrophotometer. A few grains of solid Na-dithionite or Na-ascorbate for reduction of the cytochromes and of K-ferricyanide for oxidation of them were directly added to cuvettes containing sample, respectively. Contents of cytochrome b and c_1 were tentatively determined using reduced minus oxidized difference absorption coefficients of 25.6 $mM^{-1}cm^{-1}$ for the wavelength pairs 563-577 and 20.1 $mM^{-1}cm^{-1}$ for 553-539 nm, respectively, for those of bovine heart mitochondria (39) and applying them to corresponding wavelength pairs on difference spectra.

The molecular mass of complex III with bound Triton X-100 was estimated by gel filtration chromatography. Purified complex III(4 mg) was applied on a Toyopearl HW-65F column(2 x 70 cm) equilibrated with buffer III and eluted with the same buffer. The fractions eluted were analyzed on an SDS-gel, and the elution volume of the complex III was compared with that of molecular standards (thyroglobulin, 670 k; ferritin, 470 k; catalase, 230 k; γ-globulin, 160 k; and bovine serum albumin, 67 k).

The effect of addition of crude phospholipid, cardiolipin, and ubiquinone on the ubiquinol-cytochrome c reductase activity was investigated as follows. Crude phospholipid was homogenized with acetone and dried, and then dissolved in chloroform. After evaporation, the material was suspended and homogenized in a

buffer containing 40 mM Na-phosphate, pH 7.5, 150 mM NaCl, and 0.5 % Na-cholate. Complex III at 1.0 μM was mixed with ubiquinone-2 at a molar ratio of 10 and incubated at 0 °C for 5 min, before either the crude phospholipid or cardiolipin in methanol was added to the incubation mixture at ratio of 0.8 or 0.2 mg/mg of protein, respectively. The mixture was incubated at 0 °C for one hour. An aliquot of the mixture was withdrawn and assayed for reductase activity in a reaction mixture without phospholipids. The average molecular weight of the phospholipid was assumed to be 775.

The effect of Triton X-100 concentration on the reductase activity was investigated. The isolated complex III was mixed with reaction mixtures containing up to 0.1 % Triton X-100 and the activity was assayed immediately.

RESULTS

Purification of complex III from Euglena submitochondrial particles

In a preliminary experiment, mitochondrial membranes prepared from fresh Euglena cells showed fully antimycin-sensitive ubiquinol-cytochrome c reductase activity. But during storage of cells at -20 °C over several weeks, antimycin-sensitivity of the ubiquinol-cytochrome c reductase was lost in parallel with activity. Myxothiazol, another specific inhibitor of ubiquinol-cytochrome c reductase, had less effect on the activity, though preparations were still fully antimycin-sensitive. For subsequent work, Stored cells whose antimycin-sensitivity had been lost were used.

Table I-1 summarizes the purification of complex III. Analysis by SDS-PAGE at each step of the preparation is shown in Fig. I-1. The ubiquinol-cytochrome *c* reductase was solubilized with Triton X-100 in a two-step extraction. Approximately two thirds of the protein was removed in the first extraction. The ubiquinol-cytochrome *c* reductase was solubilized from the subsequent precipitate in the second extraction step. This solubilization process achieved a 2.5-fold purification of the ubiquinol-cytochrome *c* reductase. The supernatant from the second extraction was applied to a DEAE-Toyopearl column (Fig. I-2). Most of the protein was washed out with the equilibration buffer, and the absorbance at 415 nm in these fractions was attributed to caroteno-proteins and was not due to cytochromes. The ubiquinol-cytochrome *c* reductase was eluted in the middle of the salt gradient corresponding to absorption due to cytochrome. Analysis of the column fractions by SDS-PAGE showed that 10 polypeptides were eluted in the fractions containing ubiquinol-cytochrome *c* reductase (Fig. I-1). At this stage, the complex III was purified 22-fold on the basis of the content of cytochrome c_1. Further chromatography using either hydroxylapatite or Toyopearl HW-65F did not alter the polypeptide pattern or the content with respect to cytochromes.

Table I-2 summarizes the composition of the isolated *Euglena* complex III. When estimated by a protein assay and using the difference absorption coefficients for bovine heart mitochondrial cytochrome *b* and c_1, the contents of the cytochromes were 2.6 and 2.5 nmol/mg of protein, respectively. These values indicated that

Table I-1. Purification of ubiquinol-cytochrome c reductase from Euglena submitochondrial particles

Protein was determined by the Lowry procedure. Units of activity were measured in the ubiquinol-cytochrome c reductase assay as described in Materials and Methods. Extent of purification was calculated from the relative amount of cytochrome c_1 measured spectrophotometrically. The values in parentheses were assumed to be the same as those for SMP.

Purification step	Protein	Cytochrome c_1	Purification	Activity
	(mg)	(%)	(-fold)	(units)
Submitochondrial particles (SMP)	2660	100	1	44.1
First extraction				
SMP+Triton X-100	(2660)	(100)	(1)	139
Precipitate	972	90	2.5	89.5
Second extraction				
Supernatant	614	78	3.4	61.6
DEAE-Toyopearl	69	54	21	28.6

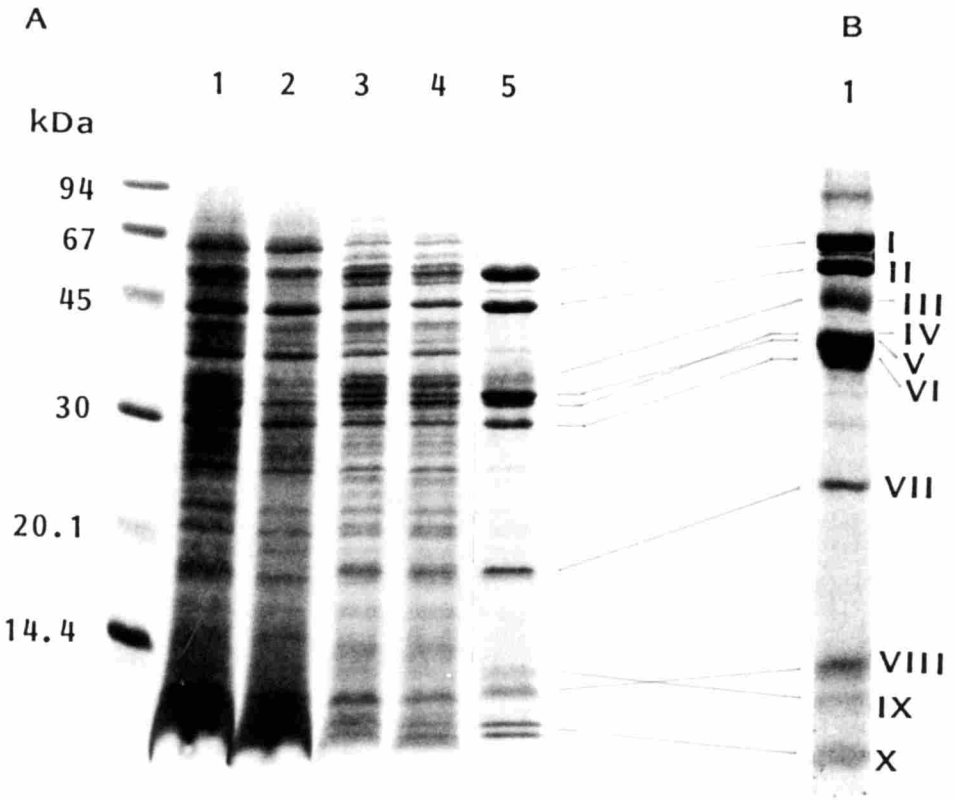

Fig. I-1. SDS-polyacrylamide gel analysis of fractions at different stages in the purification of ubiquinol-cytochrome c reductase from Euglena submitochondrial particles. The gels were stained for protein with Coomassie Brilliant Blue R-250. A) Purification of Euglena ubiquinol-cytochrome c reductase is shown on a 15 % acrylamide gel using SDS-PAGE system A as described in Materials and Methods: Lane 1, submitochondrial particles, 80 µg of protein; Lane 2, supernatant from the first extraction, 54 µg of protein; Lane 3, precipitate from the first extraction; 32 µg of protein. Lane 4, supernatant from the second extraction, 24 µg of protein; and Lane 5, ubiquinol-cytochrome c reductase from the DEAE-Toyopearl column, 9 µg of protein. B) Isolated ubiquinol-cytochrome c reductase (20 µg protein) was analyzed on a 18 % acrylamide gel containing 6 M urea using SDS-PAGE system B as described in Materials and Methods. Correspondence of the bands from gel system B to those from the gel system A was carried out with a two-dimensional gel, where the isolated ubiquinol-cytochrome c reductase was subjected to SDS-PAGE in system B for the first dimension and the resulting gel was dialyzed against the SDS-sample buffer (described in Materials and Methods) for 30 min, and separated in the second dimension by SDS-PAGE system A. Correspondence of each band of the isolated ubiquinol-cytochrome c reductase on these two gel systems is indicated by the lines between A) and B).

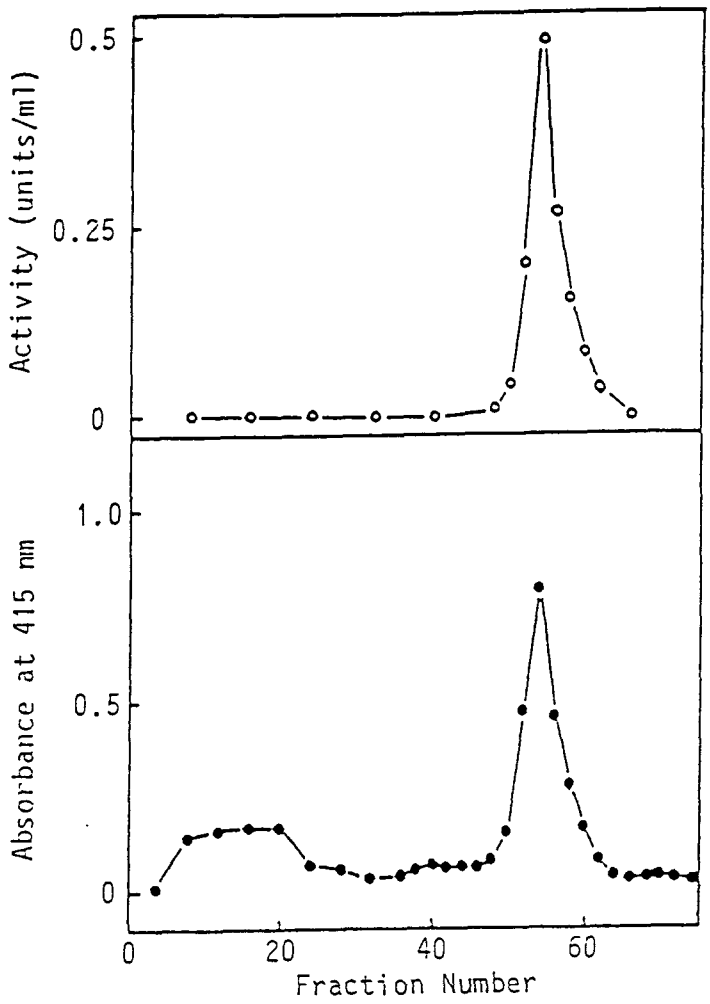

Fig. I-2. DEAE-Toyopearl chromatography of Triton X-100 extract of submitochondrial particles. After the first extraction of the Euglena submitochondrial particles, 972 mg protein was further extracted with Triton X-100 (1.9 mg/mg protein) and applied to a 3.6 x 14-cm DEAE-Toyopearl column. The column was washed with 150 ml of buffer III (described in Materials and Methods), and proteins were eluted with a gradient of 100 to 300 mM NaCl in buffer III. Fractions containing 15 ml of the eluate were collected and absorbance at 415 nm was recorded. Ubiquinol-cytochrome c reductase was assayed as described in Materials and Methods. In the chromatogram the washing buffer and the salt gradient began to elute at around fractions 20 and 30, respectively.

Table I-2. Composition of the isolated complex III from Euglena. Protein concentration and amounts of each component were determined as described in Materials and Methods. Molar ratio is based on comparison with cytochrome $\underline{c}_1$, assuming one complex III monomer contains one cytochrome $\underline{c}_1$.

Component	Content	Molar ratio
	(nmol/mg protein)	(mol/mol)
cytochrome $\underline{c}_1$	2.6	1.0
cytochrome $\underline{b}$	2.4	0.9
acid-labile sulfur	2.9	1.1
ubiquinone	<0.4	<0.2
phospholipid	16 (12 μg)	6
Triton X-100	400 (260 μg)	150

one mol of isolated Euglena complex III contained one mol of each cytochrome. The ratio of $\underline{b}/\underline{c}_1$ was one and thus differed from the ratio of two reported for the $\underline{bc}_1$ complexes from other sources (1,2,5-19). The content of acid-labile sulfur indicated the presence of a Rieske iron-sulfur protein with a 2Fe-2S cluster (1-4). The lower contents of ubiquinone and phospholipids seemed to be common properties of the complexes isolated using Triton X-100 (6,54). The molar ratio of bound Triton X-100 to complex III indicated that the solubilized complex III molecules were buried in micelles of Triton X-100 (6,13).

Absorption spectra

Figure I-3A shows the absoption spectra of the isolated complex III. Difference absorption spectra are shown in Fig. I-3B. The difference spectrum of the fully reduced minus fully oxidized complex (Fig. I-3B, top) showed an absorption maximum at 562 nm similar to that of other complexes III. However, no shoulder at 552-553 nm due to a typical cytochrome $\underline{c}_1$ (1,2,5-19) was found. The spectrum also shows that the purified complex III was free from a-type cytochromes. A difference spectrum obtained for the ascorbate reduced minus ferricyanide oxidized complex (Fig. I-3B, bottom) showed the absorption maximum at 561 nm, which was unusual for cytochrome $\underline{c}_1$, this wavelength being shifted by 8 nm to the red (45-49). The absorption maximum of cytochrome $\underline{c}_1$ was interesting when considered in terms of the nature of the heme binding. This will be discussed in part II. The cytochrome $\underline{b}$ was characterized by an absorption maximum at 563 nm, shown by a difference spectrum for the dithionite reduced

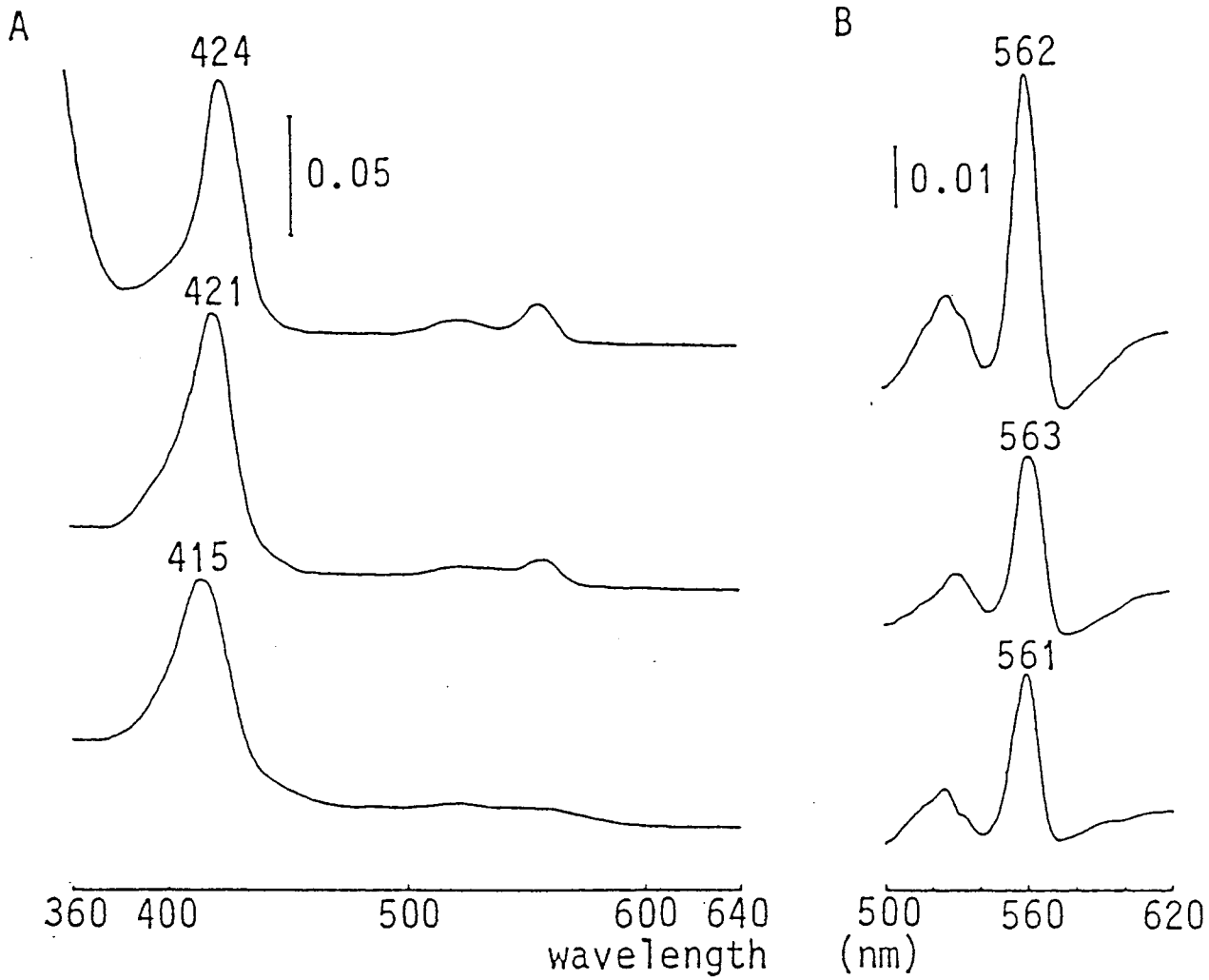

Fig. I-3. Visible spectra of the isolated complex III from Euglena. A) Absorption spectra of the isolated complex III (0.15 mg protein/ml). The top spectrum is of a dithionite reduced sample; the middle, an ascorbate reduced sample; and the bottom, an air oxidized sample. B) Difference absorption spectra of the isolated complex III (0.5 mg protein/ml). The top spectrum shows a difference spectrum of dithionite reduced minus ferricyanide oxidized form; the middle, a difference spectrum of dithionite reduced minus ascorbate reduced form; and the bottom, a difference spectrum of ascorbate minus ferricyanide oxidized form.

minus ascorbate reduced complex (Fig. I-3B, middle).

Identification of the subunits of Euglena complex III

The subunit structure of the Euglena complex III(Fig. I-1) was essentially similar to that from other eukaryotic sources (1,2,6-15) and was apparently different from that of prokaryotic cytochrome bc_1 complexes (8,9,16-18). Purified complex III was analyzed on two SDS-PAGE systems (systems A and B) (Fig. I-1). The relative positions of bands IV and V and bands VIII and IX on gel system B were reversed on gel system A. The band X on gel system B occasionally formed doublet bands on gel system A. In Euglena complex III there were two larger subunits, called core proteins, and a number of smaller subunits present in the complexes from mitochondria of other eukaryotes. The isolated complex consisted of 10 subunits, and their relative molecular masses on an SDS-urea gel (Fig. I-1B) were: subunit I, 50; II, 42; III, 37; IV, 32.5; V, 32; VI, 30; VII, 18; VIII, 9.6; IX, 8.8; X, 7.2 kDa.

Staining of gels for peroxidase activity indicated the presence of cytochromes. In gel system A only subunit IV showed peroxidase activity (Fig. I-4A, lane 2), whereas in gel system B not only subunit IV but also subunit III were stained. Subunit III on gel system A was a faint band and was occasionally not detected by protein staining (Fig. I-4A, lane 1). The highly hydrophobic amino acid composition of isolated subunit III and its amino-terminal sequence analysis described below supported the identification of this as cytochrome b. Subunit IV was

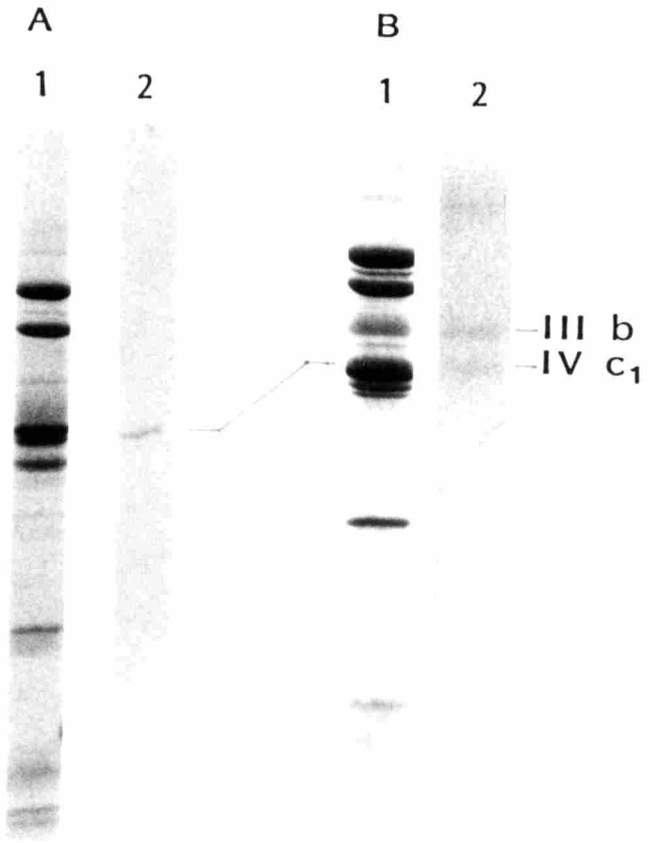

Fig. I-4. SDS-polyacrylamide gel analysis of the isolated Euglena complex III showing protein and peroxidase staining. A) SDS-PAGE system A was used to analyze 10 µg of the Euglena complex III. B) System B was used to analyze 20 µg of the Euglena complex III on each lane. In each case: Lane 1, the gels were stained for protein with Coomassie Brilliant Blue R-250; and Lane 2, the gels were stained for peroxidase activity. See Fig. I-1 for the identification of subunits in gel systems A and B.

identified as cytochrome $\underline{c}_1$ from its amino-terminal sequence analysis (51) and cDNA cloning described in the following part II and III, respectively.

Amino acid compositions of the isolated 10 subunits are given in Table I-3, and the N-terminal sequences are listed in Table I-4. Subunit III, cytochrome b, consisted of relatively higher content of hydrophobic amino acids than the other subunits. The N-terminal residues of the subunits I, III, V, VII, and VIII were probably blocked. In the case of subunit III, the N-terminal residue is probably formylMet, as phenylthiohydantoin derivatives beginning with Met were identified after treatment with methanol containing 1 N HCl (58). N-terminal sequences were determined for the subunits II, III, IV, VI, IX, and X. Homologies were found between the sequence of the subunit III and that of yeast or human cytochrome b. Apparent homology was not found when the sequences of the other subunits were compared with those of the subunits constituting bovine or yeast complex III.

Some properties of the isolated complex III

The ubiquinol-cytochrome c reductase activity of the isolated complex III from Euglena showed up to 10 % of the antimycin-sensitive activity reported for isolated $\underline{bc}_1$ complexes from other sources (1,2,5-19). The low activity corresponded to the 'antimycin-inhibited' ubiquinol-cytochrome c reductase activity shown by bovine enzyme (49). In a typical preparation either antimycin or myxothiazol at a molar ratio of a thousand had almost no effect on the ubiquinol-cytochrome c reductase activity of the isolated complex III. Another specific inhibitor of

Table I-3. Amino acid compositions of the 10 subunits of Euglena complex III

Values in mol % are listed. Cmc, carboxymethyl cysteine; n.d., not determined.

	Subunit									
	I	II	III	IV	V	VI	VII	VIII	IX	X
cyt.			b	c_1						
kDa:	50	42	37	32.5	32	30	18	9.6	8.8	7.2
Cmc	n.d.	n.d.	n.d.	1.1	n.d.	n.d.	0.3	n.d.	n.d.	n.d.
Asx	11.0	10.0	11.2	9.9	10.1	9.9	7.4	8.8	7.5	8.7
Thr	5.6	7.0	4.9	4.5	4.6	4.7	8.3	5.1	6.3	5.7
Ser	6.6	8.6	9.0	5.9	4.7	6.3	3.5	5.2	5.2	5.4
Glx	12.1	9.5	5.8	10.0	13.1	9.8	9.1	12.0	10.3	13.0
Pro	4.6	4.5	4.4	8.3	7.3	8.3	4.8	4.3	6.9	2.5
Gly	8.4	7.5	7.7	8.2	4.7	7.8	6.9	9.7	8.1	5.8
Ala	9.5	12.5	6.6	8.0	8.1	9.8	13.4	10.1	10.5	14.6
Val	5.4	7.3	4.6	6.1	4.5	6.0	7.6	6.7	6.0	4.6
Met	1.3	0	2.0	2.0	2.4	3.3	1.8	2.0	1.2	1.2
Ile	5.2	4.0	9.3	2.9	3.7	4.6	2.5	4.9	4.4	4.6
Leu	8.9	9.7	13.2	7.6	11.6	8.2	11.6	10.3	9.6	10.0
Tyr	2.9	1.5	4.5	5.5	4.3	4.0	2.0	0.9	2.9	3.3
Phe	4.8	5.2	6.2	5.5	3.7	3.7	7.2	4.8	4.5	7.7
Lys	5.6	7.1	3.2	5.2	4.7	5.3	5.2	4.9	5.9	6.5
His	3.3	2.7	3.1	2.3	3.8	2.0	2.7	2.5	2.8	0.6
Arg	5.7	2.9	4.3	7.0	8.8	6.3	5.7	7.8	7.9	5.8
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table I-4. Amino-terminal sequences of the subunits of the Euglena complex III

Isolated 10 subunits were analyzed for amino-terminal sequence. The sequence of subunit III was determined after treatment with methanol containing 1 N HCl (58). X, an unidentified residue; f, formyl.

Subunit		Amino-terminal sequence
	(kDa)	
I	50	not detected
		10
II	42	LFEVDKVQEK GL
		10
III	37	fMKISLYNHYH TWIL
		20 30
IV	32.5	GVDSHPPALP WPHFQWFQGL DWRSVRRGKE VYEQVFAP[a]
V	32	not detected
		10 20 30
VI	30	STSPAVNNAV GGLATSQIRD LVGNPAKSGK
VII	18	not detected
VIII	9.6	not detected
		10 20
IX	8.8	ATTAVSGELL TKTPYTRPGY AAQ
		20 40
X	7.2	MLANFSRFFY KTFLQSNATL IPLTLAFTYI VVEKGVXEGI DNA

a) Compete sequence will be presented in part III.

cytochrome $\underline{bc}_1$ and also of cytochrome $\underline{b}_6\underline{f}$ complex of photosynthetic membranes, DBMIB (1,52,53), inhibited the isolated Euglena ubiquinol-cytochrome c reductase. The inhibitor is known to act on electron transfer from ubiquinol to the Rieske iron-sulfur protein. The titration curve obtained (Fig. I-5) was very similar to that reported for the bovine complex (52).

The relative molecular mass of the isolated complex III in the Triton X-100 micelle was estimated to be 570 kDa using a gel filtration column of Toyopearl HW-65F (Fig. I-6). This indicated that the complex III was a dimer in a micelle of Triton X-100 if each subunit existed in one mol per mol of complex, as reported for complex III from other sources (6,11,13).

Some kinetic parameters were determined from the results shown in Fig. I-7. The maximal turnover number was 5.9 sec^{-1} and the K_m values for ubiquinol-2 and for horse heart cytochrome c were 5.4 and 7.6 μM, respectively. The parallel plots in Fig. I-7 indicate that the catalytic mechanism is a double-displacement reaction.

The maximal turnover number and K_m values for cytochromes c from horse heart, Yeast and Euglena at a fixed concentration of ubiquinol-2 were determined to be 4.8 and 6.5, 6.5 and 5.4, and 9.0 sec^{-1} and 1.1 μM, respectively (Table I-5). Euglena complex III transferred electrons to its physiological acceptor, cytochrome c(c-558), with the highest rate and the lowest K_m among these three.

Since most of phospholipids and ubiquinone seemed to be removed from the isolated complex III during isolation (Table I-

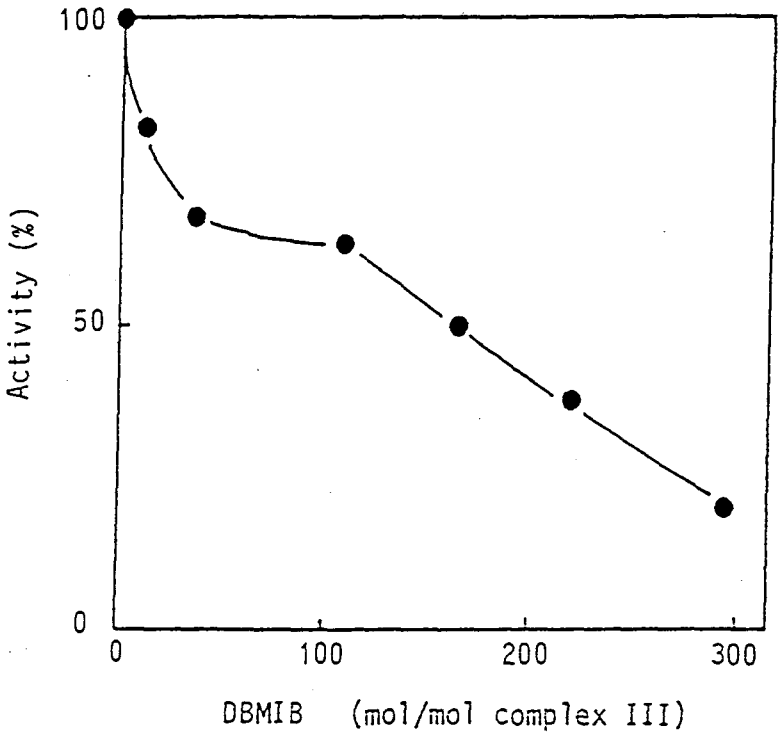

Fig. I-5. Inhibition of ubiquinol-cytochrome c reductase of the isolated complex III by dibromothymoquinone(DBMIB). The assay mixtures contained 0.54 nM of the isolated complex III (as cytochrome c_1 content) and indicated amounts of DBMIB. The rate in the absence of the inhibitor was 2.9 sec^{-1}.

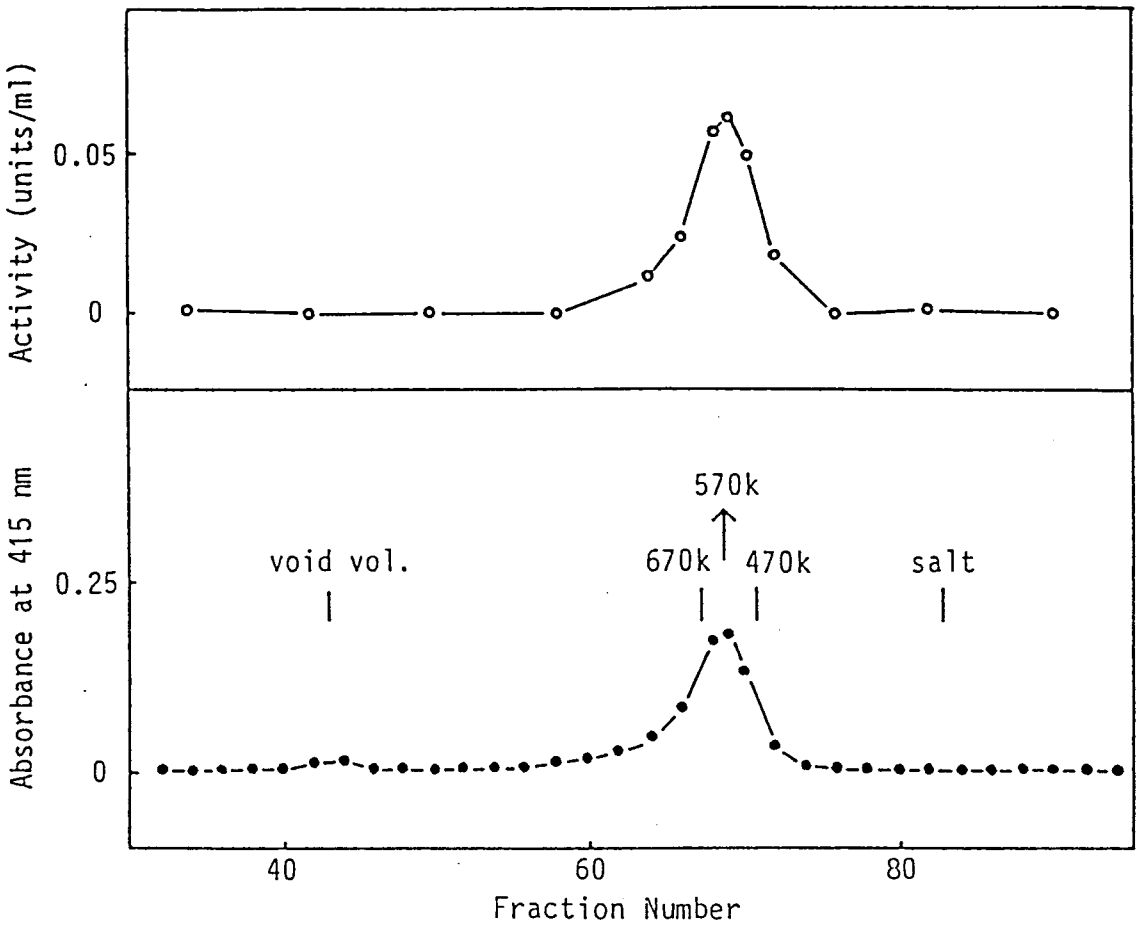

Fig. I-6. Gel filtration chromatography of the isolated Euglena complex III on a Toyopearl HW-65F column. The isolated Euglena complex III (4 mg protein) was applied to a Toyopearl HW-65F column (2.1 x 70 cm) equilibrated with buffer III (described in Materials and Methods), and eluted with the same buffer. The 3-ml fractions were collected, and both the absorbance at 415 mn and the ubiquinol-cytochrome c reductase activity were measured as described in Materials and Methods.

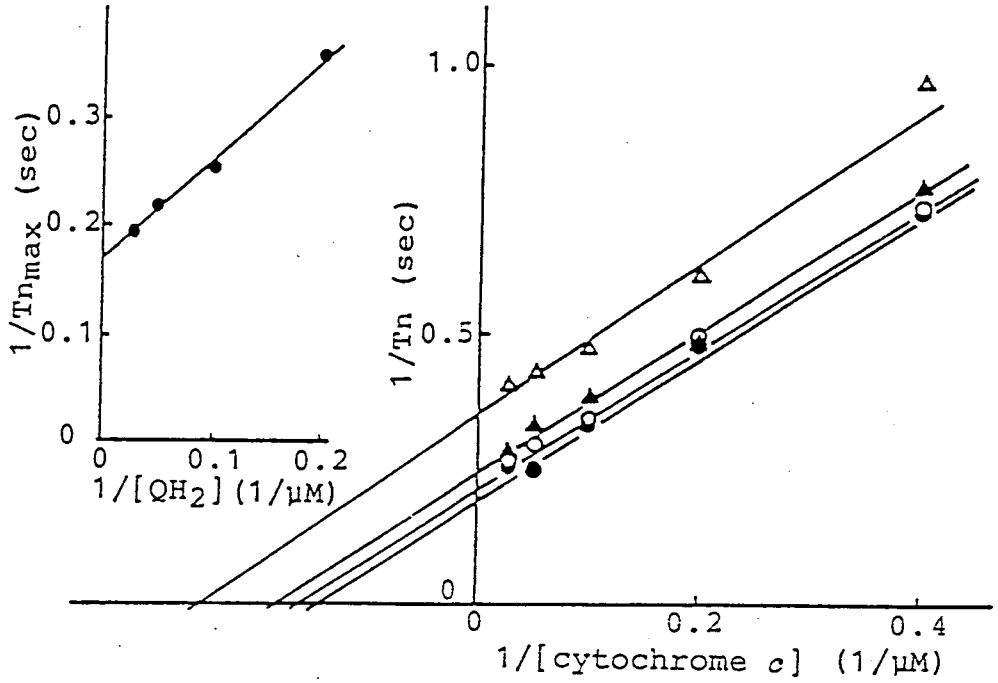

Fig. I-7. Kinetic analysis for the ubiquinol-cytochrome c reductase of Euglena complex III. The assay mixture contained ubiquinol-2 at the concentrations of 37.5 (closed circle), 20 (open circle), 10 (closed triangle), or 5 μM (open triangle), and cytochrome c at the concentrations of 40, 20, 10, 5, or 2.5 μM. The inset shows extrapolation of the maximal turnover number versus the reciprocal of the concentration of ubiquinol-2.

Table I-5. Comparison of the kinetic parameters of ubiquinol-cytochrome c reductase of isolated Euglena complex III using horse heart cytochrome c, yeast cytochrome c, and Euglena cytochrome c(c-558)

Ubiquinol-cytochrome c reductase was measured in an assay mixture containing 37.5 μM ubiquinol as described in Materials and Methods. The concentrations of cytochrome c used were 20, 10, 5, and 2.5 μM for both horse heart and yeast cytochrome c, and 10, 5, 3.3, and 2.5 μM for Euglena cytochrome c. The maximal turnover number and the Michaelis constant(K_m) were determined by graphical procedures.

Cytochrome c source	Turnover number	K_m
	(sec^{-1})	(μM)
Horse heart	4.8	6.5
Yeast	6.5	5.4
Euglena	9.0	1.1

2), the effect of replenishment with these was tested (Table I-6). The ubiquinol-cytochrome c reductase activity of the isolated complex III was activated up to 2.3-fold by addition of either crude phospholipid mixture or cardiolipin to the same extent. Addition of ubiquinol-2 prior to phospholipids, however, had no effect on the activity (cf. 55). The insensitivity of the ubiquinol-cytochrome c reductase activity of the isolated complex to antimycin and myxothiazol was not restored by these additions.

The ubiquinol-cytochrome c reductase activity of the isolated complex III was maximal at 0.006 % Triton X-100 in the assay medium. This concentration seemed to be independent on the concentration of isolated complex III, whereas the rate of non-enzymic reduction of cytochrome c decreased with increasing concentration of the detergent. Thus, some factor other than the state of complex III, such as the solubility of ubiquinol-2, may be involved in the effect of Triton X-100 on the activity.

DISCUSSION

The greatest difference in the isolated *Euglena* complex III compared to the cytochrome bc_1 complexes from other sources, whether mitochondria or prokaryotic cells, is the spectrophotometric properties of cytochrome c_1. The absorption maximum of the slightly asymmetrical α-band of *Euglena* cytochrome c_1 was at 561 nm and was shifted by 8 nm to the red (Fig. I-3) by comparison with other cytochromes c_1 whose symmetrical α-peak was at 552-553 nm (45-49). These spectrophotometric properties are similar to those for *Euglena* cytochrome c (c-558) (26-28), the physiological electron acceptor of the ubiquinol-cytochrome c

Table I-6. Effect of addition of crude phospholipid, cardiolipin, and ubiquinone-2 on the ubiquinol-cytochrome c reductase activity of the isolated Euglena complex III

The activity was assayed as described in Materials and Methods. The amounts of crude phospholipid(PL) was 0.8 mg/mg protein; cardiolipin(CL), 0.2 mg/mg protein; and ubiquinone-2(UQ), 10 mol/mol complex III.

Addition	Turnover number
	(sec^{-1})
none	2.4
PL	5.5
CL	5.3
PL+CL	5.4
UQ	2.4
UQ+PL	5.4
UQ+CL	5.6
UQ+PL+CL	5.3

reductase. This has an asymmetrical α-peak at 558 nm which is shifted by 8 nm to the red, and binds a heme to a single cysteine through a thioether bond. In the following part, evidence indicating that Euglena cytochrome c_1 binds its heme in this same way (51) will be provided.

The isolated complex consisted of 10 polypeptides (Fig. I-1), similar to the complex III from other eukaryotic cells (1,2,6-15). Two large subunits, the so-called core proteins I and II, and several small subunits were present in the complex with ubiquinol-cytochrome c reductase activity. This polypeptide composition appear to be common to the complexes from mitochondria and have not been found in the bc_1 complexes isolated from prokaryotes (1,2,8,9,16-19). Subunits III and IV were identified as the redox carrier subunits, cytochrome b and cytochrome c_1, respectively, by peroxidase staining of SDS-gels (Fig. I-4) and amino-terminal sequence analysis. The molecular masses of cytochrome c_1, 32.5 kDa, and cytochrome b, 37 kDa, on SDS-urea gels were similar to those of the subunits from other sources except for the 60-68 kDa cytochrome c_1 of Paracoccus (8,18,47). Cytochrome b had an α-peak at 563 nm, similar to that reported for other sources. The iron-sulfur protein was not identified, but the presence of this subunit was suggested by the content of acid-labile sulfur (Table I-2); the inhibition of ubiquinol-cytochrome c reductase with DBMIB also supports this view (Fig. I-5).

The isolated ubiquinol-cytochrome c reductase was not inhibited by antimycin, and its activity corresponded to the

level reported for the complex of other sources in the presence of antimycin. A similarly slow rate of antimycin-insensitive activity was reported for a reconstituted system consisting of iron-sulfur protein and cytochrome $\underline{c}_1$ (49). This antimycin-inhibited or insensitive ubiquinol-cytochrome $\underline{c}$ reductase activity was regarded as the electron transfer catalyzed by only the iron-sulfur protein and cytochrome $\underline{c}_1$, in which cytochrome $\underline{b}$ was not involved (49). According to the Q-cycle or related mechanisms (1-4), the iron-sulfur protein catalyzes oxidation of ubiquinol to ubisemiquinone and the electron on the iron-sulfur protein is transferred through cytochrome $\underline{c}_1$ to cytochrome $\underline{c}$. Normally, the electron on ubisemiquinone is transferred to cytochrome $\underline{b}_1$ and then $\underline{b}_h$ and is used for reduction of quinone; this process is highly sensitive to antimycin and electron transport through cytochromes $\underline{b}$ does not then occur in the presence of the inhibitor. Myxothiazol, another potent inhibitor, is considered to inhibit oxidation of ubisemiquinone by cytochrome $\underline{b}_1$, whose site is on the low potential side of electron flow through the antimycin-blocking site. This inhibitor partially inhibited the ubiquinol-cytochrome $\underline{c}$ reductase of only freshly prepared mitochondria from Euglena. The isolated complex III showed almost no myxothiazol-sensitive activity. The loss of sensitivity to antimycin and myxothiazol was concomitant. Thus, the ubiquinol-cytochrome $\underline{c}$ reductase of the isolated Euglena complex III appeared to be of this 'antimycin-inhibited' activity where cytochrome $\underline{b}$ was not involved. The insensitivity of the isolated Euglena complex III to these two antibiotics might be explained by lack of a functional route through cytochromes $\underline{b}$.

The low content of cytochrome b heme based on spectrophotometric analysis is possibly related to the characteristic sensitivity to both antimycin and myxothiazol effective on the electron transfer around the cytochrome(s) b. The inhibition of the isolated Euglena ubiquinol-cytochrome c reductase by DBMIB supports this view, since DBMIB is considered to inhibit electron transfer from ubiquinol to the iron-sulfur protein (1,52,53). The double-displacement reaction (Fig. I-7) probably explains the mechanism of electron transfer without cytochrome b involvement.

It was reassuring that the isolated Euglena complex III had the highest turnover number and the lowest K_m for Euglena cytochrome c (c-558), the physiological substrate (Table I-5). The other properties of the isolated complex III were essentially common to those reported for the bc_1 complexes isolated from other sources. A dimeric structure of complex III (Fig. I-6) has been reported for bovine, yeast and Neurospora enzyme (6,11,13). Replenishment with crude phospholipids or cardiolipin gave enhanced activity (Table I-6), suggesting a requirement for cardiolipin (54).

The growth of Euglena cells in the presence of antimycin was reported and was explained by an inducible alternative succinate oxidase (25). In fact, Euglena cells grew slowly in the presence of antimycin, but the mitochondrial membranes prepared from these cells showed almost no ubiquinol-cytochrome c reductase activity (unpublished data), confirming that the antimycin-insensitive growth was not due to the presence of an active but antimycin-insensitive ubiquinol-cytochrome c reductase.

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PART II

Amino-terminal sequence of *Euglena gracilis* cytochrome c_1 including an atypical heme-binding structure

SUMMARY

In the previous part, it was found that Euglena gracilis mitochondrial complex III showed an atypical α-band at 561 nm in the difference spectrum for cytochrome c_1. The pyridine ferrohemochrome prepared from covalently bound heme in the Euglena complex III had an α-peak at 553 nm. This wavelength is the same as that of pyridine ferrohemochrome prepared from Euglena cytochrome c (c-558), the heme of which is linked to only a single cysteine residue through a thioether bond.

The N-terminal 46-amino-acid sequence of cytochrome c_1 from Euglena was determined. On the basis of apparent homology to cytochromes c_1 from other sources, this sequence included the heme-binding region. However, the amino acid at position 36, corresponding to the first cysteine involved in heme linkage in other cytochromes c_1, was phenylalanine. Position 39, corresponding to the second cysteine, was not identified despite treatment for removal of the heme and carboxymethylation of the expected cysteine. The unidentified amino acid is assumed to be a derivative of cysteine to which the heme is linked through a single thioether bond. The histidine-40 corresponding to the probable fifth ligand for heme iron was conserved in Euglena cytochrome c_1.

INTRODUCTION

Typical C-type cytochromes have a covalently bound heme as prosthetic group(1,2). The heme binds to the polypeptide chain through two thioether bonds with a conserved sequence of -Cys-X-

Y-Cys-His-(1). Mitochondrial typical cytochromes *c* and c_1 show their characteristic visible spectra, where α-band maxima are at 550 (1) and 553 nm (3-7), respectively. An atypical cytochrome *c*(*c*-558) was found from *Euglena gracilis* mitochondria (8,9). The cytochrome shows its α-band maximum at 558 nm and the heme links to the polypeptide chain by a single cysteine with a sequence of -Ala-Ala-Gln-Cys-His- (10). Such an atypical cytochrome *c*(*c*-557) from *Crithidia*, another protozoan, mitochondria was also reported (10). Amino acid sequences were determined for cytochromes c_1 from mitochondria and some bacteria (11-17). There has been no report on the structure of protozoan cytochrome c_1.

In part I, *Euglena* cytochrome c_1 was found to show an α-band absorption maximum at 561 nm in the difference spectrum for purified complex III (18). The α-peak showed a red shift of 8 nm in comparison with those of cytochromes c_1 from other sources (3-7). This red shift is comparable to that of *Euglena* cytochrome *c*-558. This part provides the N-terminal 46-amino-acid sequence of *Euglena gracilis* cytochrome c_1 including the heme-binding region, which indicates that the heme in this case also binds to the polypeptide chain through a single thioether linkage (19).

MATERIALS AND METHODS

Materials

Complex III and cytochrome *c* (*c*-558) were isolated from *Euglena gracilis* SM-ZK, a chloroplast-lacking mutant, as described in the previous part (19). Bovine heart cytochrome c_1 was isolated as a subcomplex with the hinge protein, as previously reported (20,21). Hydroxylapatite, 2-

nitrophenylsulphenyl chloride, 2-mercaptoethanol and pyridine were products of Nakarai Chemicals (Kyoto). Iodoacetic acid and lysyl endopeptidase were from Wako Pure Chemical Industries (Osaka). Trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) was from Worthington Diagnostic Systems. An octadecylsilane column (TSKgel ODS-120T, 4.6 x 250 mm) for HPLC and Toyopearl HW-55F were from Toyo Soda (Tokyo). Sephadex G-150 was from Pharmacia. Diaflo XM300 ultrafiltration membranes from Amicon were used for concentration of the isolated complex III. UP-20 ultrafiltration membranes from Advantec (Tokyo) were used for concentration of cytochrome $\underline{c}_1$.

Purification of cytochrome c_1

The isolated complex III (60 mg) was mixed with a 1/49 volume of 0.5 M Na-phosphate, pH 7.2, and applied to a hydroxylapatite column (2.6 x 9 cm) equilibrated with a buffer composed of 10 mM Na-phosphate, pH 7.2, 50 mM NaCl and 0.1% Triton X-100, at 4 °C. The column was washed with 50 ml of the same buffer. Most of the subunits except cytochrome $\underline{c}_1$ were eluted from the column with 50 ml of a buffer composed of 6.7 mM Na-phosphate, pH 7.2, 2.0 M guanidine-HCl, 0.2 M NaCl, 0.1% Triton X-100 and 0.5% Na cholate, followed by washing with 50 ml of a buffer composed of 10 mM Na-phosphate, pH 7.2, 0.2 M NaCl and 0.1% Triton X-100. Next, 50 ml of a buffer containing 10 mM Na-phosphate, pH 7.2, 2% SDS and 1% 2-mercaptoethanol was passed through the column at room temperature, followed by a linear gradient between 200 ml each of 10 and 500 mM Na-phosphate, pH 7.2, in the presence of 0.1% SDS.

The fractions containing cytochrome $\underline{c}_1$ were pooled and concentrated on an ultrafiltration membrane. 2-Mercaptoethanol was added to the cytochrome solution to give a final concentration of 1% and the mixture was applied to a column of Toyopearl HW-55F (2 x 120 cm) (column I) equilibrated with 0.2 M Na-phosphate, pH 7.2, containing 0.1% SDS (buffer A) and then eluted with the same buffer. The fractions containing cytochrome $\underline{c}_1$ were combined and concentrated, and 2-mercaptoethanol was added in the same way as previously. The sample was applied again to column I and similarly eluted. After concentration of the eluate containing cytochrome $\underline{c}_1$ and addition of 2-mercaptoethanol, the sample was applied to a Sephadex G-150 column (2.1 x 77 cm) (column II) equilibrated and eluted with buffer A. The purified cytochrome $\underline{c}_1$ was dialyzed thoroughly against 0.3% ammonium bicarbonate to remove SDS, and then stored at -80 °C.

Preparation of CM-cytochrome c_1

In order to release the covalently bound heme, the isolated complex III (120 mg) was treated with nitrophenylsulphenyl chloride in 70% formic acid instead of acetic acid (22). The mixture was incubated for 20 min at 37 °C with gentle stirring, then diluted three-fold with water and extracted four times with ethylacetate using low-speed centrifugation. The aqueous phase was dialyzed against water and lyophilized. The dried sample was suspended in 8 ml of 6 M guanidine-HCl containing 1 M Tris-HCl, pH 8.0, and 5 mM Na-EDTA, and reduced with 200 µl of 2-mercaptoethanol. The mixture was incubated at 30 °C for 4 h and

carboxymethylated with 507 mg of iodoacetic acid (23). It was dialyzed against 0.2 M Na-phosphate, pH 7.2, to remove guanidine-HCl, and 2 ml of 20% SDS was added into the dialysis bag to dissolve the precipitate. The material was concentrated and chromatographed on column II as described earlier. The fractions containing cytochrome $\underline{c}_1$ were pooled and concentrated, and then chromatographed successively on columns I (twice) and II. The pure CM-cytochrome $\underline{c}_1$ was concentrated by ultrafiltration to 2 ml and dialyzed thoroughly against 0.2% ammonium bicarbonate to remove SDS. After concentration by lyophilization, the sample was again carboxymethylated using 100 µl of 2-mercaptoethanol and 253 mg of iodoacetic acid. The reaction mixture was dialyzed against 0.1% ammonium bicarbonate and 600 µl of 20% SDS was added into the dialysis bag. The dialysate was applied to column II and chromatographed again to remove any excess SDS. The fractions containing cytochrome $\underline{c}_1$ were pooled, concentrated on an ultrafiltration membrane, and dialyzed thoroughly against 0.2% ammonium bicarbonate.

Purification of a peptide containing the heme-binding region

CM-cytochrome $\underline{c}_1$ (50 nmol) in 2.4 ml of 0.2% ammonium bicarbonate was digested with 70 µg (1/20, w/w) of trypsin and 3 µg (1/500, w/w) of lysyl endopeptidase for 3 h at 37 °C. After lyophilization, the dried material was dissolved in a small volume of a solution containing 0.1% trifluoroacetic acid (TFA) and 0.2% ammonium bicarbonate. The digest was applied to an octadecylsilane column equilibrated with 0.1% TFA and the

peptides were separated with a linear gradient of 0 - 47% acetonitrile in 0.1% TFA using a Gilson HPLC system. The fractions showing absorbance at 220 nm were collected and analyzed for amino acid composition.

Analytical procedures

Protein was determined by the Lowry procedure (24) using bovine serum albumin as a standard. Isolated cytochrome $\underline{c}_1$ was determined by amino acid analysis assuming that its molecular weight was about 28,000. The amino acid composition was analyzed after hydrolysis of samples with 6 N HCl in an evacuated, sealed tube at 110 °C for 21 h. For hydrolysis of carboxymethylated samples, a small amount of thioglycolic acid was added to prevent decomposition of carboxymethylcysteine. The hydrolysates were analyzed with an amino acid analyzer (Irica model A-5500, Irica Instruments Inc., Kyoto, Japan), essentially as reported previously (25). Amino acid sequences were determined by Edman degradation (26) using a gas-phase sequencer (Applied Biosystems, model 470A) equipped with an on-line-connected HPLC, model 120A.

Preparation of pyridine ferrohemochromes

The isolated Euglena complex III (0.5 mg) was mixed with ice-cold acetone containing 2% (v/v) conc. HCl to give a 90% (v/v) acetone mixture and incubated for 10 min at 0 °C. The mixture was centrifuged and the supernatant and the precipitate were dried separately. The dried materials, hemin (1.0 nmol), horse heart cytochrome $\underline{c}$ (1.0 nmol), bovine heart cytochrome $\underline{c}_1$ (1.0 nmol), Euglena cytochrome $\underline{c}$ ($\underline{c}$-558) (1.0 nmol), and the isolated Euglena

complex III (0.5 mg) were converted to pyridine hemochromes (27) in 2.0 ml of a mixture containing 1 N NaOH and 10% pyridine. A small amount of solid Na-dithionite was added and the spectra of the ferrohemochromes of these materials were immediately recorded at room temperature using a Hitachi spectrophotometer, model 220A or U3200.

RESULTS

Pyridine ferrohemochromes prepared from isolated Euglena gracilis complex III

In Table II-1, the α-band maxima of pyridine ferrohemochromes prepared from isolated Euglena complex III are compared with those prepared from hemin, horse heart cytochrome c, bovine heart cytochrome c_1, and Euglena cytochrome c (c-558). The pyridine ferrohemochrome prepared directly from the isolated Euglena complex III had an α-band maximum at 554.8 nm. Upon acidic acetone extraction, noncovalently bound heme from cytochrome(s) b was extracted and the pyridine ferrohemochrome prepared from this extract showed an α-band maximum at 555.6 nm, similar to that from hemin. The pyridine ferrohemochrome prepared from the resulting orange precipitate had an α-band maximum at 553.0 nm (Fig. II-1). This indicated the presence of a covalently bound heme of cytochrome c_1 in the isolated Euglena complex III. The α-peak was the same as that of the pyridine ferrohemochrome prepared from Euglena cytochrome c (c-558) (10) and showed a red shift of 3 nm when compared with those of horse heart cytochrome c and bovine heart cytochrome c_1, the hemes of which were bound

Table II-1. Comparison of wavelengths of the α-peaks of pyridine ferrohemochromes

Hemin, horse heart cytochrome *c*, bovine heart cytochrome c_1, *Euglena* cytochrome *c* (*c*-558), and *Euglena* complex III were converted directly to the respective pyridine ferrohemochromes. *Euglena* complex III was treated with acidic acetone to extract non-covalently bound heme, and the supernatant and precipitate were converted to pyridine ferrohemochrome after evaporation (see Materials and Methods).

Sample	α-peak
	(nm)
Hemin	555.9
Horse heart cytochrome *c*	550.0
Bovine heart cytochrome c_1	550.0
Euglena cytochrome *c* (*c*-558)	553.1
Euglena complex III	554.8
Euglena complex III	
Acidic acetone extraction	
Supernatant	555.6
Precipitate	553.0

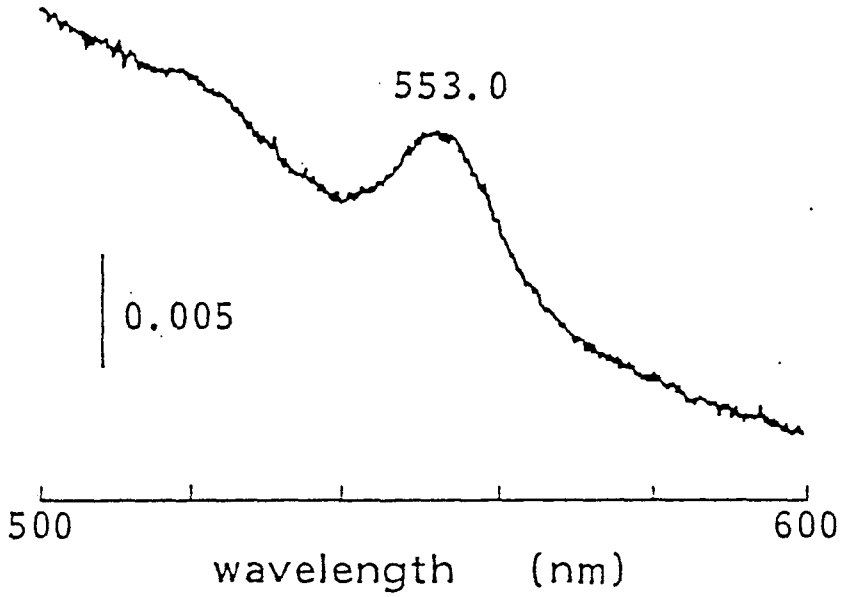

Fig. II-1. Absorption spectrum of pyridine ferrohemochrome prepared from acidic acetone-extracted Euglena gracilis complex III. Isolated complex III (0.5 mg) from Euglena gracilis was extracted with acidic acetone. The pyridine hemochrome was prepared from the precipitate and reduced with Na dithionite.

to the polypeptide chain through two thioether linkages.

Purification of cytochrome c_1 and CM-cytochrome c_1

Cytochrome $\underline{c}_1$, the subunit IV of the isolated complex III, was purified in the presence of denaturing reagents. Cytochrome $\underline{c}_1$ was eluted from a hydroxylapatite column with SDS after elution of most of the other subunits with 2 M guanidine-HCl (28). A combination of gel filtration chromatography on a Toyopearl HW-55F column and on a Sephadex G-150 column in the presence of SDS was effective for the separation of cytochrome $\underline{c}_1$. 12 nmol of cytochrome $\underline{c}_1$ was purified to homogeneity from 60 mg of complex III, and was almost colorless. It did not show peroxidase activity on SDS gels.

CM-cytochrome $\underline{c}_1$ was prepared from the complex III as described in the Materials and Methods and separated from other subunits by a combination of gel filtration chromatography on two different columns, as described above. Starting with 120 mg of complex III, 90 nmol of CM-cytochrome $\underline{c}_1$ was purified.

The isolated cytochrome $\underline{c}_1$ and CM-cytochrome $\underline{c}_1$ had almost the same amino acid composition (Table II-2) similar to those of eukaryotic cytochromes $\underline{c}_1$ (11-13,17).

N-Terminal amino acid sequence of the isolated Euglena cytochrome c_1

Direct N-terminal sequence analysis of the isolated Euglena cytochrome $\underline{c}_1$ determined the sequence of the first 38 amino acids (Fig. II-2). The residue at position 36, corresponding to the first cysteine involved in the heme linkage in cytochromes from

Table II-2. Amino acid compositions of cytochrome c_1, carboxymethyl (CM-) cytochrome c_1, and an isolated peptide including the heme-binding region

Samples were hydrolyzed and analyzed as described in Materials and Methods. Cysteine, cystine and tryptophan were not determined. The number of total amino acids per molecule was assumed to be about 240, excluding tryptophan. The numbers in parentheses were determined by sequence analysis. Cmc, carboxymethyl cysteine; n.d., not determined.

Amino acid	Cytochrome c_1		CM-cytochrome c_1		Heme peptide	
	(mol%)	(mol/mol)	(mol%)	(mol/mol)	(mol/mol)	
Cmc	n.d.	n.d.	1.1	3	0.0	(0)
Asx	10.3	24	9.9	24	0.59	(0)
Thr	4.4	10	4.5	11	0.27	(0)
Ser	6.4	15	5.9	14	2.0	(2)
Glx	10.4	25	10.0	24	3.1	(3)
Pro	8.9	21	8.3	20	1.4	(1)
Gly	9.1	21	8.2	20	0.64	(0)
Ala	8.0	19	8.0	19	1.2	(1)
Val	6.1	14	6.1	15	1.7	(2)
Met	0.7	2	2.0	5	0.19	(0)
Ile	3.2	8	2.9	7	0.85	(1)
Leu	7.5	18	7.6	18	1.1	(1)
Tyr	4.6	11	5.5	13	0.98	(1)
Phe	5.6	13	5.5	13	1.9	(2)
Lys	5.2	12	5.2	12	0.94	(1)
His	2.8	7	2.3	5	0.96	(1)
Arg	6.8	16	7.0	17	0.10	(0)
X						(1)
Total residues		236		240		17
Yield (%)					28	

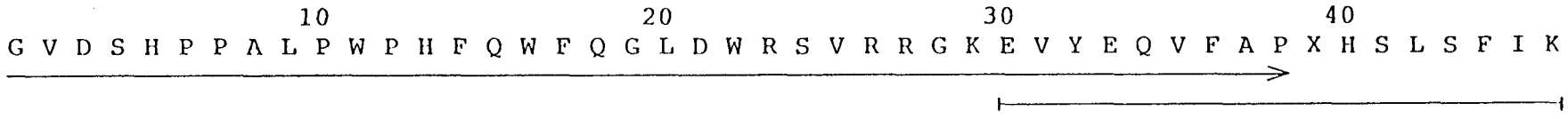

Fig. II-2. N-terminal Amino Acid Sequence Study of Euglena gracilis Cytochrome c_1. Direct N-terminal amino acid sequence analysis for isolated cytochrome c_1 is indicated by an arrow up to position 38. The bar indicates an isolated heptadecapeptide including the heme- binding region obtained from a digest of isolated carboxymethyl-cytochrome c_1. X represents an unidentified amino acid.

other sources (11-17), was replaced by phenylalanine. The amino acid sequence after position 39 was not determined for this preparation.

A heptadecapeptide was purified from a digest of CM-cytochrome $\underline{c}_1$ with a mixture of trypsin and lysyl endopeptidase by reverse-phase HPLC with a yield of 28%. Its amino acid composition is shown in Table II-2 and the amino acid sequence was determined except for position 39 (Fig. II-2). Amino acids, such as Asx and Gly, present in its composition and not identified by sequence analysis, might have been due to a small quantity of a large contaminating peptide(s) rich in these amino acids. The sequence of the isolated peptide overlapped with the direct N-terminal sequence from Glu-30. Thus, the N-terminal 46-amino-acid sequence was established. However, the residue at position 39 was not identified. The histidine-40, the probable fifth ligand for heme iron in cytochromes $\underline{c}_1$, was found to be conserved when the sequence was aligned with those of cytochromes $\underline{c}_1$ from other sources (11-17).

These results indicated that the N-terminal sequence included the heme-binding region and suggested that the unidentified amino acid was a derivative of cysteine, to which the heme was originally attached through a thioether bond. The reason why the amino acid at position 39 could not be determined, despite carboxymethylation after heme release, is not known. As will be discussed later, other evidence supported the contention that the unidentified amino acid was cysteine.

DISCUSSION

When salt-washed submitochondrial particles prepared from Euglena gracilis were extracted with acidic acetone, the pyridine ferrohemochrome prepared from the resulting pellet did not show the typical spectrum of c-type cytochrome (not shown). This suggested the presence of an atypical cytochrome $\underline{c}_1$. The part I reported that cytochrome $\underline{c}_1$ in the Euglena complex III had an atypical α-band, as revealed by the difference absorption spectrum (19). It was slightly asymmetrical with a maximum at 561 nm, shifted by 7-8 nm toward the red in comparison with cytochromes $\underline{c}_1$ from other sources (3-7). Such a red shift of the α-band by 7-8 nm has also been found for two other protozoan cytochromes $\underline{c}$ (cytochrome $\underline{c}$-558 from Euglena and cytochrome $\underline{c}$-557 from Crithidia)(10). These cytochromes $\underline{c}$ bind a heme to only a single cysteine through a thioether bond (8-10).

The pyridine ferrohemochrome of the covalently bound heme in the isolated Euglena complex III also showed an atypical cytochrome $\underline{c}_1$ (Fig. II-1 and Table II-1). The red-shift in both the difference absorption spectrum (19), and the absorption spectrum of pyridine ferrohemochrome (Fig. II-1) indicated a similarity of structure between the heme-binding site in cytochrome $\underline{c}_1$ and that in cytochrome $\underline{c}$ (8-10) of Euglena mitochondria.

The N-terminal 46-amino-acid sequence of Euglena cytochrome $\underline{c}_1$ indicated that the heme was bound to only a single cysteine residue, although the amino acid corresponding to the second cysteine was not positively identified (Fig. II-2). The sequence included a region with apparent homologies to the heme-binding

sequence of cytochromes $\underline{c}_1$ from other sources (11-17). Furthermore, Euglena cytochrome $\underline{c}_1$ had phenylalanine-36 at the position corresponding to the first cysteine in other cytochromes $\underline{c}_1$. The histidine, a probable fifth ligand for the heme iron, was also conserved in Euglena cytochrome $\underline{c}_1$. Thus, the amino acid at position 39 was probably cysteine and the heme of Euglena cytochrome $\underline{c}_1$ was linked to only a single cysteine.

Other evidence supported the likelihood that the unidentified amino acid at position 39 was a cysteine derivative. Mass spectrometric analysis of the isolated heptadecapeptide indicated the residue weight of the amino acid to be 147, corresponding to that of phenylalanine or methionine sulfoxide, but these amino acids were not consistent with the amino acid composition and the sequence of the peptide. S-(2-Hydroxyethyl) cysteine, with the same residue weight, was synthesized chemically from ethylene bromohydrin and cysteine. Amino acid analysis of the hydrolysate of the isolated peptide gave an almost stoichiometric amount of an unidentified peak at the same retention time between those of aspartic acid and threonine as that of S-(2-hydroxyethyl)cysteine. The PTH-derivative of this amino acid also showed the same retention time as the unidentified PTH-derivative at the 10th step of sequence analysis of the heptadecapeptide upon HPLC connected with the gas-phase sequencer. S-(2-Hydroxyethyl)cysteine might be formed by cleavage between a pyrrole ring of the heme and a vinyl moiety linking the sulfur of cysteine, although this would be an unusual case. As to the isolated peptide, treatment with 2-nitrophenylsulphenyl chloride

released the heme in the different way from the cases of other cytochromes, although the color due to the covalently bound heme in the isolated Euglena complex III disappeared in a similar way to that in horse cytochrome c and bovine cytochrome c_1.

The N-terminal amino acid sequence of Euglena cytochrome c_1 is compared with those of other cytochromes from mitochondria and prokaryotes in Fig. II-3, where the unidentified amino acid at position 39 is assumed to be cysteine. While the sequence of Euglena cytochrome c_1 has apparent homologies to those from other sources (11-17), the homologies are lower than those among other cytochromes c_1. On the other hand, cytochrome c_1 from Euglena, one of the most primitive eukaryotic cells, is more similar to those from other mitochondria (11-13) rather than to those from prokaryotes (14-16).

In the case of Euglena cytochrome c, the cysteine binding the heme was identified (8-10) and the chemical structure of the bound porphyrin was established as 2-vinyl-4-(α-S-cysteinylethyl) deuteroporphyrin IX (29). The single thioether linkage of heme was found for both membraneous cytochrome c_1 and soluble cytochrome c in Euglena mitochondria, while the heme of Euglena chloroplast cytochrome c-552 is bound via two thioether bonds (30). This might apply to other protozoan c-type cytochromes. The unique structure of the heme linkage in these different c-type mitochondrial cytochromes might reflect a common attachment mechanism of the heme to the polypeptide chain.

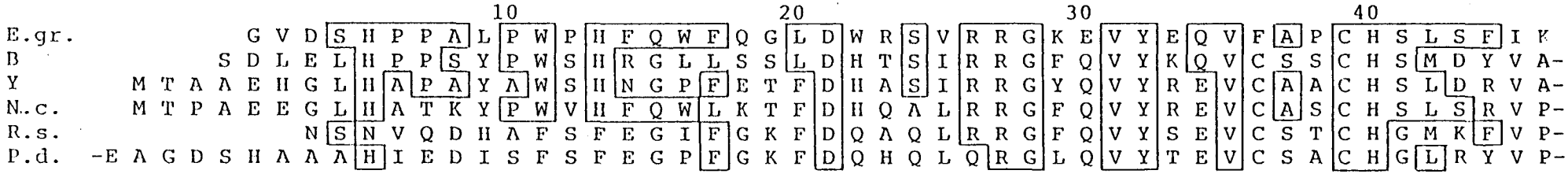

Fig. II-3. Comparison of N-terminal Amino Acid Sequences Including Heme-binding Region of Cytochromes c_1 from Mitochondria (Euglena gracilis, E.gr.; bovine (11), B; yeast (12), Y; Neurospora crassa (13), N.c.) and Prokaryotes (Rhodobacter sphaeroides (14), R.s.; Paracoccus denitrificans (16), P.d.). The numbers pertain to the sequence of Euglena cytochrome c_1. The residues identical with those of Euglena cytochrome c_1 are boxed.

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PART III

Molecular Cloning and Nucleotide Sequence of a cDNA encoding *Euglena gracilis* Cytochrome c_1

SUMMARY

The amino acid sequence of the mature protein of Euglena gracilis cytochrome c_1 was determined by sequencing of its cDNA. A cDNA expression library was constructed from Euglena poly(A)$^+$ RNA in phage λgt11 and screened with an antiserum raised against cytochrome c_1 polypeptide isolated from purified Euglena gracilis complex III. An isolated cDNA clone consisted of 872 base pairs and encoded the mature protein with 243 amino acids. The deduced amino acid sequence contained the unusual heme binding sequence -Phe-Ala-Pro-Cys-His- instead the typical sequence, -Cys- X - Y - Cys-His-, found in C-type cytochromes as reported in part II.

INTRODUCTION

Cytochrome c_1 is a subunit of the cytochrome bc_1 complex (complex III, ubiquinol-cytochrome c oxidoreductase, EC 1.10.2.2), which is one of the oligomeric enzymes in the respiratory chain of the mitochondrial inner membrane and some bacterial cell membranes (1,2). Cytochrome c_1 accepts electrons from Rieske iron-sulfur protein, another subunit in the complex, and transfers electrons to cytochrome c on the outer surface of the inner membrane (3,4).

Amino acid sequences have been established for cytochromes c_1 of bovine heart (5), yeast (6), Neurospora crassa (7), Rhodobacter sphaeroides (8), Rhodobacter capsulatus (9) and Paracoccus denitrificans (10), and human (11). Recent studies on the synthesis of cytochrome c_1 in yeast or Neurospora showed that it was synthesized as a precursor whose amino-terminal

presequence was cleaved in two successive steps coupling with import and sorting into mitochondria (12,13).

As described in part I, an unusual cytochrome $\underline{c}_1$ was found in Euglena gracilis mitochondria (14). Its α-band exists at 561 nm instead 553 nm (14). The heme is linked through a single thioether bond to the polypeptide chain with a sequence of -Phe-Ala-Pro-Cys-His- as described in the previous part (15). This part describes cDNA cloning and nucleotide sequence encoding Euglena cytochrome $\underline{c}_1$ to obtain more information on the structure of the unique cytochrome $\underline{c}_1$.

MATERIALS AND METHODS

Materials

Euglena gracilis complex III and cytochrome $\underline{c}_1$ were isolated as described methods (15). (α-^{32}P)dCTP (3000 or 800 Ci/mmol) were products of Amersham Corp. Restriction enzymes were obtained from Toyobo Co. (Osaka) or Nippon Gene Co. (Toyama, Japan). A DNA ligation kit was purchased from Takara Shuzo Co. (Kyoto). Nitrocellulose filters were Millipore HATF. Unless specified, all recombinant DNA techniques followed the procedures described by Maniatis et al. (16).

Construction of a cDNA library

Euglena gracilis SM-ZK, a stable chloroplast-lacking mutant, was grown aerobically on Oda medium lacking glucose (17), and the cells were collected at the middle exponentially growing stage. Total RNA was extracted with the guanidine thiocyanate method (18) using Amersham's RNA extraction kit. Poly(A)$^+$ RNA was

prepared by chromatography on an oligo(dT)-cellulose column (Collaborative Research, type 3) (19). A cDNA library in λgt11 was prepared by the methods of Gubler and Hoffman (20) and Huynh *et al.* (21) using Amersham's kits.

Immunoscreening

An antiserum was raised against purified *Euglena* cytochrome c_1 polypeptide in a rabbit. The cDNA library was screened following the procedure of Huynh *et al.* (21). The antiserum was diluted 200-fold and treated with an extract of *E. coli* Y1090 before use. Bound antibodies were visualized using an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antibody (Bio-Rad) (22). Affinity-preparation of antibodies cross-reacting with expressed fusion protein was performed as described (23). The antibodies thus obtained were used for immunoblot analysis.

DNA Sequencing

Appropriate restriction fragments of cDNA inserts were subcloned into M13mp18 (24). DNA sequencing was carried out by the dideoxy method of Sanger *et al.* (25) using Sequenase™ sequence kits for dGTP and 7-deaza-dGTP (U. S. Biochemical) (26).

Peptide Sequencing

Several peptides were isolated from a digest of *Euglena* carboxymethyl cytochrome c_1 with a mixture of trypsin and lysylendopeptidase as in the previous part (15). Amino acid sequence was determined (27) using a gas phase sequencer (Applied Biosystems Inc., model 470A) equipped with an on-line

phenylthiohydantoin derivative analyzer.

RESULTS AND DISCUSSION

Isolation of cDNA clones encoding for Euglena cytochrome c_1

A λgt11 cDNA library constructed from poly(A)$^+$ RNA of a bleached mutant of Euglena was screened with rabbit polyclonal antibodies raised against the purified cytochrome c_1 polypeptide. Among 68,000 recombinant clones 24 positive clones were found and isolated. Antibodies cross-reacting with the expressed fusion proteins by the individual positive clones were prepared and used for immunoblot analysis of isolated Euglena complex III. The affinity-prepared antibodies recognized a subunit corresponding to cytochrome c_1. The insert size of the positive clones varied from 0.4 to 1.8 kilobase pairs (kbp), and 22 clones out of 24 are 0.8 to 0.9 kbp in size.

Nucleotide Sequencing

One of the 0.9-kbp inserts was subcloned into a phage vector M13mp18 and sequenced. Figure III-1 shows the restriction map and the strategy for nucleotide sequencing of the insert. The nucleotide sequence of 872-bp insert was determined as shown in Fig. III-2 with deduced amino acid sequence. A coding region is flanked by a 3'-untranslational region which is followed by poly(A). The longest 1.8-kbp insert was also subcloned and sequenced. This insert started at the forth nucleotide of the 872-bp insert, and the identical sequence of the 846 nucleotides was followed with a poly(A) and an unrelated sequence. The 1.8-

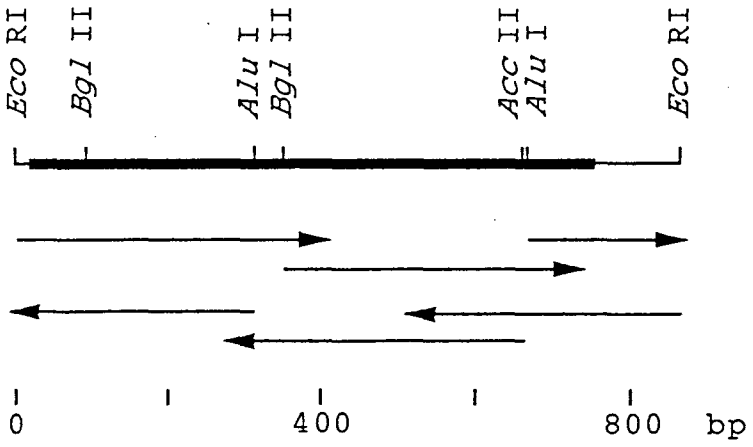

Fig. III-1 Restriction map and sequencing strategy for a cDNA encoding Euglena gracilis cytochrome c_1. The mature protein encoding region is shown by the solid line. Arrows indicate the direction and extent of the sequence determinations.

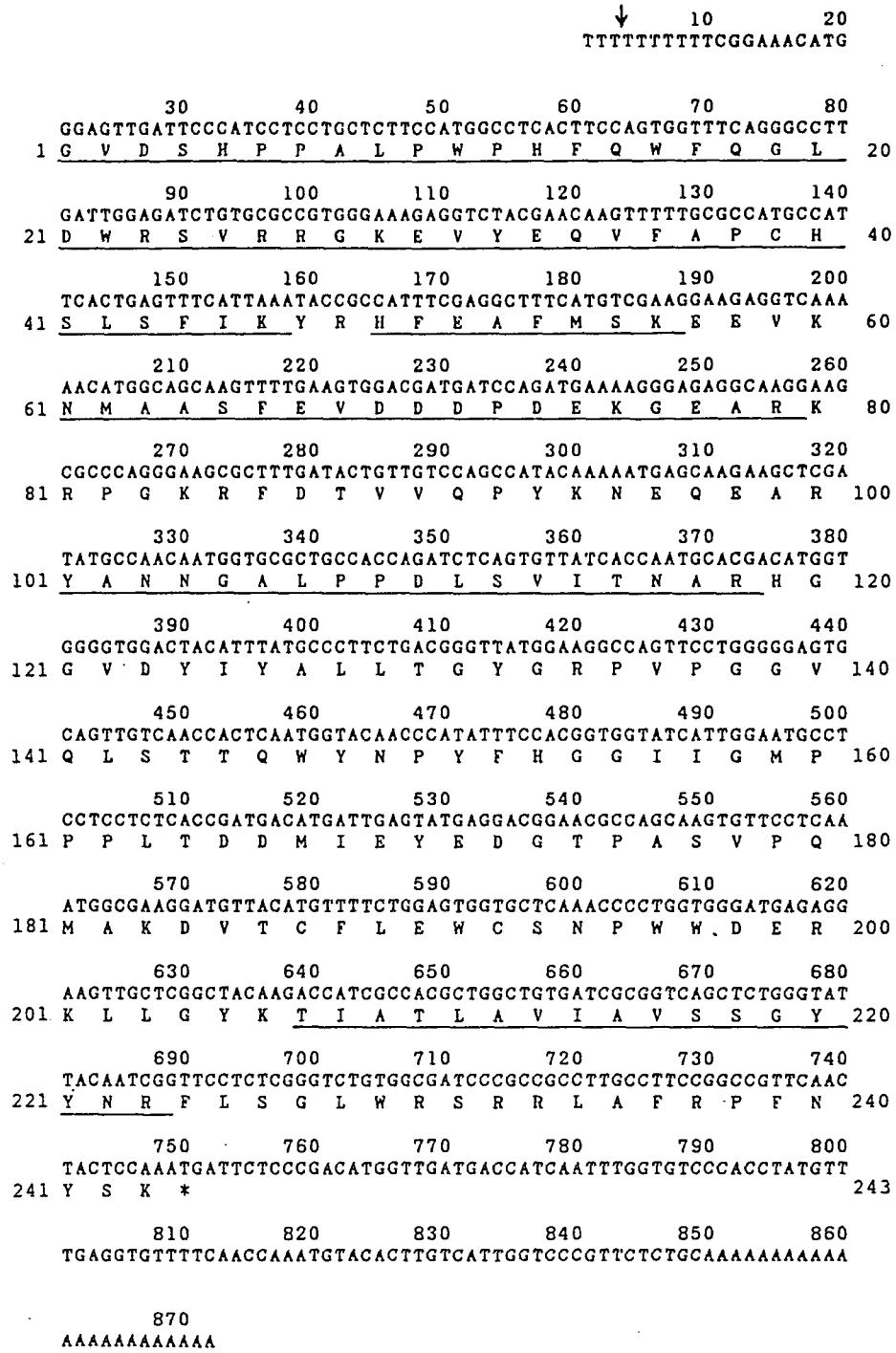

Fig. III-2 Nucleotide sequence of Euglena cytochrome c_1 cDNA and deduced amino acid sequence for the mature protein. Linker sequences at the 5'- and 3'-end of the clone are omitted from the chart. Amino acid sequence is numbered beginning with the amino-terminal residue determined by chemical analysis of the purified mature protein. Underlines show the regions identical to sequences determined with the purified protein or the isolated peptide fragments from the carboxymethylated protein. An arrow indicates the 5'-end of another insert.

kbp insert presumably resulted from blunt-end ligation during construction of the cDNA library.

Deduced Amino Acid Sequence

The deduced amino acid sequence included the mature protein of Euglena cytochrome c_1. The amino-terminal sequence of the purified protein analyzed chemically (15) started at the 21st nucleotide. Amino acid sequences determined with four purified peptides from a digest of the purified protein were also assigned in the deduced sequence. The results of amino acid analysis of the peptides are shown in Table III-1. Peptide 2 and 3 corresponded to residues 61 to 79 and 101 to 118, respectively. Peptide 1 corresponding to residues 49 to 56 was contaminated with peptide 2. Peptide 4, residues 207 to 223, was contaminated with a peptide beginning with residue 135 and other unidentified peptide(s). The deduced amino acid sequence of the mature protein of Euglena cytochrome c_1 consisted of 243 amino acids, and the molecular weight was 27,855, excluding the heme.

Comparison of the deduced amino acid sequence of the mature protein with those of other cytochromes c_1 is shown in Fig. III-3. An atypical heme-binding sequence -Phe-Ala-Pro-Cys-His- was at positions 36 to 40 (15) instead -Cys- X - Y -Cys-His- (1,2). The amino acid at 39 was unambiguously determined to be cysteine, for which evidence indicating a cysteine derivative had been obtained in the previous part (15). The heme is covalently linked by the single cysteine at position 39, probably chelated by His-40 as the fifth ligand just like other C-type cytochromes. The sixth ligand is most likely Met-159, which is conserved in all the

Table III-1. Amino acid compositions of peptides isolated from digest of CM-cytochrome c_1. Preparation and digestion of CM-cytochrome c_1 and separation of peptides were described in part II. Amounts of tryptophan were not determined. Cmc, carboxymethylcysteine. The numbers in parentheses were determined by peptide sequencing.

	Peptide			
Amino acid	1	2	3	4
Cmc	0.1(0)	0 (0)	0 (0)	0 (0)
Asx	1.8(0)	4.7(5)	3.6(4)	2.0(1)
Thr	0.2(0)	0.2(0)	1.0(1)	1.3(2)
Ser	1.2(1)	1.1(1)	1.1(1)	2.3(2)
Glx	1.9(1)	3.1(3)	0.5(0)	2.5(0)
Pro	0.5(0)	0.9(1)	2.0(2)	0.8(0)
Gly	0.4(0)	1.1(1)	1.2(1)	3.0(1)
Ala	1.8(1)	3.0(3)	2.8(3)	1.8(3)
Val	0.4(0)	0.9(1)	0.7(1)	1.1(2)
Met	1.0(1)	0.9(1)	0.1(0)	0.4(0)
Ile	0.2(0)	0 (0)	0.7(1)	1.1(2)
Leu	0.2(0)	0.3(0)	1.9(2)	1.7(1)
Tyr	0.3(0)	0.2(0)	1.1(1)	1.3(2)
Phe	2.0(2)	1.1(1)	0.3(0)	0.8(0)
Lys	1.3(1)	1.1(1)	0.2(0)	0.9(0)
His	0.8(1)	0.1(0)	0.1(0)	0.4(0)
Arg	0.2(0)	0.9(1)	1.0(1)	1.1(1)
Total	8	19	18	17
Residues	49-56	61-79	101-118	207-223

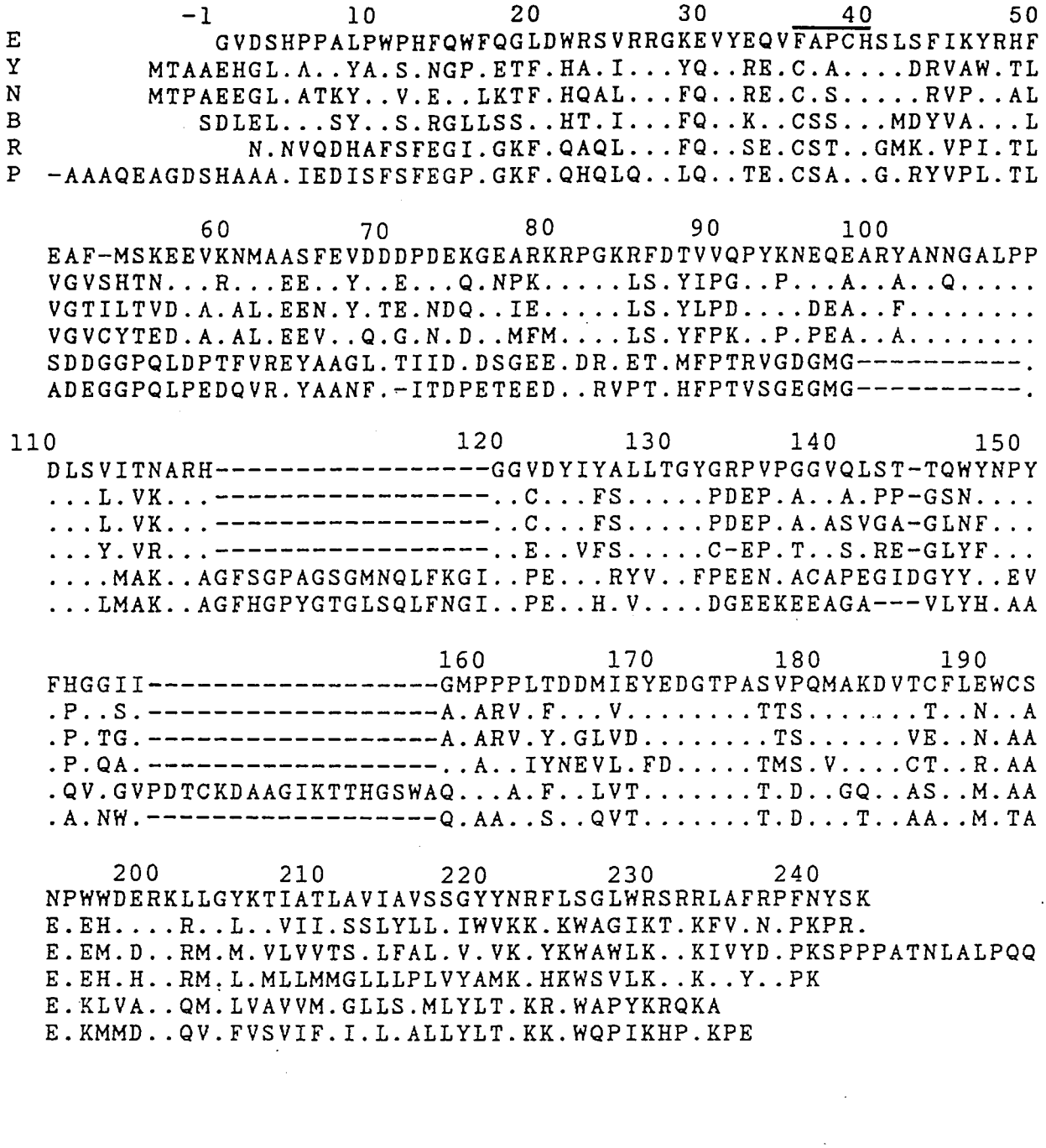

Fig. III-3 Comparison of amino acid sequences of mature cytochrome c_1 from various origins. E, Y, N, and B indicate mitochondrial cytochromes c_1 from E. gracilis, yeast (6), Neurospora crassa (7), and bovine (5); R and P indicate prokaryotic cytochromes c_1 from Rhodobacter sphaeroides (8) and Paracoccus denitrificans (10). The numbering system is that of the Euglena sequence. Alignments are optimized for maximal homology by inserting gaps indicated as hyphens. Identical amino acids at the same position with the Euglena protein are indicated as dots. The heme binding site is indicated as a solid bar.

sequences of cytochromes $\underline{c}_1$ reported so far (5-11). Two negatively charged regions which were reported as the sites for interaction with cytochrome $\underline{c}$ are also conserved in the Euglena protein at 57 to 74 and at 165 to 172 (28,29). A stretch of 16 uncharged amino acids regarded as a membrane anchor is located at 207 to 222.

The amino acid sequence of the mature protein of Euglena has 50 % homologies to that of yeast (6), 44 % to that of Neurospora (7) and bovine (5), 26 % to that of Rhodobacter sphaeroides (8), and 25 % to that of Paracoccus (9) (Fig. III-3). The sequences of Rhodobacter capsulatus and human cytochrome $\underline{c}_1$ are not compared with that of the Euglena protein, since these are highly homologous to those of R. sphaeroides and bovine, respectively (9,11). The homologies between the sequence of the Euglena protein and those from the other eukaryotes are lower than those among the three eukaryotic proteins, approximately 60 %. When compared with prokaryotic cytochromes $\underline{c}_1$, the slightly lower values are also calculated for Euglena cytochrome $\underline{c}_1$ than for the other eukaryotic cytochromes $\underline{c}_1$. The lower homologies of the sequence of Euglena cytochrome $\underline{c}_1$ are due to amino acid replacements in highly conserved positions: 10 replacements are found in the positions conserved in all of the other 5 sequences and 29 replacements in the positions conserved among the other three eukaryotic cytochromes $\underline{c}_1$. Amino acids around these replacements are often highly conserved in the sequence of Euglena cytochrome $\underline{c}_1$, giving 44 to 50 % homologies to those of mitochondrial cytochromes $\underline{c}_1$. Thus, the global structure of the

Euglena protein will be similar to those of the other mitochondrial cytochromes $\underline{c}_1$. An amino acid replacement in a conserved region may destabilize the polypeptide folding or domain interactions common to the structure of cytochrome $\underline{c}_1$. Yet, these amino acid replacements apart on the primary structure might stabilize the global structure each other: An amino acid replacement in a conserved region possibly compensates for destabilization caused by another region with such an amino acid replacement.

Regarding the biosynthesis of the *Euglena* cytochrome $\underline{c}_1$, the 20 nucleotides upstream of the mature protein coding region probably code a part of the amino acid sequence of the precursor protein. Amino-terminal presequences were reported for mitochondrial cytochromes $\underline{c}_1$, and was consisted of 61 amino acids for yeast (6), 70 for *Neurospora* (7), and 84 for human cytochrome $\underline{c}_1$ precursor (11). In the case of *Euglena* there is possibility of the absence of the precursor polypeptide with such a long amino-terminal prepiece which is cleaved coupling with import (12,13). The ATG just before the mature protein coding sequence might be the initiation codon. In this case, the nucleotide sequence surrounding the ATG is partially in agreement with Kozak's consensus sequence for higher eukaryotic initiation sites (30). The 5'-end $(T)_{10}$ in the upstream region is something unusual within a translational region. To elucidate this possibility, cloning and sequencing of the whole gene should be performed.

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PUBLICATIONS

I. Publications related to the thesis.

1. Kuniaki MUKAI, Masaki YOSHIDA, Yoshio YAO, Sadao WAKABAYASHI, and Hiroshi MATSUBARA (1988)
   Evidence for a Single Thioether Bond in Heme Binding of Cytochrome c_1 from Euglena gracilis
   Proc. Jpn. Acad. 64, Ser. B, 41-44

2. K. MUKAI, M. YOSHIDA, Y. YAO, S. WAKABAYASHI, and H. MATSUBARA (1988)
   EUGLENA CYTOCHROME C_1 HEME MOIETY IS COVALENTLY BOUND TO ITS POLYPEPTIDE CHAIN THROUGH A SINGLE THIOETHER BOND
   14th Int'l Cong. Biochem.(1988), vol.IV, p.174
   Prague, Czechoslovakia

3. Kuniaki MUKAI, Masaki YOSHIDA, Hiroshi TOYOSAKI, Yoshio YAO, Sadao WAKABAYASHI, and Hiroshi MATSUBARA (1989)
   An Atypical Heme-Binding Structure of Cytochrome c_1 of Euglena gracilis Mitochondrial Complex III
   Eur. J. Biochem. in press

4. Kuniaki MUKAI, Sadao WAKABAYASHI, and Hiroshi MATSUBARA (1989)
   Molecular Cloning and Nucleotide Sequence of a cDNA encoding Euglena gracilis Cytochrome c_1
   in preparation

II. Other publications.

1. Kuniaki MUKAI, Toshiyuki MIYAZAKI, Sadao WAKABAYASHI, Seiki KURAMITSU, and Hiroshi MATSUBARA (1985)
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2. Kuniaki MUKAI and Hiroshi MATSUBARA (1986)
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