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THE ISOLATION OF BOVINE-HEART CYTOCHROME *c* OXIDASE SUBUNITS

DEPENDENCE ON PHOSPHOLIPID AND CHOLATE CONTENT

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Summary

The polypeptide chains of bovine-heart cytochrome *c* oxidase were preparatively isolated by a simple large-scale procedure based on gel permeation chromatography in the presence of sodium dodecyl sulphate.

The resolution of the subunits as a function of the cholate and phospholipid content of the preparation was investigated.

Cholate, and to a lesser extent, phospholipids interfere with the separation of the subunits; however, they do not prevent dissociation of the enzyme by SDS.

Bovine-heart cytochrome *c* oxidase consists of six major subunits (estimated molecular weights in thousands: 40, 25, 20, 14, 12 and 10). In addition, the enzyme preparation contains at least five minor constituents, present in less than stoichiometric amounts.

The first two of the three large subunits, all of which are hydrophobic, have amino-terminal *N*-formylmethionine. Subunit III, however, has a free methionine N-terminus.

Abbreviations: SDS, sodium dodecyl sulphate; TEMED, *N,N,N',N'*-tetramethylethylenediamine; I, II, III, IV, V, VI, VII and VIII, subunits (polypeptide chains) of cytochrome *c* oxidase, I, II, ..., VIII.

Introduction

Cytochrome *c* oxidase (cytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1) is located in the mitochondrial inner membrane and is evidently a transmembrane protein [1,2]. Oxygen supposedly interacts with the enzyme from the matrix side and cytochrome *c* from the outer surface of the inner membrane [3]. Both of the enzyme's heme groups are probably located in the hydrophobic interior of the enzyme [4–6].

Cytochrome *c* oxidase is composed of several different polypeptide chains, as amply demonstrated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Up to seven protein bands are observed [7,8], particularly when 8 M urea is used in addition to SDS [9]. The subunits *, designated by Roman numerals, are estimated to have the following molecular weights (in thousands) I, 40; II, 25; III, 20; IV, 14; V and VI, 12; VII, 10; and VIII, ·6. The three largest are hydrophobic, and are probably translated on mitochondrial ribosomes and encoded by the mitochondrial genome, as has been shown for yeast and neurospora [10,11]. The smaller, hydrophilic subunits are products of cytoplasmic protein synthesis [12].

Confusion about the number of subunits present in cytochrome *c* oxidase may be attributed to different techniques for dissociating the enzyme with SDS and carrying out the SDS-polyacrylamide gel electrophoresis. In the absence of urea, subunits I and III tend to form aggregates which do not penetrate the gel, suggesting that they do not exist.

Depending upon conditions, it is frequently observed that several polypeptides co-migrate and anomalous electrophoretic behaviour of the large subunits has also been described [13,14]. After these problems had been recognized it still appeared that the beef oxidase lacked the third large subunit present in the yeast and neurospora enzymes. However, the presence of subunit III in bovine-heart cytochrome *c* oxidase has since been demonstrated by Briggs et al. [15]. The number of seven subunits now has been generally accepted (for an extreme exception see Ref. 16); this does not mean, however, that all the investigators agree as to the identity of all the subunits they observe, or as to the total number required for the functional enzyme [7–9,17,18].

For physico-chemical characterization and the determination of the primary structures, the polypeptides of bovine-heart cytochrome *c* oxidase must be purified on a preparative scale. A single-step procedure has been the primary goal of the present study. Analytical SDS-polyacrylamide gel electrophoresis proved difficult to scale up, so we turned to gel permeation chromatography in SDS. With this method it was found that the mitochondrial phospholipid as well as the cholate used in the isolation of the enzyme had dramatic effects on the chromatographic resolution. These observations allowed the development of a satisfactory preparation procedure, similar to that of Steffens and Buse [19].

Our results suggest that bovine-heart cytochrome *c* oxidase preparations con-

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* The use of 'subunits' for polypeptide chains of cytochrome *c* oxidase, in accordance with general practice, does not imply a functional entity.

sist of six different major polypeptides and also contain at least five minor constituents in non-stoichiometric amounts.

Experimental

Bovine-heart cytochrome *c* oxidase was prepared according to the method of Fowler et al. [20] as modified in the Amsterdam laboratory [21]. The preparations contain about 10 μmol heme *a* per gram protein and possess a molecular activity of 250 $s^{-1} \cdot mol^{-1}$ aa_3 at pH 7.0 and 25°C. The extinction coefficient [22] used to determine the concentration of cytochrome *c* oxidase was 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm (reduced minus oxidized).

Analytical methods

Protein concentrations were determined either by a biuret method [23] or by amino acid analysis, with comparable results. SDS however interferes, particularly with the biuret method.

Polyacrylamide gel electrophoresis

System 1. Preparative electrophoresis was carried out according to the procedure described by Laemmli [24]. Drawings of our preparative electrophoresis apparatus, which is made of glass and Perspex are available upon request. Experimental details are given in the legend to Fig. 2.

System 2. For routine analyses samples were electrophoresed by the method of Swank and Munkres [25,26] using slab gels of 12 X 7 X 0.3 cm containing 10% acrylamide, 1% *N,N'*-methylene-bis-acrylamide, 0.1% SDS and 8 M urea, in a GE-4 apparatus (Pharmacia). Occasionally, the system of Weber and Osborn [27] was used. Protein bands were fixed in 10% trichloroacetic acid for 30 min at room temperature and stained with 0.25% Coomassie brilliant blue in 10% acetic acid/10% methanol for 1 h at 60°C; the gels were destained overnight in 10% acetic acid/10% methanol at 60°C.

Densitometric traces of slab slices at 550 nm were obtained with a gel scan attachment on a Zeiss PMQ III spectrophotometer.

Removal of lipids and cholate

In order to extract lipids and bile acids protein samples were treated five times with *n*-butanol at 0°C. Lyophilized material (400 mg) was extracted with 10 ml *n*-butanol in a Potter-Elvehjelm homogenizer for 30 min and insoluble protein was collected by centrifugation. To further decrease the lipid and cholate concentrations, butanol-extracted protein samples were subsequently treated with either acetone or chloroform/methanol 2 : 1 (v/v).

Separation of polypeptides using gel filtration methods

Up to 350 mg protein was incubated in a total volume of 20 ml with SDS (5—10 mg/mg protein) and 2—4% β-mercaptoethanol (v/v) for 4—20 h at either 20 or 50°C. Columns of 5 X 150 cm were used with either Ultrogel AcA 54 (LKB) or Bio-Gel P-60 (-400 mesh) (Bio-Rad). Before packing the columns, the finest particles were removed by repeated settling and decantation and the gel

matrices were equilibrated with a solution of 3% SDS, 1 mM EDTA and 0.01% sodium azide. Chromatography was usually performed with a migration velocity of about 0.4 cm/h. All 5 and 2.5 cm columns were the Glenco glass-teflon system 3500. The gels were regenerated by washing with 1 M NaCl, followed by extensive washing with water and equilibrated with the chromatography solution. For rechromatography and SDS removal, lyophilized fractions were submitted to gel permeation chromatography either on Bio-Gel P-6 or P-10 (200—400 mesh) or on Ultrogel AcA 54 in 50 mM sodium phosphate, pH 7.0, and 8 M urea. Urea was removed by dialysis.

For rechromatography on porous glass beads (CPG-170, 200—400 mesh) (Pierce) in 88% formic acid, a sequence of 4 columns of 0.9 X 90 cm (Altex) was used. Column eluates were monitored at 280 nm with a Micromedic MS 2 spectrophotometer.

Phospholipid assay

Phospholipids were determined as phosphate, after digestion with perchloric acid, by the method of Fiske and Subbarow, as described by Böttcher et al. [28].

Determination of bile acids

Bile acids were determined with the enzymic method of Turnberg and Anthony-Mote [29], using 3α-hydroxysteroid dehydrogenase (Worthington).

Amino acid analysis

Amino acid analyses were performed according to the method of Spackman, Stein and Moore [30,31]. Protein samples were hydrolyzed with 6 N HCl for 24, 40 and 72 h in vacuo at 110°C and analyzed on a Beckman Multichrom-M amino acid analyzer. Cysteine was determined as cysteic acid according to Moore [32].

Determination of aminoterminal residues

The Edman degradation, as modified by Tarr [33,34] was used, followed by identification of phenylthiohydantoin-amino acids by thin layer chromatography. The identification was supplemented by amino acid analysis, after hydrolysis of the phenylthiohydantoin-amino acids with HI according to Smithies et al. [35]. *N*-Formylmethionyl polypeptides were deformylated with HCl/methanol according to Sheehan and Yang [36], prior to Edman degradation.

Chemicals

Acrylamide, *N,N'*-methylene-bis-acrylamide, TEMED, ammonium persulphate and Coomassie brilliant blue of electrophoretic purity were purchased from Bio-Rad, Tris from Sigma, SDS (sequanal-grade) from Pierce or ('specially pure') from British Drug Houses. Chemicals used for amino acid sequencing were from Pierce (sequanal grade). All other chemicals were analytical grade.

Results

Separation of subunits by SDS-polyacrylamide electrophoresis

Fig. 1 shows typical SDS-polyacrylamide electrophoresis profiles of bovine-

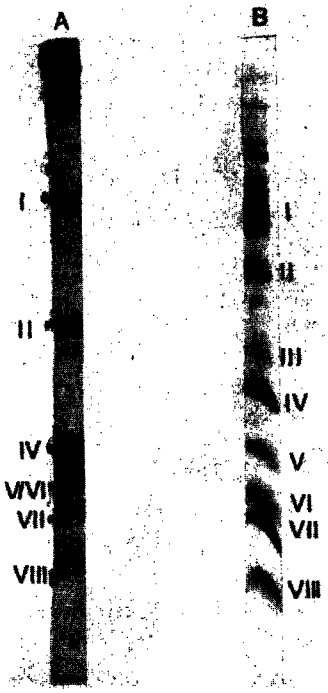

Fig. 1. A. Polyacrylamide gel electrophoretogram of cytochrome *c* oxidase in the presence of SDS. The gel contains 12% acrylamide, 0.4% *N,N'*-methylene-bis-acrylamide, 0.5% SDS, 0.3 M Tris-Cl, pH 8.8. Sample: 10 μg protein incubated with 10-fold excess SDS and 5% v/v β-mercaptoethanol forl h at room temperature. B. Polyacrylamide gel electrophoresis of cytochrome *c* oxidase in the presence of SDS and 8 M urea. The gel contains 10% acrylamide, 1% *N,N'*-methylene-bis-acrylamide, 0.1% SDS, 8 M urea and 250 mM Tris-phosphate, pH 6.8. Sample as in (A) with 8 M urea.

heart cytochrome *c* oxidase in the absence (Fig. 1A) and in the presence (Fig. 1B) of 8 M urea. In the absence of urea, most of subunits I and III aggregate and do not enter the gel. The remaining subunit III material comigrates with Subunit II, and V comigrates with VI. When 8 M urea is present, no aggregated material is found at the top of the gel, III is visible as a diffuse band, and V and VI separate from each other. Moreover, traces of low mobility components are often visible above I in gels with or without urea.

We tried scaling up this electrophoresis system for preparative separation of the chains. Using a custom-made apparatus (see Methods) 75 mg cytochrome *c* oxidase protein could be processed on a cylindrical gel of 5 X 12 cm. The heat exchange capacity of the cooling jacket was insufficient at the higher amperage of urea gels, so only SDS-polyacrylamide gel electrophoresis was used. A typical preparative run is shown in Fig. 2. Witli the large diameter gel the greenish protein bands are visible without staining. Some protein has not penetrated the gel (Band A in Fig. 2A), two sharp bands, B and C, are observed in the upper half and several fused bands are visible in the lower part of the gel. The polypeptide bands were eluted from the sliced gel and identified on a SDS-urea slab gel as shown in Fig. 2B. From these and similar experiments it would appear that preparative gel electrophoresis could be used for the isolation of subunits I

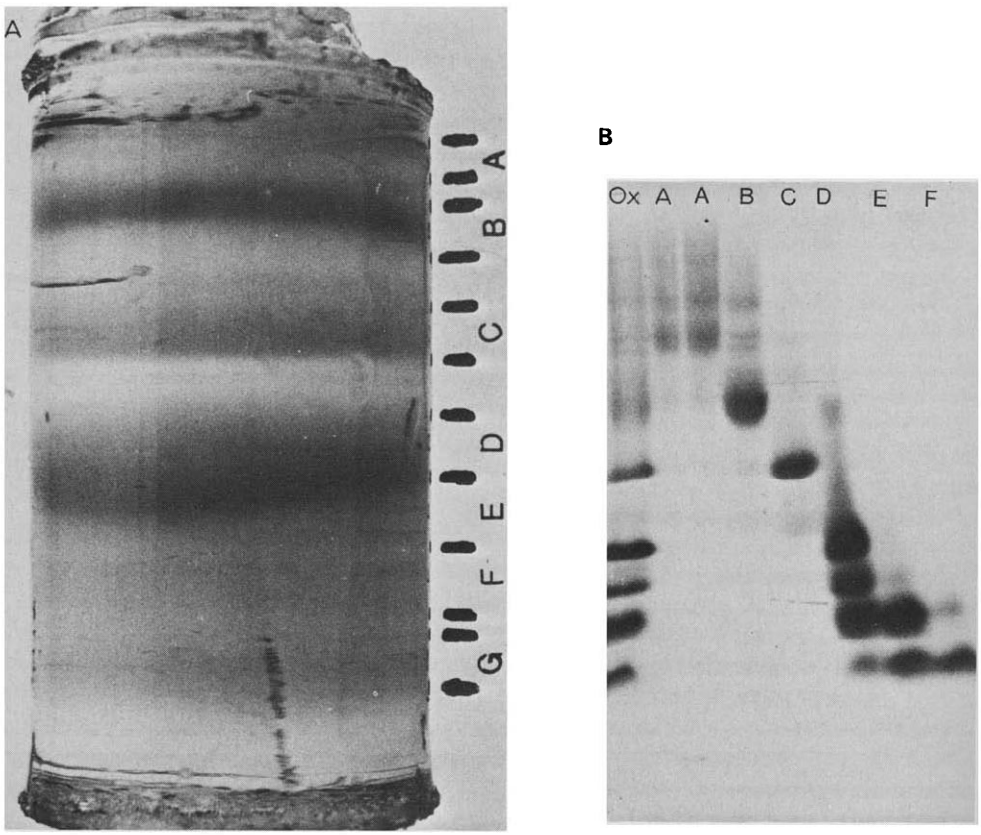

Fig. 2. A. Preparative polyacrylamide gel electrophoresis of cytochrome *c* oxidase. Running gel: 15% acrylamide, 0.4% *N,N'*-methylene-bis-acrylamide, 0.5% SDS and 0.3 M Tris-Cl, pH 8.8; stacking gel 2.5% acrylamide, 0.06% *N,N'*-methylene-bis-acrylamide, pH 6.8. Sample: 75 mg protein in 4 ml, incubated as for Fig. 1A, protein bands extracted from sliced gel. B. SDS-urea slab gel of bands A—F from the preparative gel of (A). 10% acrylamide, 1% *N,N*-methylene-bis-acrylamide, 0.1% SDS, 8 M urea and 250 mM Tris-phosphate pH 6.8. The sample labelled 'Ox' contains the dissociated holoenzyme.

and II (fractions B and C in Fig. 2, respectively) but not for III (aggregates in fraction A) or for the smaller polypeptides (cf. also Refs. 7, 8, 19, 37).

Separation of subunits by SDS gel permeation chromatography; the effect of cholate and phospholipid

An alternative to electrophoresis is SDS-gel permeation chromatography. The result with ultrogel AcA 54 (polyacrylamide/agarose), is shown in Fig. 3A. Even though the enzyme is fully soluble in the running buffer after preincubation in SDS and β-mercaptoethanol at 45°C, no satisfactory separation of subunits could be achieved beyond a division into large hydrophobic and small hydrophilic polypeptides. The separation on Bio-Gel P-60 (minus 400 mesh) (Polyacrylamide) or Sephadex G-150 superfine (dextran) is equal or worse (not shown). Samples from the column effluent (marked by a lettered bar in Fig. 3A) were examined by slab gel electrophoresis in SDS/urea (Fig. 3B). Although little separation was achieved, all the polypeptides eluted from the

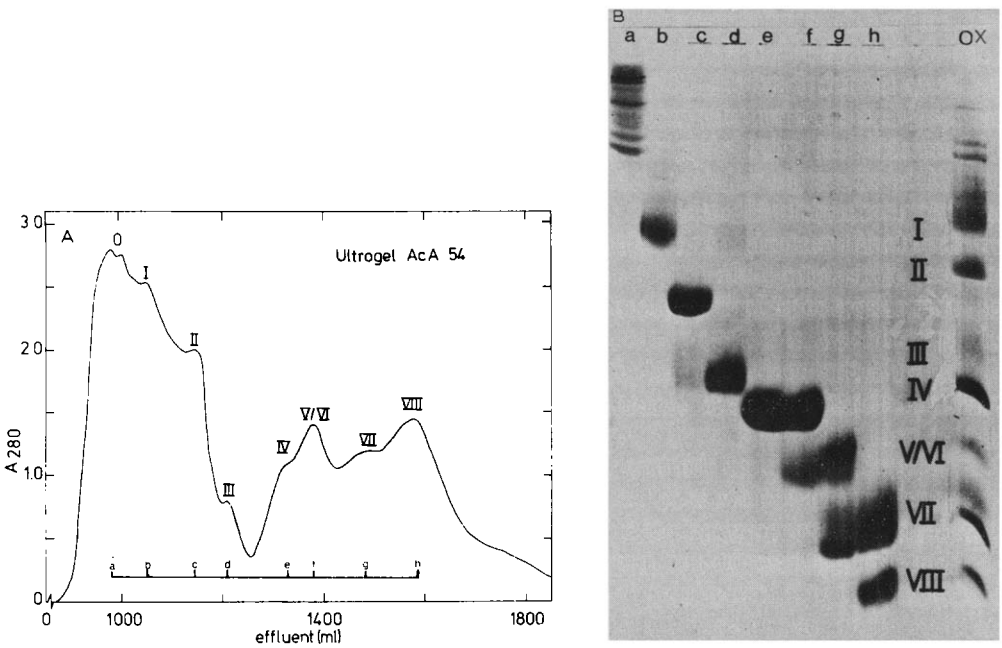

Fig. 3. A. Chromatogram of the subunits of unextracted cytochrome *c* oxidase on Ultrogel AcA 54 (5 X 147 cm column). Sample: 500 mg protein in 45 ml incubated with SDS (5 mg/mg protein) and 4.5% β-mercaptoethanol for 5 h at 50°C. Elution with 0.5% SDS, 1 mM EDTA and 0.01% NaN_3 at 0.5 cm/h. B. SDS-urea slab polyacrylamide gel electrophoresis of peak fractions of the separation shown in (A). The sample slots are marked by letters a—h, corresponding to those in (A). At 'Ox' the dissociated holoenzyme was applied.

column in order of decreasing molecular weights, from I to VIII. No specific complexes of different subunits were detected, except perhaps in the void fraction. This fraction contained aggregates of I and III, similar to the top layer of the electrophoretic separations, and traces of other material, which are presumably the large subunits of contaminating mitochondrial ATPase-F_1 or NADH dehydrogenase.

In general, each polypeptide had a characteristic amino acid composition (see below), which together with its mobility in urea-SDS-polyacrylamide gel electrophoresis, served to identify it.

Reynolds and Tanford [38] found that proteins bind 1.4 times their weight of SDS, but in our gel filtration experiments the subunits of cytochrome *c* oxidase apparently behave as smaller complexes than expected. We suppose the properties of the gel matrices change in the presence of 3% SDS, though we have not determined the amount of SDS bound to the oxidase subunits.

The results depicted in Fig. 3 show that cytochrome *c* oxidase was dissociated into its polypeptide constituents under these conditions, but that the subunits failed to separate completely. Other investigators [9,15,39,40] have unsuccessfully attempted similar separations. Furthermore, there was a high degree of variability in the elution profiles, even though the distribution of subunits of preparations from different sources were identical when analyzed together by SDS-polyacrylamide gel electrophoresis [41]. One possibility for

TABLE I

CHOLATE AND PHOSPHOLIPID CONTENT OF PREPARATIONS OF CYTOCHROME *c* OXIDASE FOLLOWING ORGANIC SOLVENT EXTRACTION

Results expressed in mg/100 mg protein.

Treatment	Phospholipid	Cholate
None	8.4	44
n-Butanol	6.4	23
n-Butanol, followed by acetone	5.0	0.9
n-Butanol, followed by chloroform/methanol (2 : 1, v/v)	0.5	0.4

these discrepancies might be that the elution profiles are influenced by either residual phospholipid or detergents used in the preparation of the enzyme.

Most investigators do not specify the lipid and detergent content of their preparations. The type used in the present investigation is lipid-rich, and is catalytically very active. As shown in Table I 24% of the phospholipid and 48% of the cholate was removed from the lyophilized enzyme by extraction with *n*-butanol. Subsequent extractions with acetone practically eliminated the cholate but had much less effect on the phospholipid. Extraction with chloroform/methanol after the butanol treatment removed nearly all of the remaining phospholipid.

Extraction with butanol renders 10% of the enzyme insoluble in the SDS dissociation mixture; the identification of this fraction as a mixture of III and I will be discussed below. The SDS-soluble material was chromatographed on Bio-Gel P-60 with a dramatic improvement in the separation (Fig. 4). The large peak with a trailing shoulder just behind the void volume is largely aggregated subunit I with considerable III. Non-aggregated I is represented by the shoulder

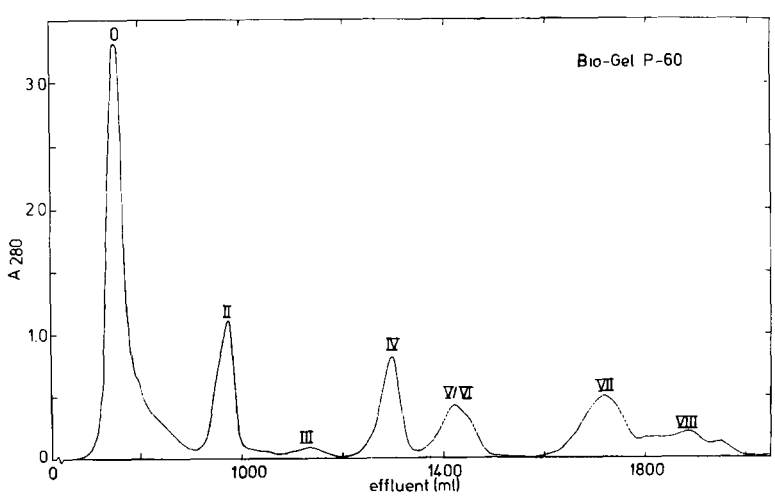

Fig. 4. Chromatogram of the subunits of butanol-extracted cytochrome *c* oxidase on Bio-Gel P-60 (--400 mesh, 5 X 145 cm column). Sample: 200 mg protein in 18 ml, incubated with SDS (10 mg/mg protein) and 4% β-mercaptoethanol for 16 h at 50°C. Elution with 3% SDS, 1 mM EDTA and 0.01% NaN_3 at 0.4 cm/h.

after the first peak and II is well resolved, while monomeric III is hardly present. Bio-Gel P-60 better resolved the smaller subunits, but is inferior to the Ultrogel AcA 54 for the larger polypeptides. The peak containing IV shows baseline resolution, while VIII has split into three components, each having a different amino acid composition (see below). This last observation is in agreement with the results of Steffens and Buse [19], who found three different amino terminal residues in their heterogeneous fraction VIII. In Fig. 4 the fused peak containing V and VI is not resolved into its constituents, though its shape indicates the presence of more than one polypeptide. This conclusion is supported by the frequently observed splitting of the corresponding band in analytical SDS-polyacrylamide gel electrophoresis. After dialysis and lyophilization the V/VI mixture could largely be separated on a set of 4 coupled columns of porous glass beads (360 cm total bed length) in 88% formic acid.

The SDS-insoluble part of the butanol-extracted cytochrome *c* oxidase was washed with water to remove traces of soluble protein and the SDS. The dried material then contained more than 95% protein, an indication that unlike other proteins, it is unable to bind SDS. The protein was fully soluble in 88% formic acid and the clear solution was then chromatographed on controlled pore glass columns in 88% formic acid. As shown by Fig. 5 this otherwise intractable protein aggregate completely dissociated and separated into pure subunits I and III. The small shoulder in Fig. 5 preceding I represents the residual aggregate moving at the void volume and the last peak represents non-protein material migrating with the total bed volume. Subunit I eluted as a pink solution with a peak at 558 nm, probably caused by interaction of tryptophan residues with formic acid. The 280 nm absorbance decreased.

When the butanol-treated cytochrome *c* oxidase was extracted with acetone, the preparation still contained 60% of its original phospholipid but only 2% of the cholate that had been present (Table I). The treated protein was largely soluble in the SDS dissociation medium; it yielded an improved separation of subunits, when chromatographed on Bio-Gel P-60 (Fig. 6). Subunits I and III are present in separate peaks although not in stoichiometric amounts, the balance probably occurring in the void volume peak in the form of aggregates. At this point a satisfactory separation of the small subunits has been achieved. The

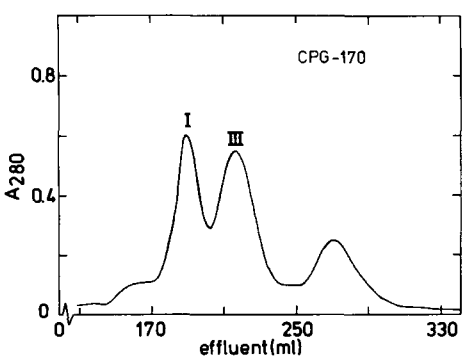

Fig. 5. Chromatogram of SDS-insoluble fraction of butanol-extracted cytochrome *c* oxidase on porous glass CPG-170 (200—400 mesh, 0.9 X 360 cm column) in 88% formic acid. Sample: 36 mg in 500 l. Flow 6 ml/h, pressure drop over column 1.2 atm (Milton-Roy pump).

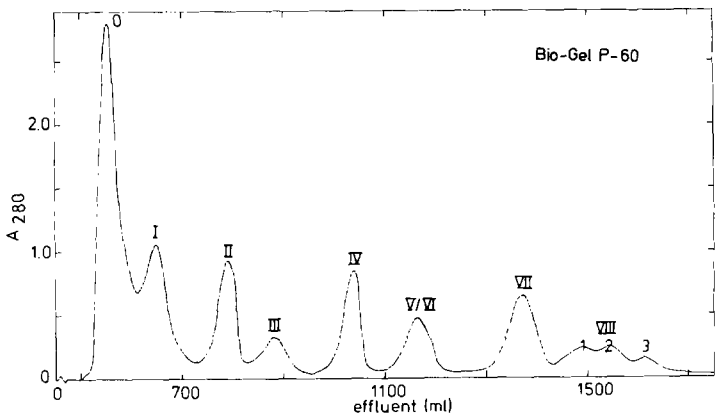

Fig. 6. Chromatogram of butanol/acetone-extracted cytochrome *c* oxidase on Bio Gel P-60 (—400 mesh, 5 X 145 cm column). Sample: 225 mg protein in 18 ml, incubated with SDS (9 mg/mg protein) and 5% β-mercaptoethanol for 5 h at 50°C. Elution with 3% SDS, 1 mM EDTA and 0.01% NaN_3 at 0.2 cm/h.

remaining lipid was removed from the butanol-treated enzyme with chloroform/methanol (Table I). After incubation with SDS at 50°C, the separation on Ultrogel AcA 54 (Fig. 7) showed a relative decrease of the void volume peak, separate peaks representing I and III, which remained rather low despite nearly complete removal of lipid and cholate.

The effect of incubation temperature

Temperatures at which cytochrome *c* oxidase preparations have been dissociated ranging from 20 to 100°C have been reported [8,9,39,42]. Because we suspected that high temperatures promoted aggregation, even in the presence of SDS, experiments in which temperature was lowered from 50°C to 20°C were performed. As shown in Fig. 8 the yield of monomeric subunit III doubled as compared to that shown in Fig. 7. The shoulder preceding the peak of I increased to the point that it was now a partially separated peak; both peaks contained only subunit I.

Second runs on previously used Bio-Gel P-60 columns, and to a lesser extent

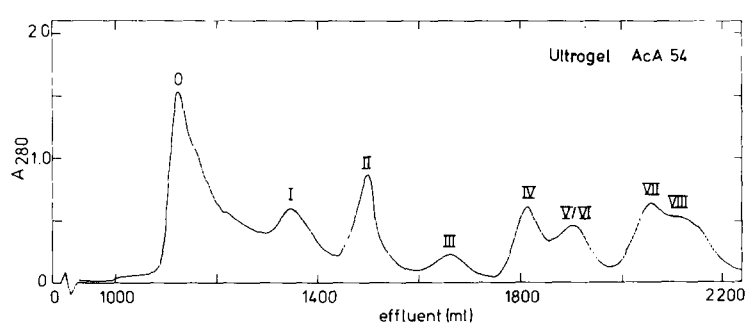

Fig. 7. Chromatogram of subunits of butanol/chloroform/methanol (2 : 1, v/v)-extracted cytochrome *c* oxidase on Ultrogel AcA 54 (5 X 143 cm column). Sample: 350 mg protein in 20 ml, incubated with SDS (5 mg/mg protein) and 2% β-mercaptoethanol for 5 h at 50°C. Elution with 3% SDS, 1 mM EDTA and 0.01 NaN_3 at 0.45 cm/h.

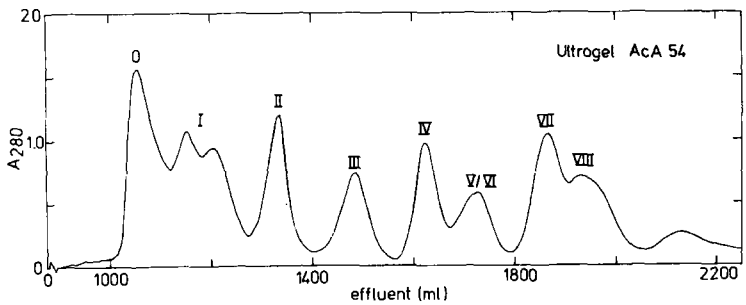

Fig. 8. Chromatogram of subunits of butanol/chloroform/methanol (2 : 1, v/v)-extracted cytochrome *c* oxidase on regenerated Ultrogel AcA 54 (5 × 147 cm column). Sample: 350 mg protein in 20 ml, incubated with SDS (5 mg/mg protein) and 2% β-mercaptoethanol for 16 h at 20°C. Elution with 3% SDS, 1 mM EDTA and 0.1% NaN_3 at 0.45 cm/h.

also on Ultrogel AcA 54 columns, resulted in poorer resolutions (not shown). A regenerated column, even after packing to the same height, had a compressed fractionation range, having lost about 30% of the separation distance between the first peak and subunit VIII. Rapid deterioration of the polyacrylamide matrix makes large scale use of Bio-Gel P-60 impractical. Although the apparent pore size of Ultrogel AcA 54 changed after regeneration under our conditions, the separation distance between two subunits was hardly affected, so it is a more stable and reproducible matrix for gel filtration in SDS.

The gradual increase in yield of non-aggregated I and III in the separation experiments just described, concomitant with the decline of the peak of aggregated material, is shown in Table II, where peak height of relevant subunits measured from Figs. 4, 6, 7 and 8 and other chromatograms are compared.

TABLE II

RELATIVE HEIGHTS OF SUBUNIT PEAKS SHOWING THE EFFECT OF PRETREATMENT OF THE PROTEIN PRIOR TO CHROMATOGRAPHY

The values are normalized to a unit peak height of subunit IV.

Pretreatment	Subunit peaks					
	0	A [d]	I	II	III	IV
I. Ultrogel AcA 54						
1. BuOH-prep. SDS inc., 50°C [a]	4.8	0.5	0.3	1.2	0.1	1.0
2. BCM-prep. SDS inc., 50°C [b]	2.5	0.9	1.0	1.4	0.4	1.0
3. BCM-prep. SDS inc., 20°C [c]	1.6	1.1	1.0	1.2	0.8	1.0
II. Bio-Gel P-60						
1. BuOH-prep. SDS inc., 50°C [a]	4.2	—	0.4	1.4	0.1	1.0
2. BuOH acetone prep. SDS inc. 50°C [b]	3.3	—	1.3	1.1	0.4	1.0
3. BCM-prep. SDS inc., 20°C [c]	1.8	—	1.6	1.0	0.8	1.0

[a] Butanol-extracted preparation, incubated at 50°C with SDS.
[b] As for [a], except that the extraction was with butanol followed by chloroform/methanol.
[c] As for b, but incubated at 20°C.
[d] Where subunit I elutes as twin peak, the designation A and I is used.

Removal of SDS and rechromatography of impure subunits

Following preparative SDS-gel permeation chromatography fractionation the pooled and lyophilized samples of each subunit must be freed of most of the SDS, since large amounts of this detergent interfere with enzymic digestion, peptide mapping, Edman degradation, accurate amino acid analysis and rechromatography of impure polypeptides. Typically, lyophilized residues contained less than 1% protein, the remainder being SDS.

Simple dialysis of SDS was ineffective because of the high particle weight of SDS micelles ($20 \cdot 10^3$) [43] and though the detergent can be extracted with acetone most cytochrome *c* oxidase subunit-SDS complexes were found to be soluble in this solvent. Anion exchange on Dowex 1 in 8 M urea, according to Weber and Kuter [44], has also been used for the removal of SDS, but led to irreversible binding of cytochrome *c* oxidase polypeptides to the resin and loss of protein. Gel filtration in deionized 8 M urea proved to be a simple method for SDS removal, as the urea disrupts the micelles and can itself be removed by dialysis.

The need for further purification of some fractions is illustrated for the separation of butanol-acetone extracted oxidase on Bio-Gel P-60 shown in Fig. 6. Densitometric scans of samples from complete pooled peaks (not merely top fractions), on a urea SDS slab gel, are depicted in Fig. 9. Subunits I, II, IV, VIIIb and VIIIc appear to be adequately pure after the initial separation, but subunits III, V/VI, VII and VIIIa clearly require further purification. The gel scan also shows that the peak immediately following the void volume of the column consists primarily of aggregated subunits I and III with traces of smaller polypeptides. It must be emphasized that I, II and particularly III have low affinities for the Coomassie blue stain and that therefore the tracings do not

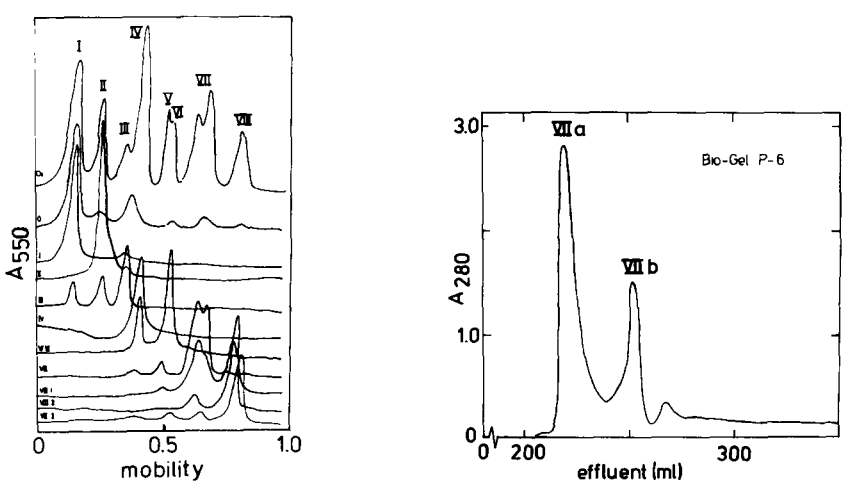

Fig. 9. Densitometric tracings of a sliced polyacrylamide gel electrophoresis slab gel (SDS and urea) of pooled subunit fractions from the Bio-Gel P-60 separation depicted in Fig. 6. The dissociation holoenzyme is marked 'Ox'; the numerals correspond to subunit notation used in Fig. 6.

Fig. 10. Elution profile of the rechromatography of subunit VII on Bio-Gel P-6 (200—400 mesh, 2.5 X 160 cm column) in 50 mM sodium phosphate pH 7.0, 8 M urea and 0.01% NaN_3. Sample: 10 mg protein in 15 ml; flow 0.4 cm/h.

permit quantification of the degree of contamination of the polypeptides.

Subunits I, II, III and IV were rechromatographed on Ultrogel AcA 54 in 8 M urea for repurification and/or SDS removal. As mentioned above, aggregates of I and III and the mixture of V and VI were separated by chromatography on porous glass beads in formic acid. The small polypeptides VIIIa, b, and c were rechromatographed in 8 M urea on Bio-Gel P-6 or P-10. In all cases the urea was removed by dialysis against deionized water.

Purified subunit VII showed a split peak on SDS-urea gel electrophoresis (Fig. 9) as it did in whole oxidase. It also showed two peaks upon rechromatography in 8 M urea on Bio-Gel P-6 (Fig. 10). The second peak, VIIb, contained 20—25% of the total material of VII and had a free amino-terminal residue (see below). The major fraction, VIIa, was N-terminally blocked and had a similar amino acid composition (Table III). The composition differences between V and VI and between the three components of VIII were greater and therefore did not suggest a relationship.

Amino acid composition of subunits

The amino acid compositions of all polypeptides, as determined with our purified chains, are given in Table III. A number of conclusions may be drawn. First, there are clear distinctions between the large hydrophobic subunits, which have high leucine and methionine contents and low values for lysine and arginine, and the smaller more hydrophilic chains. Furthermore, it is obvious that there are three different hydrophobic subunits (I, II, III), in agreement with most authors but in contrast to the opinion recently expressed by Yu and Yu [18], who do not find a material corresponding to subunit III. The very reproducible differences in amino acid composition between I and III, such as the much higher glutamate and histidine contents of III, show that I is not a dimer of III.

The amino acid compositions of the large void volume peak of the butanol-extracted preparation (Fig. 4) and the corresponding SDS-insoluble fraction showed that both were mixtures of I and III, as previously demonstrated by chromatography in formic acid (Fig. 5).

Comparison of the amino acid composition of the fused V/VI band with those of the purified subunits V and VI showed that the total amount of VI was only 20% of that of V. In contrast with VIIa and b, the conspicuous differences in amino acid compositions (see, for example the contents of aspartate, serine, glycine and leucine) suggested that V and VI were similar in molecular size but not in structure.

The amino acid compositions of the cytochrome *c* oxidase subunits determined here are in general agreement with those reported by Steffens and Buse [19]. For subunits I to IV they are also in fair agreement with those reported by Briggs et al. [15] and Downer et al. [9], although the values given by these authors suggest mutual contamination of II and III. For the smaller subunits the similarities are rather vague. Our results can also not be completely reconciled with those of Tanaka et al. [45] and Yu and Yu [18], even if allowance is made for the elimination of subunit III. The amino acid compositions given in Table III for subunits I, II, IV and V are similar to those reported by these authors.

TABLE III

AMINO ACID COMPOSITION OF POLYPEPTIDES PRESENT IN CYTOCHROME *c* OXIDASE FROM BOVINE HEART

The polarity % was taken to be the sum of the molar percent values for Asx, Thr, Ser, Glx, Lys, His and Arg. Trp was not determined. Ser, Thr and Tyr were corrected for decomposition during hydrolysis.

	Enzyme	Major constituents						Minor constituents					Other fractions			
		I	II	III	IV	V	VIIa	VI	VIIb	VIIIa	VIIIb	VIIIc	Void peak	SDS insoluble	V/VI peak	
Asx	7.6	6.9	6.9	4.9	8.5	10.6	7.6	7.4	8.7	8.2	6.9	5.9	7.0	6.2	10.0	Asx
Thr	7.2	7.6	7.7	9.0	5.6	5.6	6.0	5.6	6.0	6.4	6.9	5.1	7.5	9.3	5.5	Thr
Ser	6.9	6.1	10.0	7.5	7.4	4.2	5.3	8.1	5.9	5.5	6.4	8.4	6.1	7.4	4.8	Ser
Glx	8.2	4.6	10.4	7.0	8.9	12.7	6.7	12.2	8.1	9.5	8.5	8.9	5.8	4.8	12.5	Glx
Pro	5.4	5.5	5.5	5.2	4.7	6.9	5.3	5.5	5.5	6.5	6.6	6.4	4.3	5.4	6.7	Pro
Gly	7.5	9.2	4.1	7.9	6.5	7.1	8.4	12.3	7.5	9.1	7.9	8.9	9.7	9.0	7.3	Gly
Ala	7.5	7.6	3.7	6.2	8.1	7.9	10.3	9.9	9.7	9.2	9.3	8.8	8.2	7.6	7.8	Ala
Cys	n.d.	n.d.	n.d.	0.9	n.d.	n.d.	2.2	n.d.	2.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Cys
Val	6.5	7.2	5.2	6.5	7.4	6.7	4.1	5.5	4.4	6.9	5.9	4.9	7.4	6.7	6.5	Val
Met	4.1	5.0	6.3	3.7	3.2	1.2	2.3	2.0	2.2	1.4	1.5	2.1	3.9	5.4	1.5	Met
Ile	5.4	6.1	6.1	5.4	4.7	5.8	2.9	4.3	3.3	3.7	3.5	3.5	5.6	5.9	5.5	Ile
Leu	10.3	10.6	15.4	12.4	7.6	9.0	7.6	6.5	6.3	8.1	11.1	10.4	14.9	11.8	8.8	Leu
Tyr	3.7	3.4	4.7	4.3	3.7	2.9	3.8	2.6	4.1	3.1	3.6	1.8	2.8	4.0	3.2	Tyr
Phe	6.2	8.0	3.3	8.9	4.4	2.6	8.2	3.2	7.3	5.4	5.4	8.3	7.6	8.4	2.7	Phe
Lys	5.1	2.3	2.9	2.1	10.7	6.4	7.0	7.0	7.2	6.5	7.9	8.5	2.1	1.6	6.9	Lys
His	3.8	3.7	3.1	5.7	3.4	2.8	4.0	2.5	3.1	2.8	2.9	2.5	3.2	2.8	3.0	His
Arg	3.9	2.0	2.7	2.4	3.1	5.6	8.5	3.4	8.2	5.6	3.6	3.4	1.9	1.4	5.3	Arg
Polarity %	43	33	43	38	48	50	45	47	47	45	43	43	34	34	49	Pol. %

The composition of subunit V (Table III) corresponds closely to that of the subunit, the amino acid sequence of which was determined by Tanaka et al. [45]. Subunit VIIa is probably identical to VII of Buse et al. [46]. Subunit VIIIc is clearly the chain obtained from the mixture termed VIII, carrying an N-terminal serine and whose sequence was established by Buse and Steffens [47].

The rather large differences between the amino acid compositions given by different authors for preparations of the same subunit indicates either different degrees of purity or differences in the amino acid analysis technique. In particular, the presence of SDS in the protein samples to be hydrolyzed leads to disproportionate losses of some amino acids, notably tyrosine, serine and threonine.

Amino terminal residues of subunits

The amino terminal residues of the subunits were determined by Edman degradation as modified by Tarr [34]. Subunits I and II were found to have *N*-formylmethionine, confirming the results of Steffens and Buse [19]. For subunit III, however, free methionine was found to be the amino terminal residue, and the yield of methionine did not increase after treatment with HCl/methanol by the usual deformylation procedure.

Subunit IV is apparently blocked but in some samples alanine was found in substoichiometric amounts. Subunit V has serine as amino terminus, as already reported by Tanaka et al. [45]. Subunit VIIa is blocked and VIIb begins with alanine. The three polypeptides VIIIa, b, c have amino terminal phenylalanine, isoleucine and serine, respectively, as reported by Steffens and Buse [19].

Discussion

Detergent solubilization of subunits

Cytochrome *c* oxidase is firmly anchored in the mitochondrial inner membrane and can only be extracted by anionic detergents such as cholate and deoxycholate or the non ionic detergents Triton X100 and X114. The isolation procedure employed here involves solubilization of the enzyme from the membrane with deoxycholate, and cholate is used in the later purification steps [20,21]. The protein molecules at this stage can be visualized as ellipsoids with a thin coat of membrane phospholipid around the middle part of the protein where, according to Eytan et al. [1,48], the large hydrophobic subunit I is located. Part of the original phospholipid has been replaced by the negatively charged cholate.

SDS is able to dissociate the oxidase molecule into its subunits (Fig. 3) and the experiments described above can be interpreted in the following fashion. The solubilized polypeptides of the non-extracted enzyme elute more rapidly from a gel permeation column than after extraction of phospholipids and cholate by chloroform/methanol (cf. Fig. 3 with Figs. 7, 8), indicating the larger size of the protein/cholate/phospholipid/SDS complexes. Likely there are different degrees of SDS-binding as a result of varying amounts of cholate and phospholipid attached to individual subunit molecules, and this causes heterogeneity in size and shape of the molecules of the same subunit. The

result is a severe peak broadening which explains the lack of resolution of subunits in the experiments of Fig. 3. Removal of cholate, with its repelling negative charge, would make the subunits more accessible to SDS. Consequently, molecules have a more uniform SDS coating and are better resolved. Nonionic detergents such as Tween or Triton should result in fewer difficulties than the anionic cholate.

After chloroform/methanol extraction the small subunits probably bind their full complement of SDS to become monodisperse protein-SDS complexes. However, the large subunits may aggregate by the strong hydrophobic surface interactions. The molecules of the hydrophobic subunits may even stick together to an extent that little or no SDS is bound and the subunits remain insoluble. Usually, optimal SDS binding requires full denaturation and unfolding of the protein chain such as is achieved by heating to 100°C in the presence of SDS. This procedure is unsatisfactory for very hydrophobic polypeptides as the strength of hydrophobic interactions increases with temperature. Thus, incubation of the enzyme with SDS at a lower temperature would prevent the aggregation of the hydrophobic chains as was observed in the experiment depicted in Figs. 8 and 9.

Separation of subunits

The various results reported for the separation of cytochrome *c* oxidase subunits by different investigators can be attributed to several factors. The poor resolution of subunits obtained by Hundt and Kadenbach [39], using a column of Ultrogel AcA 54 150 cm long, may have been caused by the high phospholipid content (19%) of their rat-liver cytochrome *c* oxidase preparation as well as the high flow rate of 16 cm/h as compared to the 0.4 cm/h used here. Yu and Yu [18] employed a lipid-depleted preparation, dialyzed to remove excess cholate, which should have been effective. However, the chromatographic conditions (80 cm column of Sephadex G-150 superfine, eluted with 0.1% SDS at 1.5 cm/h) prevented higher resolution. Downer et al. [9], did not specify the lipid content of their bovine-heart enzyme preparation, but the chromatography through an 84 cm bed of Sephadex G-100 in 0.15% SDS is insufficient for separation. However, the addition of 8 M urea led to partial resolution of subunits III and II. Some separation of the small subunits by gel filtration on Sephadex G-100 in 6 M guanidine-HCl was earlier reported by the same authors [45]. The higher degree of resolution obtained by Steffens and Buse [19] can be attributed to the lipid-poor preparation (5% phospholipid), dialysis against the nonionic detergent Tween-80 to replace cholate, and suitable conditions for gel filtration (184 cm Bio-Gel P-60, —400 mesh in 2—3% SDS at 0.4 cm/h). Their results are in agreement with our conclusion that removal of cholate is more important than removal of phospholipid.

An example of the isolation of one particular subunit is the procedure of Yu et al. [49]. Subunit V was obtained from beef-heart cytochrome *c* oxidase in a pure form suitable for amino acid sequence determination, by a procedure consisting of pyridine extraction, acid precipitation, ammonium sulphate fractionation and DEAE cellulose chromatography.

For the baker's yeast enzyme, Poyton and Schatz [8] have developed a system for the isolation of 7 subunits utilizing treatment with guanidine HCl,

gel filtration in SDS and in urea, DEAE-cellulose and hydroxylapatite chromatography and preparative SDS gel electrophoresis. The procedure of Werner and Neuner-Wild [37] for the purification of the 7 subunits of *Neurospora crassa* cytochrome *c* oxidase is similar but consists mainly of SDS gel chromatography with a variety of column materials. A common aspect of both methods is the isolation of the largest subunits by preparative SDS gel electrophoresis as described earlier by Sebald et al. [7]. The hydrophobic subunits of yeast and neurospora cytochrome *c* oxidase obviously have little tendency to aggregate, in contrast to those of the mammalian enzyme.

Which subunits belong to cytochrome c oxidase?

Notwithstanding numerous investigations, there are still divergent opinions as to which subunits constitute fully functional cytochrome *c* oxidase and what are their relative molecular proportions. With regard to the 3 large hydrophobic subunits there is little disagreement. With few exceptions, it is generally accepted that they are present in 1 : 1 : 1 ratios, as has been conclusively proven only for the *Neurospora crassa* [7]. One of these exceptions is the claim of Phan and Mahler [40,50] that all three large subunits can be removed without loss of prosthetic groups or of ferrocytochrome c-O_2 catalytic activity. We have been unable to reproduce these results. Wikström and coworkers [51], although they accept that subunits I, II and III play a role, consider them to be present in half the stoichiometric amounts of the 4 small subunits and to consist of phospholipid-stabilized aggregates of $6-12 \cdot 10^3$ dalton polypeptides which can be dissociated under certain conditions. Another opinion is that of Yu and Yu [18] who conclude that there are only 2 large hydrophobic subunits. Notwithstanding these differences, we consider it sufficiently well documented that cytochrome *c* oxidase does contain 3 large hydrophobic subunits. Polypeptides larger than $40 \cdot 10^3$, present in most preparations, are generally considered to be contaminants or aggregates of other subunits.

If the well-known analogy between bacterial and mitochondrial protein synthesis [52] is believed to extend to cytochrome *c* oxidase, the occurrence of N-formylmethionine termini in subunits I and II of the beef-heart enzyme supports the idea that these subunits are translated on mitochondrial ribosomes. In baker's yeast, loci coding for cytochrome *c* oxidase subunits have in fact been located in the mitochondrial genome [53,54]. Our demonstration that the amino-terminus of III is free methionine does not negate the idea that subunit III is also synthesized inside the mitochondrion. However, if this were the case, a mitochondrial deformylase must be assumed to have acted on it and it is not clear why the termini of subunits I and II should not have been deformylated at all.

The picture with regard to the small subunits is even more complex, although all authors agree that the enzyme does contain 3 or more essential small subunits. As pointed out above, it is difficult to compare the individual small subunits described by different investigators. The subunits we identify as VI, VIIb and VIII a, b, c, are present in smaller amounts than the others (I, II, III, IV, V and VIIa), which, based on preliminary calculations of the total amino acid content of subunits peaks from a column separation, appear to be present in equal molecular ratios. Many authors report additional peaks or

shoulders in SDS-polyacrylamide gel electrophoresis or the splitting of bands under certain conditions [1,7,9,13,39—41,50,55,56,58]. A heterogeneity of the smallest subunit (VIII a, b, c) has been reported by Steffens and Buse [19] for bovine heart, by Hundt and Kadenbach [39] for rat liver and by Sebald et al. [7] for the neurospora enzyme. For yeast cytochrome *c* oxidase, similar observations have been ascribed to heterogeneity of the yeast strain [59]. Labelling studies with *Neurospora crassa* cytochrome *c* oxidase [7,60] have made it likely that this enzyme contains 7 or 8 subunits in equimolar amounts. After incorporation of radioactive leucine followed by polyacrylamide gel electrophoresis, the amount of label and the molar percentage of leucine was determined in each subunit band. Other investigators [18] extracted protein from bands after polyacrylamide gel electrophoresis of bovine-heart cytochrome *c* oxidase and reached the same conclusion of 1 : 1 ratios. In this case the evidence appears less convincing, however. Both stoichiometry calculations are based on assumed molecular weights of polypeptides, obtained from relative mobilities in polyacrylamide gel electrophoresis experiments.

As a working hypothesis we consider cytochrome *c* oxidase to contain 6 major polypeptides which are here denoted as I, II, III, IV, V and VIIa, and at least 5 minor constituents. The subunit nomenclature used here appears to be arbitrary by retaining the designation of VII after delegation of VI of the minor chains. However, we have chosen to adhere closely to the notation used by Steffens and Buse [19,47] in order not to compound the confusion. At present we do not know whether these minor chains are copurifying contaminants or intrinsic parts of the cytochrome *c* oxidase, or whether they are remnants of the biosynthetic process. Sequence heterogeneity of some subunits may contribute to the multiplicity. A final answer to these questions has to await determination of the amino acid sequences of individual chains, a process currently underway in several laboratories including our own.

Addendum

After submission of the present paper the proceedings of a congress appeared [61], in which additional information about the topics discussed in our study can be found.

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