The Polypeptide Composition of Ubiquinone–Cytochrome *c* Reductase (Complex III) from Beef Heart Mitochondria†

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ABSTRACT: The subunit structure of ubiquinone–cytochrome *c* reductase (complex III) has been examined and eight different polypeptides have been identified. Apparent molecular weights for each have been obtained by one or more methods including polyacrylamide gel electrophoresis in sodium dodecyl sulfate and in sodium dodecyl sulfate–8 M urea and by gel filtration in sodium dodecyl sulfate and in 6 M guanidine hydrochloride. Values obtained are as follows: I, 47 500; II, 45 500; III, 29 500; IV, 27 800; V, 24 800; VI, 13 900; VII, 10 700; VIII, 4 800–9 000. Individual polypeptides have been purified and the amino acid composition of several of these have been determined. At least one polypeptide, the apoprotein of cytochrome *b*, is hydrophobic in character and this is a mitochondrially synthesized component (B. Lorenz, W. Kleinow, and H. Weiss (1974), *Hoppe-Seyler's Z. Physiol. Chem. 355*, 300). Other polypeptides including the hemoprotein of cytochrome c_1 are more hydrophilic in amino acid composition.

Ubiquinone–cytochrome *c* reductase or complex III is a structural element of the mitochondrial inner membrane, an important part of the electron transport chain, and an integral part of coupling site II. The enzymic complex, as isolated with detergents, contains a number of different polypeptides associated with *b* and c_1 heme, as well as non-heme iron moieties (Hatefi et al., 1962; Baum et al., 1967; Goldberger et al., 1961; Ohnishi, 1966; Rieske et al., 1964). In order to understand the organization of complex III in the membrane, as well as its role in both electron transport and energy coupling, it is necessary to have available a detailed chemical analysis of each of the components of the complex. In this paper we describe identification of eight different polypeptides in complex III, and methods devised to allow purification of each. Those components associated with *b* and c_1 heme are characterized.

Experimental Section

Beef heart mitochondria were prepared by the method of Crane et al. (1956). Submitochondrial particles (ETP[1]) were obtained as described by Crane et al. (1956). Complex III was made from ETP as described by Rieske (1967) but with the following modifications.

The red pellet (S1), obtained by dialysis [step 3 in the method of Rieske (1967)], was resuspended in Tris–sucrose–histidine solution to a protein concentration of 10 mg/ml and potassium deoxycholate (0.5 mg/mg of protein) and 50% saturated ammonium acetate (8.2 ml/100 ml) were added. After standing for 15 min at 0 °C, the turbid solution was centrifuged for 30 min at 108 000 *g* and the pellet was discarded. Saturated ammonium sulfate (1 ml per 1 ml) was added to the supernatant and a precipitate was collected by centrifugation for 30 min at 108 000 *g*. This pellet was resuspended in Tris–sucrose–histidine buffer and adjusted to a protein concentration of 10 mg/ml. A solution of 20% (w/v) of potassium cholate was added to a final concentration of 0.4 mg of cholate per mg of protein, cold saturated ammonium sulfate was added (0.5 ml/ml), and the solution was incubated on ice for 15 min. A supernatant and pellet were separated by centrifuging the turbid solution for 15 min at 108 000*g*. The supernatant was collected and saved as complex III. The pellet was resuspended to 10 mg/ml and the final purification step repeated to increase the yield of the complex. The yield from ETP was 1–2% of the starting material. The complex was stored in small aliquots at −20 °C.

Fractionation of the Complex into Cytochrome c_1 Rich and Cytochrome b Rich Fractions. Complex III suspended at 3 mg/ml in 0.05 M Tris-HCl (pH 8.0) containing 2 M Gdn·HCl, 30% glycerol, and 2% β-mercaptoethanol was incubated for 1 h at 4 °C and then centrifuged at 150 000*g* for 1 h. The pellet containing almost all of the cytochrome *b* was separated and resuspended in Tris buffer for further analysis. The supernatant containing almost all of the cytochrome c_1 was dialyzed against 0.05 M Tris-HCl (pH 8.0) for 12 h, a step which precipitated protein containing only small amounts of heme. This "cytochrome free fraction" was separated from the cytochrome c_1 rich supernatant by centrifuging at 150 000 *g* for 1 h.

Assay Methods. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Heme concentrations were determined spectrally as described by Williams (1964).

Duroquinone–cytochrome *c* reductase activity was measured using a modification of the method of Guerrieri and Nelson (1975). The reaction medium contained 150 mM KCl, 15 μM cytochrome *c*, 1 mM KCN, and 5 mM $MgCl_2$ in phosphate buffer (pH 7.4). Complex III at varying concentrations was added to one cuvette. Duroquinone (40 μM) reduced by borohydride was added into both the refer-

† From the Institute of Molecular Biology and Department of Biology, University of Oregon, Eugene, Oregon 97403. *Received September 11, 1975.* This investigation was supported by National Science Foundation Grant GB41619 and by a National Institutes of Health Grant GM 00715. This paper is no. 2 in a series on Components of the Mitochondrial Inner Membrane; paper 1 is by Briggs et al., 1975; see references. This work has been submitted (by R.L.B.) in partial fulfillment of the requirements for a Ph.D. degree at the University of Oregon.

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[1] Abbreviations used are: ETP, beef heart submitochondrial particles; Gdn·HCl, guanidine hydrochloride.

ence and sample cuvettes and the reaction thus started. Absorbance at 550 nm was followed with time using a Cary 14 recording spectrophotometer. Activities were calculated from 30 to 90 s during which time rates were linear.

Polyacrylamide Gel Electrophoresis. Samples to be run in dodecyl sulfate alone were electrophoresed on Biophore preformed gels (12% acrylamide monomer gel dimensions 5.5 mm diam., 100 mm length) in a buffer system containing 0.205 M Tris, 0.205 M acetic acid, and 0.1% w/v dodecyl sulfate (pH 6.4).

Gel electrophoresis in dodecyl sulfate–8 M urea was performed as described by Swank and Munkres (1971) with gels formed with 12.0% acrylamide and bisacrylamide at one-tenth the amount of acrylamide. The buffer system contained 0.1 M Tris-phosphate, 0.1% dodecyl sulfate (w/v), and 8 M urea (pH 7.1). Samples were prepared for electrophoresis by heating at 100 °C for 1 min in the presence of 3% dodecyl sulfate, 8 M urea, and 2% β-mercaptoethanol.

Gels were stained by incubating for 20 min at 60 °C with a solution of 0.2% Coomassie Brilliant Blue dissolved in methanol–acetic acid–water (5:1:5). This process was repeated three times with a change of dye solution each time. The gels were destained by diffusion in a Bio-Rad Model 172 diffusion destainer using methanol–acetic acid–water in the proportions of the staining solution. Densitometric traces of the gels were obtained at 500 nm with a Gilford linear scanning attachment to a Beckman DU spectrometer using a 5 mm × 10 cm quartz cuvette.

Gel Chromatography in Sodium Dodecyl Sulfate. Complex III or fractions derived from this complex were dissolved in 1 ml of dodecyl sulfate (10% w/v) containing 1 mM dithiothreitol and heated at 100 °C for 1 min. Gel chromatography was carried out in a column (1.5 × 84 cm) of Sephadex G-200, equilibrated with 10 mM dodecyl sulfate, 0.09 M NaCl, and 0.05 M Tris-acetate (pH 8.0). The column was calibrated with bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c* as standards. Blue dextran was used to determine the void volume and a drop of mercaptoethanol was included in samples as a marker for the total volume of the column.

Gel Chromatography in 6 M Guanidine Hydrochloride. Complex III was dissolved at 2 mg/ml in 8 M Gdn·HCl, 50 mM Tris-HCl, 50 mM dithiothreitol, and 2 mM EDTA (pH 8.0) by heating to 50 °C for 4 h. After this dissociation step, the protein was reacted with 0.2 M iodoacetamide for 20 min in the dark. The reaction was stopped by bringing the pH down to pH 6.0 with HCl and the sample was concentrated into 0.5–1.0 ml by repeated additions of dry Sephadex G-25 (0.3 g/ml of solution).

The concentrated sample was applied to a column (1.5 × 88 cm) of Sephadex G-100 superfine and eluted with 6 M Gdn·HCl in 10 mM Tris-HCl–0.5 mM dithiothreitol (pH 8.0) as described by Fish et al. (1970). Cytochrome *c*, lysozyme, bovine trypsin inhibitor, insulin A and B chain, and the three cyanogen bromide fragments of cytochrome *c*, each reduced and alkylated with iodoacetamide, were used to calibrate the column. The column was monitored for protein by absorbance at 280 nm.

Amino Acid Analyses. Samples were hydrolyzed for 24 h with 6 N HCl in a sealed tube under high vacuum in a 110 °C oven. HCl was removed by rotary evaporation at 30 °C and samples were analyzed in a Technicon automatic amino acid analyzer, according to the method of Spackman et al. (1958).

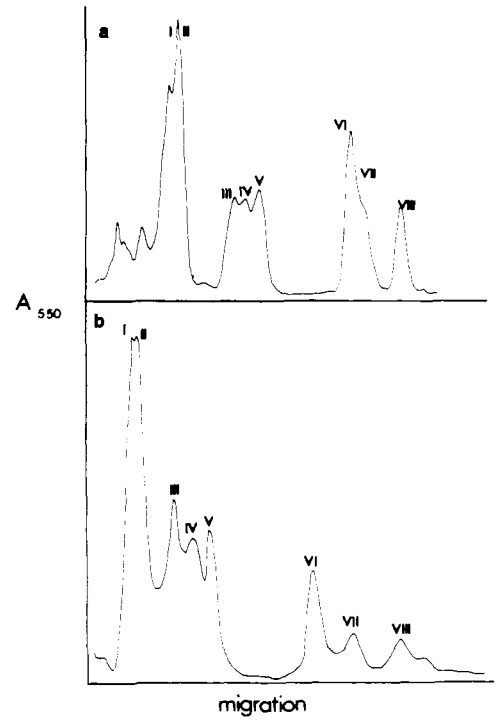

FIGURE 1: Densitometric tracings of polyacrylamide gels of beef heart complex III. (a) 12% gel run in buffer containing dodecyl sulfate. (b) 12% gel run in buffer containing dodecyl sulfate and 8 M urea. Polypeptides are labeled I through VIII in order of their apparent molecular weights.

Results

Characteristics of the Complex III Preparation. Complex III as isolated was soluble in Tris buffer (pH 8.0) without addition of further detergents. Preparations contained cytochrome *b* and c_1 at concentrations of 5.0–6.5 μmol/g of protein and 3.0–3.5 μmol/g of protein, respectively, and showed a duroquinone–cytochrome *c* reductase activity of between 15 and 19 μmol of cytochrome *c* reduced per min per mg of protein. This activity was greater than 92% antimycin sensitive.

The Number of Polypeptides in Complex III. The polypeptide composition of complex III was determined using dodecyl sulfate polyacrylamide gel electrophoresis. Different polypeptide patterns were seen depending on the percentage of acrylamide monomer used in making the gels. On 12% acrylamide gels, preparations of complex III with the highest amount of *b* and c_1 hemes showed bands in three molecular weight ranges (Figure 1, trace a). There was a doublet with apparent molecular weights of 50 000 and 46 000, a triplet with molecular weights of 31 500, 29 000, and 25 000, and two fast migrating bands, one of molecular weight 14 000 which has a distinct shoulder on the trailing edge and a second of molecular weight around 9 000. Less pure preparations contained additional components in small amounts, migrating at positions typical of the major subunit of succinate dehydrogenase, NADH dehydrogenase, and the large molecular weight polypeptides of ATPase (F_1) (Capaldi, 1974). These enzymes are found commonly as contaminants of complex III samples (Gellerfors and Nelson, 1975; Hare and Crane, 1974). Impure preparations also contained minor components with molecular weights of 37 000 and 18 000.

No better resolution of the fast migrating components of complex III was obtained at higher concentrations of acryl-

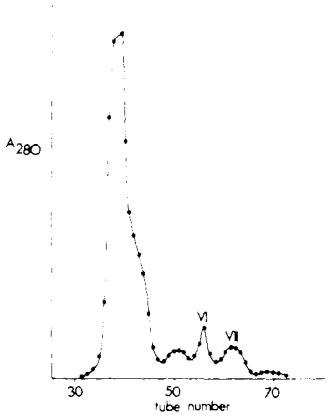

FIGURE 2: The elution profile of complex III eluted through Sephadex G-100 superfine in 6 M Gdn·HCl. The first peak eluting after the void volume was a polypeptide(s) of molecular weight 18 000. This is an impurity or possibly an aggregate of smaller subunits which is found in some preparations (see the text). Polypeptides VI and VII were separated. Polypeptide VIII was not detected off the column probably because of the low amount by weight in the complex.

Table I: Apparent Molecular Weights of Polypeptides in Complex III.

Component	Dodecyl Sulfate Gels	Dodecyl Sulfate–Urea Gels	6 M Gdn·HCl Columns
I	50 000	45 000	
II	46 000	45 000	
III	31 500	28 600	
IV	29 000	26 700	
V	25 000	24 600	
VI	14 000	15 000	12 700
VII	12 500	9 000	9 800
VIII	9 000	4 800	

amide. The reason for this is that they migrate in the region of the standard curve in which changes in molecular weight have very little effect on the size of the dodecyl sulfate–protein aggregates (Reynolds and Tanford, 1970). It has been shown that small molecular weight polypeptides are separated better when 8 M urea is included in gels along with dodecyl sulfate (Swank and Munkres, 1971), and we have used this buffer system to get additional estimates of the molecular weights of components, and also to ask if there are extra polypeptides of small molecular weight in complex III which are not resolved in the conventional dodecyl sulfate system. The electrophoretic profile of complex III, run on 12% gels with 0.1% dodecyl sulfate and 8 M urea, is shown in Figure 1 (trace b). The large molecular weight components I and II ran together in this system, with an apparent molecular weight of 45 000. The triplet including polypeptides III, IV, and V was resolved and migrated with apparent molecular weights close to those found with gels including dodecyl sulfate alone. The fast migrating doublet was separated into five different bands. Three of these (labeled VI, VII, and VIII in Figure 1) were found in approximately the same relative amounts in all samples. These have apparent molecular weights of 15 000, 9 000, and 4 800 on the highly resolving gel system. In addition, bands were resolved with molecular weights of 12 000 and 3 000 but these were present in variable amounts in different preparations and were virtually absent from preparations of the highest purity (as shown in Figure 1).

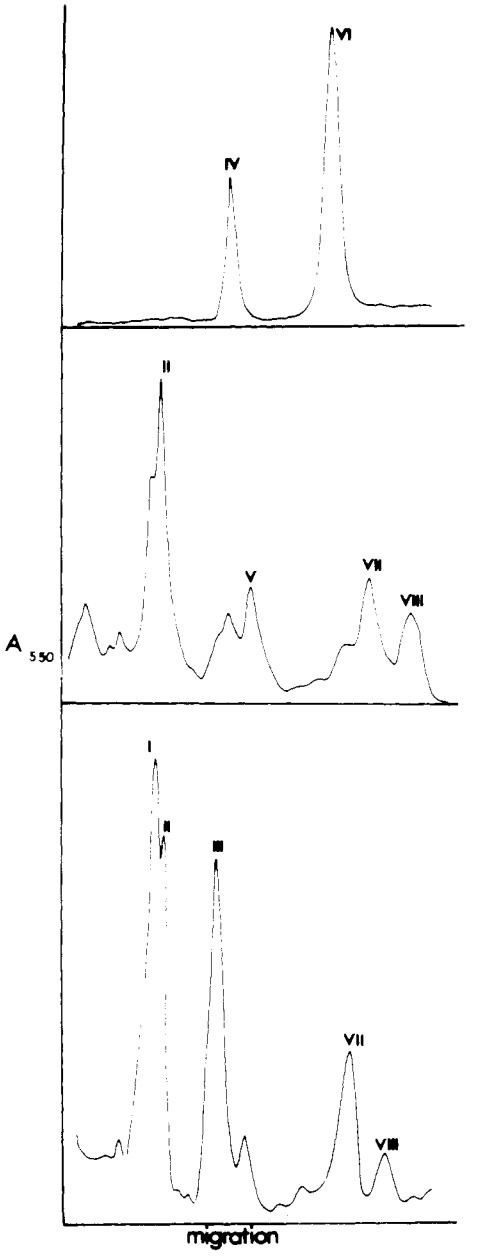

FIGURE 3: Densitometric tracings of the fractions obtained by 2 M Gdn·HCl treatment of complex III. The cytochrome c_1 rich fraction is shown in the top tracing; the cytochrome free fraction is in the middle; the cytochrome *b* rich fraction is the bottom tracing.

As an additional approach to estimating the molecular weights of the smaller polypeptides, complex III was eluted through Sephadex G-100 superfine in 6 M Gdn·HCl. Polypeptides I through V eluted in or very close to the void volume of this column. Polypeptides VI and VII entered the gel and eluted as two sharp peaks with apparent molecular weights of 12 700 and 9 800, values somewhat lower than those estimated by gel electrophoresis (Figure 2).

In summary, then, eight different polypeptides are found routinely in preparations of complex III. The apparent molecular weights of these as obtained by various methods of analysis are summarized in Table I.

Identification of Polypeptides with Heme Moieties. Complex III was separated into cytochrome *b* and cytochrome c_1 rich fractions by a modification of the method of Yamashita and Racker (1969). The cytochrome *b* rich fraction showed several bands on 12% gels. Polypeptides I, III,

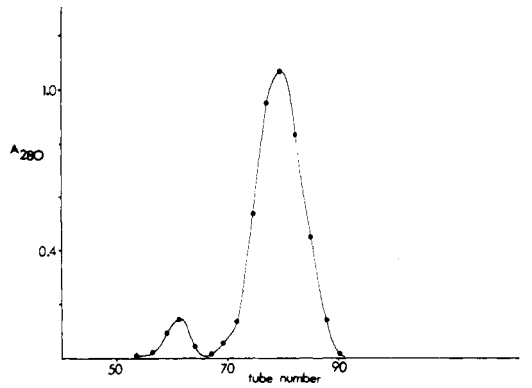

FIGURE 4: The elution profile of the cytochrome c_1 rich fraction run on Sephadex G-200 in 10 mM dodecyl sulfate. Polypeptides present in the various fractions were identified by dodecyl sulfate gel electrophoresis. Peak samples were pooled for amino acid analyses of individual polypeptides.

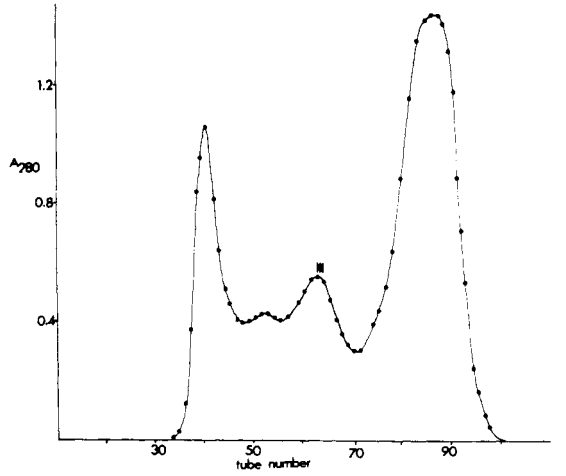

FIGURE 5: The elution profile of the cytochrome *b* rich fraction run on Sephadex G-200 in 10 mM dodecyl sulfate.

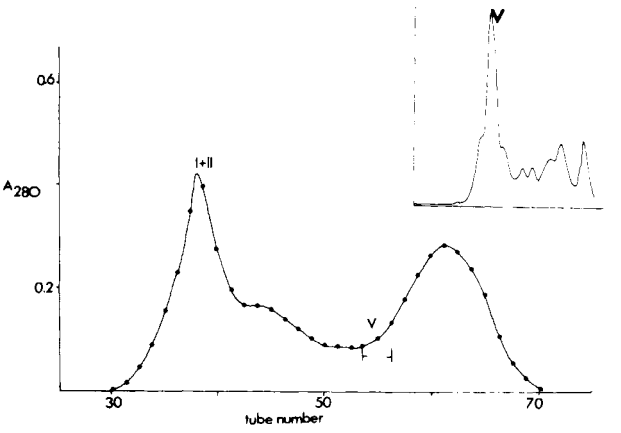

FIGURE 6: The elution profile of the cytochrome free fraction run on Sephadex G-200 in 10 mM dodecyl sulfate. The insert shows a 12% dodecyl sulfate polyacrylamide gel of the pooled fractions 53–55.

and VII were present in increased amounts compared with their concentration in complex III (Figure 3, bottom trace).

The cytochrome c_1 rich fraction contained all of the polypeptides of complex III, but was enriched in polypeptides II, IV, V, VI, and VIII. This fraction could be further subdivided by dialyzing against buffer. A pink supernatant and a white pellet were separated by centrifugation. The supernatant, reduced with dithionite, had an absorbance spectrum typical of native cytochrome c_1. This fraction contained polypeptides IV and VI (Figure 3, top trace), and, in some samples, small amounts of polypeptides I and V were also present. The heme of cytochrome c_1 is covalently attached to protein. It was seen in association with polypeptide IV on unstained gels overloaded with protein. When the cytochrome c_1 rich supernatant was chromatographed through Sephadex G-200 in the presence of dodecyl sulfate, the heme eluted with polypeptide IV.

The white precipitate generated by dialysis contained only very small amounts of either cytochrome *b* or c_1. This fraction was heavily enriched in polypeptides V and VIII (Figure 3, middle trace).

As a second approach to identifying polypeptides associated with type *b* heme, cytochrome *b* was purified as described by Goldberger et al. (1961). Polypeptides III and VII were greatly enriched in this fraction.

Purification of Individual Components. The selective enrichment of polypeptides in different fractions formed the basis of the protocol for purifying individual components. Polypeptides I and II as a mixture, and polypeptides III, IV, V, and VI separately, were isolated by gel filtration on a G-200 column eluted with 10 mM dodecyl sulfate. Polypeptides I + II and V were obtained from the cytochrome free fraction; polypeptide III was isolated from the cytochrome *b* rich fraction and polypeptides IV and VI were purified from the cytochrome c_1 rich fraction.

These experiments are shown in Figures 4–6. Polypeptides I + II, III, IV, and VI were obtained in pure form in peak samples off the column and were characterized without further purification. Polypeptide V was not fractionated cleanly but could be obtained in pure form by pooling and concentrating peak samples (Figure 6) and rechromatographing them through the column.

Polypeptides VI and VII could be isolated by gel filtration in Gdn·HCl. These two polypeptides were separated from each other and from other components when complex III was chromatographed through Sephadex G-100 superfine in 6 M Gdn·HCl (Figure 2). Peak fractions contained pure polypeptides.

Amino Acid Composition of Polypeptides. The amino acid composition of polypeptides I + II, III, IV, and VI have been determined. These are listed in Table II. Polypeptide III is hydrophobic in character with a polarity (Capaldi and Vanderkooi, 1972) of 35%; other polypeptides all have polarities of between 45 and 50%, values typical of water-soluble proteins.

Discussion

Our results show that complex III contains at least eight different polypeptides with apparent molecular weights ranging from 50 000 down to around 5 000 (Table I). This polypeptide profile is similar to that obtained in other laboratories (Hare and Crane, 1974; Yu et al., 1974; Gellerfors and Nelson, 1975; Das Gupta and Rieske, 1973) with the exception that we resolve additional low molecular weight components by running dodecyl sulfate–8 M urea gels. It is important to determine the association of the various polypeptides with the several spectrally distinct heme and nonheme iron moieties identified as part of complex III by electron paramagnetic resonance and adsorption spectroscopic studies (Davies et al., 1972; Wikström, 1973). Our fractionation procedure and purification of individual components

Table II: Amino Acid Composition of the Polypeptides of Complex III.[a]

Amino Acid	I + II This Study	I + II *b*	III	IV This Study	IV *c*	VI
Lys	5.6	4.5	4.7	5.5	4.9	5.8
His	3.4	3.3	2.8	2.6	3.6	0.5
Arg	4.8	5.3	3.5	5.7	6.3	6.0
Asp	9.2	9.5	7.8	8.9	8.8	9.4
Thr	5.0	4.5	6.8	3.9	3.5	3.8
Ser	5.4	6.4	6.1	6.6	5.9	6.8
Glu	12.1	10.9	4.9	10.8	10.5	17.5
Pro	4.9	4.6	7.2	9.5	9.4	6.0
Gly	8.8	7.9	7.5	8.3	7.3	6.5
Ala	10.9	13.1	7.7	8.7	8.3	8.3
Val	7.9	7.1	5.8	6.5	6.3	6.4
Met	1.1	1.8	2.5	2.8	3.2	3.3
Ile	5.6	3.0	8.5	3.0	1.9	3.6
Leu	10.9	10.8	15.0	10.8	10.7	11.1
Tyr	N.D.[d]	3.6	2.2	2.7	5.7	2.4
Phe	4.4	3.8	6.9	3.7	3.7	2.6
Polarity	45.5	44.4	36.7	44.0	43.5	49.8

[a] Compositions are reported as moles/100 moles of amino acids detected. [b] From Silman et al. (1967). [c] From Trumpower and Katki (1975). [d] Not determined.

allow us to identify some but not all of these functionally important moieties with different polypeptides.

The site of binding of c_1 heme is most easily identified because the heme is covalently bound to protein. On polyacrylamide gels and in gel filtration experiments, c_1 heme can be visualized in association with polypeptide IV. The amino acid composition of this polypeptide as determined here is similar to that reported recently for a purified cytochrome c_1 hemoprotein by Trumpower and Katki (1975). There is some indication that cytochrome c_1 is an aggregate of two dissimilar polypeptides. A second component, polypeptide VI, remains associated with the cytochrome c_1 hemoprotein after its solubilization by Gdn·HCl (this study and Trumpower and Katki, 1975). This polypeptide is also found in association with the hemoprotein after solubilization of cytochrome c_1 with cholate and ammonium sulfate (Yu et al., 1972). However, Ross et al. (1974) claim to have separated polypeptide VI from the hemoprotein of cytochrome c_1 in yeast, by Sephadex DEAE column chromatography in the presence of cholate, and this fractionation apparently does not alter the spectral properties of the hemoprotein. A role for polypeptide VI in cytochrome c_1 function then remains to be established.

Under the denaturing conditions we use in fractionating complex III, the spectral characteristics of cytochrome *b* are altered and eventually the heme moiety is dissociated from protein. For this reason it is difficult to assess which polypeptides bear *b* type heme. However, of the polypeptides concentrated into a cytochrome *b* rich fraction in our study, polypeptide III would appear to be a prime candidate for an apoprotein of cytochrome *b*. This polypeptide is found in large amounts in cytochrome *b* prepared as described by Goldberger et al. (1961). It has a molecular weight close to that reported for cytochrome *b* from *Neurospora crassa* (Weiss and Ziganke, 1974). Also polypeptide III is hydrophobic in character (polarity 36.7) and this is a feature which might be expected of a mitochondrially synthesized component (Tzagoloff et al., 1972; Schatz and Mason, 1974). By spectral analysis there are at least two and possibly three different cytochromes *b* in complex III (Davies et al., 1972; for review see Wikström, 1973). If these are indeed distinct proteins and not a single component in different environments, then polypeptide VII is the most likely candidate for a second apoprotein of cytochrome *b*. This polypeptide is concentrated into the cytochrome *b* containing fraction separated by Gdn·HCl treatment of complex III. Polypeptide VII is also highly concentrated in the cytochrome *b* preparation obtained by the method of Goldberger et al. (1961).

Again because of the denaturing conditions used, identification of polypeptides bearing non-heme iron moieties is difficult. On the basis of molecular weight alone, polypeptide V can be tentatively identified as the Rieske non-heme iron protein (Rieske et al., 1964). Among other polypeptides, I and II can be equated with the so called "core protein" fraction identified by Silman et al. (1967). These polypeptides have molecular weights close to that reported for core protein and, analyzed together, they have an amino acid composition very similar to that reported for core protein by Silman et al. (1967). Das Gupta and Rieske (1973) have identified a protein involved in antimycin action by covalently labeling the component with an azido derivative of the inhibitor. Based on its migration in gels, polypeptide VIII is most likely the antimycin binding protein.

An understanding of the function of different polypeptides of complex III is clearly far from complete and must await the isolation of the various cytochromes and non-heme iron moieties in a reconstitutionally active form. Our analysis of the polypeptide composition of the complex and the development of procedures for isolating individual polypeptides in a denatured form does, however, allow us to begin analysis of the topology of complex III, and this work is in progress. Also, with the compositional data available, we can ask the important question of whether different electron transfer complexes have any polypeptides in common. Recently we have completed an analysis of the subunit structure of complex IV, the cytochrome *c* oxidase complex. This complex contains seven different polypeptides ranging in molecular weight from 36 000 to 5 300 (Briggs et al., 1975; Downer, N. W., Robinson, N. C., and Capaldi, R. A., manuscript in preparation). Each of the components has been isolated and characterized. These polypeptides are different both in terms of size and amino acid composition from any of the components of complex III. In common, both complexes III and IV contain hydrophobic polypeptides which are synthesized on mitochondrial ribosomes and hydrophilic polypeptides made in the cytoplasm (Mason et al., 1973; Schatz, 1975; Lorenz et al., 1974). There is now good evidence that complex IV is organized with the hydrophilic subunits on the outside and the hydrophobic polypeptides more buried within the lipid bilayer (Poyton and Schatz, 1975; Carroll and Eytan, 1975; Downer, N. W., unpublished results). It will be interesting to see if complex III is organized in a similar way.

Acknowledgment

We are grateful to Mr. Stephen Cross for his assistance with amino acid analyses and to Dr. E. Herbert for the use of his amino acid analyser.

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Structural Identification and Synthesis of Luciferin from the Bioluminescent Earthworm, *Diplocardia longa*†

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ABSTRACT: For the first time, luciferin from a bioluminescent earthworm has been purified, identified, and synthesized. This luciferin from the North American species, *Diplocardia longa,* is a simple aldehyde compound, *N*-isovaleryl-3-aminopropanal, with an amide functional group. It is a clear, odorless oil at room temperature. It is nonvolatile and has no near-uv–visible absorption or fluorescence. Derivatives of this compound were made to facilitate its identification: the luciferin 2,4-dinitrophenylhydrazone (mp 174 °C), a yellow crystalline solid; and the luciferin alcohol, a clear oil. Synthesis of *Diplocardia* luciferin yielded an oil of identical spectroscopic (proton nuclear magnetic resonance (NMR), ^{13}C NMR, mass, and ir), chemical (dinitrophenylhydrazone and alcohol derivatives, bioluminescence activity), and physical (thin-layer chromatography, volatility) properties to those of the purified native *Diplocardia* luciferin.

Bioluminescent earthworm species (Oligochaete) are found worldwide but few detailed studies of their luminescence system have been carried out. Harvey (1952) has reviewed the published work on earthworm bioluminescence and since that time only a few additional studies have been performed (Johnson et al., 1966; Cormier et al., 1966; Bellisario et al., 1972). In general these reports have indicated that earthworm luminescence originates from exuded coelomic fluid and that it involves a classical luciferin–luciferase reaction. More recently the work on the North American species, *Diplocardia longa,* has indicated that, at least in the case of this worm, the luminescence system requires

† From the Bioluminescence Laboratory, Department of Biochemistry, University of Georgia, Athens, Georgia 30602. *Received September 19, 1975.* This work was supported by National Science Foundation Grant GB-43804. The University of Texas HRM spectrometer was purchased with funds provided by the National Science Foundation (GP-8509). The Joel PFT-100 spectrometer was purchased by the University of Georgia with matching funds from the National Science Foundation.