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The role of subunit III in bovine cytochrome *c* oxidase. Comparison between native, subunit III-depleted and *Paracoccus denitrificans* enzymes

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In order to obtain information on the role of subunit III in the function and aggregation state of cytochrome *c* oxidase, the kinetics of ferrocytochrome *c* oxidation by the bovine cytochrome *c* oxidase depleted of its subunit III were studied and compared with those of the oxidase isolated from *P. denitrificans* which contains only two subunits. The aggregation state of both enzymes dispersed in dodecyl maltoside was also compared. The two-subunit oxidase from *P. denitrificans* gave linear Eadie-Hofstee plots and the enzyme resulted to be monomeric (M_r = 82 000) both, in gel filtration and sucrose gradient centrifugation studies. The bovine heart subunit III depleted enzyme, under conditions when the *P. denitrificans* cytochrome *c* oxidase was in the form of monomers, was found to be dimeric by sucrose gradient centrifugation analysis. At lower enzyme concentrations monomers were, however, detected by gel filtration. Depletion of subunit III was accompanied by the loss of small polypeptides (VIa, VIb and VIIa) and of almost all phospholipid (1–2 molecules were left per molecule of enzyme). The electron-transfer activity of the subunit III-depleted enzyme showed a monophasic Eadie-Hofstee plot, which upon addition of phospholipids became non-linear, similar to that of the control bovine cytochrome *c* oxidase. One of the roles of subunit III may be that of stabilising the dimers of cytochrome *c* oxidase. Lack of this subunit and loss of phospholipid is accompanied by a change in the kinetics of electron transfer, which might be the consequence of enzyme monomerisation.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal component of the mitochondrial respiratory chain. The enzyme contains two coupled activities (for a review, see Ref. 1), namely electron transfer from cytochrome *c* to molecular oxygen [2] and proton translocation through the membrane where the enzyme is located [3–5]. Purified mammalian cytochrome *c* oxidases contain 13 polypeptides [6], with a stoichiometry close to 1 [7,8]. It is still a matter of debate whether all these polypeptides are true subunits and what is their contribution to the function of the enzyme.

The four prostetic groups of cytochrome *c* oxidase, two hemes *a* and two coppers, are located within the two largest subunits [9–12]. Cross-linking studies with lysine-13 modified aryl-azido-cytochrome *c* indicate that subunit II and in particular its negatively charged hydrophilic domain represent the high afinity binding site for cytochrome

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Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

c [13–15]. The side of yeast cytochrome *c* opposite to the one interacting with the oxidase could be covalently linked to subunit III of the enzyme [16]. This indicates that the bound cytochrome *c* is in close contact with subunit II and III of the cytochrome *c* oxidase complex [17]. These subunits might be located one in each monomer of the dimeric form of the enzyme and the cytochrome *c* binding site would be in a cleft between the two monomers [18]. The involvement of some of the smaller subunits in cytochrome *c* binding is not excluded [19–21].

Strong evidence has been accumulated that subunit III plays an important role in the proton translocating activity of cytochrome *c* oxidase [22–26]. However, the lack of this subunit in some bacterial oxidases does not prevent these enzymes from translocating protons [27–29]. Some of the prokaryotic cytochrome *c* oxidases (aa_3-type oxidases), similar to the mitochondrial enzyme in their spectral properties, but much simpler in their subunit composition (see Ref. 30), resulted to be a useful model system for the studies on mammalian enzymes (for a review, see Ref. 31). Unfortunately, for those enzymes, only limited information is available concerning their electron-transfer kinetics [27,32].

In the mammalian enzyme, the oxidation of reduced cytochrome *c* does not have simple kinetics [33]. From cross-linking studies, as well as from spectral perturbation measurements after binding of cytochrome *c* [34], only one catalytic site per enzyme monomer is proposed (see also ref. 35). Monophasic Eadie-Hofstee plots were observed for the monomeric form of the enzyme and the biphasic ones were explained in terms of a homotropic negative cooperativity, an exclusive property of the dimers [36].

Cytochrome *c* oxidase depleted of subunit III has been described to be monomeric [37] as well as dimeric [24] or a mixture of both [25]. The activity of this enzyme has not been unequivocally characterised. A lack of agreement may have originated by the fact that the kinetics of electron transfer was measured under conditions different from those in which the molecular weight was estimated and by the use of different detergents. This paper presents the characterisation of a subunit III-depleted enzyme obtained by the covalent chromatography procedure described previously [38]. Both, molecular weight studies, as well as kinetic experiments were performed using dodecyl maltoside as a detergent known to reveal highest cytochrome *c* oxidase activity. A structural role of subunit III, that of stabilising the enzyme dimer, is discussed. The two subunit cytochrome *c* oxidase from *P. denitrificans* was also studied, for comparison, since this preparation lacks as well subunit III. Preliminary data were presented elsewhere [39].

Materials and Methods

Materials. TMPD and cytochromes *c* from horse heart, and yeast were from Sigma. Catalase, ferritin and aldolase were from Serva, immunoglobulin G was a gift from Dr. F. Hesford. Blue dextran, Sephadex G-100 and Sephadex G-25, cyanide bromide activated Sepharose 4B were from Pharmacia, Ultrogel AcA 34 was from LKB, DEAE-Bio-Gel from Biorad. Cholate and Triton X-100 were purchased by Fluka. Dodecyl maltoside was synthesised according to Rosevear et al. [40]. [^{3}H]Dodecyl maltoside was obtained from Amersham, after catalytic exchange in solution with tritium (TR. 7) and was purified further from the degradation products by thin layer chromatography in ethyl acetate/methanol 4:1 (v/v) and next separated from the residual maltose by gel filtration on Sephadex G-100 equilibrated with 10 mM Tris-HCl (pH 7.4). Asolectin was purchased by Associated Concentrates. All other reagents were of the highest analytical grade commercially available.

Ferrocytochrome c was obtained by reducing ferricytochrome *c* with sodium dithionite and passed through Sephadex G-25 to remove excess of dithionite and aggregates of cytochrome *c*.

Cytochrome c oxidase from bovine heart was prepared according to the method of Yu et al. [41] and stored highly concentrated in small portions in 50 mM phosphate buffer at −80°C. Depletion of subunit III was obtained after binding of the bovine heart enzyme to a Sepharose 4B column with covalently bound yeast cytochrome *c* and further elution with 1.5% Triton X-100, 200 mM NaCl, 10 mM sodium phosphate buffer (pH 7.4), according to Bill and Azzi [38]. In order to ex-

change Triton X-100 for dodecyl maltoside, the oxidase preparation was first passed through a Sephadex G-25 column (2 × 30 cm) equilibrated with 10 mM Tris-HCl (pH 7.4), 0.1% Triton X-100 to decrease the ionic strength of the buffer. The enzyme was next bound to DEAE-Bio-Gel equilibrated in a column (1 × 10 cm) with 10 mM Tris-HCl (pH 7.4)/0.25% dodecyl maltoside, and after extensive washing with the same buffer the protein was eluted with 300 mM KCl/10 mM Tris-HCl (pH 7.4)/0.25% dodecyl maltoside. In order to remove KCl, finally, the enzyme was passed through Sephadex G-25 equilibrated with 10 mM Tris-HCl (pH 7.4) and 0.1% dodecyl maltoside. KCl was added whenever indicated in the text.

The cytochrome *c* oxidase from *P. denitrificans* was isolated as described by Ludwig and Schatz [42] and equilibrated with the appropriate medium by dialysis in the presence of dodecyl maltoside.

Spectral analysis was made using an Aminco DW-2a spectrophotometer. The measurements were performed in 10 mM Tris-HCl (pH 7.4)/50 mM KCl/0.1% dodecyl maltoside, at 25°C. The following extinction coefficients were used: $\epsilon_{605-630} = 11.7\ mM^{-1} \cdot cm^{-1}$ and $\epsilon_{605} = 12\ mM^{-1} \cdot cm^{-1}$ for the heme *a* content in the *P. denitrificans* [42] and the bovine [43] enzyme, respectively, for the reduced-minus-oxidized spectrum. For reduced-CO-minus-reduced spectra of the bovine enzymes coefficients for heme a_3 given by Vanneste [44] were applied, $\epsilon_{428.5-445} = 148\ mM^{-1} \cdot cm^{-1}$ or $\epsilon_{590-606} = 9.9\ mM^{-1} \cdot cm^{-1}$. In case of the oxidase from *P. denitrificans* $\epsilon_{592-608} = 7.0\ mM^{-1} \cdot cm^{-1}$ was used [42].

Gel filtration studies were carried out at 4°C on Ultrogel AcA 34 using a 1 × 45 cm column, equilibrated with 10 mM Tris-HCl (pH 7.4) 0.1% dodecyl maltoside and 50 mM KCl. The column was protected from light to prevent the loss of heme during chromatography. The column was calibrated with ferritin, catalase, immunoglobulin G, aldolase, the void volume was determined with blue dextran and the total volume with ferricyanide.

Binding of dodecyl maltoside to bovine subunit III depleted oxidase was determined by gel filtration on Ultrogel AcA 34 in the presence of [^{3}H] dodecyl maltoside (50 Ci/mol). The content of heme and protein, estimated in all fractions, was determined spectrophotometerically and by the method of Lowry et al. [45], respectively. In the fractions containing protein the heme to protein ratio was constant (12–13 nmol heme *a* per mg protein). The binding was expressed in moles of dodecyl maltoside per mol of cytochrome *c* oxidase. Since the *P. denitrificans* oxidase peak was partially overlapping with the micelles of dodecyl maltoside, the binding of detergent to this enzyme was determined on Sephadex G-100 equilibrated with 10 mM Tris-HCl (pH 7.4)/50 mM KCl/0.1% [^{3}H] dodecyl maltoside of the same specific radioactivity.

The phospholipid content in different oxidase preparations was calculated on the basis of phosphate content, assuming an average molecular weight of 750 for the phospholipid molecule. Phospholipid extraction was carried out three times with chloroform/methanol (2 : 1), the extracts were pooled, evaporated to dryness and ashed with concentrated sulphuric acid. The amount of phosphorus was estimated by the method of Chen et al. [46].

The density measurements for protein, detergent and the buffer solutions were performed with a mechanical oscillator densitometer (Präzisions-Dichtemesseinrichtung DMA-50, Paar) by varying the concentration of the solute, at 20°C. The partial specific volumes (v) of oxidase as well as of the detergent were calculated from the following equation [47]:

$$v = \frac{1}{\rho_0}\left(1 - \frac{d\rho}{dc}\right) \quad (1)$$

where $d\rho/dc$ is the deviation of the density of the solution with the concentration of the solute and ρ_0 is the density of the solvent. For the partial specific volume of the lipid the one for cardiolipin (1.002 ml/g) was taken from Steele et al. [47]. The partial specific volume of the multicomponent complex (v^*) was calculated from the sum of its individual components

$$v^* = \Sigma x_{i,j,k} v_{i,j,k} \quad (2)$$

where x and v are the weight fractions and partial specific volumes of the individual components (protein, detergent, phospholipid), respectively.

Surcose gradient centrifugation was run in a Beckman SW 60 rotor on a Beckman L-8 ultracentrifuge. Sucrose solutions contained 10 mM Tris-HCl) (pH 7.4)/0.1% dodecyl maltoside/50 mM KCl. Linear gradients (5–20%) were subsequently formed using a self-made mixing chamber. 150 μl of oxidase samples (3–7 μM heme aa_3) were layered on top of the gradient and centrifuged at 55000 rpm for 4 h 45 min, at 5°C. The gradients were analysed by puncturing the bottoms of the tubes and monitoring the effluent at 280 nm with an Uvicord I ultraviolet analyzer (LKB). The sucrose concentrations were checked by density measurements using the mechanical oscillator densitometer (DMA-50, Paar). The relative mobility of the oxidase was calculated and the sedimentation coefficients, normalized to the density and viscosity of water and to 20°C, were then calculated using the tables of McEven [48].

The molecular weight M of the protein was calculated after subtracting all bound components (phospholipid, detergent) from the molecular weight of the complex, M^*. The molecular weight of the complex, M^*, was calculated from the following equation:

$$M^* = \frac{s_{20,w} R_S N 6\pi\eta}{1 - v^* \rho_s} \quad (3)$$

where $s_{20,w}$ is the sedimentation coefficient, R_S is the Stokes radius taken from gel filtration experiments, N is Avogadro's number, η is the viscosity and v^* is the partial specific volume of the complex, ρ_s – the density of the solvent. All parameters were normalised to 20°C and water conditions (index 20, w).

The activity of cytochrome c oxidase was assayed spectrophotometrically in an Aminco DW-2a spectrophotometer at 550–540 nm using the extinction coefficients $\epsilon_{550-540} = 19.4\ mM^{-1} \cdot cm^{-1}$ [49] and $\epsilon_{605} = 24\ mM^{-1} \cdot cm^{-1}$ [43] for cytochrome *c* and cytochrome *c* oxidase, respectively. The cytochrome *c* oxidase concentration was 0.25 nM and that of cytochrome *c* was 0.2–30 μM. When the assay was performed in the presence of asolectin, the enzyme (0.3 μM) was preincubated for 1 h with 0.75 mg/ml of asolectin. The velocity is expressed as molecular activity (s^{-1}).

The rates of oxygen uptake were measured polarographically using a Yellow Springs Instrument oxygen electrode supplemented with an LKB recorder. The assay conditions are described in the legend to Fig. 6.

Polyacrylamide gel electrophoresis was carried out according to Kadenbach et al. [6]. Gels were stained with Coomassie blue and next were scanned in an Aminco DW-2a spectrophotometer at 570–530 nm.

Curve-fitting was carried out in Commodore 2001 and 8032 computers equipped with a BBC-Servogor digital plotter.

Results

Subunit composition and spectral analysis.

The polypeptide pattern of the protein complexes discussed here is presented in Fig. 1. By

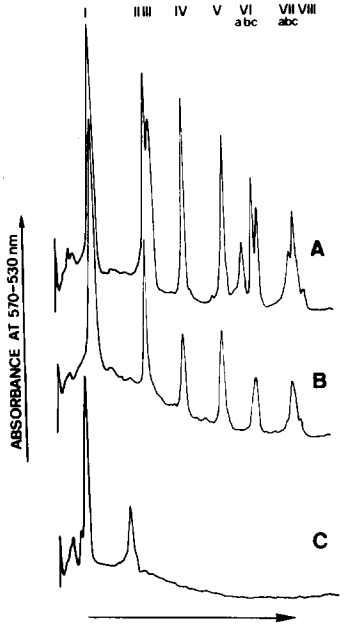

Fig. 1. Subunit composition of different preparations of cytochrome *c* oxidase. Polyacrylamide gel electrophoresis in the presence of dodecylsulphate was performed according to Kadenbach [6], stained with Coomassie blue and scanned as described in Materials and Methods. The direction of migration of the dodecylsulphate protein complexes is indicated by the arrow. The subunits of bovine cytochrome *c* oxidase according to Kadenbach's nomenclature are indicated above the bands. (A) Bovine heart cytochrome *c* oxidase. (B) Subunit III-depleted cytochrome *c* oxidase isolated according to Bill and Azzi [38]. Before loading Triton X-100 was exchanged for dodecyl maltoside as described in Materials and Methods. (C) Cytochrome *c* oxidase isolated from *P. denitrificans*.

SDS polyacrylamide gel electrophoresis using the system developed by Kadenbach et al. [6], 12 polypeptides in the case of the intact bovine cytochrome *c* oxidase could be well separated. Fig. 1B shows that the subunit III-depleted cytochrome *c* oxidase, isolated by covalent chromatography on a yeast cytochrome *c* derivatised Sepharose 4B column [38], was not only devoid of subunit III, but also subunit VIa and VIb were missing and the amount of subunit VIIa was strongly decreased. Loss of these polypeptides occurs as well when the extraction of subunit III is obtained with other methods [24,25]. Cytochrome *c* oxidase from *P. denitrificans* is purified in a form which contains only two subunits (Fig. 1C); the electrophoretic characteristics after SDS solubilisation as well as their amino acid composition is comparable with that of subunit I and II of the bovine enzyme [42].

Also the spectral properties of *P. denitrificans* cytochrome *c* oxidase are similar to those of the mammalian enzyme (Table I, Ref. 42), with the exception of a slight red shift of the α-band of its absorption spectrum. A comparison of this enzyme with the mammalian one, and especially with the bovine oxidase depleted of subunit III may lead to some indication as to the molecular significance of the subunits in the more complicated complex. To achieve such a comparison, the enzymes, whenever possible, were studied under the same experimental conditions.

To extract subunit III from the enzyme, covalent chromatography [38] was used, in which pH, ionic strength or detergent concentrations were kept at levels known not to alter the protein.

The reduced-minus-oxidised spectrum of the subunit III depleted bovine oxidase, obtained in the Titron X-100 eluate (not shown) as well as after exchange of Triton X-100 for dodecyl maltoside (Fig. 2, trace B) showed only a very small blue shift of 0.5 nm for the Soret and the α-band compared with the control bovine enzyme (Fig. 2, trace A). The absorbance ratios between these two maxima were, within experimental error, the same in the control (6.1) and the subunit III depleted enzyme (6.0, Table I), which indicated that the two

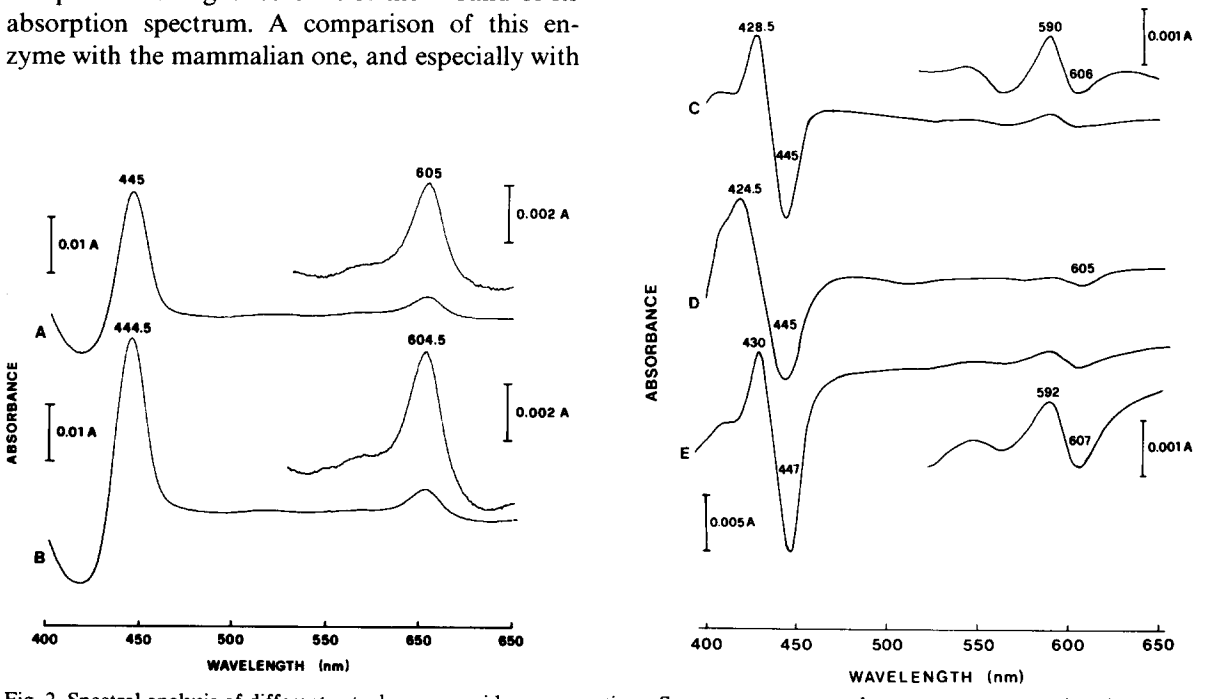

Fig. 2. Spectral analysis of different cytochrome *c* oxidase preparations. Spectra were measured at enzyme concentrations between 0.1 and 0.9 μM heme aa_3 in 10 mM-Tris-HCl (pH 7.4)/50 mM KCl/0.1% dodecyl maltoside as described in Materials and Methods. Reduced carbon monoxide-minus-reduced spectra were recorded after the sample cuvette containing the dithionite reduced enzyme was bubbled for 2 min with a stream of carbon monoxide. Dithionite reduced-minus-oxidised spectra: (A) control bovine heart cytochrome *c* oxidase; (B) bovine heart subunit III depleted cytochrome *c* oxidase. Dithionite reduced-carbon monoxide-minus-dithionite reduced spectra: (C) control bovine heart cytochrome *c* oxidase; (D) subunit III-depleted cytochrome *c* oxidase; (E) cytochrome *c* oxidase from *P. denitrificans*.

TABLE I

SPECTRAL PROPERTIES OF CYTOCHROME *c* OXIDASE SPECIES FROM BOVINE HEART AND *P. DENITRIFICANS*

Spectra were recorded as described in Materials and Methods and in the legend to Fig. 2. Cytochrome *a* concentration was calculated from the reduced-minus-oxidised spectrum and carbon monoxide binding was evaluated from the reduced CO-minus-reduced spectrum, using the extinction coefficients indicated in Materials and Methods.

	Reduced-oxidised		CO-reduced-reduced		
	Maxima (nm)	Absoxbance ratio γ/α	Maxima (nm)	Minima (nm)	CO-binding (mol CO/mol heme *a*)
Control (bovine)	445, 605	6.1	428.5, 590	445, 606	0.42
Subunit III-depleted (bovine)	444.5, 604.5	6.0	424.5, 594	445, 605	0.42
P. denitrificans	446.5, 606.5	5.9	430, 592	447, 607	0.48

hemes were still present in their 1 : 1 natural ratio. Other spectral differences could not be detected. A certain modification of the heme properties was observed in the CO-reduced-minus-reduced spectra (Fig. 2, traces C and D, Table I). The Soret maximum (at 424.5 nm) of the subunit III depleted enzyme was 4 nm blue-shifted relative to the control, whilst the α-peak (594 nm) was 4 nm red-shifted. A shoulder of the Soret band at about 410 nm indicated a spectral component, which might have been responsible for the apparent shift of the γ-band. In fact the through at 445 nm was normally located. Reoxidation of cytochrome *c* oxidase could not have caused such an effect, since further addition of dithionite was without effect. Carbon monoxide binding, calculated by using the same extinction coefficients as for the control cytochrome *c* oxidase [44], gave normal values for the subunit III depleted enzyme (0.42 mole of CO per mole of total heme *a*, Table I), when calculated from the α-band, but a too high value (0.58), when determined from the Soret region. This supports the idea of a perturbation in this spectral region. With cytochrome *c* oxidase from *P. denitrificans* (Fig. 2, trace E, Table I) the spectra obtained in the presence of CO were similar to those of the control bovine cytochrome *c* oxidase, in agreement with earlier reported measurements [42]. The reduced-minus-oxidised spectra showed a slight red shift of the Soret and α-bands.

Aggregation state and molecular-weight estimation.

Gel filtration. All the gel filtration experiments presented in Fig. 3 were carried out under the same experimental conditions, using a 45 × 1 cm Ultrogel AcA 34 column, of which the resolution capabilities between M_r 100000 and 400000 are apt to separate cytochrome *c* oxidase monomers from dimers. However, it is known that the column is able to separate macromolecules on the basis of their hydrodynamic radii rather than of their molecular weights, a property which is documented by the fact that two calibration proteins, aldolase and immunoglobulin G, which have approximately the same molecular weight (150000) were well separated. Due to these properties of the gel, one cannot expect that detergent solubilised membrane proteins are eluted according to their precise molecular weight. Rather, gel chromatography might be only used for qualitative separation of proteins having different molecular size.

Bovine subunit III depleted cytochrome *c* oxidase, loaded on Ultrogel AcA 34 directly after obtaining it from the affinity column, showed a single peak in its elution profile (when the activity was employed to follow the peaks) with a relative elution value $K_{AV} = 0.41$ (Fig. 3A, Table II). The absorbance profile at 280 nm showed, beside the protein peak, also a second peak with a $K_{AV} = 0.6$ corresponding to the Triton X-100 micelles. Rechromatography of the peak fraction which had enzymatic activity gave the elution profile shown in Fig. 3B. Two species were separated ($K_{AV} = 0.41$ and $K_{AV} = 0.59$) both absorbing at 280 nm and enzymatically active. The relative high absorbance to activity ratio of the more retarded species might

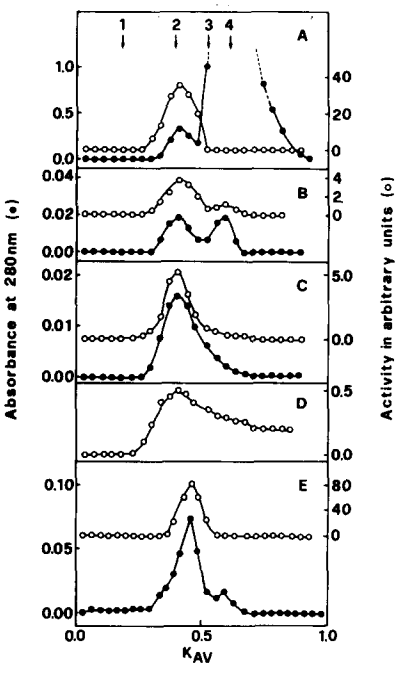

Fig. 3. Exclusion chromatography of cytochrome *c* oxidase preparations devoid of subunit III. Experiments were run on an Ultrogel AcA 34 column in 10 mM Tris-HCl (pH 7.4)/50 mM KCl/0.1% dodecyl maltoside as described in Materials and Methods. 100–200 μl of the following enzyme preparations were loaded (concentrations of heme aa_3 are indicated): (A) subunit III-depleted cytochrome *c* oxidase (25 μM) in Triton X-100 isolated according to Bill and Azzi [38] and concentrated by ultrafiltration; (B) rechromatography of the peak fractions from (A) revealing cytochrome *c* oxidase activity; (C) bovine heart subunit III-depleted cytochrome *c* oxidase (3 μM), which was passed through a DEAE-Bio-Gel column to exchange Triton X-100 for dodecyl maltoside; (D) the same as in (C), but only 1 μM was loaded; (E) cytochrome *c* oxidase from *P. denitrificans* (20 μM). The cytochrome *c* oxidase activity was measured spectrophotometrically at 0.5 μM ferrocytochrome *c* in the elution buffer, and is expressed in nmols of cytochrome *c* per s per 100 μl of the fraction.

have been due to the presence of residual Triton X-100, which absorbs at 280 nm and inhibits enzymatic activity at the same time. Complete removal of Triton X-100 by exchange with dodecyl maltoside using DEAE-Bio-Gel chromatography resulted in the disappearance of the second species, at enzyme concentrations above 3 μM (Fig. 3C). At lower concentrations (1 μM), the second peak started to reappear (Fig. 3D).

The elution profile of cytochrome *c* oxidase from *P. denitrificans* (Fig. 3E) shows that also this enzyme was homogeneously dispersed in dodecyl maltoside. The second inactive peak was again a trace of residual Triton X-100 left over from the isolation. It disappeared when the enzyme was rechromatographed (not shown).

The elution position (K_{AV}) and estimated Stokes radii are given in Table II. The Stokes radii for the species of the subunit III-depleted oxidase were somewhat smaller than that (6.5 nm) of the oxidase Triton X-100 complex depleted of subunit III by high pH treatment [24].

Sucrose gradient centrifugation analysis. In order to determine the molecular weights of the

TABLE II

PROPERTIES OF THE DETERGENT-LIPID-PROTEIN COMPLEXES OF CYTOCHROME *c* OXIDASE DEVOID OF SUBUNIT III FROM BOVINE HEART AND *P. DENITRIFICANS*

The distribution coefficients (K_{AV}) and Stoke's radii were evaluated from gel filtration experiments described in Materials and Methods and in the legend to Fig. 3. (A) and (B) are the two oxidase species observed in gel chromatography of the subunit III-depleted bovine cytochrome *c* oxidase as shown in Fig. 3. The estimation of phospholipid content, detergent binding, partial specific volumes and molecular weights are described in Material and Methods. Sedimentation coefficients were calculated from sucrose gradient centrifugation analysis.

	Cytochrome *c* oxidase	
	Bovine, subunit III-depleted	*Paracoccus denitrificans*
K_{AV}	(A) 0.41 (B) 0.59	0.46
R_S (nm)	(A) 5.40 (B) 4.80	5.25
Phospholipid content (mol/mol)	< 2	< 1
Bound dodecyl-β-D-maltoside (mol/mol)	(A) 165 ± 11 (B) not determined	270
Partial specific volume of the protein moiety (ml/g)	0.768	0.551
Partial specific volume of the complex (ml/g)	0.787	0.715
$S_{20,w}$ (S)	14.7	12.2
Molecular weight of the complex	(A) 430000 (B) not determined	220800
Molecular weight of the enzyme	(A) 272000 (B) not determined	82000
Aggregation state	(A) dimer (B) monomer	monomer

enzyme-detergent complex according to Eqn. 3, the sedimentation coefficients were calculated from the position of the complex in a linear density gradient (5–20% sucrose), after centrifugation at high angular velocity. The ionic strength (50 mM KCl), the buffer (10 mM Tris-HCl, pH 7.4) and the type of detergent (0.1% maltoside) were kept the same as in the gel filtration experiments. The sedimentation analysis gave the same picture as the gel permeation chromatography: at enhanced protein concentration a single peak for all enzyme species was observed. The sedimentation velocities increased in the following order: the enzyme from *P. denitrificans*, subunit III-depleted and the control bovine cytochrome *c* oxidase. The sedimentation coefficients of these species determined according to the tables of McEven [48] are surprisingly high (14.7 S for the subunit III-depleted and 12.2 S for the bacterial cytochrome *c* oxidases, respectively; Table II) when compared with the value to be expected from the polypeptide pattern (subunit III-depleted enzyme) or from sedimentation analysis (*P. denitrificans* enzyme (4.1 S [50]). However, the amount of bound detergent and the density of the protein-detergent complex had a strong influence on the observed sedimentation velocity; two factors which do not appear in the mentioned calculations.

Phospholipid and detergent binding. The content of phospholipid and the amount of dodecyl maltoside bound to the enzyme complexes were estimated by phosphorous determination and chromatography on Ultrogel AcA 34 in the presence of [^{3}H] dodecyl maltoside, respectively, as described in Materials and Methods. The obtained values are listed in Table II. The removal of subunit III from the bovine cytochrome *c* oxidase was accompanied by an efficient delipidation of the enzyme. The phosphorous content in the chloroform/methanol extract from 6 nmol subunit III-depleted oxidase was below the detection limit of 1–2 mol per mol heme aa_3. A similar low lipid content was detected in cytochrome *c* oxidase from *P. denitrificans* (Table II, [50]) and reported for the subunit III-lacking cytochrome *c* oxidase from rat liver (3 mol/mol [51]).

It seems, therefore, that the low lipid content is a property of subunit III-missing enzymes rather than a specific effect of the procedure used for depletion. It is especially not an effect of the detergent (Triton X-100) used in our procedure, since it is known that this detergent leaves not less than about 15 mol of phospholipid per mol of the native enzyme [52].

In the subunit III-depleted oxidase the lost lipid seemed not to be substituted by additional binding of detergent. The detergent bound was only about 50% (165 mol/mol heme aa_3, Table II) that of the control cytochrome *c* oxidase (320 mol/mol [53]), which might indicate that the loss of subunit III made the enzyme less hydrophobic. Cytochrome *c* oxidase from *P. denitrificans*, although containing only about 60% of the protein of subunit III-depleted oxidase, had much more dodecyl maltoside bound (270 mol/mol of oxidase, Table II). The weight fraction of the protein in the complex was therefore quite low (0.375 g protein/g complex).

Partial specific volume. The partial specific volumes of the protein moieties of their detergent complexes, given in Table II, were calculated from the density measurements of solutions containing different protein concentrations (Fig. 4), according to Eqn. 1 in Materials and Methods. The density, as a function of the solute's concentration, was linear for dodecyl matloside (Fig. 4A), the subunit III-depleted oxidase (Fig. 4B) and the *Paracoccus* enzyme (Fig. 4C), which allowed to use the slopes instead of the deviations $d\rho/dc$. The partial specific volume for the subunit III-depleted oxidase (0.768 g/cm^3) was similar to the one of the holoenzyme (0.763 g/cm^3, [53]), whilst that of the complex was slightly smaller, because of the different amounts of bound detergent and phospholipid. For cytochrome *c* oxidase from *P. denitrificans* the partial specific volume for the pure protein was found to be rather small (0.56 g/cm^3). A value for the partial specific volume for this protein was also calculated from its amino acid composition, according to Cohn and Edsall (54) (0.69 g/cm^3) and it was found also low relative to water soluble proteins (above 0.7 g/cm^3 [55]). This approach, however, of summing of the individual partial specific volumes of the amino acids according to their weight fractions, does not take any structural parameters of the protein into account, and one may expect some discrepancies with the experimental values, especially for very hydrophobic proteins.

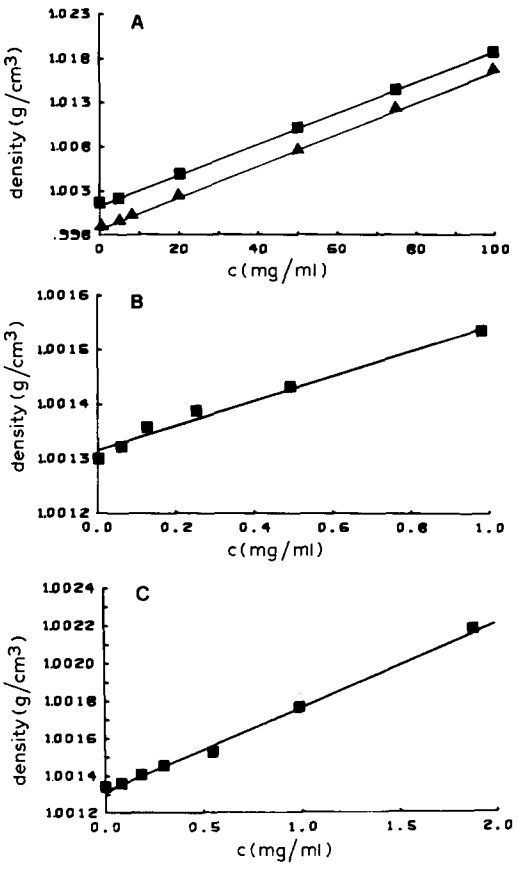

Fig. 4. Density measurements of different solutions to estimate the partial specific volume of the solutes. The densities of solutions at different solute's concentrations were measured at 20°C as described in Materials and Methods. The media contained 10 mM Tris-HCl (pH 7.4) (▲) or 10 mM Tris-HCl (pH 7.4)/50 mM KCl (■) in the presence of dodecyl maltoside as indicated. (A) dodecyl maltoside. (B) Subunit III-depleted cytochrome *c* oxidase in 0.1% dodecyl maltoside. (C) Cytochrome *c* oxidase from *P. denitrificans* in 0.1% dodecyl maltoside.

The higher value for the partial specific volume of the detergent protein complex can be attributed to the strong influence of the bound dodecyl maltoside, which itself has a much higher partial specific volume (0.825 g/cm^3).

Molecular weight and aggregation states. The molecular weight of the enzyme-detergent complexes and of their protein moieties (Table II) were obtained according to Eqn. 3 in Materials and Methods without and with subtraction of the bound dodecyl maltoside, respectively. The values introduced into the equation were the sedimentation coefficients, estimated by sucrose gradient centrifugation, and the Stokes radius, obtained from gel filtration. Since the latter method rather underestimates Stokes radii of asymmetric proteins solubilised in detergents [53,56,57], the result of the calculation will be more likely an underestimation of the molecular weight. After subtraction of the bound detergent the molecular weight of the observed species from *P. denitrificans* was 82000, consistent with the notion that the monomeric enzyme is composed of two subunits with molecular weights of 45000 and 28000. A similar molecular weight (79000) was calculated from a sedimentation coefficient of 4.1 S for the pure protein [50], which indicates that the high sedimentation coefficient of 12.2 S obtained for the protein dodecyl maltoside complex was only due to the high amount of bound detergent.

The main species of subunit III depleted oxidase, obtained by sucrose gradient centrifugation (14.7 S, Table II), corresponded to a molecular weight of 430000, consistent with a dimeric enzyme in which the protein contributes with 272000 (the sum of two sets of subunits, each having a molecular weight of 136000) to the total molecular weight. The molecular weight estimated from a single set of the known amino acid sequences of the subunits is 145000. In conclusion, the evidence indicates that the major species observed in the gel permeation chromatography (species A, Table II) as well as in the sucrose gradient centrifugation was a dimeric enzyme. The minor species (species B), observed only in the gel chromatography at low protein concentration, was not visible by sedimentation analysis, probably because in this technique a higher protein concentration was required, which shifted the equilibrium towards dimers. As a consequence of this, an exact molecular weight of this species could not be established and its aggregation state remains unknown. Possibly, it was a monomer, appearing only at very low enzyme concentrations (see Fig. 3B and D).

The steady-state kinetics of cytochrome c oxidase activity

The steady-state kinetics of the bovine subunit III-depleted oxidase and the *P. denitrificans* enzyme were analysed either by measuring ferrocytochrome *c* oxidation spectrophotometrically (Fig.

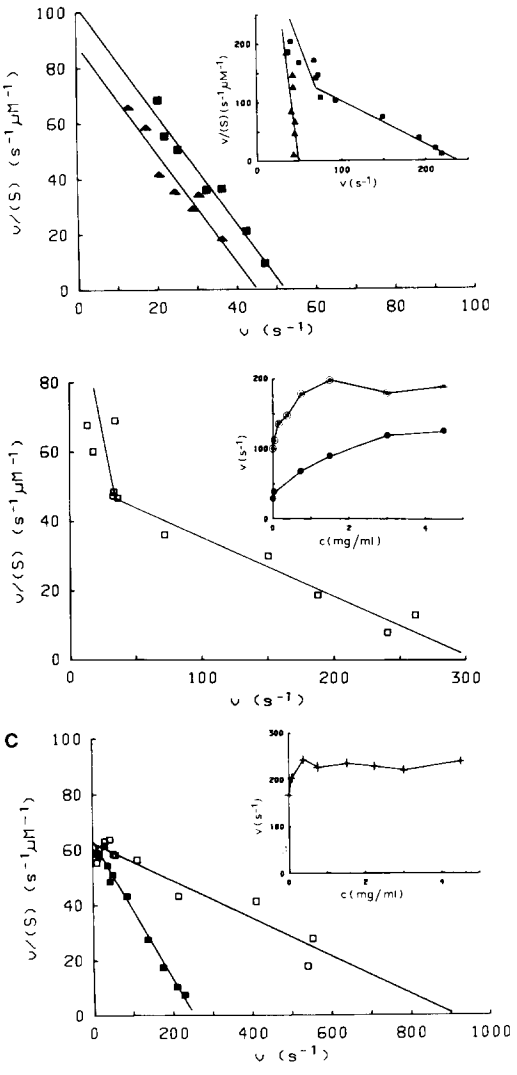

Fig. 5. Eadie-Hofstee plots of cytochrome *c* oxidase activity measured spectrophotometrically. The activity measurements are described in Materials and Methods. Ferrocytochrome *c* concentrations were between 0.3 and 20 μM. The molecular activity (v) is expressed as mol cytochrome *c* oxidised per s and per mol heme aa_3. (A) Subunit III-depleted cytochrome *c* oxidase after passing through a DEAE Bio-Gel column to exchange Triton X-100 for dodecyl maltoside. Inset: Control bovine heart cytochrome *c* oxidase. The assay media contained 10mM Tris-HCl (pH 7.4)/0.1% dodecyl maltoside in the absence (▲) or in the presence of 50 mM KCl (■). (B) Subunit III-depleted oxidase after passing through a DEAE-Bio-Gel column and incubated with 0.75 mg/ml asolectin for 1 h. The assay medium contained 10 mM Tris-HCl (pH 7.4)/50 mM KCl/0.1% dodecyl maltoside and 4.5 mg/ml asolectin. Inset: Titration of subunit III depleted (●) and control cytochrome *c* oxidase (⊙) with increasing amount of asolectin, at 5 μM ferrocytochrome *c*. Except for the lipid the assay medium was the same as in (B). (C) Cytochrome *c* oxidase from *P. denitrificans* in the absence (■) and in the presence (□) of 0.75 mg/ml asolectin. Inset: Titration with increasing amount of asolectin. Assay medium was the same as in B.

5) or by following oxygen reduction polarographically (Fig. 6). In Fig. 5A the kinetics of subunit III-depleted oxidase, presented as Eadie-Hofstee plots, was compared with that of the intact bovine enzyme. The activity was measured in the presence of 0.1% dodecyl maltoside at low ionic strength (only 10 mM Tris-HCl, pH 7.4), or after addition of 50 mM KCl. Under both conditions subunit III depleted oxidase gave monophasic Eadie-Hofstee plots. No change in K_m ($5 \cdot 10^{-7}$ M) and only a slight increase in V_{max} at higher ionic strength were observed. The control enzyme (Fig. 5A, inset) showed monophasic kinetics at low ionic strength with similar V_{max} but lower K_m ($0.5 \cdot 10^{-7}$ M). A change from monophasic to biphasic Eadie-Hofstee plots, as a consequence of increased salt concentration in the medium, was discussed earlier for the native bovine heart oxidase and attributed to a change in the aggregation state of the enzyme [36]. There is no kinetic indication, however, that the subunit III-depeleted enzyme did undergo such a change under the influence of salts. Since the affinity for cytochrome *c* was not influenced by the amount of salt present (no change in K_m), the electrostatic interaction between cytochrome *c* and the subunit III-depleted oxidase seemed not to be a limiting factor in the electron-transfer activity, at least not in the dodecyl maltoside solubilised enzyme and under the ionic strength conditions used.

Addition of asolectin to the assay medium stimulated the enzymatic activity of subunit III depleted oxidase at a certain cytochrome *c* concentration by a factor of 4, whilst the control enzyme increased its activity only twice in the presence of lipids (Fig. 5B, inset). The stimulation of the activity was present at much lower asolectin concentrations in case of the intact enzyme (1 mg/ml) than in case of the subunit III-deplete oxidase (3–4 mg/ml). In the presence of asolectin the Eadie-Hofstee plot of subunit III depleted oxidase became biphasic and V_{max} increased about 6 times reaching values, exceeding those obtained for the dodecyl maltoside solubilised control enzyme (250 s^{-1}) and which were in the range of those of cytochrome *c* oxidase reconstituted into phospholipid vesicles [58].

Cytochrome *c* oxidase from *P. denitrificans* was highly active in the presence of dodecyl maltoside. A linear Eadie-Hofstee plot was observed with $K_m = 4 \cdot 10^{-6}$ M and $V_{max} = 260$ s^{-1} (Fig. 5C). Even at very low phospholipid concentrations (0.4 mg/ml), a stimulation of its activity was observed due to an increase of V_{max} (approx. 900 s^{-1}) although a parallel increase of K_m ($1.4 \cdot 10^{-5}$ M) was occurring. However, contrary to subunit III-depleted oxidase, cytochrome *c* oxidase from *P. denitrificans* never exhibited biphasic Eadie-Hofstee plot, even in the presence of lipids (Fig. 5C).

Cytochrome *c* oxidase activity was also studied polarographically, where ascorbate/TMPD were used as electron donors for horse heart cytochrome *c*. The rate of oxygen reduction was measured. The assay medium was the same as in the spectrophotometric assay. The kinetics for subunit III-depleted oxidase and the *Paracoccus* enzyme are presented as Eadie-Hofstee plots in Fig. 6. For both enzymes, monophasic plots were observed. The maximal velocities were in the same range as in the spectrophotometric assays, the apparent K_m values, however, were increased by a factor of 2–3 ($K_m = 1.3 \cdot 10^{-6}$ M for subunit III-depleted oxidase; $K_m = 1 \cdot 10^{-5}$ M for the *Paracoccus* oxidase). Bovine cytochrome *c* oxidase showed negligible electron transfer associated with the TMPD oxidase activity observable in the absence of cytochrome *c*, and this was also the case for the subunit III-depleted enzyme. In both cases, correction for the TMPD oxidase activity did not change K_m and increased V_{max} only slightly (Fig. 6, inset). *Paracoccus* oxidase, however, had quite a high TMPD oxidase activity, which resulted in the Eadie-Hofstee plot as an apparent second, high affinity phase appearing at low substrate concentrations (Fig. 6, inset). Subtraction of the TMPD oxidase activity abolished this high affinity phase. A biphasic kinetics with a high affinity phase was reported for the monomer of cytochrome *c* oxidase from *Nitrobacter agilis* [32], which could have been a TMPD effect rather than an intrinsic property of the enzyme.

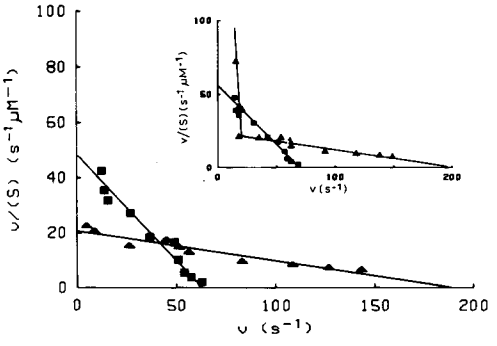

Fig. 6. Eadie-Hofstee plot of cytochrome *c* oxidase activity measured polarographically. Oxygen consumption was measured at 25°C with an oxygen electrode in 10 mM Tris-HCl (pH 7.4)/50 mM KCl/0.1% dodecyl maltoside/10 mM ascorbate/1.1 mM TMPD. Enzymes' concentrations were 15 nM or 30 nM for the bovine subunit III depleted (■) or *P. denitrificans* oxidase (▲), respectively. The cytochrome *c* concentrations were varied between 0.2 and 40 μM. The reaction was started with the addition of cytochrome *c*. The inset shows the data plotted without subtraction of the TMPD oxidase activity. The molecular activities are expressed as nmol of electrons transferred to oxygen per s and per nmol of heme aa_3.

Discussion

The preparation of cytochrome *c* oxidase depleted of subunit-III utilised in this study is very similar to that obtained by Saraste et al. [24] and Penttilä [25]. It reveals essentially the same spectral properties, although the blue shift shown by this preparation is smaller. More obscure is the explanation of the difference in CO binding relative to the native enzyme. It seems that the amount of cytochrome a_3 does not change, but either another species appears or some conformational changes affect the redox behaviour of the cytochrome *a*.

Subunit III-depleted enzymes, either from rat liver [51] or from bovine heart after chymotrypsin digestion [59] were reported to be monomeric. Saraste et al. [24], by equilibrium centrifugation and sedimentation velocity measurements, reported that a species of molecular weight 208 000 was a dimer. Penttilä [25], however, proposed later that this value could have incidentally represented a mixed population of monomers and dimers. Our preparation showed only dimers in centrifugation studies. Monomers could be visualised only in gel filtration experiments, when lower concentrations of the enzyme were employed. The binding of the two non-ionic detergents, Triton X-100 and dodecyl maltoside, was similar and quite high (165 mol dodecyl maltoside/mol aa_3), but still lower than

the amount bound to the dimeric control bovine enzyme (320 mol dodecyl maltoside/mol aa_3 [53]). This result is expected, since depletion of subunit III, a strongly hydrophobic polypeptide, should result in decreased detergent binding.

A lack of proton pumping was observed, as a consequence of the removing of subunit III [24,25] or as a result of the reaction of oxidase with antibodies against subunit III [26]. Since the inhibition of proton pumping by N,N'-dicyclohexylcarbodiimide was correlated with binding of this reagent to subunit III [22], it was proposed that this subunit might be involved in proton translocation. Depletion of subunit III changes as well the electron-transfer activity. A decreased V_{max} and monophasic Eadie-Hofstee plots were obtained both using the spectrophotometric as well as the polarographic assay. Also the subunit III-depleted bovine enzyme obtained by high pH treatment revealed one phase when the activity was measured in Tween-80 [25].

Similarly, it was found that the maximal molecular activity of the subunit III-depleted rat liver enzyme, when measured in Tris buffer, was about 30% of that measured in solubilised membranes (Fig. 4A and C in Ref. 51) with a tendency to monophasic kinetics.

The nonlinear Eadie-Hofstee plot obtained when the kinetics of ferrocytochrome *c* oxidation was measured under steady-state conditions was interpreted in terms of two [33] or more [60] catalytic sites for cytochrome *c* on the enzyme's molecule. From cross-linking experiments [14,15] and spectral perturbation measurements [34] there is increasing evidence that there exists only one catalytic site for cytochrome *c* per aa_3 unit (see also Margoliash and Bosshard [35]). Moreover, the low-affinity enzymic reaction seems to be influenced to the same extent as the high-affinity reaction (see a detailed analysis of Sinjorgo et al. [61]) and as it was concluded from the pre-steady-state measurements, the low and the high affinity interaction domains are indistinguishable [62]. As a consequence of this, assuming one catalytic site per monomer of cytochrome *c* oxidase, the biphasic Eadie-Hofstee plots can be interpreted at least in two different ways:

(1) The low-affinity phase could result from cytochrome *c* binding to a non-catalytic site(s), presumably negatively charged phospholipids [63]. The charge modification (from negative to positive) occuring upon binding of the substrate to one or more sites near the catalytic one on the enzyme molecule decreases the binding constant for the ferrocytochrome *c*, resulting in an increase of the first-order rate constant of the enzyme-product complex [64].

(2) There is only one catalytic site per heme aa_3 unit (monomer), that means two catalytic and binding sites per dimer of cytochrome *c* oxidase. Occupation of one of those sites will hinder further cytochrome *c* binding to the second site, what in terms of kinetics can be described as a homotropic negative cooperativity [36,65].

Since it seems impossible to distinguish between the above explanations only on the basis of kinetic data, we tried to correlate the kinetic pattern of the steady-state reaction with the molecular form of the enzyme. We ascribed a monophasic Eadie-Hofstee plot to monomers and a nonlinear one to the dimeric form of the enzyme [36,65]. In case of both preparations lacking subunit III, under all applied conditions, the kinetics of electron transfer by *P. denitrificans* resulted in a monophasic Eadie-Hofstee plot, consistent with the finding that *P. denitrificans* oxidase is a monomer. Also when the enzyme was assayed in the presence of asolectin, only one phase in the Eadie-Hofstee plot, with quite low affinity ($K_m = 4 \cdot 10^{-6}$ M), was observed, indicating that the addition of phospholipid was not constantly accompanied by the appearance of the second site. The enzyme has been reported to pump protons with a stoichiometry of about half that of the native mammalian enzyme. Whether this phenomenon can be correlated with the lack of subunit III or with the monomeric state of the oxidase cannot be established at present.

The preparation of subunit III depleted oxidase studied here is highly delipidated and contains only 1–2 residual phospholipid molecules. This indicates that not more than one cardiolipin molecule per heme aa_3 unit could be present. As confirmed by NMR analysis of our preparation [66] already in the starting enzyme, which contained totally 14 phospholipid molecules per monomer, only one cardiolipin was present. A similar low phospholipid content, with one

cardiolipin molecule, was reported for the subunit III-depleted rat liver enzyme [51].

The removal of subunit III-obtained here with the technique described [38] is associated with the removal of phospholipid. Which of the two events, phospholipid or subunit III-removal, is responsible for the tendency of the enzyme to monomerisation cannot at present be established. Similarly to the *P. denitrificans* oxidase, it is difficult to establish, in this case as well, which of the two is the actual cause of the diminution [67] or absence [24–26] of a proton pump. In our enzyme preparation, the Eadie-Hofstee plots are, at both high and low ionic strength, monophasic. Moreover, the effect of ionic strength on the rate constants of the reaction between ferrocytochrome *c* and subunit III-depleted enzyme is not evident. The reasons for this phenomenon remain obscure; however, since it was demonstrated that small polypeptides are involved in binding of cytochrome *c* [21] as well, it cannot be excluded that the parallel removal of three small subunits in our preparation eliminates part of a charged binding site on the enzyme molecule; therefore the charge-charge or dipole-dipole interaction between cytochrome *c* and subunit III-depleted oxidase are diminished. It is to be noticed that, although the V_{max} is relatively low, the enzyme is not permanently inactive and that a 3- to 6-fold stimulation of the electron-transfer rate can be obtained with addition of phospholipids. The fact that the increase in activity in the presence of phospholipids is associated with the appearance of a second phase in the Eadie-Hofstee plot may be interpreted in the following ways: (1) phospholipid induces a structural alteration, i.e., dimerisation if the enzyme in solution is monomeric, or closer interaction between sites in the dimer; and (2) phospholipid provides the second site. Further studies on the role of phospholipid will try to clarify which of these two alternatives is correct.

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