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Studies on the Resolution of Cytochrome Oxidase

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Abstract

Cytochrome oxidase has been resolved in acetic acid and high salt/detergent media. In 0.5% acetic acid, the smaller subunits of the enzyme are selectively extracted with retention of an insoluble protein fraction containing subunits I–IV, VII. This fraction retains all the heme and copper of the original enzyme in a spectrally unaltered state, and possesses enzymic activity comparable to the unresolved enzyme. The further removal of subunit IV from this fraction results in migration of heme and copper and modification of their spectral characteristics. Resolution of the enzyme in a high salt/detergent medium extracts smaller subunits (V–VII) together with subunit IV and some heme and copper. The heme associated with this enzymically active extract has spectral characteristics that are partially suggestive of heme a_3. It is suggested that the fraction of subunits I–IV,VII, resolved in dilute acetic acid, may represent the limit of resolution of the cytochrome oxidase complex that remains actively and spectrally indistinguishable from the original enzyme.

Key Words: cytochrome oxidase; copper; spectral characteristics; heme a_3; electron-transport.

Introduction

Cytochrome oxidase (ferrocytochrome c:O_2 oxidoreductase, E.C. 1.9.3.1) is the terminal member of the mitochondrial electron-transport chain, catalyzing the donation of electrons from reduced cytochrome c to the reduction of dioxygen to water. Resolution of the beef-heart cytochrome oxidase in highly cross-linked gels containing urea and sodium dodecyl sulfate has shown the enzyme to be composed of some seven subunits ranging in molecular weight from 40,000 to 4000 [1]. In this respect the beef-heart enzyme has a similar subunit composition to cytochrome oxidase isolated from other sources including *Neurospora crassa* [2] and *Saccharomyces cerevisiae* [3]. A question that is often posed is whether all of the subunits of cytochrome

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oxidase are necessary for electron-transfer activity. There are now a number of reports in the literature which suggest that not all the subunits in the cytochrome oxidase complex are absolutely necessary for electron-transfer activity [4–8]. Using detergent solubilization and ammonium sulfate precipitation, Komai and Capaldi [4] and Hare and Crane [5] extracted the smaller subunits of the enzyme which retained enzymic activity. However, the low heme content of such preparations (less than 14 nmol heme/mg protein) clearly suggests that such fractions contained only a portion of the original heme of the enzyme. In another study, Yamamoto and Orii [6] used proteolytic cleavage to remove subunits I, II, III, V, and VII from the enzyme, apparently without loss of electron-transfer activity. Phan and Mahler [7] have used exclusion chromatography in the presence of 0.1% sodium dodecyl sulfate to obtain an active four-subunit enzyme devoid of the larger hydrophobic subunits. More recently Ozawa *et al.* [8] have described the resolution of cytochrome oxidase into two fractions, components A and B, by the use of chromatography in the presence of sodium dodecyl sulfate and urea. Component A contained heme, copper, and subunits I and II, while component B contained heme, copper, and subunits III–VI; both components were reported to have catalytic activity although no absolute values of activity were given. However, from the gel photographs given it is clear that component A also contained additional subunits and is, therefore, probably better described as containing subunits I, II, III, and IV (by the nomenclature of Downer *et al.* [1]). The need for seven subunits in the beef heart cytochrome oxidase has recently become even more questionable with several reports on the subunit compositions of terminal oxidases from other sources. Ludwig and Schatz [9] have described the isolation of a cytochrome oxidase from the bacterium *Paracoccus denitrificans* containing only two large subunits that appear to correspond to the two largest mitochondrially synthesized subunits of the yeast cytochrome oxidase. Yamanaka *et al.* [10] have described a cytochrome *a*-type terminal oxidase from the prokaryotes *Thiobacillus novellus* and *Nitrobacter agilis,* also composed of only two large subunits. A three-subunit cytochrome oxidase has been isolated from the extreme thermophile, *Thermus thermophilus* HB8 [11], one of the subunits being apparently responsible for the binding of cytochrome *c* in the enzyme.

It has been established that each of the four metal components of cytochrome oxidase, two heme *a*-bound iron atoms and two copper atoms, exist in a unique environment. Observations based principally on absorption spectroscopy [12] have distinguished between the two heme *a* components on the basis of their respective ligand binding properties; cytochrome a_3 binds ligands in both the oxidized and reduced state, whereas cytochrome *a* does not. Magnetic circular dichroism [13] has indicated that cytochrome *a* is low-spin and cytochrome a_3 high-spin. On the basis of electron paramagnetic studies (EPR), Van Gelder and Beinert [14] postulated an antiferromagnetic

coupling between high-spin cytochrome a_3 and one of the oxidized copper atoms, such that the expected EPR signals of these two metal atoms are quenched. Thus in the oxidized cytochrome oxidase the EPR-detectable species represent no more than 50% of the potentially paramagnetic components of the enzyme [15].

Current views of cytochrome oxidase generally regard cytochrome a_3 as the site of dioxygen reduction and cytochrome a as the site of cytochrome c oxidation [16]. However, despite the enormous effort so far expended in an attempt to understand the structure of cytochrome oxidase, the location of heme and copper within the enzyme complex remains a mystery. This has largely been because the conditions needed to separate the subunits of the enzyme are those that have led to the release and consequent migration of the noncovalently bound prosthetic groups. There is some evidence for subunit I being a heme protein [17–19], although the studies of Phan and Mahler [7] and Komai and Capaldi [4] argue for association of at least a part of the heme with the smaller subunits of the enzyme. Using conditions designed to militate against the migration of free heme, Freedman *et al.* [20] have identified a heme-associated complex of subunits I, II, and IV. Identification of the copper-associated subunits of the enzyme is even less clear although subunit II [21, 22] may be a likely candidate.

It is significant that most resolutions of cytochrome oxidase begin with or result in a separation of the complex into two fractions, one fraction containing the largest subunits and the other fraction the smallest subunits. Such resolutions can be reconciled with the different chemical properties of these two subunit classes, i.e., the high apolar nature of subunits I–III and the more hydrophilic nature of subunits IV–VII [23]. Further reconciliation comes from the dual biosynthetic origins of the cytochrome oxidase complex. Cytochrome oxidase is composed of subunits synthesized on both cytoplasmic and mitochondrial ribosomes [24]. The largest subunits (I–III) are coded for by mitochondrial DNA, whereas the smaller subunits (IV–VII) are coded for by cytoplasmic DNA. Thus the two subunit classes of cytochrome oxidase must be synthesized in separate locations and brought together under close cooperation for assembly into the functional enzyme. This requirement for close cooperation during biosynthesis has been exemplified in a heme-deficient mutant of *Saccharomyces cerevisiae* [25]. Heme deficiency affected synthesis of both mitochondrial and cytoplasmic subunits, and those subunits that were synthesized in detectable amounts were apparently not assembled with each other.

If it is assumed that the four metal prosthetic groups of cytochrome oxidase are only loosely bound to their apoproteins or simply caged between certain subunits, then their correct location in the enzyme will only be deduced through the careful stepwise resolution of the subunit complex. Clearly, procedures that completely dissociate or denature the enzyme are

unlikely to lead to an unequivocal assignment of heme or copper location. With this view in mind, we have been engaged in developing a number of methods designed to partially resolve cytochrome oxidase under the mildest conditions possible in an attempt to exploit natural lines of resolution within the enzyme complex, such as might be assumed to exist between the mitochondrially synthesized and cytoplasmically synthesized subunits. Here we report on two resolution procedures, one resulting in the removal of the smaller subunits from the enzyme with retention of a two heme–two copper complex of subunits I–IV (VII), and another resolving the smaller subunits along with subunit IV and one heme and one copper group. Using the criteria of enzymic activity and spectral analysis we have shown that the minimum functional complex of cytochrome oxidase is a subunit I–IV (VII) complex containing the full complement of heme and copper. Further resolution of this complex may possibly take place with a separation of heme a and a_3.

Materials

Cholic acid was recrystallized twice from ethanol before use. Triton X-100 (polyoxyethylene *p-t*-octyl phenol) was purchased from Rohm and Haas (Philadelphia, Pennsylvania). Cytochrome *c* (horse heart) and L-α-lysophosphatidyl choline (egg yolk) were from Sigma Chemical Company. Glacial acetic acid (99.7% pure) was purchased from Hi-Pure Chemicals, Inc. (Nazareth, Pennsylvania). All other chemicals used were of the purest grades available.

Methods

Cytochrome oxidase from beef heart was prepared according to Fowler et al. [26] as modified by Capaldi and Hayashi [27] and further fractionated according to Fry et al. [28]. Based on protein determination by the method of Lowry *et al.* [29], preparations of cytochrome oxidase contained an average of 12 nmol heme a/mg protein and 14 nmol Cu/mg protein and were about 6% by weight in phospholipid as determined by the method of Chen *et al.* [30] and assuming 4% phosphorus content in phospholipids. Heme determination was as previously described [28] assuming a mM extinction coefficient of 21.7 for the difference in absorption between 585 and 650 nm. Copper was determined by the method of Felsenfeld [31].

Cytochrome oxidase activity was assayed by measurement of oxygen uptake using a Clark-type oxygen electrode. The assay mixture contained 7 ml of 50 mM potassium phosphate buffer, pH 7.0, 25 mM ascorbate, 70 μM

cytochrome *c*, and 0.7 mg/ml of lysolecithin [32]. Activity was assayed at 38°C.

The method developed by Swank and Munkres [33] for sodium dodecyl sulfate/urea/gel electrophoresis in highly cross-linked gels was employed for the analysis of the subunit composition of cytochrome oxidase and fractions derived therefrom. Procedures used were identical to those recommended by Downer *et al.* [1] with the exceptions that sample dissociation in sodium dodecyl sulfate was extended for 2 h at 38°C and the staining time was doubled. Electrophoresis was carried out in a Bio-Rad model 150A gel electrophoresis cell at a constant current of 2.5 mA per tube for 22–24 h. Densitometric traces of the gels were made at 550 nm in a Gilford linear transport and recorder apparatus fitted with a Varian CDS 101 Data system for computation of peak areas.

Visible spectra were recorded in a Cary Model 118 spectrophotometer. Protein samples were solubilized in 50 mM sodium phosphate buffer, pH 7.0, containing 1% (v/v) cholate and clarified where necessary by brief sonication in a bath-type sonifyer. All readings were made at room temperature. Samples were reduced with sodium dithionite and recorded 30 min later, bubbled with carbon monoxide for 10 min, or reacted with cyanide for 15 min (added from a neutralized stock solution of sodium cyanide, final concentration 1×10^{-4} M).

Prior to resolution, cytochrome oxidase (as prepared) was dialyzed for 12 h against 200 volumes of 50 mM sodium phosphate buffer, pH 7.0, at 4°C. The particulate protein was washed once by suspension in and centrifugation from distilled water. A wet pellet containing about 100 mg protein (for large-scale procedures) or about 10 mg protein (for small-scale procedures) was used per resolution procedure.

Resolution of Cytochrome Oxidase with Acetic Acid

To approximately 100 mg of particulate enzyme was added 10 ml of 0.5% (v/v) acetic acid (or higher concentrations where indicated). The suspension was homogenized in a Potter-Elvehjem homogenizer rotating at 750 rpm with 30 up-and-down strokes. Samples were immediately centrifuged in a Spinco No. 40 rotor at 40,000 rpm for 10 min (including acceleration and deceleration times). The supernatant was removed, neutralized to pH 7–8 by addition of a solution of saturated Tris-base, and warmed at 38°C for about 10 min or until the solution became cloudy and precipitation was induced. The supernatant precipitate was centrifuged for 10 min (No. 40 rotor), solubilized in a small volume of 20% (v/v) cholate, and finally diluted with 50 mM Tris-HCl, pH 7.0, to give a protein solution 1% in cholate. The insoluble protein fraction from the first centrifugation was

immediately resuspended in 50 mM Tris-HCl, pH 7.0, centrifuged, and likewise solubilized in cholate. All operations were made at 4°C.

Where retention of enzymic activity in the insoluble protein fraction was the main objective, a scaled-down procedure was used. About 10 mg of protein was homogenized in 1 ml of 0.5% acetic acid and the insoluble fraction separated by a 2-min centrifugation in a Brinkmann 3200 microcentrifuge.

Resolution of Cytochrome Oxidase in a High Salt/Detergent Solution

(1) NaCl/Cholate. To about 100 mg of particulate cytochrome oxidase was added 10 ml of a solution 4 M in NaCl and 1% (v/v) in cholate. The suspension was thoroughly homogenized and set to stand on ice for 1 h. After this time, the suspension was briefly warmed at 38°C for 5 min and then centrifuged at 40,000 rpm for 15 min. The light-green supernatant was decanted, the pH adjusted to 7.0 with 2 M Tris-HCl, pH 7.0, and stored on ice or frozen at −100°C until required. Prior to incubation at 38°C, all solutions were maintained at 4°C.

(2) NaCl/Triton X-100. To about 100 mg of particulate cytochrome oxidase was added 10 ml of a solution 4 M in NaCl, 0.5% (v/v) in Triton X-100, and 50 mM in sodium phosphate, pH 4.3. Homogenization of this mixture first caused the protein to become completely solubilized; homogenization was continued until the mixture became cloudy. The suspension was warmed at 38°C for 15 min and then centrifuged at 40,000 rpm for 15 min. The light-green supernatant was decanted and stored on ice or frozen at −100°C until required. All steps were made at 4°C up until the warming stage.

Results

Dilute Acetic Acid Resolution of Cytochrome Oxidase

When a dialyzed and particulate suspension of cytochrome oxidase was homogenized in a dilute acetic acid solution (0.5–1%), the smaller subunits of the enzyme (subunits V–VII) could be removed through their solubilization in this acid. Following centrifugation the acid supernatant was neutralized to pH 7.0, causing the complete precipitation of a colorless protein fraction that contained these subunits. The insoluble protein residue from this procedure comprised subunits I–IV and a lesser amount of subunit VII. The gel profiles of these resolved fractions are shown in Fig. 1. This resolution procedure results in a sharp separation of these two groups of subunits provided certain precautions are taken. These include the proper preparation of the enzyme

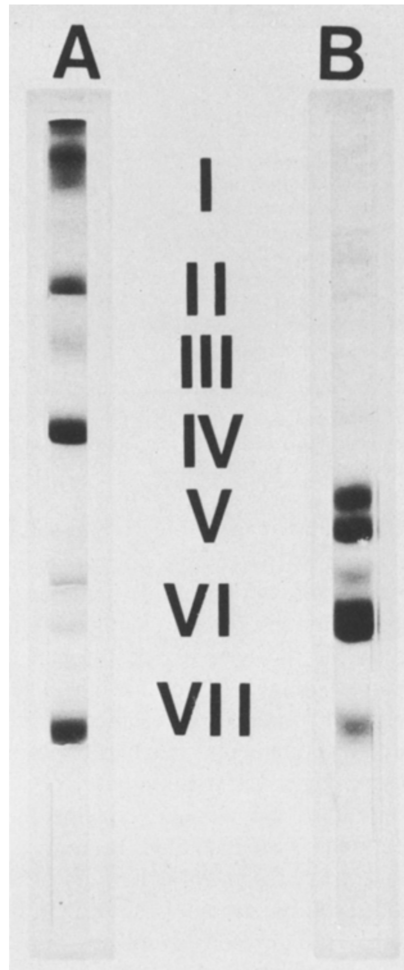

Fig. 1. Gel profiles of cytochrome oxidase fractions resolved in 0.5% acetic acid. (A) Insoluble fraction (subunits I–IV,VII); (B) soluble fraction (subunits V–VII).

prior to resolution (i.e., dialysis and washing), maintenance of low temperature during all steps, and minimization of the time of exposure of the enzyme to acid. This last precaution is probably the most important and demands a relatively rapid centrifugation step to separate the insoluble protein from the solubilized fraction followed by immediate neutralization of the pelleted insoluble protein fraction. A scaled-down procedure, employing a Brinkmann 3200 microcentrifuge, has proved particularly advantageous since exposure of the enzyme to acid can be limited to about 3.5 min, including homogenization and centrifugation.

Analyses of the resolved fractions from the dilute acetic acid procedure

Table I. Resolution of Cytochrome Oxidase in 0.5% Acetic Acid[a]

Parameter measured	Enzyme fraction: Dialyzed cyt. oxidase	Insoluble fraction	Soluble fraction
Total protein (mg)	100	74	25
Subunit distribution	I–VII	I–IV, VII	V–VII
Total phospholipid (μg/mg protein)	58.5	39.2	54
% phospholipid of original enzyme	100	49.5	23
Heme content (nmol/mg protein)	12.2	16.3	0.3
Copper content (nmol/mg protein)	13.6	18.3	0.2

[a]Results are averaged from four resolutions in 0.5% acetic acid on different preparations of cytochrome oxidase. The insoluble and soluble fractions were washed twice in 50 mM Tris-HCl, pH 7.0, prior to analysis.

are tabulated in Table I. The soluble fraction (subunits V–VII) contained little or no detectable copper or heme. In contrast, the heme and copper content of the insoluble fraction (subunits I–IV, VII) are increased to approximately 16 and 18 nmol/mg protein, respectively. This represents close to 99.5% recovery of the original copper of the enzyme and >98% of the original heme of the enzyme. The combined protein contents of the two resolved fractions represented close to 100% recovery, the protein content of each fraction being clearly rationalized on the basis of its subunit composition (assuming a 1:1 stoichiometry of subunits). About 50% of the phospholipid content of the original enzyme, generally between 5–6% by weight, was retained by the insoluble fraction.

Assay for enzymic activity of the insoluble fraction was made following initial solubilization of the protein in 20% cholate with buffer dilution to 1%. Unlike the unresolved enzyme, removal of the smaller subunits of the enzyme renders the insoluble protein fraction more difficult to solubilize in detergent, and it was for this reason that 20% cholate was used to compel complete solubilization. Activity of the insoluble fraction (Table II) was generally between 75–85% of the activity of the unresolved enzyme, although in our best preparations it could exceed 90%. These percentages refer to the comparison with the unresolved and dialyzed cytochrome oxidase. Dialysis and washing can result in a loss of activity of almost 50% compared to the enzyme as prepared. The enzymic activity of the insoluble fraction behaved, in all respects, in a similar fashion to that of the original enzyme, including stimulation of activity by lysolecithin or Tween-20 and sensitivity to cyanide (Table II). The inclusion of catalase in the assay mixture (10 μg/ml) neither increased nor decreased the respiratory rates of the insoluble fraction,

Table II. Enzymic Activity of Dialyzed Cytochrome Oxidase and Fractions Resolved in 0.5% Acetic Acid[a]

	Activity of enzyme fraction (μmol O/min/mg protein)		
Assay conditions	Dialyzed cyt. oxidase	Insoluble fraction	Soluble fraction
No lysolecithin	2.1	0.5	0
+ lysolecithin (0.7 mg/ml)	21.8	18.6	0
+ Tween-20[b] (0.3% w/v)	19.3	15.7	0
+ cyanide (5 μM)	9.4	7.8	0
+ catalase (10 μg/ml)	21.6	18.4	0

[a]Approximately 50 μg of protein, solubilized in 1% v/v cholate, was added to 7 ml of assay mixture at 38°C. Concentrations given refer to final concentration in assay bowl. Rates measured in presence of cyanide and catalase refer to those in the presence of lysolecithin. Results are the average from three separate resolutions.
[b]Tween-20 = polyoxyethyleneglycol (20) sorbitan monolaurate.

suggesting the product formation of H_2O rather than H_2O_2. The pH optimum of the insoluble fraction was identical to that of the unresolved enzyme, pH 7.0. All enzymic activities were corrected for the small blank in the presence of enzyme and ascorbate and in the absence of cytochrome *c*. The soluble fraction of subunits V–VII showed no enzymic activity under these assay conditions.

The visible spectrum of the insoluble fraction is shown in Fig. 2. The oxidized and reduced spectra are almost identical to that of the original enzyme, with absorption maxima for the reduced form at 602 nm (603 in the unresolved enzyme) and 444 nm and a weak absorption at 518 nm. The γ/α ratio for the reduced form was 5.0. The reduced pyridine hemochromogen

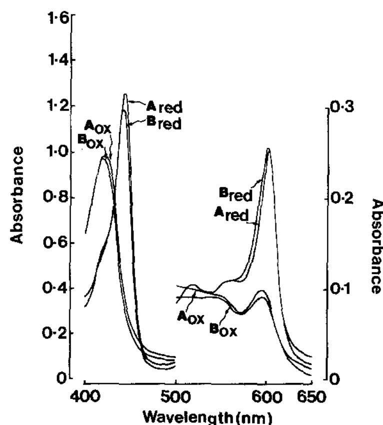

Fig. 2. Absorption spectra of cytochrome oxidase (A) and insoluble fraction (B) derived by resolution in 0.5% acetic acid. red. = reduced spectra, ox. = oxidized spectra. Samples were reduced by addition of 1 mg sodium dithionite to 1 ml solution (1 mg protein/ml) and recorded 30 min later. Absorbance scale 0–0.3 from 500–650 nm, and scale 0–1.6 from 400–500 nm.

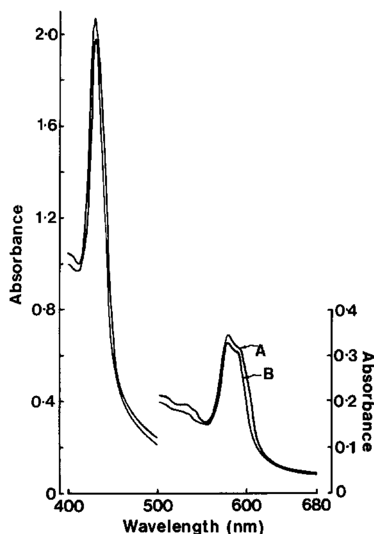

Fig. 3. Reduced pyridine hemochromogen spectra of cytochrome oxidase (A) and insoluble fraction (B) derived by resolution in 0.5% acetic acid. The pyridine hemochromogen was reduced by sodium dithionite and recorded 30 min later. Absorbance scale 0–0.4 (500–680 nm) and 0–2.0 (400–500 nm).

(Fig. 3) of the insoluble fraction and original enzyme were identical, with absorption maxima at 585, 573, and 423 nm. The EPR spectra of the original dialyzed cytochrome oxidase and the insoluble fraction are compared in Fig. 4. The EPR spectrum of the oxidized insoluble fraction (subunits I–IV, VII) shown in Fig. 4B has the characteristic resonances observed for the oxidized cytochrome oxidase (Fig. 4A [15]). Resonance at $g = 3$ is attributed to low-spin ferric heme. Signals at $g \simeq 6$ and 4.3 are slightly increased for the insoluble fraction. There are always weak signals at these resonances that vary in intensity between different enzyme preparations; these signals may be due to denatured material or partial reduction of high-spin heme.

We believe that the retention of enzymic activity and unaltered spectral characteristics are a token of the "completeness" of the subunit I–IV, VII complex. The question remains whether this simpler two-heme two-copper complex can be further resolved. Below are some detailed studies that bear on this question.

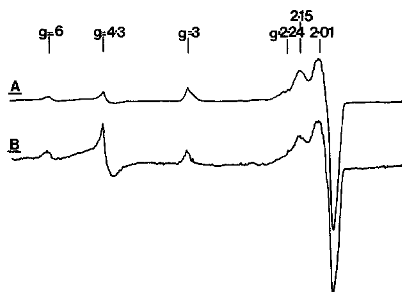

Fig. 4. EPR (oxidized) spectrum of cytochrome oxidase (A) and insoluble fraction (B) derived by resolution in 0.5% acetic acid. Microwave power and frequency, respectively, 1 mW and 8 GHz. Temperature 13 K. Protein was solubilized in 5% cholate and 50 mM Tris-HCl, pH 7.0, to approximately 28 mg/ml.

Further Resolution with Acetic Acid

Of the subunits of the resolved insoluble fraction, subunit IV is the most susceptible to further extraction with acetic acid. If the concentration of acetic acid is increased or if the time of exposure to acid is prolonged, subunit IV tends to distribute between the resolved fractions. For example, upon homogenization of the particulate enzyme in 10% acetic acid, almost all of the enzyme is solubilized. Stepwise adjustment of the pH of such a solution towards neutrality results in precipitation of distinct subunit fractions (Fig. 5) containing either subunits I–IV or IV–VII. While demonstrating the separability of these two groups of subunits, it is clear that some rupture of the [I–III]–IV link has occurred. Concomitant with the migration of subunit IV there is also a migration of heme and copper. Homogenization of particulate cytochrome oxidase in 5% acetic acid results in resolution of the enzyme into two fractions, an insoluble fraction containing subunits I–IV and a soluble fraction containing subunits IV–VII (Fig. 6). Both fractions contain heme and copper; the sum of the hemes approximately accounts for all the heme of the original enzyme, while some 30% of the original copper of the enzyme is lost (Table III). The insoluble fraction derived in 5% acetic acid (Fig. 6) can be further extracted with the almost complete removal of subunit IV (Fig. 7) along with some heme and copper (Table IV). Collection and neutralization of the combined extractions from the above can yield an almost pure preparation of subunit IV. However, despite the almost parallel loss of copper and subunit IV from the insoluble fraction, the recovered subunit IV has only a low copper content. It is clear from the data presented in Table IV that

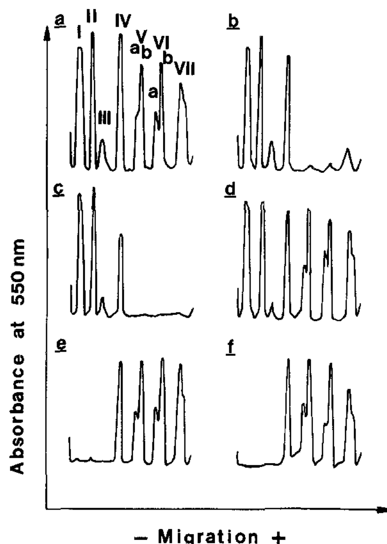

Fig. 5. Densitometric gel traces of cytochrome oxidase fractions precipitated from 10% acetic acid solution by readjustment of pH. (a) Gel profile of unresolved cytochrome oxidase; (b) first insoluble fraction (that fraction insoluble in 10% acetic acid and removed by centrifugation prior to readjustment of pH); (c) second insoluble fraction at pH 3.7; (d) third insoluble fraction at pH 5.5; (e) fourth insoluble fraction at pH 8.1; (f) final insoluble fraction precipitated by addition of solid ammonium sulfate to 30% saturation. pH adjustments were made by the addition of a saturated solution of Tris base. All precipitates were sedimented by centrifugation at 30,000 rpm for 20 min, 10 min after the mixture had been allowed to stand on ice at the required pH.

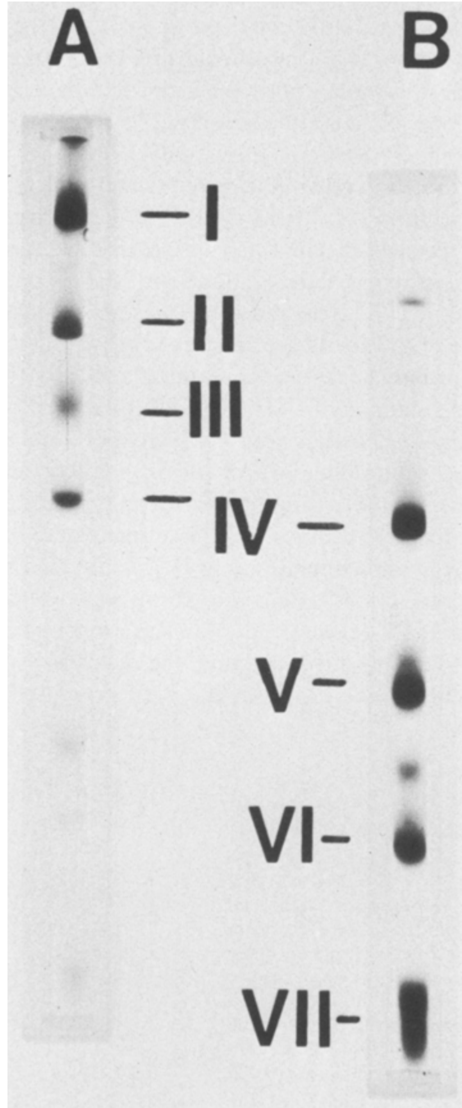

Fig. 6. Gel profiles of cytochrome oxidase fractions resolved in 5% acetic acid. (A) insoluble fraction; (B) soluble fraction.

Table III. Resolution of Cytochrome Oxidase in 5% Acetic Acid[a]

Parameter measured	Enzyme fraction: Dialyzed cyt. oxidase	Insoluble fraction	Soluble fraction
Total protein (mg)	100	68	32
Subunit distribution	I–VII	I–IV	IV–VII
Heme content (nmol/mg protein)	12.4	9.7	17.8
% of original heme of enzyme	100	53	46
Copper content (nmol/mg protein)	13.5	8.4	12.5
% of original copper of enzyme	100	42	30

[a]Results are the average of three resolutions in 5% acetic acid on different preparations of cytochrome oxidase. The insoluble and soluble fractions were washed twice in 50 mM Tris-HCl, pH 7.0, prior to analysis.

during such extractions there is a release and consequent loss of copper from the recoverable fractions; thus the balance sheet for copper (Table IV) shows that only some 56% of the original copper of the enzyme is recoverable. In contrast, recovery of protein and heme was close to 100%. Following a first resolution in 5% acetic acid and one extraction of the insoluble fraction, further extractions of the insoluble fraction remove very little more heme (Table IV). Thus after repeated extractions, essentially a subunit I–III

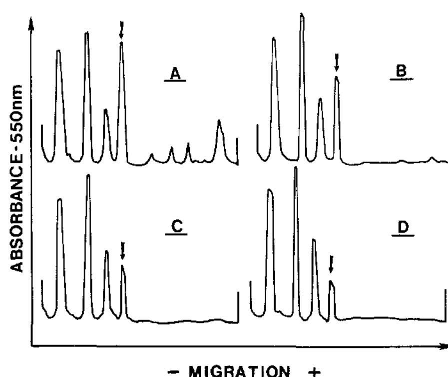

Fig. 7. Densitometric gel traces of insoluble fraction (subunits I–IV,VII) repeatedly extracted in 5% acetic acid. The insoluble fraction of cytochrome oxidase resolved in 5% acetic acid (A, Fig. 6) was re-extracted with 5% acetic acid one to three times (B–D). Arrow marks the position of subunit IV.

Table IV. Extraction of Insoluble Fraction (Subunits I–IV, VII) with 5% Acetic Acid[a]

Parameter measured	Insoluble fraction			
	A	B	C	D
Total protein of fraction (mg)	68	62	58	56
Total heme of fraction (nmol)	660	648	646	645
Total Cu of fraction (nmol)	571	210	98	24
	Derived soluble fraction			
	A	B	C	D
Total protein of fraction (mg)	30	5.5	3.5	1.5
Total heme of fraction (nmoles)	570	11	2	1.5
Total Cu of fraction (nmoles)	400	82	30	8
% recovery of protein from all fractions = 96.5				
% recovery of heme from all fractions = 99				
% recovery of copper from all fractions = 56				

[a]Insoluble-fractions A–D refer to those after one to four extractions in 5% acetic acid (see fractions A–D, Fig. 7). The derived soluble fractions are those extracted from the insoluble fraction with each separate extraction. Thus initial resolution of the enzyme in 5% acetic acid gives insoluble-fraction A and soluble-fraction A; repeated extractions of the insoluble fraction give rise to soluble-fractions B–D. Results are the mean of two such experiments on different enzyme preparations.

complex remains containing approximately 11.5 nmol heme a/mg protein and representing about 52% of the total heme of the original enzyme.

Removal of subunit IV from the [I–IV, VII] complex results in altered spectral characteristics of the remaining heme (Fig. 8). The oxidized spectrum gives broad absorption maxima at 620.5 and 585 nm and a Soret peak at 416 nm. Upon reduction the intensity of the α-peak increases but is only

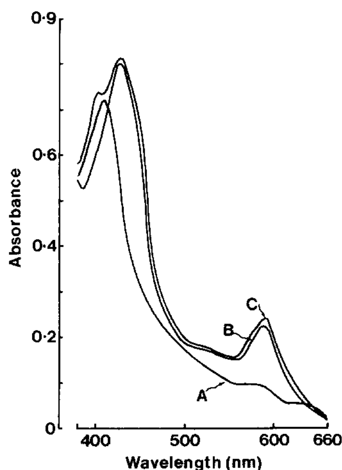

Fig. 8. Absorption spectra of insoluble fraction resolved in 5% acetic acid. Insoluble fraction (subunits I–III[IV]) derived from three extractions in 5% acetic acid (see Fig. 7D). (A) Oxidized spectrum; (B) reduced spectrum with sodium dithionite; (C) reduced + carbon monoxide spectrum.

shifted to 590 nm while the Soret peak appears at 435 nm. The γ/α peak ratio for the reduced form was approximately 3.6. Upon interaction with carbon monoxide and reduction, the position and intensity of the α-peak is virtually unchanged and the position of the 435 nm peak also remains similar but for the appearance of a band at 410.5 nm.

Resolution of Cytochrome Oxidase in High Salt/Detergent

Two variations on the same theme have been developed in an attempt to resolve cytochrome oxidase under the mildest conditions. This involves

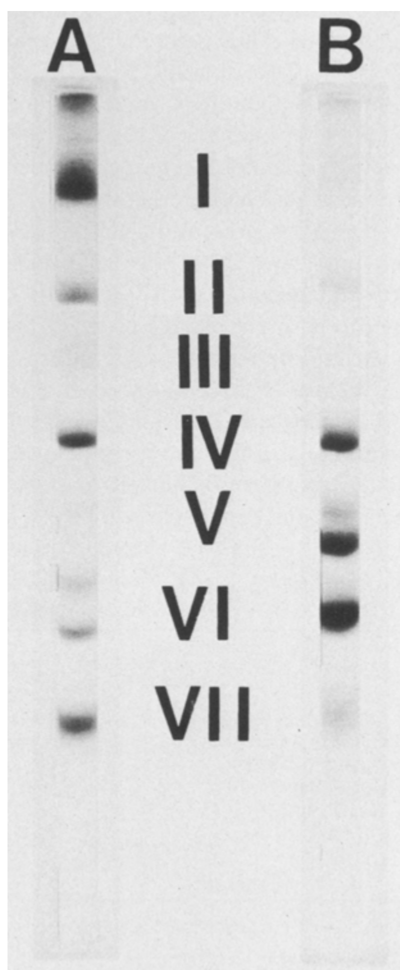

Fig. 9. Gel profiles of cytochrome oxidase fractions resolved in 4 M NaCl/1% cholate. (A) Insoluble fraction; (B) soluble fraction.

extracting the particulate enzyme preparation in a medium of high salt concentration and low detergent concentration. Qualitatively, the resolution of cytochrome oxidase achieved by this method is similar to that found in strong acetic acid solutions. Extraction of cytochrome oxidase in 4M NaCl containing either 1% cholate or 0.5% Triton X-100 solubilizes a part of the enzyme containing subunits IV–VII. It can be seen from the gel profiles given in Fig. 9 that subunit V is the most readily extracted and is almost completely absent from the insoluble fraction. Very similar results are obtained for either cholate or Triton X-100, the only difference being the amount of subunit VII extracted into the soluble fraction (higher in the case of Triton X-100). From a direct comparison of fractions resolved by this procedure through computation of relative peak areas of gel profiles, a preferential extraction of subunits is indicated. Thus extraction of subunit V is almost 95% complete, subunit VI 65% complete, subunit VII 50% complete (in presence of Triton X-100), subunit IV about 40% complete, and subunit VII 10% complete (in presence of cholate). Analyses of the two resolved fractions are given in Table V. Both fractions contained heme and copper, although the content of these prosthetic groups in the soluble fraction was low (about 6 nmol/mg protein). Heme and copper were present in the soluble fraction in approximately equimolar amounts, and there was an almost 100% recovery in heme and copper between the two resolved fractions. As was shown for the case of acetic acid resolution, maintenance of the subunit I–IV complex appears critical if the heme and copper of the enzyme are to be retained unaltered. With strong acid the further extraction of subunit IV leads to a redistribution of heme and copper between the insoluble and soluble fractions. The low heme and copper content of the salt/detergent resolved soluble fraction is probably a reflection of the less than stoichiometric amount of subunit IV in this fraction. Indeed, the heme and copper content of this fraction would appear to be relative to the amount of subunit IV extracted (Table V).

Table V. Resolution of Cytochrome Oxidase in 4 M NaCl/1% Cholate[a]

	Enzyme fraction		
Parameter measured	Dialyzed cyt. oxidase	Insoluble fraction	Soluble fraction
Total protein (mg)	100	83.5	15.8
Subunit distribution[b]	I–VII	I–III [IV, VI, VII]	V [IV, VI, VII]
Heme content (nmol/mg protein)	12.8	14.2	6
Cu content (nmol/mg protein)	13.7	15.1	6.7

[a]Results are the mean of four resolutions in 4 M NaCl/1% cholate on different enzyme preparations.
[b]See gel profiles in Fig. 9.

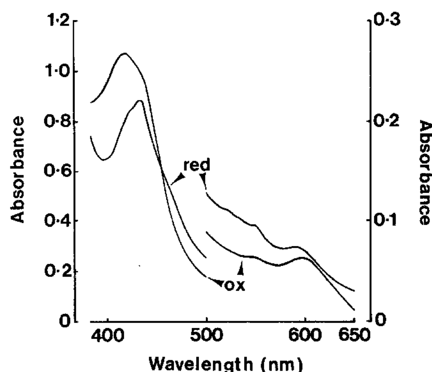

Fig. 10. Absorption spectra of soluble fraction resolved in 4 M NaCl/1% cholate. red. = reduced by sodium dithionite, ox. = oxidized as prepared. Protein = 2.18 mg/ml. Absorbance scale 0–0.3 (500–650 nm) and 0–1.2 (400–500 nm).

Unlike the resolution in dilute acetic acid, the method of high salt/detergent is less efficient in terms of a clean separation of subunits. After a first extraction, repeated extractions of the insoluble fraction remove no further material. So far, all attempts to increase the efficiency of extraction have failed, although we have found indications that the initial lipid content of the enzyme preparation may be of some importance. Extraction of the enzyme in high salt/detergent effectively delipidates the insoluble fraction; readdition of lipid to this fraction slightly increased the amount of material resolved in a second extraction.

With the possibility that there exists two distinct heme *a* prosthetic groups in cytochrome oxidase (heme *a* and a_3), we have been interested in determining the spectral characteristics of the heme associated with the high salt/detergent soluble fraction. The visible spectrum of a salt/cholate soluble fraction is shown in Fig. 10. According to the spectral characteristics proposed for heme a_3 [12], spectra of the soluble fraction would appear to be consistent with a major proportion of the heme in this fraction being of the a_3-type. Thus the α-peak of the reduced heme (594 nm) has only a weak absorbance; Wickstrom et al. [34] have calculated a no more than 20% contribution of heme a_3 to the α-peak. The γ/α ratio was about 11.7 compared to 5.0 for the unresolved enzyme and for the resolved I–IV complex (Fig. 2), and 2.9 and 14 for heme *a* and a_3, respectively [12]. This fraction was only slowly reduced by sodium dithionite, taking about 30 min for full reduction after which the sample was slowly autooxidized. Treatment of a salt/Triton X-100 soluble fraction with carbon monoxide or cyanide produces significant spectral changes (Fig. 11). The reduced CO spectrum shows an

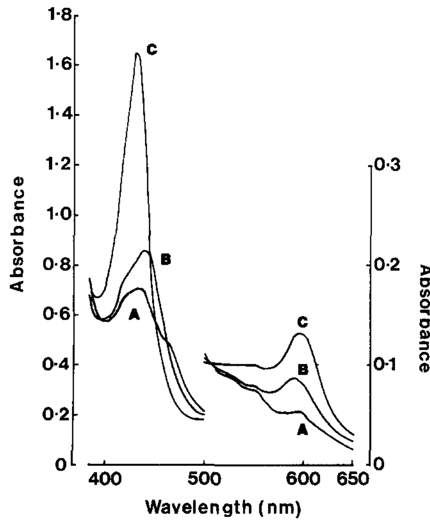

Fig. 11. Absorption spectra of soluble fraction resolved in 4 M NaCl/0.5% Triton X-100. (A) Reduced spectrum with sodium dithionite; (B) reduced + cyanide spectrum; (C) reduced + carbon monoxide spectrum. Protein = 1.82 mg/ml. Absorbance scale 0–0.3 (500–650 nm) and 0–1.8 (400–500 nm).

increase in absorbance of both the α- and Soret peaks, the α-peak remaining at 594 nm and the Soret peak being shifted to 427 nm. The reduced CN spectra shows an α-peak at 588 nm and a Soret peak that is shifted to a longer wavelength (437 nm). According to Hartzell *et al.* [35] and Beinert *et al.* [36], an absorption band at 655 nm is a manifestation of heme a_3. This band is difficult to visualize in conventional absorption spectroscopy because of the increasing absorption over this range. However, a 655 nm band was visible in the oxidized soluble fraction that was decreased in absorption and shifted to 660 nm for the reduced form (Fig. 12). A reduced pyridine hemochromogen spectrum of the soluble fraction produced absorption maxima at 585, 573,

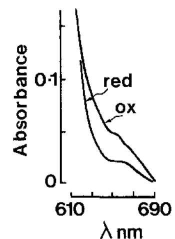

Fig. 12. Adsorption spectrum of soluble fraction resolved in 4 M NaCl/1% cholate in 610–690 nm range. red. = reduced with dithionite, ox. = oxidized.

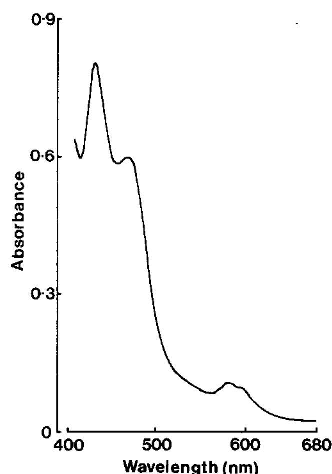

Fig. 13. Reduced pyridine hemochromogen spectrum of soluble fraction resolved in 4 M NaCl/1% cholate.

and 424.5 nm with a strong shoulder at 461.5 nm (Fig. 13). We had hoped that an EPR analysis of this fraction might have indicated a heme a_3–Cu component to be antiferromagnetically coupled and therefore, EPR-undetectable. Unfortunately, because of the low heme and copper content of the soluble fraction we have not been able to obtain a solution concentrated enough to give a reliable EPR analysis. An EPR spectrum obtained for the dilute fraction (Fig. 14) certainly indicates that a portion of the iron present is in a high-spin detectable state ($g = 4.3$) with a weak copper signal.

The soluble fraction obtained by NaCl/cholate extraction has a measurable and apparently bona fide enzymic activity (Table VI). Activities as high as 5 μmol O/min/mg protein were measured in the presence of lysolecithin. The enzyme-catalyzed reaction had an absolute requirement for cytochrome

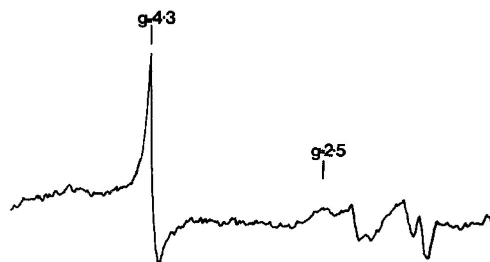

Fig. 14. EPR (oxidized) spectrum of soluble fraction resolved in 4 M NaCl/1% cholate. Protein concentration of sample was 11.5 mg/ml, containing approximately 6 nmol heme and 6.6 nmol copper/mg protein. EPR conditions as for legend to Fig. 4.

Table VI. Enzymic Activity of Soluble Fractions Extracted in 4 M NaCl/1% Cholate or 4 M NaCl/0.5% Triton X-100[a]

Assay conditions	Activity of enzyme fraction (μmol O/min/mg protein) Soluble cholate fraction	Soluble Triton X-100 fraction
No lysolecithin	0.5(0)	0
+ lysolecithin (0.7 mg/ml)	5(0.9)	0.2(0)
+ Tween-20 (0.3% w/v)	3.8(0.9)	0.1(0)
+ cyanide (5 mM)	2.1(0.3)	0
+ catalase (10 μg/ml)	0.9	0
+ H_2O_2 (5 μmol)	0.9	0

[a]Activities were measured in the presence of catalase (10 μg/ml following addition of 5 μmol of H_2O_2 to fully oxygenate the system, in the presence or absence of lysolecithin or Tween-20. Values given for + cyanide, catalase, or H_2O_2 were measured in the presence of lysolecithin; in the case of catalase without addition of H_2O_2, and in the case of H_2O_2 without addition of catalase (i.e., in air-saturated media). Figures in brackets are those activities measured in air-saturated media in both the absence of catalase and H_2O_2. Results are averaged from five separate experiments on different enzyme preparations.

c. Since the presence of catalase had no effect on the reaction rate we assume the reaction product was H_2O rather than H_2O_2. However, the highest respiratory rates were obtained in media of high O_2 tension, generated by a catalase–H_2O_2 system. In media equilibrated with air, respiratory rates of less than 1 μmol O/min/mg protein were usual. The reaction was cyanide sensitive although unusually high concentrations of cyanide were required (about 5 mM for 50% inhibition compared to about 5 μM for the original enzyme). In contrast, the soluble fraction obtained by NaCl/Triton X-100 extraction had negligible activity in the presence or absence of lysolecithin or Tween-20. The characteristics of these two soluble fractions are very similar, and an explanation for the differences in activity probably resides in the state of aggregation of the particles. Electron microscopic examination of a soluble cholate fraction revealed the particles to be associated in large linear aggregates; on addition of lysolecithin these aggregates were effectively dispersed with the appearance of more ordered and crystalline arrays. The soluble Triton X-100-fraction also contained large aggregates which, though dispersed by lysolecithin, did not assume the more ordered structures observed for the cholate fraction.

Discussion

The methods and results presented in this paper represent an attempt to explore and define the limitations of resolution of cytochrome oxidase. In the

past, structural studies on cytochrome oxidase have often started with near complete resolution of the enzyme complex and employing such denaturing reagents as sodium dodecyl sulfate and urea. On the basis of such studies, attempts have been made to assign specific subunits as responsible for the binding of heme and copper, although it is widely recognized that under such conditions the noncovalently linked prosthetic groups are readily dissociated and free to migrate. Any study that claims to define a resolved fraction of the original enzyme as a functionally active "cytochrome oxidase" must initially satisfy certain criteria. Chief among these criteria are the demonstration of enzymic activity and the retention of heme and copper in a spectrally unaltered state. Resolution of cytochrome oxidase in dilute acetic acid, which results in removal of subunits V, VI, and some VII, meets all these criteria. On the basis of spectral analysis, the complex of subunits I–IV (VII) is essentially indistinguishable from the original enzyme. Likewise, this subunit fraction effectively catalyzes the donation of electrons from cytochrome *c* to the reduction of dioxygen.

It is clear from the gel profiles shown in Fig. 1 that subunit VII is partitioned between the insoluble and soluble fractions resolved in 0.5% acetic acid; in fact, approximately 70% of subunit VII (calculated from relative peak areas) remains associated with the insoluble fraction. Since we have yet to devise a method to fully extract subunit VII from the insoluble fraction, while still retaining the critical properties of this fraction, we cannot say with certainty that subunit VII is of absolute necessity to the structure and function of subunits I–IV. However, we can say with some certainty that subunit VII is unlikely to be involved with maintenance of heme or copper prosthetic groups, as the heme and copper of the insoluble fraction are fully accounted for and retain their spectral characteristics, despite the fact that some 30% of subunit VII is missing from this fraction. It is possible that the partitioning of subunit VII with this resolution procedure simply reflects the physical and chemical properties of this protein, resulting in incomplete solubilization in this medium or nonspecific association with the insoluble and more hydrophobic fraction of subunits I–IV. This phenomenon is also seen in the resolution of cytochrome oxidase in high salt/detergent media, subunit VII being largely associated with the insoluble fraction resolved in NaCl/cholate (Fig. 9), but largely associated with the soluble fraction resolved in NaCl/Triton X-100. With this uncertainty in mind, we feel justified in concluding that subunits I–IV(VII) probably represent the limit of resolution of cytochrome oxidase that can satisfy the imposed criteria. The tight association of subunits I–IV in the insoluble fraction of subunits I–IV and VII (as indicated by the retention of enzymic activity and heme/copper characteristics) justify the use of the term "complex" in referring to this fraction. With prolonged exposure to acid, the use of stronger acid or high salt/

detergent media, the integrity of this complex is destroyed. These results are suggestive of a critical role of subunit IV in maintaining the intactness of this complex, and procedures that result in dissociation of this subunit inevitably lead to release of heme and copper with consequent changes in their spectral properties. However, an equally viable alternative to this suggestion is that the methods employed to remove subunit IV are also ones that could lead to possible denaturation of the remaining subunits and their heme and copper prosthetic groups. For example, cytochrome oxidase resolved in 5% acetic acid, which leads to a partioning of subunit IV between fractions, contains no measurable enzymic activity in either the insoluble or soluble fractions. To decide between these possibilities, alternative methods will need to be devised to more gently extract subunit IV.

The resolution of cytochrome oxidase in 0.5% acetic acid gives an insoluble protein fraction with a potential enzymic activity of some 75–85% of the original enzyme; this percentage activity is referable to that of the washed and dialyzed enzyme immediately prior to resolution. Given that the resolved insoluble fraction is depleted some 25% in protein relative to the unresolved enzyme, its enzymic activity per milligram of protein is about 60% of the original enzyme. Still, with the additional handling involved with this fraction, we feel that this degree of activity still represents a significant retention of this functional parameter. Furthermore, a lower enzymic activity may be a real characteristic of this fraction, and one that is not directly comparable to the unresolved enzyme. For example, removal of the smaller hydrophillic subunits may result in altered physical and chemical properties of the subunit I–IV(VII) fraction, such as solubility and dispersion in detergent or possibly a lowered affinity for oxygen.

If subunits I–IV(VII) do indeed represent the "heart" of the cytochrome oxidase complex, then it is reasonable to question the role of the smaller subunits of the isolated complex. A number of observations may provide clues to the role(s) of the small subunits. All of the smaller subunits of the complex (including subunit IV) are synthesized on cytoplasmic ribosomes [24], some of which may need to be assembled in precursor form prior to transport across the mitochondrial membranes and incorporation into the enzyme complex [37]. For example, preliminary results on cytochrome oxidase from *Neurospora crassa* suggested a preassembly form of subunit I, precipitated by a specific antibody that recognized a small portion of subunit I to which either subunit V or VI (or possibly both) are attached. This intermediate complex was labile and readily dissociated in 2 M KCl [38]. Furthermore, Saltzgaber-Müller and Schatz [25] have shown that in a heme-deficient mutant of *Saccharomyces cerevisiae* synthesis of both mitochondrial and cytoplasmic subunits of cytochrome oxidase were affected with only minimal assembly of existing subunits. Such observations clearly indicate a role for the smaller

subunits in controlling and mediating in the proper construction of the cytochrome oxidase complex. Most labeling studies on cytochrome oxidase support the idea that the complex is asymmetric, with the small subunits being concentrated on one side of the enzyme [39–41]. Given the more hydrophilic character of these subunits [23] it is conceivable that they play a role in maintaining the correct orientation of cytochrome oxidase across the membrane for its proper interaction with cytochrome *c* and oxygen, as well as for its interaction with other electron-transport complexes and the F_1–F_0 ATPase. Thus the small subunits of the complex may mediate in its assembly (while helping to maintain its correct membrane orientation) and be retained by the assembled complex to impart asymmetry. This view would at least partly explain their ease of removal in dilute acetic acid, as well as in high salt/detergent media. Of course, it is also possible that some of the smaller subunits merely represent impurities in the cytochrome oxidase preparation (e.g., [42]), although only impurities in the sense that they are not functionally required by the isolated soluble enzyme and can be removed from such (but presumably are required by the enzyme *in situ?*). It must be remembered that we are dealing with the isolated cytochrome oxidase; what is therefore true for the solubilized enzyme need not necessarily apply to the membrane-incorporated form. A further role for some of the small subunits might be in the maintenance of an "oxygen pocket." De Fonseka and Chance [43] have recently presented evidence for the existence of an O_2 pocket in cytochrome oxidase that might be filled with a phospholipid environment. From the molecular model of cytochrome oxidase proposed by Fuller *et al.* [44] it is tempting to speculate that such a pocket could exist between the arms of the "Y" of the Y-shaped complex, the arms presumably being constructed from the small subunits of the enzyme.

Resolution of the subunit I–IV complex, by either strong acetic acid or high salt/detergent, gives rise to heme fractions that have altered spectral characteristics and that according to Lemberg [12] are a reflection of heme denaturation or modification. It may be difficult, if not impossible, to isolate the purified heme components from the enzyme in a state that reflects their spectral characteristics observed in the native enzyme. Consequently, we believe that this potential drawback to an identification of a suspected heme type should not prevent a rationalization of the data in this respect. Compared to the spectral characteristics expected of heme *a* and a_3 [12] we would assume a major portion of the heme associated with the high salt/detergent soluble-fraction to be of the a_3-type. This heme fraction has a low α-peak absorption, high γ/α ratio, 655 nm absorption, undergoes significant spectral changes upon binding of carbon monoxide or cyanide, and is autooxidizable. A less convincing argument may be made on identification of the heme (about 50% of the original in the enzyme) that remains tightly associated

with the insoluble fraction following repeated extractions in acetic acid to remove subunit IV. The high α-peak absorption and low γ/α ratio are suggestive of heme a. We may further conclude that heme a_3 is most closely associated with subunit IV or at least more readily released upon dissociation of this subunit. Resolution of subunit IV from the I–IV complex also results in the loss of copper.

These studies on methods of resolving cytochrome oxidase are readily reconciled with resolutions of the enzyme reported by other workers. Thus the complex of subunits I–IV containing >90% of the heme of the enzyme identified by Freedman *et al.* [20] corresponds closely to the subunit I–IV enzyme described herein. The resolutions of cytochrome oxidase described by Ozawa *et al.* [8] are similar in many respects to the present studies. These authors have used sodium dodecyl sulfate and urea to resolve the enzyme into two fractions, containing subunits I–III and subunits IV–VII; subunit IV was partitioned between the two fractions each of which contained heme and copper, and was probably responsible for the enzymic activity of each fraction. On the other hand, ammonium sulfate precipitation from cholate apparently led to removal of larger subunits with retention of enzymic activity [4]; in partial support of these findings were the studies of Phan and Mahler [7] that succeeded in the isolation of an active four-subunit enzyme also devoid of the larger hydrophobic subunits. These latter findings would seem to be in conflict with the present studies, although such conflict may only be apparent. Assuming the most classical picture of cytochrome oxidase as containing four metal groups arranged in series, only one or two of these groups would actually constitute the "oxidase" of the enzyme, i.e., that part responsible for the actual reduction of molecular oxygen. Therefore, it should be theoretically possible to isolate this part of the enzyme in an active form, either devoid of the additional heme and copper of the enzyme or of the subunit framework for this heme and copper, and in a form still capable of electron acceptance from cytochrome c. It is our belief that the soluble fraction derived by high salt/cholate extraction constitutes just such a preparation. Similarly, other resolution procedures that have fractionated the smaller subunits in active form may constitute resolution of the oxygen-reductase proper of cytochrome oxidase; presumably, a minimal requirement of such preparations would be the presence of subunit IV. The low activities measured for the high salt/cholate soluble fraction may be a reflection of the low content of subunit IV in these preparations; on a specific activity basis the actual activity of this fraction may be much higher (about threefold higher) if we correct for the nonessential presence of subunits V–VII.

The activity of the high salt/cholate soluble fraction was dependent on the presence of cytochrome c. We have tried to replace cytochrome c with

other potential electron donors such as phenazine methosulfate, but so far we have met without success. As far as we are aware, other studies that have purported to show enzymic activity for similar fractions (e.g., [4, 7, 8] have also required the presence of cytochrome *c* as electron donor. This raises the question as to what constitutes the cytochrome *c*-binding site of cytochrome oxidase. A number of studies have implicated subunit II as the binding site [45, 46] or subunit III [47]. However, the studies of Erecinska [48, 49] implicate one of the smaller subunits of the enzyme, possibly V or VII. Steady-state kinetic studies with cytochrome oxidase have revealed two binding sites, one high affinity and one low affinity [50, 51]. Two cytochrome *c* binding sites on cytochrome oxidase, one associated with the larger subunits and one with the smaller subunits, might well explain the observations in these and other studies on a cytochrome *c* requirement. Alternatively, the cytochrome *c* requirement may simply be a reflection of the molecular architecture of the cytochrome *c* molecule, allowing for complex formation with the enzyme, inducement of fit and necessary conformational changes in the enzyme, and the ability to associate and dissociate from the enzyme with oxidation-reduction changes.

Without ignoring the function of some or all of the smaller subunits of the enzyme, the present studies present a strong argument that subunits I–IV constitute a minimal functionally intact complex of cytochrome oxidase. Further resolution of this complex may occur with a physical separation of heme *a* and a_3 (possibly together with their associated copper groups). These studies provide a more simplistic view of cytochrome oxidase that will hopefully accelerate an understanding of those critical subunits in the enzyme and their relation to heme or copper groups within the complex. Recent studies in our laboratory have emphasized some of the important properties of the subunits of this complex, such as the ion-transport capabilities of subunit I [52] and the absolute requirement for cardiolipin by the enzyme [53] that may be found associated with subunit I [54].

Acknowledgments

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Note Added in Proof

Using specific mixtures of ionic and nonionic detergents, temperatures below 0°C, and in buffer of low ionic strength, Winter *et al.* [55] have studied the association of heme and copper with cytochrome oxidase subunits following subunit dissociation by polyacrylamide gel electrophoresis. Some 90% of the copper was associated with subunit II, and equal amounts of heme *a* were associated with subunits I and II. The oxidized-reduced and reduced-CO absorption spectra of these subunits resembled those of cytochrome oxidase. Thus, between them, subunits I and II contained essentially all the heme and copper of the enzyme. These results at least partly support our present findings with regard to the minimum cytochrome oxidase complex of subunits I–IV(VII).

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