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Studies on the role of the oligomeric state and subunit III of cytochrome oxidase in proton translocation *

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1. Anion-exchange fast protein liquid chromatography in the presence of lauryldimethylamine *N*-oxide (LDAO) was introduced to separate cytochrome oxidase into different complexes that either did or did not contain subunit III. 2. Both kinds of enzyme complex exhibited H^+ translocation after reconstitution into phospholipid vesicles, but with a significantly (approx. 50–60%) reduced H^+/e^- ratio as compared with unchromatographed enzyme. 3. The anion-exchange FPLC fractions of the enzyme (with or without subunit III) sedimented more slowly than the control enzyme upon sucrose gradient centrifugation in the presence of cholate and a high potassium phosphate concentration. 4. When the control enzyme was subjected to the sucrose gradient centrifugation in the presence of LDAO or Triton X-100, instead of cholate, one band containing all subunits was observed, which sedimented slowly like the FPLC fractions. Transfer of this band to cholate medium, and reapplication on the sucrose gradient (with cholate), yielded both a slow- and a fast-migrating band after centrifugation. 5. Enzyme complexes that sedimented slowly or rapidly in the sucrose gradients revealed longer and shorter elution times, respectively, in gel filtration FPLC. This suggests that these complexes corresponds to monomers and dimers of cytochrome oxidase. 6. Solubilization of proteoliposomes and subsequent sucrose gradient centrifugation in cholate yielded one fast-migrating band for the untreated enzyme, but both a fast- and a slow-migrating band for the anion-exchange FPLC-treated enzyme, which was exclusively slow-migrating before reconstitution into liposomes. 7. It is suggested that dimerisation of monomeric cytochrome oxidase may be favoured when the enzyme encounters a membranous milieu, and that the dimeric structure might be necessary for proton translocation.

Introduction

Cytochrome oxidase (EC 1.9.3.1) is the oxygen-reducing enzyme complex of the respiratory chains of mitochondria and some bacteria (see Ref. 1). Transfer of electrons from cytochrome *c* to O_2 is linked to electrogenic translocation of protons across the mitochondrial or bacterial membrane [2], generating an electrochemical proton gradient. The latter may be used subsequently for the synthesis of ATP.

Mitochondrial cytochrome oxidase is, as isolated, composed of several different polypeptide chains per monomer. Of these the three heaviest (I–III) are coded by mtDNA and synthesised in the mitochondrion, whereas the others are produced by the nuclear-cytoplasmic route. The pros-

* This paper is dedicated to the memory of Dr. Robert P. Casey, to whom we owe much of our knowledge about reconstitution of cytochrome oxidase into liposomes.

Abbreviations: DCCD, dicyclohexyl carbodiimide; FCCP, *p*-trifluoromethoxycarbonylcyanidephenylhydrazone; HPLC, high performance (pressure) liquid chromatography; FPLC, fast protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

thetic groups, two haems (a and a_3) and two copper ions (Cu_A and Cu_B) are associated with subunits I and II [3].

Subunit III was previously suggested to play an important role in the catalysis of proton translocation for two reasons. First, covalent modification of a glutamic acid residue in this subunit by dicyclohexylcarbodiimide (DCCD) results in inhibition of H^+ translocation without much effect on electron transfer [4]. Secondly, removal of subunit III by alkaline treatment of the enzyme in the presence of Triton X-100 was reported to have a similar effect [5]. However, in more recent work proton translocation has been reported to occur, albeit significantly reduced, even after removal of subunit III [6,7].

The isolated mammalian enzyme is dimeric when dissolved in nonionic detergents at neutral pH [8]. It has been suggested to be dimeric also in the mitochondrial membrane [9], but there is no clearcut proof for this. However, it is clear that monomeric cytochrome oxidase, which may be produced by incubation at high pH [10,11], chymotrypsin treatment [12], or low concentrations of dodecyl sulphate or guanidine hydrochloride [13], is active in electron transfer. Moreover, cytochrome oxidase from sharks [11,14] and from *Paracoccus denitrificans* [15] have been reported to be monomeric in nonionic detergent, and also to catalyse proton translocation after reconstitution into phospholipid vesicles [16,17].

Here we report on a new method to remove subunit III, which differs from previous ones in that a single treatment of the enzyme yields separable enzyme complexes that either contain or lack this subunit. It will be demonstrated that treatments used to remove subunit III tend to monomerize the enzyme, but that monomerization is nevertheless not an obligatory or necessarily permanent consequence of the removal of subunit III. Such treatments lead to reduced proton pumping after reconstitution, but this is again independent of whether subunit III was actually removed or not.

Reconstitution of monomeric enzyme into phospholipid vesicles results in partial dimerisation. The observed reduction in proton-pumping efficiency correlates at least qualitatively with the monomer/dimer ratio of the enzyme after reconstitution, but not with the occurrence of subunit III. It is possible, therefore, that the dimeric structure is necessary for proton translocation.

Materials and Methods

Materials. LDAO (purum) was purchased from Fluka as a 30% solution. Commercial grade soy bean lecitin (L-α-phosphatidylcholine, Type II-S) was purchased from Sigma and washed with acetone. Cholic acid was from Merck and was recrystallised from ethanolic solution. Valinomycin and cytochrome *c* (type VI) were from Sigma. Triton X-100 was purchased from Boehringer and SDS and acrylamide from BDH. FCCP was a gift from Dr. P.G. Heytler. All other materials were of analytical grade and obtained commercially.

Enzyme preparation. Two batches of cytochrome oxidase from bovine heart were used in parallel throughout this study. One was prepared according to Kuboyama et al. [18], as modified by Saari et al. [19], and stored under liquid nitrogen. This preparation had very little high molecular mass bands on SDS-polyacrylamide gel electrophoresis (band h in Fig. 1B, lane 1). The other was prepared by a slight modification of the procedure of Yu et al. [20]. The 'red-green split' was achieved by incubation with only 0.8% (w/v) sodium cholate for 10 min, followed by slow addition of ammonium sulphate. All subsequent incubation and centrifugation times were reduced by one half. This method gave a good yield of active oxidase but contained more high molecular mass contamination (band h, Fig. 1, lane 2).

Fast protein liquid chromatography (FPLC) – anion exchange FPLC. The FPLC system used was equipped with the anion exchange column MONO-Q (Pharmacia, Sweden). Buffer A contained 0.08% (w/v) LDAO, 40 mM Tris-Cl (pH 7.4). 20 nmol cytochrome aa_3 were dissolved in 1 ml of buffer A and LDAO was added to give 2% (w/v). The mixture was incubated for 30 min on ice and then briefly centrifugated to remove undissolved material before chromatography. Particularly with less pure enzyme preparations the results could be improved by centrifugation of the sample on a sucrose gradient in the presence of LDAO (see legend to Fig. 4) and dilution with

buffer A to 100 mM potassium phosphate before application on the column.

Gel filtration FPLC. A Superose 12 column (Pharmacia, Sweden) was pre-equilibrated with 1% (w/v) sodium cholate, 300 mM NaCl and 100 mM potassium phosphate (pH 7.4). 3 nmol of cytochrome aa_3 was diluted to 0.5 ml and chromatographed in this medium at a flow rate of 0.3 ml/min.

Reconstitution into phospholipid vesicles. Reconstitution was carried out according to Casey et al. [21], but at pH 7.4 and with a final dialysis medium containing only 0.1 mM potassium-Hepes. Equal volumes of anion-exchange FPLC (or control) fractions of enzyme, containing 5–10 μM cytochrome aa_3, and a sonicated suspension containing 80 mg/ml purified soy bean lecitin, 2% (w/v) sodium cholate and 200 mM potassium-Hepes (pH 7.4) were mixed and dialysed.

Solubilization of vesicles. 0.25 ml of proteoliposomes were mixed with 0.25 ml of 20% (w/v) sodium cholate and 0.5 ml of water. The mixture was incubated for 1 h on ice before loading on a sucrose gradient.

Sucrose gradient centrifugation. The gradients contained 0.5 M potassium phosphate (pH 7.4) and detergent. For detailed conditions, see legends to Figs. 4 and 7. Note that centrifugation was not performed until equilibrium. Longer centrifugation times yielded further sedimentation. If the potassium phosphate concentration was reduced to 50 mM all different preparations tested (see below) were pelleted in the described conditions. After centrifugation 0.25 ml fractions were collected manually from the top.

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. In order to achieve good separation between subunits II and III, and reasonable separation of the small subunits on the same gel, we employed a linear gradient of acrylamide (10–20%) in the separating gel. The Laemmli [22] buffer system was used, but 6 M urea was included in the separating gel.

Electron transfer and proton translocation. Oxygen consumption was assayed polarographically at 25 °C using a Clark oxygen electrode in a medium containing 50 mM KCl/50 mM potassium phosphate (pH 7.4)/15 mM potassium ascorbate/20 μM cytochrome *c*. Respiratory control ratios were determined as the ratio between velocities of oxygen consumption in the presence and absence of valinomycin plus FCCP.

Proton translocation was measured potentiometrically from acidification of the extravesicular medium in response to initiation of respiration by a pulse of ferrocytochrome *c* at 25 °C. The reaction medium contained 50 mM KCl/50 mM choline chloride/0.1 mM potassium-Hepes (pH 7.4). Cytochrome *c* was suspended in the reaction medium, reduced with dithionite, and passed twice through a Sephadex G-25 'centricolumn' [23]. Such preparations were generally 90–95% reduced. The pH of the cytochrome *c* solution was very carefully adjusted to the pH of the reaction vessel so that no pH change occurred on addition of an aliquot of cytochrome *c* to the latter in the absence of vesicles. Further details are specified in the figure legends.

Spectrophotometry. Spectral analyses were carried out using a Shimadzu UV-3000 spectrophotometer. The concentration of ferrous and ferric cytochrome *c* was determined by oxidation with an excess of potassium ferricyanide and subsequent full reduction with excess dithionite, at 550–540 nm using a molar absorptivity (reduced minus oxidised) of 21 000 cm^{-1}. The concentration of cytochrome aa_3 was determined by reduction with dithionite at 605–630 nm using a molar absorptivity of 27 000 cm^{-1} for the reduced-minus-oxidised absorption change.

Results

Fig. 1A shows how anion-exchange FPLC fractionates the cytochrome oxidase preparation in the presence of LDAO. Elution with a shallow salt gradient yields cytochrome oxidase complexes with different polypeptide compositions (cf. 24). Peak b (Fig. 1) represents a complex that lacks both subunits II and III. In some cases this peak was split into two, of which the one that eluted first lacked both subunits II and III, while the second one lacked only subunit II. The amount of complex lacking subunit II was low, and depended on the batch of enzyme used. It was not detected at all, for example, using the more highly purified enzyme shown in lane 1 of Fig. 1B. The complex lacking subunit II was unstable on reduc-

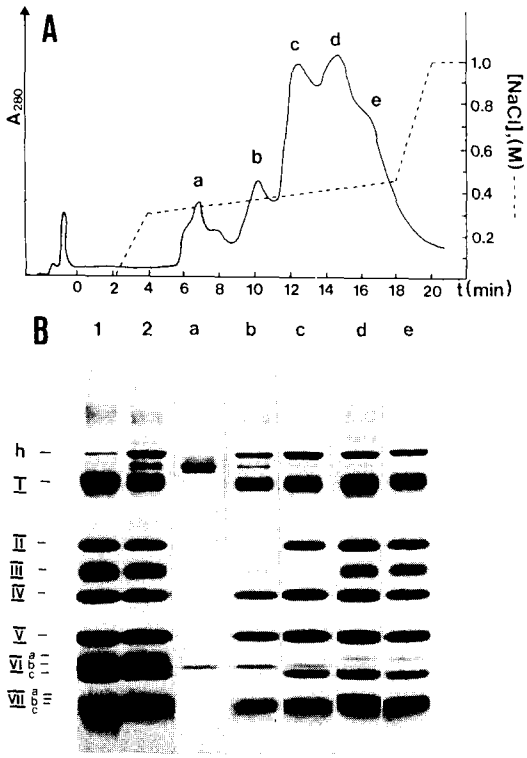

Fig. 1. Anion-exchange fast protein liquid chromatography of cytochrome oxidase. (A) Anion-exchange FPLC profile of cytochrome oxidase. The enzyme was treated as described under Materials and Methods, loaded on a MONO-Q column and chromatographed at a flow rate of 0.5 ml/min. The NaCl gradient employed (in Buffer A, see Materials and Methods) is indicated by the dashed line. (B) SDS-polyacrylamide gel electrophoresis of control oxidase and fractions from anion-exchange FPLC. Lanes 1 and 2 are the two different control enzyme preparations used in this study (see Materials and Methods). Lanes a–e correspond to peaks a–e in Fig. 1A. The preparation shown in lane 2 was used for this chromatography.

tion with dithionite with irreversible deterioration of the optical spectrum. It also showed no detectable O_2 uptake activity with ascorbate plus TMPD as the electron donor system with or without cytochrome *c*.

Fraction *c* (Fig. 1) usually lacked subunit III, as well as most of polypeptides VIa and VIb (and possibly VIIa). Fractions d and e (Fig. 1) were also deficient in these smaller cytoplasmic polypeptides, but contained subunit III. In this method the complex deficient in subunit III (sometimes traces of it were present) was prepared without raising the pH above 7.4 (contrast Refs. 5, 12 and 25). A higher pH, up to 9.0, was tested, and was found to be more effective in removing subunit III (not shown). However, pH 7.4 was chosen because it yielded roughly similar amounts of complexes containing and deficient in subunit III (Figs. 1 and 2), and because then any uncontrolled effect of high pH is thus eliminated.

Both subunit III-deficient and -containing anion-exchange FPLC fractions could be reconstituted into proteoliposomes, yielding reasonably high respiratory control ratios. The subunit III-deficient preparation often exhibited a higher ratio (up to 7.5) than the preparation containing subunit III (up to 5.0). We also found that the reconstitution of untreated enzyme was much improved by prior sucrose gradient centrifugation in the presence of 2% (w/v) cholate and 0.5 M potassium phosphate (pH 7.4). This treatment was therefore routinely employed for the control enzyme, the reconstitution of which yielded vesicles with respiratory control ratios between 5 and 7.5.

As shown in Fig. 2, H^+ translocation was observed after reconstitution of both subunit III-containing and deficient anion-exchange FPLC fractions into liposomes. The H^+/e^- ratio was similar in both cases, approx. 0.4, and much lower than with the control enzyme (Fig. 2A), where it was 0.8–1. Maximum enzyme activities were comparable for control and anion-exchange FPLC-treated enzyme (irrespective of the presence of subunit III; see Fig. 2).

Figs. 2A and 3 (reconstituted control enzyme) also demonstrate the commonly encountered very slow decay of proton ejection after complete oxidation of added ferrocytochrome *c*. Fig. 3 shows that this slow decay is accelerated by gradual addition of higher concentrations of the uncoupler FCCP, until finally no proton ejection is observed (see also Refs. 21, 26 and 27). At this point there is only consumption of about 1 proton per ferrocytochrome *c* oxidised, in accordance with the chemistry of the overall reaction.

It is clear that the low H^+/e^- ratio of the reconstituted anion-exchange FPLC fractions was not due to a low respiratory control ratio. It is also clear that the lowering of the H^+/e^- ratio did not correlate with deficiency of subunit III. These observations suggest primarily that other changes

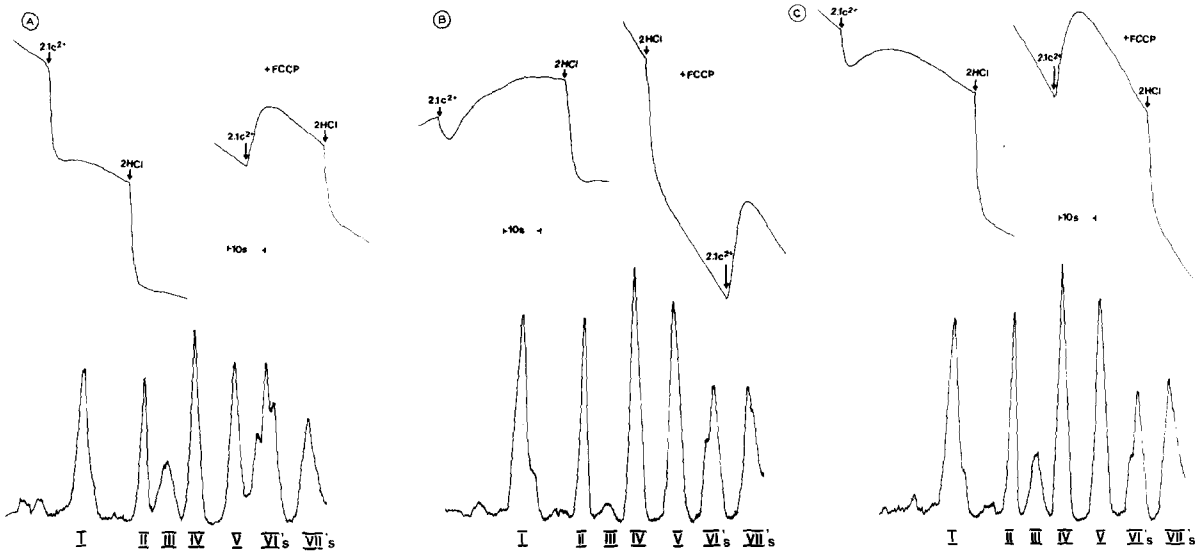

Fig. 2. Proton pumping activity of different reconstituted cytochrome oxidase preparations. The reaction vessel contained 1.4 ml of reaction medium (see Materials and Methods), 0.2 ml of vesicles (about 0.4 nmol cytochrome aa_3), and 1 μg of valinomycin. 2.1 nmol of ferrocytochrome *c* and 2 nmol of HCl were added, as indicated. For each preparation the pH change in response to the pulse of ferrocytochrome *c* is shown in the absence and presence of 0.9 μM FCCP. The densitometric traces of the corresponding Coomassie blue-stained SDS-polyacrylamide gel electrophoresis is also shown for each preparation of enzyme. (A) Control enzyme (respiratory control ratio: 5.5). (B) Subunit III-deficient enzyme fraction from anion-exchange FPLC (respiratory control ratio: 4.2). (C) Subunit III-containing enzyme fraction from anion-exchange FPLC (respiratory control ratio: 3.5).

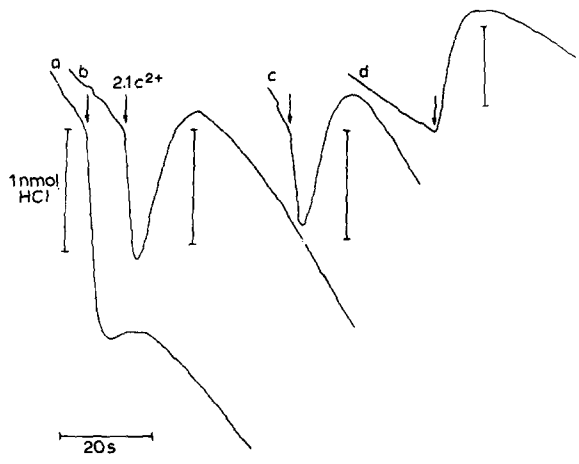

Fig. 3. Proton translocation in control cytochrome oxidase vesicles. Conditions as in Fig. 2. The arrows indicate the injection of 2.1 nmol ferrocytochrome *c*. The vertical bars show the pH response (downwards) to addition of 1 nmol of HCl. FCCP was added before the injections of cytochrome *c*, as follows: a, no FCCP; b, 0.02 nmol; c, 0.04 nmol, and d, 1.2 nmol.

in the enzyme than the removal of subunit III occurred during the treatment, causing the reduction in H^+/e^- ratio. However, the possibility that the reduced efficiency of the subunit III-containing enzyme complex after anion exchange FPLC would be due to some change in the conformation of this subunit cannot be excluded.

The possibility that the removal of the small polypeptides VIa and VIb is causing the reduced proton-translocation is unlikely, since removal of these by trypsin digestion did not have this effect [5,28].

Sucrose gradient centrifugation in the presence of cholate and at a high potassium phosphate concentration was employed earlier to exchange Triton X-100 to cholate prior to reconstitution [5]. We observed that enzyme treated with Triton X-100 at high pH according to Ref. 5 sedimented much more slowly in the sucrose gradient than did control enzyme. The enzyme fractions after anion-exchange FPLC, whether they contained subunit III or not (see above), also sedimented more slowly than control enzyme, and similarly to

the preparation treated at high pH with Triton. It should be stressed here that the sucrose gradient centrifugations were not performed isopycnically (see Materials and Methods).

The sedimentation of control enzyme in the high ionic strength sucrose gradient was strongly dependent on the detergent used, as shown in Fig. 4. If the cholate, which was used normally, was replaced either by 0.5% (w/v) LDAO or by 0.2% (w/v) Triton X-100, the sedimentation became much slower. Sucrose gradient centrifugation in LDAO did not remove subunit III (or any other subunit, as checked by SDS-polyacrylamide gel electrophoresis). The slower sedimentation is not due to higher densities of gradients containing LDAO and Triton for two reasons. First, sedimentation was similar for these two detergents (Fig. 4). Secondly, when protein that had passed an LDAO-containing gradient, where it run slowly, was transferred to cholate and run again on a cholate-containing gradient, a large part of it was still sedimenting slowly (Fig. 6).

The slower sedimentation of cytochrome oxidase on high ionic strength sucrose gradients after anion-exchange FPLC (in which LDAO was employed), or in the presence of LDAO or Triton X-100, suggests that these treatments may cause monomerisation of an originally dimeric enzyme. Gel filtration high performance liquid chromatography (HPLC; equivalent to the FPLC method employed here) was shown previously to yield a clear separation between dimeric and monomeric enzyme [13,29], and we used this to test the hypothesis. Fig. 5 shows the results of such an experiment, demonstrating a clearly different elution time for control enzyme (Fig. 5, solid line), and enzyme that has undergone one of the following treatments: anion-exchange FPLC (in the presence of LDAO), sucrose gradient centrifugation (at high ionic strength) in the presence of Triton X-100, or high pH treatment in the presence of Triton [5] (dashed line). As reported above, all the latter enzyme preparations sedimented slowly on the sucrose gradients. The two elution times (Fig.

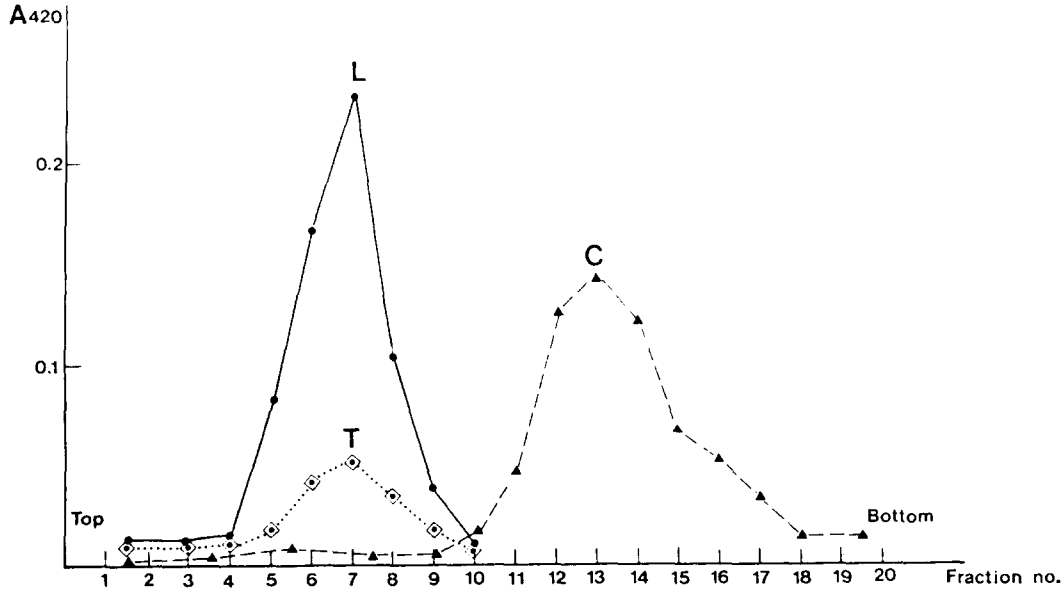

Fig. 4. Sucrose gradient centrifugation of untreated cytochrome oxidase. The centrifugation was performed in an SW 50.1 rotor at 40000 r.p.m. for 18 h at 4°C, 0.25 ml fractions were collected manually from the top. The 5–20% sucrose gradients contained 0.5 M potassium phosphate and the following detergents: C, 2% (w/v) sodium cholate; L, 0.5% (w/v) LDAO; T, 0.2% (w/v) Triton X-100.

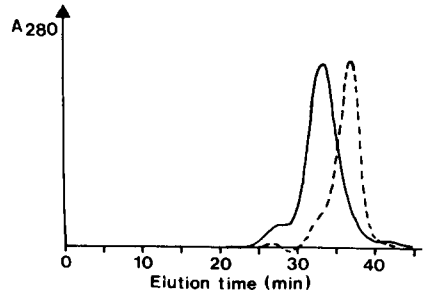

Fig. 5. Gel filtration fast protein liquid chromatography of cytochrome oxidase. Cytochrome oxidase samples that had undergone different treatments were subjected to gel filtration chromatography on a Superose 12 column (see Materials and Methods). Solid line, control enzyme (cf. fraction 'C' in Fig. 4); dashed line, the superimposed profiles of the following three cytochrome oxidase preparations: enzyme treated by anion-exchange FPLC (see Materials and Methods), control enzyme after sucrose gradient centrifugation at high ionic strength in Triton X-100 ('T' in Fig. 4), and enzyme treated with Triton X-100 at high pH according to Ref. 5.

5) agree well with those previously ascribed to dimeric and monomeric enzyme [13,29]. These results strongly support the suggestion that the slowly sedimenting green band in our sucrose gradients is monomeric enzyme, while the more rapidly sedimenting species is the dimer.

Fig. 6 schematically summarises experiments which showed that the monomeric enzyme in LDAO may be partially redimerised. The green slowly sedimenting band from the sucrose-LDAO gradient was collected, transferred to cholate-containing medium by means of a Sephadex 'centricolumn' [23], and layered on a cholate-containing sucrose gradient. After centrifugation two green bands were observed, of which the faster one sedimented like control enzyme and the slower one like the anion-exchange FPLC fractions and enzyme treated at high pH in the presence of Triton [5]. Although this phenomenon always occurred, the extent of redimerisation, as judged from these sucrose gradients, was dependent on the enzyme batch used. The enzyme preparation shown in lane 2 (Fig. 1B), for example, gave a somewhat higher extent of redimerization than did the preparation shown in lane 1 (Fig. 1B).

To test the effect of a membranous environment on the oligomeric state of the enzyme, we utilised our finding that sucrose gradient centrifugation in the presence of cholate does not monomerise the enzyme (Figs. 4 and 6). Proteoliposomes were prepared either using the control (dimeric) or the anion-exchange FPLC-treated (monomeric) oxidase (±subunit III, cf. Fig. 1).

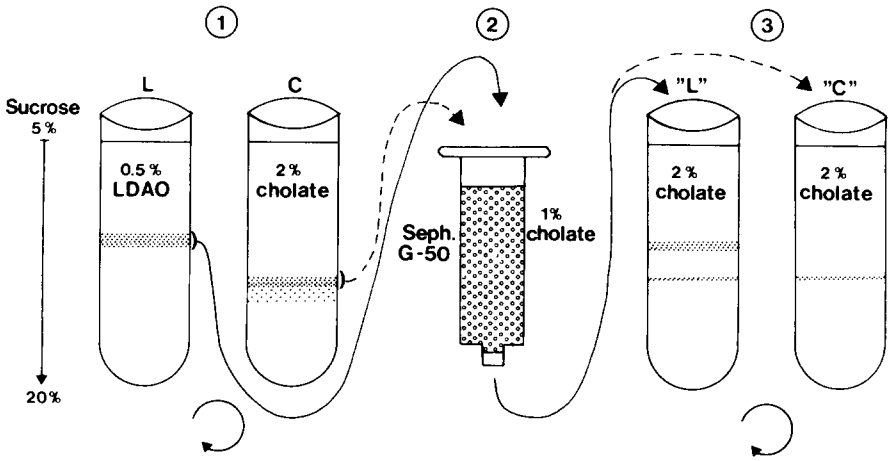

Fig. 6. Successive sucrose gradient centrifugations of cytochrome oxidase in the presence of different detergents. For the conditions of sucrose gradient centrifugations, see Materials and Methods and legend to Fig. 4. Stage 1: first centrifugation; L = 0.5% LDAO, C = 2% sodium cholate. Stage 2: the green bands from Stage 1 were collected and passed separately through a Sephadex G-50 'centricolumn' [23] preequilibrated with 1% sodium cholate and 100 mM potassium phosphate (pH 7.4). After this stage the samples were left on ice for 5–6 h. Stage 3: second centrifugation, in 2% sodium cholate. 'L' is the sample originating from L in Stage 1, and 'C' originates from C.

Then they were solubilised with cholate as described under Materials and Methods, and subjected to sucrose gradient centrifugation in the presence of cholate.

The top fractions in Fig. 7 probably correspond to a lipid-rich not fully solubilised enzyme (traces F, rec and C, rec) and were not present with unreconstituted enzyme (Fig. 7, trace C). Apart from this, the reconstituted anion-exchange FPLC-treated enzyme (trace F, rec) yielded two green bands while the reconstituted control enzyme (trace C, rec) yielded a single band. The latter sedimented in a similar way as the fast-sedimenting band of the FPLC-treated enzyme. SDS-polyacrylamide gel electrophoresis analysis (not shown) of the two bands of trace F, rec (Fig. 7) revealed identical polypeptide compositions, irrespective of whether the preparation contained subunit III.

The proteoliposome preparations analysed in Fig. 7 had the following functional properties. For the control enzyme (C, rec) the respiratory control ratio was 4.3 and the H^+/e^- ratio 0.86; for the reconstituted anion-exchange FPLC fraction (F, rec) the corresponding parameters were 6.3 and 0.44 (cf. Fig. 2 and above). From trace F, rec (Fig. 7) it may seem that there was roughly two times more fast-sedimenting than slow-sedimenting enzyme (based on areas under symmetrical peaks). However, such a conclusion would be premature because the present technique led to asymmetrical peaks, which tended to tail towards the bottom of the tube, especially with the solubilised liposome preparations. At present these results may be judged only qualitatively while measures are being taken to afford a quantitative study.

Discussion

Treatments of isolated bovine cytochrome oxidase that resulted in the removal of subunit III were originally reported to abolish the proton-translocating activity of the enzyme, as measured

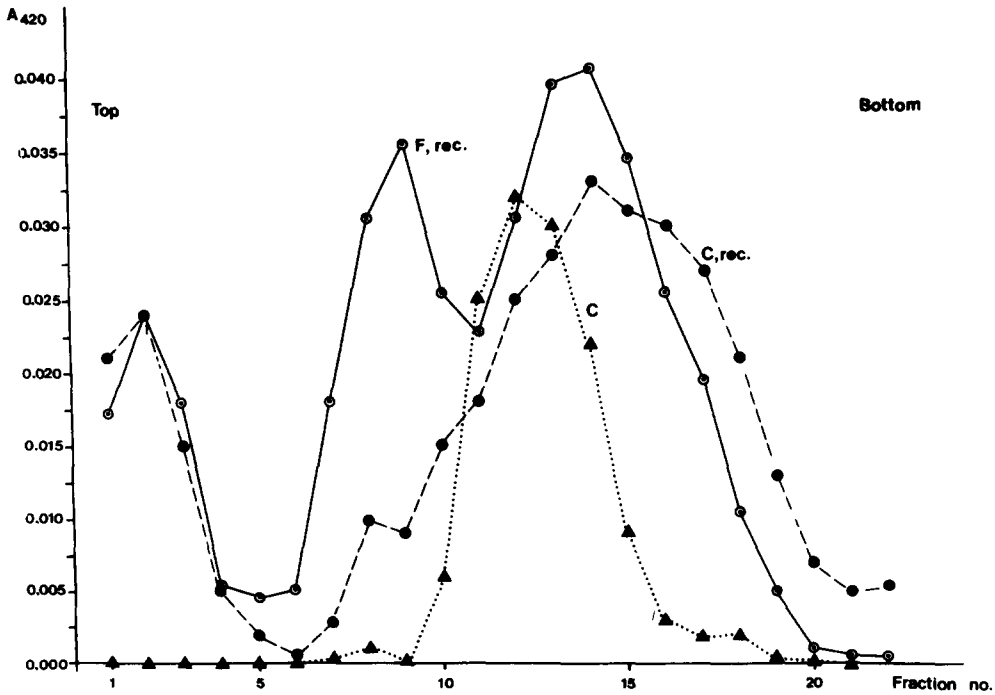

Fig. 7. Sucrose gradient centrifugation of reconstituted enzyme. Reconstituted cytochrome oxidase vesicles containing the anion-exchange FPLC-treated (F, rec) or control (C, rec) enzyme were solubilised as described under Materials and Methods, and loaded on 10 ml of 5–30% sucrose gradients containing 2% sodium cholate and 0.5 M potassium phosphate (pH 7.4). The centrifugation was performed in an SW-41-Ti rotor at 40000 r.p.m. for 20 h at 4°C. 0.5 ml fractions were collected manually from the top of the tubes and mixed with 0.5 ml of water. C = control, unreconstituted enzyme.

after its reconstitution into phospholipid vesicles [5,30,31]. This supported the proposal of a specific role of this subunit in proton translocation, as suggested on the basis of inhibition of proton pumping by covalent modification of subunit III with DCCD [4].

Subsequently, it was reported that proton translocation may still be observed, albeit with strongly reduced efficiency, also in the absence of subunit III [6,7]. The data reported here indicate that the lowering of the H^+/e^- ratio associated with treatments aimed at removing subunit III, may nevertheless not be a direct consequence of the removal of this subunit. This is because a single treatment of the enzyme can yield different fractions that either contain or are deficient in subunit III, but nevertheless show equally lowered proton translocation after reconstitution. It is probable, therefore, that the impairment of proton translocation observed here and in Refs. 6 and 7 is not simply due to the removal of subunit III. If so, it should be seriously considered whether the block of proton translocation by DCCD could not be explained by an indirect effect that may be exerted via the covalent modification of subunit III.

The sucrose gradient centrifugation and gel filtration results suggest that cytochrome oxidase is monomeric a priori when it has (i) undergone anion-exchange FPLC in the presence of LDAO, (ii) been treated at high pH with Triton, or (iii) been centrifuged at high ionic strength in sucrose with LDAO or Triton. In contrast, the control enzyme is dimeric, even in cholate at high ionic strength. Monomerisation can clearly be achieved also without raising the pH to values higher than 7.4, and without removing subunit III.

In this paper we report for the first time on an attempt to test the oligomeric state of the enzyme in proteoliposomes, viz. by sucrose gradient centrifugation of proteoliposomes that have been solubilised by cholate. Interestingly, in all cases where the enzyme was monomeric prior to reconstitution, it yielded two well-separated bands in the sucrose gradient even though it had previously yielded a single band before reconstitution. In contrast, the initially dimeric enzyme yielded a single band that sedimented similarly to the fast-sedimenting band of the enzyme reconstituted in the monomeric state.

In identical sucrose gradients the slowly sedimenting bands of the reconstituted and unreconstituted (monomeric) enzyme ran virtually identically (not shown). The fast sedimenting band of the reconstituted preparation was slightly shifted towards the bottom of the tube in comparison with the corresponding band of the unreconstituted enzyme (Fig. 7). This could be due to different extents of phospholipid binding. However, these shifts were much smaller than that between the monomeric and dimeric unreconstituted enzyme (Fig. 4). Furthermore, the latter shift was even greater for the reconstituted enzyme. On the basis of what is mentioned above it seems unlikely that the separation of reconstituted enzyme into two species in sucrose gradients (Fig. 7) would be due to different binding of phospholipids. Finally, as shown in Fig. 6, the monomeric enzyme could partially dimerise spontaneously without added phospholipids. This renders further weight to the interpretation that the monomeric enzyme preparations tended to dimerise partially when they underwent the reconstitution procedure. This could not be due to the transfer into cholate per se, which did not result in dimerisation of monomeric enzyme that had undergone anion-exchange FPLC in the presence of LDAO. Our results clearly emphasise the necessity to evaluate the oligomeric state of the enzyme afer, and not only before, reconstitution when attempts are made to correlate functions of reconstituted cytochrome oxidase with its oligomeric state (as, e.g., in Ref. 16).

Wikström et al. [1,2] suggested that whilst monomeric cytochrome oxidase is a potent catalyst of electron transfer and dioxygen reduction, the dimeric structure might be obligatory for proton translocation. The monomerisation resulting from various methods that were primarily aimed at removal of subunit III (Refs. 5 and 12, see also this paper), and the resulting poor proton-translocating efficiency, add some attraction to this suggestion.

According to the results presented in Fig. 7, and the accompanying functional data (cf. Fig. 2) it may well be that the residual proton-pumping of reconstituted monomeric enzyme preparations is, in fact, the result of various degrees of redimerisation brought about by the membrane reconstitu-

tion procedure. This could explain why some "methods of removal of subunit III" have yielded a complete loss of proton-pumping [5,31], while others (Refs. 6 and 7 and this paper) have yielded low but significant degrees of proton-pumping.

However, at the present time the evidence is not sufficient to concluded firmly that proton translocation occurs exclusively in the dimeric enzyme, even though our results are consistent with this possibility. Our data do not support the possibility that the monomeric enzyme would not reconstitute properly in the membrane, since such an effect should show up as a drastic reduction of the respiratory control ratio.

The role of subunit III is still not understood. Although methods to remove it tend to monomerise the enzyme, dimers can still be formed in the absence of this subunit [32,33], e.g., as apparently induced by a membranous milieu as shown here (Fig. 7). It seems that subunit III is not obligatorily required for proton translocation, nor for dimerisation of the enzyme. Yet, it might have a modulatory or even a regulatory role that affects these functional and structural parameters.

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References

1 Wikström, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase – A Synthesis, Academic Press, London
2 Wikström, M., Krab, K. and Saraste, M. (1981) Annu. Rev. Biochem. 50, 623–655
3 Wikström, M., Saraste, M. and Penttilä, T. (1985) in (A.N. Martonosi, ed.) The Enzymes of Biological Membranes, Vol. 4, 111–148
4 Casey, R.P., Thelen, M. and Azzi, A. (1980) J. Biol. Chem. 255, 3994–4000
5 Penttilä, T. (1983) Eur. J. Biochem. 133, 355–361
6 Puettner, I., Carafoli, E. and Malatesta, F. (1985) J. Biol. Chem. 260, 3719–3723
7 Sarti, P., Jones, M.G., Antonini, G., Malatesta, F., Colossimo, A., Wilson, M.T. and Brunori, M. (1985) Proc. Natl. Acad. Sci. USA 82, 4876–4880
8 Capaldi, R.A., Malatesta, F. and Darley-Usmar, V.M. (1983) Biochim. Biophys. Acta 726, 135–148
9 Klingenberg, M. (1981) Nature 290, 449–453
10 Love, B., Chan, S.H.P. and Stotz, E. (1970) J. Biol. Chem. 245, 6664–6668
11 Georgevich, G., Darley-Usmar, V.M. and Capaldi, R.A. (1983) Biochemistry 22, 1317–1322
12 Malatesta, F., Georgevich, G. and Capaldi, R.A. (1983) in (Quagliariello, E. and F. Palmieri, eds.) Structure and Function of Membrane Proteins, pp. 223–225, Elsevier, Amsterdam
13 Orii, Y. and Miki, T. (1983) in Frontiers in Biochemical and Biophysical Studies of Proteins and Membranes, (Liu, T.-Y., Sakakibara, S., Schechter, A.N., Yagi, K., Yajima, H. and Yasunobu, K.T., eds.) pp. 279–287, Elsevier, Amsterdam
14 Wilson, M.T., Lalla-Maharajh, W., Darley-Usmar, V., Bonaventura, J., Bonaventura, C. and Brunori, M. (1980) J. Biol. Chem. 255, 2722–2728
15 Ludwig, B., Grabo, M., Gregor, I., Lustig, A., Renass, M. and Rosenbusch, J.P. (1982) J. Biol. Chem. 257, 5576–5578
16 Bickar, D., Lehninger, A.L., Brunori, M., Bonaventura, J. and Bonaventura, C. (1985) J. Inorg. Biochem. 23, 356–372
17 Solioz, M., Carafoli, E. and Ludwig, B. (1982) J. Biol. Chem. 257, 1579–1582
18 Kuboyama, M., Choy Yong, F. and King, T.E. (1972) J. Biol. Chem. 247, 6375–6383
19 Saari, H., Penttilä, T. and Wikström, M. (1980) J. Bioenerg. Biomembr. 12, 325–338
20 Yu, C., Yu, L. and King, T.E. (1975) J. Biol. Chem. 250, 1383–1392
21 Casey, R.P., Chappell, J.B. and Azzi, A. (1979) Biochem. J. 182, 149–156
22 Laemmli, A.K. (1970) Nature 227, 680–685
23 Penefsky, H.S. (1977) J. Biol. Chem. 251, 7168–7175
24 Penttilä, T., Saraste, M. and Wikström, M. (1979) FEBS Lett. 101, 295–300
25 Bill, K. and Azzi, A. (1982) Biochem. Biophys. Res. Commun. 106, 1203–1209
26 Krab, K. and Wikström, M. (1978) Biochim. Biophys. Acta 504, 200–214
27 Casey, R.P., O'Shea, P.S., Chappell, J.B. and Azzi, A. (1984) Biochim. Biophys. Acta 765, 30–37
28 DiBiase, V.A. and Prochaska, L.J. (1985) Arch. Biochem. Biophys. 243, 668–677
29 Hakvoort, T.B.M., Sinjorgo, K.M.C., Van Gelder, B.F. and Muijsers, A.O. (1985) J. Inorg. Biochem. 23, 381–388
30 Casey, R.P. (1984) Biochim. Biophys. Acta 768, 319–347
31 Thelen, M., O'Shea, P.S., Petrone, G. and Azzi, A. (1985) J. Biol. Chem. 260, 3626–3631
32 Saraste, M., Penttilä, T. and Wikström, M. (1981) Eur. J. Biochem. 115, 261–268
33 Nalecz, K.A., Bolli, R., Ludwig, B. and Azzi, A. (1985) Biochim. Biophys. Acta 808, 259–272