Isolation, Subunit Composition, and Site of Synthesis of Human Cytochrome *c* Oxidase†

James F. Hare,‡ Edwin Ching, and Giuseppe Attardi*

ABSTRACT: Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1), the terminal oxidase of the respiratory chain in eucaryotic cells, has been purified from human placenta mitochondria. Seven polypeptides have been identified reproducibly by high-resolution electrophoresis of the enzyme complex through sodium dodecyl sulfate ($NaDodSO_4$)–urea polyacrylamide gels; these correspond closely in size to the subunits of beef heart cytochrome *c* oxidase. When HeLa cells, grown in suspension culture, were pulse-labeled with [^{35}S]methionine in the presence of cycloheximide to inhibit cytoplasmic protein synthesis and chased with an excess of unlabeled methionine in the absence of the drug, the mitochondrially synthesized polypeptides were resolved into at least 17 components by $NaDodSO_4$–urea polyacrylamide gel electrophoresis. After labeled HeLa mitochondria were mixed with human placenta mitochondria and the cytochrome *c* oxidase was isolated, three of the labeled components were found to copurify with the three largest subunits of the complex. We conclude that human cytochrome *c* oxidase contains seven subunits, the three largest of which are synthesized on mitochondrial ribosomes, while the other four are synthesized in the cytoplasm.

Historically, the stability and high content of respiratory enzymes in beef heart mitochondria have made this the material of choice for probing the physical structure and enzymology of the mitochondrial inner membrane. Recently, however, investigations on the biogenesis and genetics of mitochondria have required the purification and characterization of respiratory enzymes from other sources, in particular, cell systems which are amenable to growth in culture under defined conditions, like yeast, *Neurospora*, and various types of animal cells. Experiments from different laboratories have shown that both cytoplasmic and mitochondrial translation products contribute to the structure of respiratory complexes and the oligomycin-sensitive ATPase in yeast and *Neurospora crassa* [see review by Schatz & Mason (1974)]. The role of the mitochondrial genome in specifying at least some of the mitochondrially synthesized polypeptide components of these enzymes has been demonstrated recently (Slonimski & Tzagoloff, 1976; Tzagoloff et al., 1976; Borst & Grivell, 1978).

Previous work from this laboratory utilizing electrophoretic fractionation on cylindrical polyacrylamide gels has led to the identification, among the in vivo products of mitochondrial protein synthesis in HeLa cells, of 10 distinct components in the molecular weight range from about 11 000 to 42 000 (Costantino & Attardi, 1975, 1977). More recently, electrophoresis through slab polyacrylamide gels combined with autoradiography has further resolved the mitochondrially synthesized products into at least 17 discrete [^{35}S]-methionine-labeled polypeptide bands (Ching, 1979; Attardi & Ching, 1979).

Identification of the functional significance and of the genetic origin of these polypeptides is of primary importance for understanding the informational role of mitochondrial DNA in animal, in particular human, cells. The smaller size of the mitochondrial genome in animal cells as compared to yeast and *Neurospora* (Borst, 1972) has raised the question of whether there may be a reduced number of structural genes in mitochondrial DNA of higher eucaryotic cells relative to that of lower eucaryotic cells. Although in yeast and mammalian cell mitochondria the number of polypeptides synthesized on mitochondrial ribosomes appears to be comparable (Beattie, 1971; Borst, 1972; Schatz & Mason, 1974), only a specific identification of these components would allow direct comparisons and therefore lead to some conclusions about a possible difference in structural gene content between the mitochondrial DNAs of the two sources. A difference in the informational content of mitochondrial DNA of different lower eucaryotic cells has been already well documented in the case of one of the subunits of the ATPase complex, which is coded in mitochondrial DNA in yeast and in the nucleus in *Neurospora* (Sebald et al., 1977).

In the present work, as a preliminary for an investigation of the biogenesis of cytochrome *c* oxidase in HeLa cells, we have purified this enzyme from another, more easily available, human material, i.e., placenta mitochondria. We have probed the subunit structure of the human enzyme by direct comparison with that of the beef heart enzyme and have found that, except for minor variations, the structure of the two enzymes is identical. We have also demonstrated that, as in yeast and *Neurospora crassa* (Schatz & Mason, 1974), the three largest among the seven polypeptide components of HeLa cell cytochrome *c* oxidase are synthesized on mitochondrial ribosomes.

Experimental Procedure

Preparation of Human Placenta Mitochondria. Human placentas were obtained from a local hospital within 1 h of delivery and transported in ice to the laboratory. All manipulations were carried out at 0–4 °C. The tissue was cleaned of connective tissue and diced; about 250 g of tissue was placed in a 1-L Waring blender (2-speed) with 500 mL of 0.25 M sucrose, 0.001 M EDTA, and 0.05 M Tris–H_2SO_4 (pH 7.8 at 25 °C). This suspension was blended at low speed for 1 min and centrifuged at 250*g* for 10 min to separate unbroken cells, nuclei, and connective tissue fibers. The supernatant was poured through several layers of cheesecloth (avoiding with care the floating material consisting mostly of fat) into a flask kept in ice and centrifuged at 1300*g* for 5 min to remove any remaining red blood cells and debris. The mitochondria were pelleted by centrifuging the 1300*g* supernatant at 12000*g* for

† From the Division of Biology, California Institute of Technology, Pasadena, California 91125. *Received August 15, 1979; revised manuscript received January 4, 1980.* These investigations were supported by National Institutes of Health Grants GM-11726 and T32 GM-07616-01 and by a Damon Runyon Fellowship to J.H.

‡ Present address: Department of Biochemistry, University of Oregon School of Medicine, Portland, OR 97201.

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10 min in a Sorvall SS-34 rotor, resuspended in 0.25 M sucrose, 0.001 M EDTA and 0.01 M Tris-HCl, pH 7.4 (25 °C) (SET), and washed once. The isolated mitochondria were resuspended in 1 volume of SET and gently homogenized. Four- to five-milliliter portions of this suspension were layered onto discontinuous gradients consisting of 15 mL of 1.7 M sucrose and 15 mL of 1.0 M sucrose in 0.0001 M EDTA and 0.01 M Tris-HCl (pH 7.0), prepared in Spinco SW27 rotor tubes. The gradients were centrifuged at 70000*g* for 30–40 min. Under these conditions, the mitochondria band at the 1.0–1.7 M sucrose interface. The 1.0 M sucrose layer was removed and the interface particulate matter collected, diluted in 4–5 volumes of SET, pelleted by centrifugation for 20 min at 27000*g*, and resuspended in 1 volume of SET. The mitochondria were stored for no more than 2 weeks at −60 °C before use.

Labeling and Isolation of HeLa Cell Mitochondria. HeLa cells were grown in suspension culture (Amaldi & Attardi, 1968). Prior to labeling, the cells were grown for 16 h in the presence of 40 μg/mL chloramphenicol in order to increase the stability and, presumably, promote the integration into the inner mitochondrial membrane of the mitochondrial protein products synthesized after removal of the drug (Costantino & Attardi, 1977).

The cells were collected by centrifugation, washed twice with medium lacking methionine, and finally resuspended in the same medium to 5×10^6 cells/mL. After 5 min of adaptation, cycloheximide was added to 100 μg/mL; 5 min later, [^{35}S]-methionine (600 mCi/μmol) was added to 8.3 μCi/mL. During the labeling, filtered air containing 5% CO_2 was flushed over the medium at 2 L/min in order to keep the pH of the medium constant. After 2 h of labeling, the cells were collected, washed twice in complete unlabeled medium, resuspended in the same medium to 2×10^6 cells/mL, and incubated for 1 h under aeration conditions. This chase had the purpose of allowing integration of the mitochondrially synthesized polypeptides into the inner mitochondrial membrane under conditions of resumed cytoplasmic protein synthesis (Costantino & Attardi, 1977).

After incubation, the cells were collected and washed with 0.13 M NaCl, 0.005 M KCl, and 0.001 M $MgCl_2$, and a 12000*g* crude mitochondrial fraction was prepared as previously described (Attardi et al., 1969), using SET in the mitochondrial pellet resuspensions.

Preparation of Beef Heart Mitochondria. Beef heart mitochondria were prepared by the method of Löw & Vallin (1963).

Purification of Human Cytochrome c Oxidase. After preliminary tests, the methodology finally chosen for isolating cytochome *c* oxidase from human placenta mitochondria followed the rationale developed for yeast cytochrome *c* oxidase purification (Mason et al., 1973). All manipulations were carried out at 0–4 °C. Mitochondria from about 10 placentas were washed once in 4 volumes of SET, resuspended in SET in a final volume of 160 mL, homogenized, and sonicated in 20-mL aliquots twice for 1 min at 3-A output with a Branson sonifier (Model S-125) in 50-mL conical, plastic centrifuge tubes. The sonicated mitochondria were centrifuged in a Spinco type 65 fixed angle rotor at 160000*g* for 30 min to pellet the membrane fraction.

The membrane fraction was extracted as described by Mason et al. (1973), and 62 g of ammonium sulfate per L (40% saturation) was added to the extract under stirring. The suspension was centrifuged at 57000*g* in a Spinco 65 rotor for 15 min to remove material forming a brown-green pellet (P_2). P_2 was resuspended by gentle agitation in 15 mL of 0.5% cholate, 0.25 M sucrose, and 0.01 M Tris-HCl, pH 7.4 (CST), per g of membrane protein processed. Saturated (0 °C), neutralized ammonium sulfate solution was added dropwise under stirring to the solubilized pellet to 28% saturation. The turbid suspension was centrifuged at 100000*g* for 10 min to remove material forming a white pellet (P_3). To the supernatant was added saturated ammonium sulfate to 40% saturation; the suspension was centrifuged at 100000*g* for 10 min to remove material forming a green pellet (P_4). P_4 was resuspended in 10 mL of 0.02 M sodium phosphate (pH 7.0) and 1% Triton X-100 and diluted with 20 mL of CST buffer. Saturated ammonium sulfate was added to 30% saturation, and the suspension was centrifuged at 100000*g* for 10 min after being kept at 0 °C for 30 min. To the green supernatant (S5) was added saturated ammonium sulfate stepwise with 2% increments starting from 32% up to 40% saturation. Among the pellets obtained by centrifugation (100000*g*, 10 min) after each ammonium sulfate addition (following a 10–30-min incubation at 0 °C), those which contained most of the green material were pooled (P_6). Usually, the bulk of the enzyme was precipitated by 34–38% saturated ammonium sulfate.

P_6 was resuspended in 1.0 mL of 0.02 M sodium phosphate (pH 7.0) and 1% Triton X-100, by stirring, and desalted on a Sephadex G-25 (coarse) column (1 × 40 cm), equilibrated with the same buffer. The excluded fractions were combined and applied to a DEAE-cellulose column (Whatman DE-52; 1.2 × 6 cm), equilibrated with 0.02 M sodium phosphate (pH 7.0) and 1% Triton X-100. The column was washed successively in steps with 20-mL portions of 0.02, 0.05, and 0.10 M sodium phosphate (pH 7.0) containing 1% Triton X-100. The green absorbance at 423 nm was monitored and, of that retained, most (~60%) was found to be eluted with 0.10 M sodium phosphate. Fractions eluting with the 0.1 M sodium phosphate front were either combined and stored at −60 °C or subjected to further purification on a sucrose density gradient. For this purpose, 0.5-mL samples of DEAE-purified cytochrome *c* oxidase were layered onto 11.5 mL of 5–20% linear sucrose gradients in 0.1 M sodium phosphate (pH 7.0) and 1% Triton X-100 and centrifuged in a Spinco SW41 rotor at 150000*g* for 24 h at 2 °C; in other experiments, 0.4-mL samples were centrifuged through 5–25% sucrose gradients in the same buffer in an SW65 rotor at 250000*g* for 11 h. Fractions of 0.6 mL (SW41 tube) or 0.32 mL (SW65 tube) were collected, and their absorbance at 423 nm and protein concentration were determined.

Purification of Beef Heart Cytochrome c Oxidase. The beef heart enzyme was purified as described by Capaldi & Hayashi (1972) or by the method described above for the human enzyme. One preparation of beef enzyme prepared according to the procedure by Kuboyama et al. (1972) was kindly provided by Dr. Sunney Chan.

Polyacrylamide Gel Electrophoresis. Electrophoresis through $NaDodSO_4$–urea polyacrylamide slab gels in Tris–phosphate buffer was carried out essentially as described (Downer et al., 1976). A 1-cm stacking gel containing 5% polyacrylamide (0.133% bisacrylamide), 8 M urea, 0.03 M Tris–H_3PO_4 (pH 6.3), and 0.1% $NaDodSO_4$ was utilized. The gels were stained with Coomassie Blue and dried for autoradiography when required.

Apparent molecular weights of the cytochrome *c* oxidase subunits were determined by comparision with the migration of the following marker proteins: bovine serum albumin, mouse IgA heavy chain, ovalbumin, chymotrypsinogen A, mouse IgA light chain, beef blood globin, cytochrome *c*, insulin

Table I: Concentration of Cytochromes and ATPase Specific Activity in Beef Heart, Human Placenta, and HeLa Cell Mitochondrial Membranes

	heme specific concn[a] (nmol mg^{-1} of protein)			sp act. of ATPase (μmol of ATP min^{-1} mg^{-1} of protein)
	a, a_3	b	c_1	
beef heart	1.54	0.82	0.48	2.0
human placenta	0.16	0.14	0.089	1.0
HeLa cells	0.028	0.051	0.047	0.14

[a] Heme a/a_3, b, and c_1 concentrations were determined from the reduced minus oxidized absorption spectra at room temperature (Vanneste, 1966).

B chain, and insulin A chain. A calibration curve was constructed by drawing by the least-squares method the best fitting line through the observed migrations of the markers in all the runs, normalized to that of bovine blood globin, which was always present; from this curve the apparent molecular weights of the markers, under the elctrophoretic conditions used, were derived. These apparent molecular weights were utilized in each electrophoretic run to construct the best-fitting line to the observed migrations of the markers in that run. This procedure tended to minimize the imprecision derived from the use of less than the complete set of markers on each gel.

Analytical Procedures. Heme *a*, *b*, and c_1 concentrations were determined from the reduced minus oxidized difference spectra at room temperature (Van Gelder, 1963; Vanneste, 1966) and, in the case of heme *a*, also from the reduced absolute absorption spectrum (Yonetani, 1959) by using a Cary 15 spectrophotometer. Heme *a* concentration of purified cytochrome *c* oxidase samples was, in addition, determined by their dithionite-reduced pyridine hemochromogen (Rieske, 1967), using an $EM_{587} = 24.0$ mM^{-1} cm^{-1}. Oxygen reduction was measured polarographically (Berezney et al., 1972). ATP hydrolysis was measured colorimetrically at 37 °C, essentially as described (Ernster et al., 1962). Protein concentration was determined (Lowry et al., 1951) with 1% $NaDodSO_4$ in the assay buffer (Dulley & Grieve, 1975) and bovine serum albumin as a standard.

Chemicals. Cholic acid (Schwarz/Mann) was recrystallized (Mason et al., 1973), solubilized with potassium hydroxide, and titrated to pH 8.0. Urea (ultrapure) was obtained from Schwarz/Mann. [^{35}S]Methionine was obtained from Amersham-Searle Corp.

Results

Heme a Content and Cytochrome c Oxidase Activity of Human Mitochondria. The specific heme *a* content and the relative content of cytochromes *a*, *b*, and c_1 in human placenta or HeLa cell mitochondria were found to be lower than those measured in beef heart mitochondria (Table I). The lower specific content of heme *a* in the mitochondria from human placenta was probably not due to the lower degree of purity of the human mitochondrial preparations used here, since oligomycin-sensitive adenosine triphosphatase activity was comparable in placenta and beef heart mitochondria. Human placenta mitochondria reduced molecular oxygen in the presence of reduced cytochrome *c* (1.18 μatoms of O min^{-1} mg^{-1} of protein at 37 °C), but the activity was decreased (0.66 μatom of O min^{-1} mg^{-1} of protein) when the membrane fraction (see above) was solubilized with 6% cholate, and all activity was lost in a matter of hours when mitochondria were solubilized with Triton X-100. Since a strong nonionic detergent was essential in our fractionation procedure, we used specific heme *a* concentration as an index of purification, but we refer to the heme *a*–protein complex as cytochrome *c* oxidase.

Purification of Human Placenta Cytochrome c Oxidase. We attempted at first to purify cytochrome *c* oxidase from cytochromes *b*, *c*, and c_1 by solubilizing the latter with either Triton X-114 (Jacobs et al., 1966) or deoxycholate (Fowler et al., 1962), but a fairly wide range of concentrations of these detergents did not produce selective solubilization of the *b* and *c* cytochromes. On the contrary, we were able to solubilize fairly selectively a heme *a*–protein complex by the method utilized for the purification of yeast cytochrome *c* oxidase with slight modifications (Sekuzu et al., 1964; Mason et al., 1973). The enzyme purified through the DEAE chromatography step was subjected to several additional purification steps in an attempt to either increase the heme *a* to protein ratio or to decrease the number of components visualized on $NaDodSO_4$–urea polyacrylamide gels. Neither elution from the DEAE–cellulose column with a continuous 0.05–0.30 M sodium phosphate gradient nor gel filtration through Sepharose 4B in 1% Triton X-100 proved effective in this respect. However, a 60–130% enrichment in heme *a* to protein ratio was achieved in different experiments (Table II) by centrifuging the DEAE-purified enzyme on a 5–20% continuous sucrose gradient in the presence of 1% Triton X-100 (Figure 1).

The purification was monitored at each step by determining the heme *a* to protein ratio (Table II), by measuring oxygen consumption with ascorbate–cytochrome *c* as an electron donor (Table II), and by visualizing the polypeptide constitutents on $NaDodSO_4$–urea polyacrylamide gels (Figures 2 and 3). We obtained at least a 30-fold enrichment in the heme *a* to protein ratio from the membrane fraction to the final sucrose gradient purified enzyme. The final heme *a* to protein ratio

Table II: Summary of Cytochrome *c* Oxidase Purification

	protein (mg)	heme *a*[a] (nmol)		sp heme *a* content[a] (nmol/mg^{-1} of protein)		OD_{423} mg^{-1} of protein	sp act. (μatoms of O min^{-1} mg^{-1} of protein)
		(1)	(2)	(1)	(2)		
experiment I							
total membrane	2250	360	207	0.16	0.092		0.70
P_2	512	256	271	0.50	0.53		1.06
P_4	336	252	276	0.75	0.82		0
P_6 or G-25 excluded fraction	56.2	61	67	1.09	1.20		0
DEAE–0.10 M PO_4	10.0	35	38	3.50	3.80	0.28	0
SW41 sucrose gradient peak	5.8	28	31	4.90	5.30	0.45	0
experiment II							
DEAE–0.10 M PO_4						0.39	
SW65 sucrose gradient peak				7.4	9.4	0.90	

[a] (1) = heme *a* determined by using $\epsilon(603_{reduced} - 603_{oxidized}) = 12.0$ mM^{-1} cm^{-1}. (2) = heme *a* determined by using $\epsilon(603_{reduced} - 630_{reduced}) = 16.5$ mM^{-1} cm^{-1}.

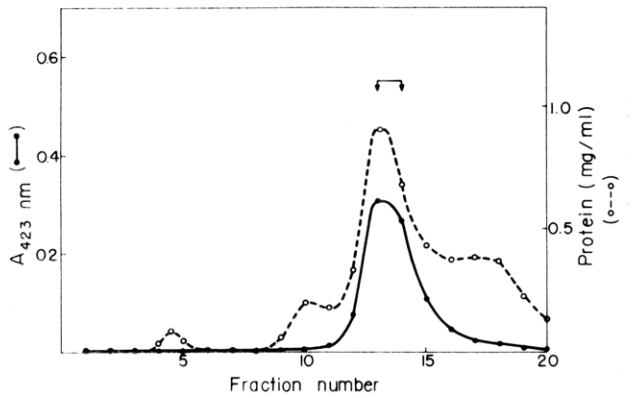

FIGURE 1: Sedimentation profile of DEAE-cellulose purified human cytochrome *c* oxidase after centrifugation in a sucrose density gradient. A 0.5-mL sample of the 0.10 M sodium phosphate eluate from the DEAE-cellulose column was run through a 5–20% sucrose gradient, as described under Experimental Procedure.

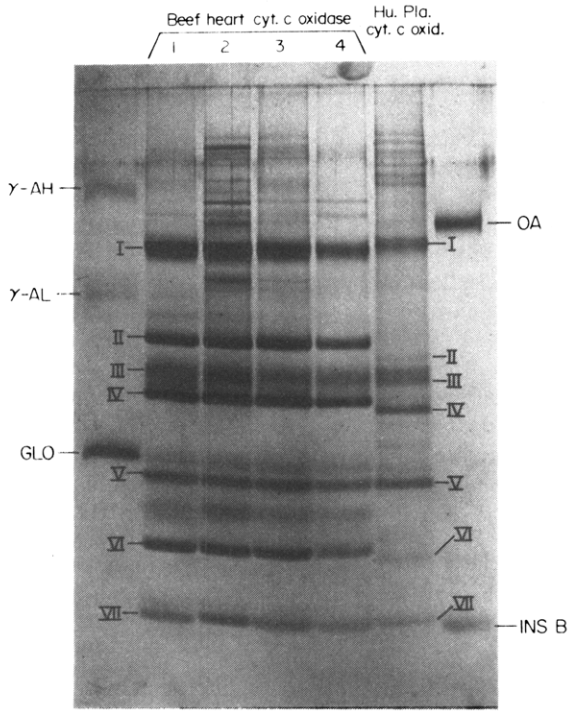

FIGURE 2: Electrophoretic patterns in $NaDodSO_4$–urea polyacrylamide gels of different preparations of beef heart cytochrome *c* oxidase and of human placenta cytochrome *c* oxidase. The beef heart enzyme samples were prepared by following the procedure of Kuboyama et al. (1972) (sample 1), Capaldi & Hayashi (1972) (sample 2), the latter procedure followed by DEAE-cellulose chromatography (0.15 M sodium phosphate eluate following the 0.05 M sodium phosphate eluate) (sample 3), and the procedure of Mason et al. (1973) (sample 4). The human placenta enzyme had been purified through the DEAE-cellulose chromatography stage (0.10 M sodium phosphate eluate). Roman numerals near slots 1 and 5 indicate the subunits of cytochrome *c* oxidase.

obtained after the sucrose gradient step, i.e., 4.9 (5.3)[1] and 7.4 (9.4) nmol of heme *a* mg^{-1} of protein, in two different experiments, was comparable to that measured in the beef heart cytochrome *c* oxidase that we purified by the same procedure (4.5 nmol of heme *a* mg^{-1} of protein) or by the Capaldi–Hayashi procedure (5.6 nmol of heme *a* mg^{-1} of

[1] Here, and below, the first estimate derives from the reduced minus oxidized A_{603} (millimolar extinction coefficient = 12) and that in parentheses, if given, from the reduced $A_{603} - A_{630}$ (millimolar extinction coefficient = 16.5). Very similar values were obtained from the dithionite-reduced pyridine hemochromogen determinations.

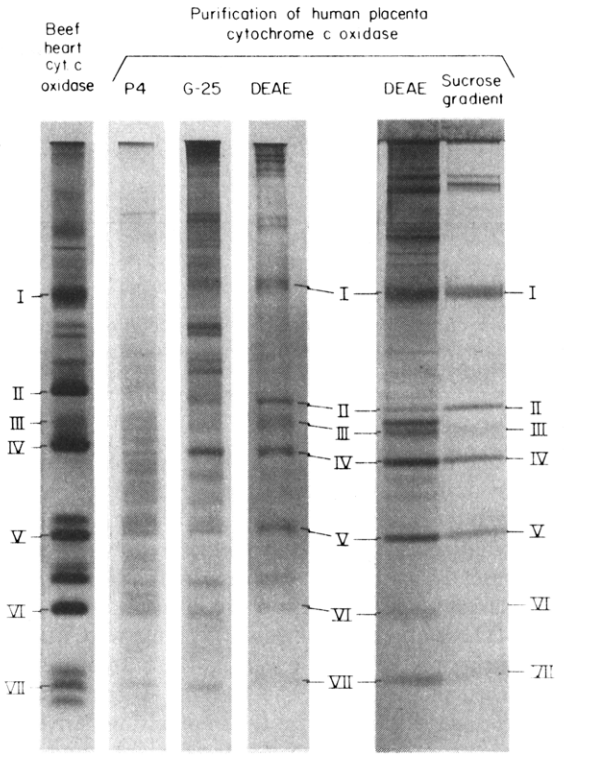

FIGURE 3: Electrophoretic patterns in $NaDodSO_4$–urea polyacrylamide gels of fractions of human placenta mitochondria containing cytochrome *c* oxidase at various stages of the purification procedure. (Slot 1) Purified beef heart cytochrome *c* oxidase (Capaldi & Hayashi, 1972). (Slots 2–6) Fractions of human placenta mitochondria: (slot 2) fraction P_4; (slot 3) Sephadex G-25 eluate; (slots 4 and 5) DEAE-cellulose–0.10 M sodium phosphate eluate; (slot 6) sucrose gradient purified cytochrome *c* oxidase. Samples in slots 2–4 and in slots 5 and 6 derive from different preparations and were run in separate gels.

protein). As mentioned above, the activity of the enzyme was extremely labile in the presence of Triton X-100, was irreversibly lost during ammonium sulfate fractionation, and could not be restored by addition of phospholipids.

Spectral Properties of the Purified Human Cytochrome c Oxidase. In the reduced form, the enzyme showed absorption maxima at 603, 520, and 444 nm. The characteristic absorbance ratios for the enzyme purified up to either the DEAE-cellulose stage or the sucrose gradient sedimentation stage were identical and are as follows: A_{603}(reduced):A_{550}(reduced), 2.8; A_{444}(reduced):A_{603}(reduced),5.1; A_{423}(reduced):A_{444}(reduced), 0.48; A_{603}(reduced):A_{599}(oxidized), 2.2. These values all fall within the acceptable limits, as defined by Lemberg (1969).

Subunit Structure of Beef Heart and Human Cytochrome c Oxidase. The seven subunits of beef heart cytochrome *c* oxidase were resolved by $NaDodSO_4$–urea polyacrylamide gel electrophoresis (Downer et al., 1976) as described under Experimental Procedure (Figure 2). The subunit polypeptides from beef heart preparations were identified by comparison with the published patterns by Capaldi and collaborators (Downer et al., 1976; Capaldi et al., 1977) on the basis of their electrophoretic mobility. No differences were seen in the mobility of the polypeptide components of the beef heart cytochrome *c* oxidase as prepared by the procedure of Kuboyama et al. (1972) (Figure 2, slot 2), by the cholate–Triton X-100 procedure (Figure 2, slot 5), by the Capaldi–Hayashi procedure (Capaldi & Hayashi, 1972) (Figure 2, slot 3), or after the enzyme prepared by the latter procedure was treated with Triton X-100 and eluted from a DEAE-cellulose column with 0.05 M sodium phosphate (10 nmol of heme *a* mg^{-1} of protein)

Table III: Estimates of Molecular Weight of Subunits of Cytochrome *c* Oxidase from Beef Heart and Human Placenta Mitochondria[a]

subunits	beef heart[b]	beef heart (this work)	human placenta (this work)
I	35 300	42 200 (41 200)	43 600 (42 400)
II	25 200	23 200 (22 700)	20 100 (21 200)
III	21 000	18 300 (18 000)	18 000 (18 900)
IV	16 200	14 300 (14 100)	13 700 (14 700)
V	12 100	9 100 (9200)	8 800 (9900)
VI	6 700	6 100 (6200)	5 600 (6600)
VII	3 400	3 800 (4000)	3 700 (4500)

[a] The molecular weights given for the subunits of the beef heart and human placenta enzymes are averages of the estimates made on the basis of standard curves constructed by the least-squares method for the individual gel runs, using for the marker proteins the apparent molecular weights derived as explained under Experimental Procedure. In parentheses are the averages of the estimates made by using the real molecular weights for the marker proteins in the construction of the individual standard curves. [b] Capaldi et al. (1977).

or 0.15 M sodium phosphate, following the 0.05 M sodium phosphate elution (9.6 nmol of heme *a* mg^{-1} of protein) (Figure 2, slot 4). Table III shows the apparent molecular weights of the seven subunits, as estimated in the present work in many experiments from their mobility relative to that of standard proteins. Apart from subunit I, all the others had apparent molecular weights fairly similar to those reported by Capaldi et al. (1977). The reason for the discrepancy in the apparent molecular weight of subunit I is unknown. The beef heart cytochrome *c* oxidase prepared by the Capaldi–Hayashi procedure showed a relatively small amount of high molecular weight contaminants, which were in most part eliminated by further purification involving treatment with 1% Triton X-100, adsorption on DEAE-cellulose, and elution with 0.05 or 0.15 M sodium phosphate. A characteristic contaminant of about 13 000 daltons was found consistently between subunits IV and V, presumably corresponding to the impurity denoted as "a" by Capaldi et al. (1977). Two other contaminants appeared in variable amounts between subunits V and VI (see also patterns in Figures 3 and 4), and they presumably correspond to the impurities with the same mobilities previously described in beef heart preparations [bands "b" and "c" in Capaldi et al. (1977)].

Two bands migrating one slightly faster and one slightly slower than subunit VII were seen with variable intensity in different preparations of beef heart cytochrome *c* oxidase and often in different runs of the same preparation (compare, for example, patterns in Figures 2–4). Identification of the middle band in the triplet as corresponding to subunit VII of Capaldi et al. (1977) was made on the basis of the observations that it was consistently the most intensely stained with Coomassie Blue and that it corresponded in mobility to the only polypeptide present in that region of the gel in the electrophoretic patterns of human cytochrome *c* oxidase (see below). The enzyme prepared by the cholate–Triton X-100 procedure showed the least degree of contamination by extraneous polypeptides.

The polypeptide composition of human placenta cytochrome *c* oxidase at various stages of the purification procedure is shown in Figure 3. The effectiveness of the DEAE-cellulose chromatograpy in removing contaminating polypeptides, especially in the high molecular weight region of the gel, is apparent (Figure 3, slots 4 and 5). A further purification was reproducibly obtained by running the DEAE-cellulose purified (0.1 M sodium phosphate eluate) enzyme through a sucrose gradient (see, for example, Figure 3, slot 6). Analysis of the

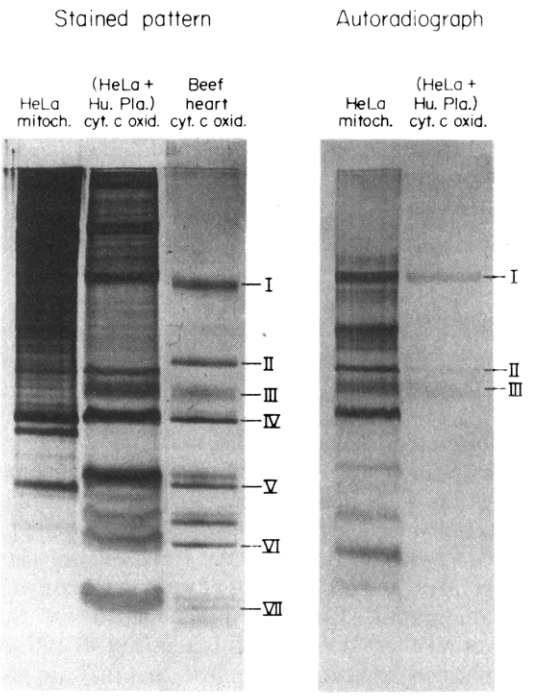

FIGURE 4: Stained pattern and autoradiograph, after $NaDodSO_4$–urea polyacrylamide gel electrophoresis, of cytochrome *c* oxidase isolated from a mixture of [^{35}S]methionine-labeled HeLa cell mitochondria and an excess of unlabeled human placenta mitochondria and run in parallel with a sample of total [^{35}S]methionine-labeled mitochondrial proteins and with a sample of beef heart cytochrome *c* oxidase [purified by the Kuboyama et al. (1972) procedure]. The HeLa cell mitochondrial sample was from cells labeled with [^{35}S]methionine for 2 h in the presence of cycloheximide and chased as described in the text.

most purified human preparations showed clearly and reproducibly seven polypeptides which migrated identically or similarly to the seven subunits of beef heart cytochrome *c* oxidase (Figures 2 and 3) and are presumably homologous to them. In particular, human subunits III, V, VI, and VII had mobility identical with that of the corresponding beef heart subunits. Subunit I migrated consistently somewhat slower and subunit IV somewhat faster than the homologous beef heart components. Subunit II, tentatively identified as the component migrating immediately behind subunit III (Figures 2 and 3), had an electrophoretic mobility distinctly lower than that of beef heart subunit II. Sometimes subunit III appeared as a doublet (cf. Figure 2, slot 6). The possibility that the slower moving band of the doublet may be a contaminant is suggested by its disappearance after sucrose gradient centrifugation (Figure 3, slot 6). As mentioned above, only one band was observed in the region of beef heart subunit VII and was identified as the homologous human component. Table III shows the apparent molecular weights of the subunits of human cytochrome *c* oxidase estimated in many experiments from their mobility relative to that of standard proteins.

Identification of Mitochondrial Translation Products Associated with Human Cytochrome c Oxidase. HeLa cells were exposed to [^{35}S]methionine under the conditions described under Experimental Procedure, designed to label specifically the polypeptides synthesized on mitochondrial ribosomes. Mitochondrial membrane protein (20 mg) derived from such cells was mixed with 580 mg of human placenta mitochondrial membrane protein, and cytochrome *c* oxidase was purified by the cholate–Triton X-100 procedure. The purification resulted in a selective enrichment of mitochondrial translation products, as judged from the amount of ^{35}S counts per minute per microgram of protein (Table IV). The purified enzyme (34

Table IV: Purification of Cytochrome *c* Oxidase from a Mixture of Human Placenta Mitochondria and HeLa Cell Mitochondria Labeled in the Organelle-Specific Translation Products[a]

	mg of protein	total cpm	cpm μg^{-1} of protein
mitochondrial membrane fraction	600 [580 (PM), 20 (HM)]	4 710 000	7.9
G-25 excluded fraction	17.5	308 000	17.6
cytochrome *c* oxidase (DEAE-0.10 M sodium phosphate)	2.5	84 000	34.0

[a] For details, see text. PM, human placenta mitochondria; HM, [^{35}S]methionine-labeled HeLa cell mitochondria.

cpm/μg of protein) was electrophoresed, and the slab gel was stained with Coomassie Blue to visualize the component polypeptides and subjected to autoradiography to visualize the labeled components (Figure 4). The electrophoretic pattern of the labeled proteins from the total HeLa cell mitochondrial fraction revealed at least 17 bands of varying intensity (Figure 4, autoradiograph). Control experiments showed that the labeling of all these components is sensitive to 100 μg/mL chloramphenicol, indicating, therefore, that they are products of mitochondrial protein synthesis (Ching, 1979; Attardi & Ching, 1979). In the gel track containing cytochrome *c* oxidase from mixed labeled HeLa mitochondria–unlabeled placenta mitochondria, three of the labeled polypeptides (Figure 4, autoradiograph) coincide with the stained cytochrome *c* oxidase subunits I, II, and III (Figure 4, stained pattern), identifying these as mitochondrial translation products. Conversely, the absence of any hint of labeled bands corresponding to subunits IV–VII strongly suggests that these components are synthesized in the cytoplasm.

Discussion

In the present work, the conventional fractionation approach which has been previously used to purify cytochrome *c* oxidase from beef heart mitochondria was not successful with the mitochondrial membranes of HeLa cell or human placenta origin. This is possibly due to the intrinsically low heme *a* content of the organelles from these sources and/or to the presence of contaminating membranes which altered the normal lipid environment during extraction. After a variety of other procedures were tested, satisfactory results were finally obtained by using a method based on a modification of that devised for the yeast enzyme isolation (Mason et al., 1973). This purification procedure resulted, however, in a final product which had lost its ability to reduce molecular oxygen. The beef heart enzyme purified in the present work by the cholate–Triton X-100 procedure was also inactive. The factor responsible for this loss of molecular activity of the cytochrome *c* oxidase appears to be, in the present work, exposure to Triton X-100. In fact, human placenta mitochondria were found to lose their ability to reduce molecular oxygen in the presence of reduced cytochrome *c* within a few hours after solubilization with this detergent. Inactivation by Triton X-100 has also been reported for the rat liver enzyme (Rascati & Parsons, 1979a). It seems possible that this loss of activity is due to a perturbation of the membrane lipid environment which results from exposure of the enzyme complex to this detergent. However, that the inactivating effect of Triton X-100 is not absolute and may depend on the previous history of the enzyme and on the actual conditions of detergent treatment is suggested by the previously reported observations of cytochrome *c* oxidase preparations from beef heart mitochondria being active in the presence of Triton X-100 (Sun et al., 1968; Briggs et al., 1975). Another report has indicated a nearly complete loss of activity of human heart cytochrome *c* oxidase during purification by a method not involving use of Triton X-100 (Jeffreys & Craig, 1977).

The most extensively purified preparations of cytochrome *c* oxidase obtained in the present work from human placenta had a specific heme *a* content [4.9–7.4 nmol mg^{-1} of protein, using red-ox A_{603} ($\Delta\epsilon$ = 12 mM^{-1} cm^{-1}); 5.3–9.4 nmol mg^{-1} of protein, using red A_{603}–red A_{630} ($\Delta\epsilon$ = 16.5 mM^{-1} cm^{-1})] comparable to that of the beef heart enzyme [present work: 4.5 nmol of heme *a* mg^{-1} of protein, using red-ox A_{603} ($\Delta\epsilon$ = 12 mM^{-1} cm^{-1})] or the yeast enzyme [Mason et al., 1973; 9.4–10.0 nmol mg^{-1} of protein, using red A_{603}–red A_{630} ($\Delta\epsilon$ = 16.5 mM^{-1} cm^{-1})] purified by the same procedure; it was also similar to that reported for the human heart enzyme [Jeffreys & Craig, 1977; 7 nmol of heme *a* mg^{-1} of protein, using red A_{603}–red A_{630} ($\Delta\epsilon$ = 16.5 mM^{-1} cm^{-1})]. There are, however, reasons to believe that the specific heme content is not necessarily a reliable indicator of the polypeptide purity of the cytochrome *c* oxidase preparations. Thus, the beef heart enzyme isolated here by the cholate–Triton X-100 method had an appreciably lower heme *a* content than the enzyme prepared by other procedures and still showed a typical pattern on $NaDodSO_4$–urea polyacrylamide gel, with the seven subunits of the enzyme in the normal relative amounts; furthermore, and most significantly, this preparation was less contaminated by extraneous polypeptides than the others. Similarly, the most purified preparations of cytochrome *c* oxidase from human placenta exhibited an electrophoretic pattern as clean as, in terms of absence of contaminating polypeptides, and probably cleaner than that of the best beef heart preparations. A decline in molecular activity of the beef enzyme during purification in the absence of any change in the essential subunit composition, as judged by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$, has been previously reported and attributed to a reorganization of the multimeric phospholipid–protein complex (Vanneste et al., 1976). It seems that heme loss can occur from the enzyme complex during purification in the absence of breakdown of the complex. On the other hand, it has been shown that even preparations of beef heart enzyme with high heme *a* content and high specific activity, apparently indicative of purity, are contaminated with NADH-acceptor reductases (Penniall et al., 1976) and many extraneous bands on polyacrylamide gels (E. Ching, Y. Hatefi, and G. Attardi, unpublished experiments). High-resolution polyacrylamide gel electrophoresis thus provides an important criterion to evaluate the polypeptide purity of the enzyme. This may be more important than the specific heme content whenever comparisons with a standard pattern and other criteria allow the identification of the enzyme subunits. Since our purpose in the present work was to be able to identify the mitochondrially and the cytoplasmically synthesized subunits of cytochrome *c* oxidase, reducing the number of contaminating polypeptides as visualized on high-resolution gels was of greater interest to us than obtaining a preparation of high specific activity or heme concentration.

The subunit structure of cytochrome *c* oxidase has been the object of great controversy. Much of the variability in results has been the consequence of differences in isolation procedures or conditions of gel electrophoresis, failure to adequately dissociate the enzyme, or selective aggregation of subunits during preparation of the samples for gel electrophoresis, and the presence of contaminating polypeptides. There is general agreement that yeast and *Neurospora* enzymes can be resolved

into seven polypeptides, each present in one copy (Rubin & Tzagoloff, 1973a; Sebald et al., 1973; Poyton & Schatz, 1975; Phan & Mahler, 1976a), with similar molecular weights of the individual subunits in the two organisms. The enzyme purified from animal cells has been resolved into six to eight subunits (Weiss et al., 1972; Koch, 1976; Rascati & Parsons, 1979a; Höchli & Hackenbrock, 1978; Rubin & Tzagoloff, 1973a; Phan & Mahler, 1976a; Yatscoff et al., 1977; Capaldi & Hayashi, 1972; Kuboyama et al., 1972; Capaldi et al., 1977; Buse & Steffens, 1976). The failure to detect one of the large subunits (subunit III) in some of these studies is due to its poor staining with Coomassie Blue or to its not being resolved from subunit II under the conditions of electrophoresis on standard $NaDodSO_4$–polyacrylamide gels (Downer et al., 1976).

The thorough investigations by Capaldi and collaborators have clearly shown the effect of different conditions of gel electrophoresis on the order of migration and the apparent molecular weights of the subunits of beef heart cytochrome *c* oxidase (Capaldi et al., 1977). Even the most purified preparations appeared to contain small amounts of other polypeptides in the 8000–16 000-dalton range, which were interpreted as impurities on the base of their nonstoichiometric amounts and variability in yield. These impurities have been observed also in the present work. There is also some question about the nature of the smallest polypeptides detected in the beef heart enzyme preparations. While the Capaldi group identified the polypeptide with an apparent molecular weight of 3400 in $NaDodSO_4$–urea gels and 4300 in standard $NaDodSO_4$ gels as a single, integral component of the enzyme (subunit VII) (Capaldi et al., 1977), in another investigation (Buse & Steffens, 1976), the presumptively homologous component separated by gel filtration on Bio-Gel P60 was found to be a nonstoichiometric mixture of polypeptides with three N-terminal amino acids.

The human cytochrome *c* oxidase purified in this work appears to contain seven major polypeptides. Identification of these as the seven subunits of the enzyme complex has been made on the basis of their size and the reproducibility of their presence and relative amounts at different stages of the purification procedure. Subunit II was missing or of low staining intensity in our early preparations, for unexplained reasons, but has consistently appeared as a major component in our more recent preparations. All these subunits migrate on $NaDodSO_4$–urea gels identically with or very similarly to the homologous beef heart subunits with the exception of subunit II, which has a slightly greater electrophoretic mobility than subunit II of the beef heart enzyme. The significance of this observation is not clear. However, the finding that this polypeptide is synthesized on mitochondrial ribosomes supports our interpretation that it is homologous to subunit II of beef heart cytochrome *c* oxidase; apparently, this component is more easily removed from the protein complex than the other subunits. We conclude, therefore, that the cytochrome *c* oxidase protein complex from human mitochondria, as that from beef heart and yeast, contains seven subunits. Fewer components may be required for electron transport function, as shown in yeast (Phan & Mahler, 1976b), but more may be required for other functions (coupling, regulation, integration, etc.). A more definitive evaluation of the number and nature of the polypeptides which are integral constituents of cytochrome *c* oxidase must await the results of reconstitution experiments.

The observation reported here of the mitochondrial biosynthetic origin of subunits I, II and III of the human cytochrome *c* oxidase extends the results obtained for the enzyme from yeast (Mason & Schatz, 1973; Rubin & Tzagoloff, 1973b), *Neurospora crassa* (Sebald et al., 1973), *Xenopus laevis* (Koch, 1976), and rat liver (Rascati & Parsons, 1979b) to the human enzyme. It is clear that in all organisms studied cytochrome *c* oxidase is a chimera of mitochondrially translated (and presumably mitochondrial DNA coded) polypeptides and cytoplasmically synthesized (nuclear DNA coded) polypeptides. Although final conclusions must await the complete identification of the mitochondrially synthesized polypeptides in yeast and animal cells, in the particular case of cytochrome *c* oxidase, there does not appear to be any difference in the structural gene content of mitochondrial DNA between yeast and animal cells. Important questions which need to be answered in the future concern the mechanism of insertion of the enzyme into the membrane and the mitochondrial and nuclear genetic control of its synthesis.

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Sedimentation Study of a Catalytically Active Form of Rabbit Muscle Phosphofructokinase at pH 8.55†

Lyndal K. Hesterberg and James C. Lee*

ABSTRACT: The enzymatic active form of rabbit muscle phosphofructokinase (PFK) was observed directly by using the method of reacting or active enzyme centrifugation (AEC). These studies were performed in two assay systems: a coupled enzyme and a pH-dependent dye-linked system in glycylglycine buffer at pH 8.55 and 23 ± 1 °C. The sedimenting band of PFK was stabilized by three solvent systems: 50% (v/v) D_2O, 10% (w/v) sucrose, and 4% (v/v) or 10% (v/v) glycerol. The active PFK species sediments as a single component with a sedimentation coefficient of 12.4 ± 0.5 S, after correcting for protein–solvent interactions. Although PFK may undergo association–dissociation, there is no observable change in the value of $s_{20,w}$ over a 57-fold range of protein concentration. Throughout this range only a single active species of PFK was observed, and within an experimental uncertainty of ±10%, the enzymatic activity observed in the sedimentation studies accounts for the total enzymatic activity observed in the steady-state kinetics. Partially purified PFK was subjected to AEC analysis. Results reveal the presence of again a single active form sedimenting at the same rate as the purified enzyme. Results from sedimentation velocity studies indicate that the stabilizing solvents employed in AEC enhance the self-association of PFK. However, such an enhancement alone cannot account for the observation of a single active species with a sedimentation coefficient of 12.4 S. The interactions between solvent additives and PFK were studied by density measurements and by the application of multicomponent theory. Results from such a preferential solvent interaction study indicate that PFK is preferentially hydrated in the presence of sucrose or glycerol. The enhancement of PFK self-association is most likely due to a nonspecific solvent–protein interaction.

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyzes the transfer of the terminal phosphate of ATP to the C-1 hydroxy of fructose 6-phosphate to produce fructose 1,6-diphosphate. This reaction represents a key control point in glycolysis. As a result of extensive investigations (Ling et al., 1965; Parmeggiani et al., 1966; Aaronson & Frieden, 1972; Pavelich & Hammes, 1973; Lad & Hammes, 1974; Leonard & Walker, 1972), it has been demonstrated clearly that rabbit muscle phosphofructokinase (PFK) is a complex regulatory enzyme capable of existing in a variety of polymeric forms.

Hammes and co-workers (Pavelich & Hammes, 1973; Parr & Hammes, 1975, 1976; Hill & Hammes, 1975; Lad et al., 1973; Lad & Hammes, 1974), in a series of reports on the

†From the E. A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104. *Received October 30, 1979.* Supported by National Institutes of Health Grants NS-14269 and AM-21489, a grant-in-aid from the American Heart Association, Missouri Affiliate, Inc., and the Council for Tobacco Research–U.S.A., Inc.

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