The Subunit Structure of the Cytochrome *c* Oxidase Complex†

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ABSTRACT: The subunit structure of the cytochrome *c* oxidase complex has been obtained for three preparations each isolated by a different detergent procedure. Six polypeptides were present in all samples with the following molecular weights: subunit I, 36000; II, 22500; III, 17100; IV, 12500; V, 9700; and VI, 5300. These subunits have been purified by gel filtration in sodium dodecyl sulfate or in 6 *M* guanidine hydrochloride and their amino acid compositions have been determined. Subunit I is hydrophobic in character with a polarity of 35.7%. Subunits II through VI are more hydrophilic with polarities of 45.5, 48.6, 47.8, 49.7, and 53.7%, respectively.

The cytochrome *c* oxidase complex is a structural element of the mitochondrial inner membrane, the terminal member of the electron transport chain, and an integral part of coupling site III. It has been isolated with detergents as a multipeptide aggregate containing heme and copper moieties (Kuboyama et al., 1972; Rubin and Tzagoloff, 1973; Capaldi and Hayashi, 1972). In order to understand its organization 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 polypeptide component of the complex. In this paper we describe methods devised to allow purification of six subunits of the beef heart cytochrome *c* oxidase complex, together with electrophoretic evidence establishing the homogeneity of each component. The amino acid composition of each individual polypeptide is also presented.

Experimental Section

Preparations. 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).

Cytochrome *c* oxidase was prepared by three different methods each using different detergents in the isolation steps.

"Cholate" cytochrome *c* oxidase was prepared from beef heart mitochondria as described by Yonetani (1961).

"Triton" cytochrome *c* oxidase was prepared from mitochondria by the general method of Sun et al. (1968).

"Deoxycholate and cholate" cytochrome *c* oxidase was prepared from ETP as described by Capaldi and Hayashi (1972).

All three preparations were stored in small aliquots at −20° and at a protein concentration of 14–20 mg/ml.

Assay Methods. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Heme *a* concentrations were estimated by the pyridine hemochromogen difference spectral method of Williams (1964), using an extinction coefficient $\epsilon_{587-620}$ 21.7.

Cytochrome *c* oxidase activity was measured spectrophotometrically by observing the course of oxidation of ferrocytochrome *c* at 550 nm in air-saturated 0.05 *M* phosphate buffer (pH 7.0) containing 0.5% Tween-80 (Smith, 1955). Aliquots of enzyme were solubilized and diluted in the assay medium. The final volume was 1.00 ml and reactions were followed at room temperature.

Cytochrome *c* oxidase samples were reconstituted into vesicles as described by Racker (1972), except that mitochondrial lipids were used instead of soybean lecithin. Respiration was measured in the presence and absence of ionophores and uncouplers as described by Hunter and Capaldi (1973).

Polyacrylamide Gel Electrophoresis. For routine analyses, samples 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% dodecyl sulfate w/v pH 6.4.

The so-called "Ferguson plots" employed to check anomalous migration were obtained from gels of varying acrylamide concentration but with a constant ratio of bisacrylamide to acrylamide (1:30) (Neville, 1971; Banker and Cotman, 1972). The buffer system employed in these studies was 0.1 *M* sodium phosphate and 0.1% dodecyl sulfate w/v at pH 7.1. Gels were stained by incubation for 10 min at 60° with a solution of 0.2% Coomassie Brilliant Blue dissolved in methanol–acetic acid–water (5:1:4). 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 same proportions as for the staining solution. Densitometric traces of the gels were obtained at 550 nm with a Gilford linear scanning attachment to a Beckman DU spectrometer, using a 5 mm × 10 cm quartz cuvet.

Gel Chromatography in Sodium Dodecyl Sulfate. Cytochrome *c* oxidase (50 mg) was dissolved in 3 ml of dodecyl sulfate, 10% w/v, containing 1 m*M* dithiothreitol and heated at 100° for 1 min. Gel chromatography was carried out in a column (1.5 × 84 cm) of Sephadex G-200, equilibrated with 10 m*M* dodecyl sulfate–0.1 *M* sodium phosphate (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 vol-

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

Table I: Molecular Characteristics of the Different Preparations of Cytochrome *c* Oxidase.

Preparation	Heme *a* Concn mμmol/mg of Protein	Activity μmol of cyt *c* oxidized sec^{-1} μmol of heme a_3^{-1}	R.C.R.[a]
Capaldi and Hayashi (1972)	9.70	110	
Yonetani (1961)	8.30	92	5.7
Sun et al. (1968)	7.95	82	

[a] The rate of respiration of the enzyme in vesicles was measured as micromoles of O per minute per milligram of protein. To obtain the respiratory control ratio (R.C.R.), the rate of respiration in the presence of valinomycin plus nigricin was divided by that without additions, as described by Hunter and Capaldi (1973).

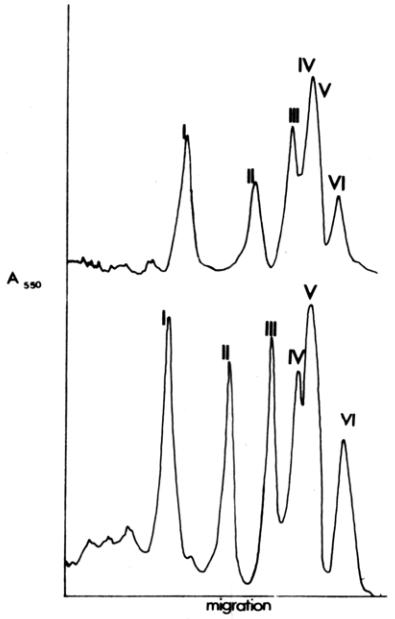

FIGURE 1: Densitometric tracings of sodium dodecyl sulfate polyacrylamide gels of beef heart cytochrome *c* oxidase. (a) Run on 10% gels; (b) run on 12% gels.

ume and a drop of mercaptoethanol was included in samples as a measure of the total volume of the column.

Gel Chromatography in 6 M Guanidine Hydrochloride. Cytochrome *c* oxidase (50 mg) was dissolved at 2 mg/ml in 8 *M* Gdn·HCl, 50 m*M* Tris-HCl, 50 m*M* dithiothreitol, and 2 m*M* EDTA (pH 8.0) by heating to 50° for 4 hr. 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 m*M* Tris-HCl–0.5 m*M* dithiothreitol (pH 6.0) 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 and mercaptoethanol was included in samples to measure for the total volume.

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

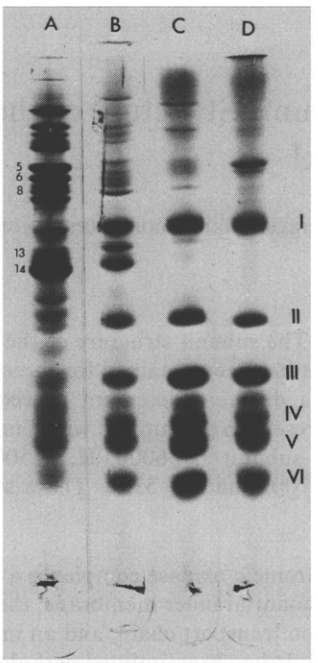

FIGURE 2: Dodecyl sulfate polyacrylamide gel electrophoreses of different samples of beef heart cytochrome *c* oxidase. (a) ETP; (b) cytochrome *c* oxidase prepared as described by Sun et al. (1968); (c) enzyme prepared by the method of Capaldi and Hayashi (1972); (d) enzyme prepared as described by Yonetani (1961). The gels are heavily overloaded to check for contaminants; 100 μg of each sample was used in this experiment. Roman numerals shown on the right designate the subunits of beef heart cytochrome *c* oxidase. Numbers on the left of the gel of ETP identify bands in the gels as described by Capaldi (1974).

Table II: Relative Amounts of the Individual Subunits.[a]

Subunit No.	Type of Preparation: Deoxycholate + Cholate	Cholate	Triton
I	18.3	18.2	18.8
II	15.6	13.8	14.0
III	18.6	19.0	19.1
IV	13.3	13.5	12.5
V	18.0	18.4	17.2
VI	16.2	17.1	18.3

[a] The amount of dye bound to each subunit as a percentage of the total dye associated with all six subunits is listed. The amount of dye adsorbed to each polypeptide was estimated by measuring the area enclosed by each peak in a densitometric tracing of a 12% gel. Peaks IV and V were separated sufficiently for an estimate of the dye bound to each to be made, either by running the gels for longer, or by including 8 *M* urea in gels as described by Swank and Munkries (1971).

Results

Characteristics of the Different Preparations of Cytochrome c Oxidase. Three preparations, one obtained with nonionic detergents of the Triton series (the method of Sun et al., 1968), a second made with cholate (Yonetani, 1961), and a third prepared with deoxycholate and cholate (Capaldi and Hayashi, 1972), were examined in order to obtain

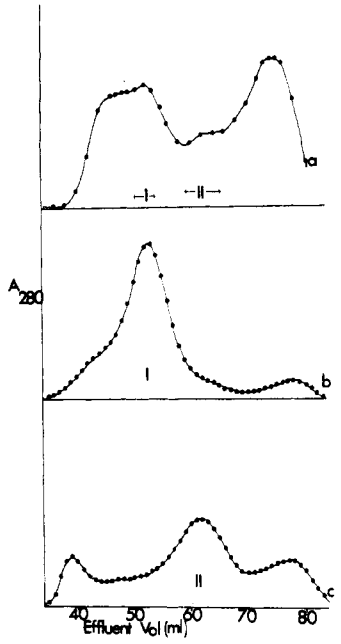

FIGURE 3: Gel filtration of cytochrome *c* oxidase and fractions enriched in higher molecular weight subunits on Sephadex G-200 in 10 m*M* dodecyl sulfate. (a) Elution profile of the enzymic complex dissociated as described in the Experimental Section. Fractions indicated by the arrows were enriched in subunits I and II, respectively, as judged by polyacrylamide gel electrophoresis. These were each pooled and concentrated. (b) The elution pattern of the fraction rich in subunit I eluted in 10 m*M* dodecyl sulfate. (c) The elution pattern of the fraction enriched in subunit II rechromatographed through the column. Protein was measured by absorbance at 280 nm.

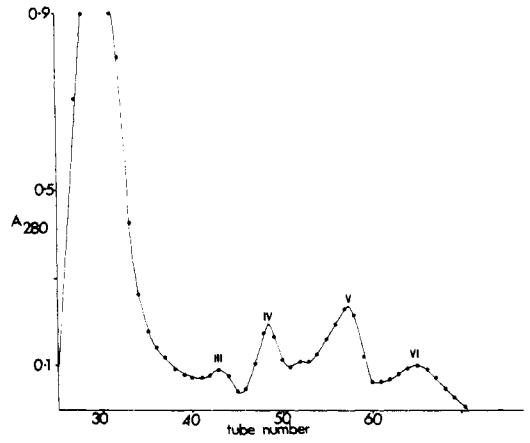

FIGURE 4: The elution profile of reduced and carboxymethylated subunits of beef heart cytochrome *c* oxidase run on Sephadex G-100 superfine in 6 *M* Gdn·HCl. The enzyme had been dissociated by incubating at 50°C in 8 *M* Gdn·HCl. The Roman numerals indicate the subunits present in each peak assessed by dodecyl sulfate gel electrophoresis after Gdn·HCl had been removed by dialysis. Absorbance of protein was measured at 280 nm.

the subunit structure of cytochrome *c* oxidase. These different samples had approximately the same heme *a* content and showed similar electron transfer activities as listed in Table I. The cholate preparation showed respiratory control when reconstituted into membrane vesicles with mitochondrial lipids. The rate of respiration of the enzyme in this system was low but could be enhanced fivefold or more by addition of valinomycin plus uncouplers or with valinomycin and nigericin. Uncouplers alone were ineffective in speeding up the rate of respiration. This is in agreement

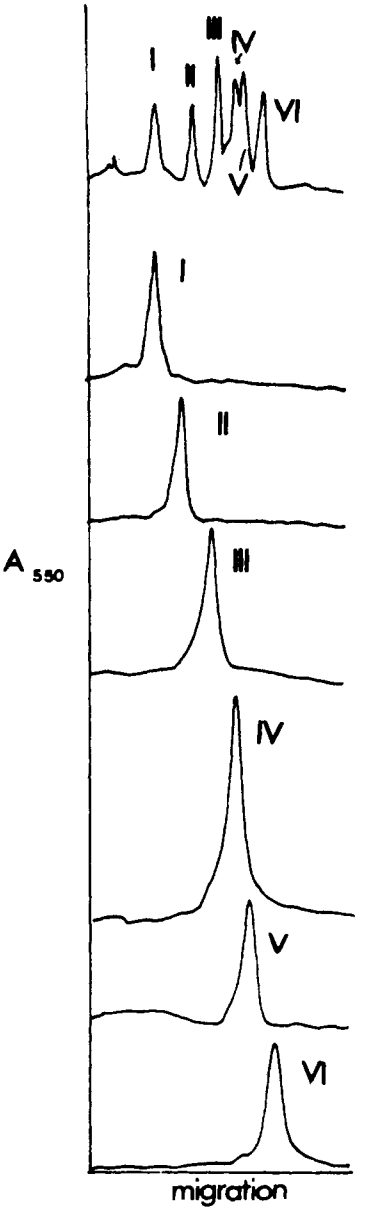

FIGURE 5: Densitometric tracings of dodecyl sulfate polyacrylamide gels of beef heart cytochrome *c* oxidase and purified subunits (I–VI).

with previous reports that a potassium carrier is required for release of respiratory control in cytochrome *c* oxidase vesicles made with mitochondrial lipids (Hunter and Capaldi, 1973; Wrigglesworth and Nicholls, 1975). The Triton and the deoxycholate plus cholate preparations did not show respiratory control, presumably because these samples contain residual detergent and membranes prepared with them are leaky to protons. More effective ways of removing Triton X-100 and deoxycholate from the reconstituted membranes are needed.

The Number of Different Subunits in the Enzyme Complex. The polypeptide composition of each preparation was determined using dodecyl sulfate polyacrylamide gel electrophoresis. Different polypeptide patterns were obtained depending on the percentage of acrylamide monomer used in making the gels. On 10% acrylamide, five major bands were seen. However, on gels containing 12% acrylamide, one of the faster migrating bands was resolved into two components (Figure 1). No further resolution of the complex was obtained at higher concentration of acrylamide,

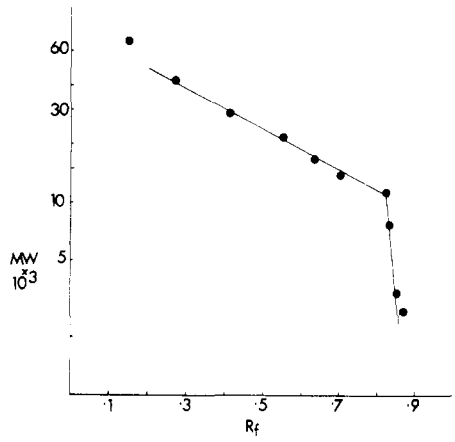

FIGURE 6: A standard curve for the 12% polyacrylamide gels constructed from the migration of a number of proteins of known molecular weight. We are consistently unable to fit bovine serum albumin to a straight line.

Table III: Molecular Weights of the Polypeptides in Beef Heart Cytochrome *c* Oxidase Obtained in This Study.

	Molecular Weight and Polypeptide No.					
Analytical Method	I	II	III	IV	V	VI
SDS[a] gel electrophoresis	36000	22500	17200	12500	11200	8000
Column chromatography in SDS	35000	23000				
Column chromatography in 6 *M* Gdn·HCl			17000	12500	9700	5300

[a] SDS, sodium dodecyl sulfate.

and therefore 12% gels were used routinely to analyze the polypeptide composition of different samples. The six major polypeptides were found to be present and in the same proportions regardless of the detergent used in the preparation of the enzyme (Figure 2 and Table II). Additional polypeptides were present but they were there in very small amounts or were found in only one of the three different preparations. We assume therefore that they are impurities adventitiously associated with the complex during purification.

Purification of Subunits. The six different subunits of cytochrome *c* oxidase were separated and purified to homogeneity by column chromatography in denaturing solvents. Gel filtration in dodecyl sulfate was used to obtain subunits I and II. These components were separated from one another and from subunits III through VI by elution through a column of Sephadex G-200 equilibrated with 10 m*M* dodecyl sulfate (Figure 3). Fractions rich in each subunit were pooled and passed through the column a second time to obtain pure samples of each component (scans 2 and 3 of Figure 5). Subunits III through VI were not resolved from each other by the column. This might be expected because dodecyl sulfate converts proteins of low molecular weight into detergent–protein aggregates of very similar size (Stokes radius) (Reynolds and Tanford, 1970).

Subunits III through VI could be separated from each other by gel filtration in 6 *M* Gdn·HCl. The cytochrome *c* oxidase complex dissolved readily in 8 *M* Gdn·HCl but was not fully dissociated by the treatment. Subunits I and II and some of III remained associated with phospholipid in an aggregate which eluted in the void volume of a Sepharose 6B column (the exclusion limit of Sepharose 6B in 6 *M* Gdn·HCl is around 200000). Some of subunit III and polypeptides IV, V, and VI were dissociated and these components entered the gel. Optimal separation of these subunits was obtained by using a column of Sephadex G-100 (superfine) equilibrated in 6 *M* Gdn·HCl (Figure 4). Each peak fraction contained a pure component as shown in gel scans 3–6 of Figure 5.

Molecular Weight of Subunits. Apparent molecular weights for the six subunits were obtained by polyacrylam-

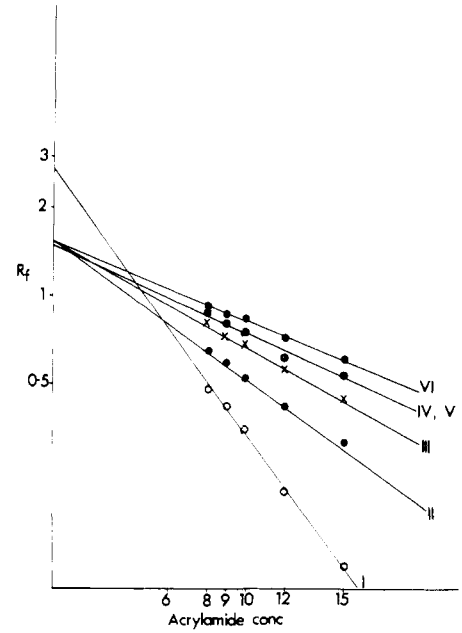

FIGURE 7: A "Ferguson plot" relating the logarithm of the relative mobility (R_f) of the six subunits of the enzyme in dodecyl sulfate to the acrylamide concentration in the gel. All lines have been extrapolated to an acrylamide concentration of zero. Each point is the mean of at least two R_f measurements. Standard proteins cytochrome *c* and ovalbumin intersect at an R_f of 1.5.

Table IV: Amino Acid Compositions of Polypeptides of Beef Heart Cytochrome *c* Oxidase.[a]

	I	II	III	IV	V	VI
Lys	2.98	5.03	11.52	8.12	5.32	8.20
His	2.52	3.82	4.18	2.64	2.11	2.57
Arg	1.97	3.52	4.55	6.87	3.85	3.56
Asp	7.09	9.03	11.24	10.68	7.00	7.08
Thr	6.90	9.37	5.38	5.18	3.68	5.19
Ser	6.80	14.01	8.50	7.42	6.64	8.15
Glu	5.18	14.00	14.38	14.10	10.03	11.61
Pro	4.46	6.91	6.98	7.83	3.13	6.23
Gly	10.00	10.00	10.00	10.00	10.00	10.00
Ala	7.82	6.87	10.56	10.06	7.21	8.84
Val	6.81	5.49	8.14	6.83	3.93	5.00
Met	4.30	6.22	5.82	1.78	0.83	3.29
Ile	6.60	6.14	4.89	6.26	2.64	3.74
Leu	10.41	18.46	9.62	10.36	5.79	9.36
Tyr	3.43	5.76	1.72	3.09	1.74	3.07
Phe	6.89	4.66	5.36	3.74	3.80	5.44
Polarity	35.7	45.5	48.6	47.8	49.7	53.7

[a] Results are presented in moles of each amino acid per 10 mol of glycine. Values are the average of duplicate samples hydrolyzed for 24 hr.

Table V: Published Data on the Subunit Structure of Beef Heart Enzyme.

Subunits						
			88000			
			55000			55000
I	37000	40000		39600	40000	
		25000				
II	19000	19000	23000	20700	22500	
III	14000	14000	17000	15200	15000	
IV–VI						26500
	10000	10000	12000	13400	11200	
		8000	9000	11700	9800	
					7300	
% acrylamide in gels	10	10	6 or 7.5	10	10	10
Ref	Kierns et al. (1971)	Yamamoto and Orli (1974)	Shakespeare and Mahler (1971)	Kuboyama et al (1972)	Rubin and Tzagoloff (1973)	Chuang and Crane (1971)

ide gel electrophoresis using the standard curve shown in Figure 6. Values between 36000 and approximately 8000 were obtained as listed in Table III. These molecular weights are only meaningful if the individual polypeptides of cytochrome *c* oxidase behave as typical soluble proteins, for example, binding 1.4 g of dodecyl sulfate/g of protein (Reynolds and Tanford, 1970). Any anomalies can be tested for by checking the migration of each subunit at different acrylamide concentrations and then determining, by extrapolation, the free electrophoretic mobility in 0% acrylamide (Banker and Cotman, 1972). The free electrophoretic mobilities of subunits II through VI were identical and similar to standard proteins analyzed in the same manner (Figure 7). These must form normal dodecyl sulfate–protein complexes and migrate as a function of their size. Subunit I, however, had a free electrophoretic mobility much higher than normal (Figure 7) and reservations must be held about the molecular weight of this component as measured by polyacrylamide gel electrophoresis.

Although subunits V and VI behave normally on gels, they migrate in a region of the standard curve where differences in molecular weight have very little effect on the mobility of the dodecyl sulfate–protein aggregate (Figure 6). Thus their apparent molecular weights are subject to a fairly large error.

Column chromatography in 6 *M* Gdn·HCl was also used to obtain the molecular weights of subunits III through VI. A Stokes radius was determined for each of these components from its elution position, on a column of Sephadex G-100 that had been calibrated with proteins of known size. Molecular weights were estimated using the relationship log Rs = 0.555 log MW − 0.775, calculated from the data of Fish et al. (1970). These values are listed in Table III. The apparent molecular weights obtained for polypeptides III and IV agree well with those estimated for these subunits by gel electrophoresis. Molecular weights obtained for subunits V and VI were significantly lower than those determined on gels.

Amino Acid Composition of Subunits. The amino acid composition of each polypeptide was determined after a 24-hr hydrolysis and these are listed in Table IV. The polarity of each subunit was calculated by summing the mole percentage of Lys, His, Arg, Asp, Thr, Ser, and Glu (Capaldi and Vanderkooi, 1972). Subunits II through VI had polarities between 44 and 54%; these are similar to the values obtained for water-soluble proteins. Subunit I is more hydrophobic with a polarity of only 35.7%.

Discussion

A number of studies of the subunit composition of the beef cardiac cytochrome *c* oxidase complex have been reported (Kierns et al., 1971; Chuang and Crane, 1971; Shakespeare and Mahler, 1971; Rubin and Tzagoloff, 1972; Kuboyama et al., 1972; Yamamoto and Orii, 1974). These do not agree in the number or molecular weights of the polypeptides in the complex (see Table V). Different laboratories have examined different preparations, but it is unlikely that this is the reason for the variations in results. As our study shows, the same subunits are presented in different preparations regardless of the detergent used in their isolation. One potential source of error is in the gel conditions used. All studies have used 7.5 or 10% dodecyl sulfate polyacrylamide gels for electrophoretic separation of polypeptides and these are conditions which do not separate the low molecular weight subunits very effectively (see Figure 1 in Results).

Using 12% polyacrylamide gels, we identify six different polypeptides in the cytochrome *c* oxidase complex with molecular weights of 36000, 22500, 17100, 12500, 9700, and 5300. These six polypeptides are isolated together and in the same proportions by three very different detergent procedures. This suggests that they are associated together to form the structural unit of cytochrome *c* oxidase in the mitochondrial inner membrane. Preparations containing all six polypeptides show *both* electron transfer activity and energy conserving functions (this study; see also Hinkle et al., 1972; Hinkle, 1974). Several preparations of beef cardiac cytochrome *c* oxidase have been described with high heme *a* content (14–15 mμmol/mg of protein) which are missing one or more of the subunits described here (Komai and Capaldi, 1973; Hare and Crane, 1974; Yamamoto and Orii, 1974). These preparations show electron transfer activity but may not retain coupled functions, and cytochrome *c* oxidase in the narrowest sense, that is, the unit that can transfer electrons from cytochrome *c* to molecular oxygen, may be only a part of the fully functional and membrane integrated "cytochrome *c* oxidase complex".

Acknowledgments

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The Linkage between Oxygenation and Subunit Dissociation in Human Hemoglobin. Consequences for the Analysis of Oxygenation Curves†

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ABSTRACT: For human hemoglobin, a pronounced dependence of oxygenation curves upon protein concentration can be demonstrated experimentally in the range between 10^{-4} and 2×10^{-6} *M* heme. The effects of such protein concentration dependence upon analysis of saturation curves have been explored using a model-independent linkage analysis which incorporates the dissociation of tetramers to dimers. We have carried out simulations of oxygenation curves representing a variety of energy distributions designed to cover a wide range of values which are relevant to known hemoglobin systems and experimental conditions. The resulting simulated oxygenation curves were analyzed by least-squares minimization procedures in terms of the tetramer binding isotherm to yield the four apparent Adair constants. These derived constants were compared with the originally assumed values used in the simulation in order to assess the extent to which their values may be altered by the presence of dimer. For each energy distribution the analysis has been carried out over a wide range of protein concentration. We have found that the presence of even small amounts of dimer that are necessarily present at the low protein concentrations commonly employed may have a devasting effect upon the reliability of Adair constant determinations. In addition to these simulated cases, we have analyzed two sets of highly precise experimental data from the literature in order to assess the degree to which constants obtained may have been influenced by the presence of dimer.

Recent technical developments have made possible the rapid determination of accurate oxygenation curves on respiratory proteins, including human and other hemoglobins (Imai et al., 1970; Sick and Gersonde, 1971; Soprounov, 1973; Kiesow et al., 1972). Most notably, the excellent automatic oxygenation apparatus developed by Imai and colleagues (Imai et al., 1970) has provided an extensive body of highly precise oxygenation curves for human hemoglobins under a wide variety of conditions. Such curves are currently being used to determine apparent stepwise ligand binding constants and to draw inferences from them regarding mechanisms of function in both normal and abnormal

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