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Separation, stability and kinetics of monomeric and dimeric bovine heart cytochrome *c* oxidase

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The stability of monomeric and dimeric bovine heart cytochrome *c* oxidase in laurylmaltoside-containing buffers of high ionic strength allowed separation of the two forms by gel-filtration high-performance liquid chromatography (HPLC). A solution of the dimeric oxidase could be diluted without monomerisation. Both monomeric and dimeric cytochrome *c* oxidase showed biphasic steady-state kinetics when assayed spectrophotometrically at low ionic strength. Thus, the biphasic kinetics did not result from negative cooperativity between the two adjacent cytochrome *c* binding sites of the monomers constituting the dimeric oxidase. On polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS) a fraction of subunit III of the dimeric enzyme migrated as a dimer, a phenomenon not seen with the monomeric enzyme. This might suggest that in the dimeric oxidase subunit III lies on the contact surface between the protomers. If so, the presumably hydrophobic interaction between the two subunits III resisted dissociation by SDS to some extent. Addition of sufficient ascorbate and cytochrome *c* to the monomeric oxidase to allow a few turnovers induced slow dimerisation (on a time-scale of hours). This probably indicates that one of the transient forms arising upon reoxidation of the reduced enzyme is more easily converted to the dimeric state than the resting enzyme. Gel-filtration HPLC proved to be a useful step in small-scale purification of cytochrome *c* oxidase. In the presence of laurylmaltoside the monomeric oxidase eluted after the usual trace contaminants, the dimeric Complex III and the much larger Complex I. The procedure is fast and non-denaturing, although limited by the capacity of available columns.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal enzyme of the mitochondrial respiratory chain. The Y-shaped multi-subunit protein is firmly anchored in the mitochondrial inner membrane and projects into the matrix as well as into the intermembrane space (for a review see Ref. 1). The three largest subunits (I, II and III) are coded for by mtDNA, whereas the smaller subunits (ten in the bovine heart enzyme) are encoded by nuclear DNA. The redox centra, haem *a* groups and copper, are located in subunits I and II. Electrons donated by ferrocytochrome *c* are used to reduce molecular oxygen to water. The required protons are taken up at the matrix side; additional protons

Abbreviation: HPLC, high-performance liquid chromatography.

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are transferred to the intermembrane space. Subunit III is probably involved in this proton pump (for a review see Ref. 2). For bovine heart cytochrome *c* oxidase a relative molecular mass of 203 000 for the sum of 13 subunits has been derived [3], on the basis of a combination of amino acid and mtDNA-sequence work.

There is much literature on the aggregation state of purified cytochrome *c*, the conclusions of which are often contradictory. Analysis of electron micrographs of two-dimensional crystalline sheets of the enzyme in cholate and Triton (reviewed in Ref. 4) has shown that both monomers (2 haems) and dimers (4 haems) can occur and that the protein easily aggregates. Determination of the molecular weight of the detergent-solubilised oxidase by centrifugation or gel filtration is complicated by several factors. The relative specific volume must be known and the determined particle size or weight must be corrected for the presence of phospholipid and detergent. Furthermore, some authors consider some of the small subunits to be contaminants [1,5], which can and should be removed by high pH or ion-exchange column treatment. Finally, it should be mentioned that subunit III is easily lost during manipulation of the enzyme.

Suarez et al. [6] made a detailed study of the residual lipid bound to the enzyme solubilised with laurylmaltoside. They found a protein molecular weight of 194 000 as determined from equilibrium sedimentation, whereas this obviously monomeric cytochrome *c* oxidase moved as a particle with an apparent molecular weight of more than 300 000 in their gel-filtration system. Robinson and Talbert [7] reported that their oxidase preparation was monomeric in the presence of high concentrations of the detergent Triton X-100, whereas dimers and aggregates were present at low detergent concentrations. According to Azzi and coworkers [8–10] the aggregation state of cytochrome *c* oxidase in the presence of laurylmaltoside depended on the ionic strength of the solution, the enzyme usually being a dimer except at very low ionic strengths, in which case the monomeric oxidase is more stable.

The importance of studies on the stability and properties of monomeric and dimeric cytochrome *c* oxidase is illustrated by the controversion about the origin of the biphasic steady-state kinetics displayed by the oxidase. The group of Azzi [8–10] proposed that the occurrence of high-affinity and low-affinity reactions is the result of negative cooperativity between the two cytochrome *c* binding sites in an oxidase dimer. Several other groups [6,7,11,12], including our own group [13], consider the biphasic kinetics to be an intrinsic property of the oxidase, regardless of its aggregation state.

In the present paper we describe the isolation of monomeric and dimeric cytochrome *c* oxidase under conditions where no rapid interconversion occurs. Some of the results have been published in a preliminary form [13].

Materials and Methods

Bovine heart cytochrome *c* oxidase was purified according to the method of Fowler et al. [14], as modified in our laboratory [15]. For some experiments a Yonetani-type preparation [16] was used.

The ultraviolet-visible absorbance spectra of cytochrome *c* oxidase samples were recorded on a Cary-17 or on a Hewlett Packard HP8451A diode array spectrophotometer. For the determinations of the cytochrome *c* oxidase concentration a molar absorption coefficient (reduced-minus-oxidised) was used of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [17].

Horse-heart cytochrome *c* was purified according to Margoliash and Walasek [18]. Concentrations of cytochrome *c* were determined using an absorption coefficient (reduced-minus-oxidised) of 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [19].

Size exclusion chromatography of bovine heart cytochrome *c* oxidase was performed on a TSK SW3000 HPLC column (Toyo Soda, Japan) or on a GF250 Bioseries column (Du Pont, U.S.A.) in an LKB HPLC system as described before [12] with minor modifications. The elution buffer contained 100 mM Tris/acetate (pH 7.5), 100 mM Na_2SO_4, 1 mM EDTA ($I = 388$ mM) and 0.1% (w/v) laurylmaltoside.

Detection of the chromatographed proteins was performed with a detector (LKB 2158 Uvicord SD, light path 2.5 mm) operating at 405 nm, in line with a second detector (Altex 153, light path 10 mm) operating at 280 nm. All results have been normalised to a light path of 10 mm and the time

difference between passage through both detectors has been corrected for.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS)/urea was performed according to Kadenbach et al. [20,21].

Turnover incubation of cytochrome *c* oxidase was performed by incubating monomeric Fowler-type cytochrome *c* oxidase (70 μM) with horse cytochrome *c* (70 μM) in 100 mM Tris/acetate (pH 7.5), 100 mM Na_2SO_4, 1 mM EDTA ($I = 388$ mM) and 0.1% (w/v) laurylmaltoside in the presence of 2 mM ascorbate, for 1 h at 4°C.

Cytochrome *c* oxidase activity was measured spectrophotometrically as described in Ref. 22, using a buffer containing 50 mM Tris/acetate (pH 7.5), 1 mM EDTA ($I = 43$ mM) and 0.1% laurylmaltoside. Reduced cytochrome *c* was added to a final concentration of 0.06–50 μM; the final concentration of cytochrome *c* oxidase was 1 nM. The absorbance decrease at 550 nm was measured with a modified Cary-14 spectrophotometer; below 5 μM cytochrome *c* a Durrum stopped-flow spectrophotometer was used.

Laurylmaltoside was obtained from Calbiochem-Behring Corp. (La Jolla, U.S.A.).

Results

Cytochrome c oxidase monomers and dimers coexist and can be separated

We have used a size exclusion HPLC column to separate the monomeric and dimeric forms present (see later) in a cytochrome *c* oxidase preparation purified from bovine heart. As can be seen in Fig. 1A, the enzyme eluted from a TSK-SW3000 column as two partially separated peaks. The buffer used for equilibration and elution had a high ionic strength (388 mM) and contained 0.1% laurylmaltoside as a detergent. From the retention times of a set of calibration proteins of known molecular mass it was inferred that the two oxidase peaks corresponded to proteins with a molecular mass of $2 \cdot 10^5$ and $4 \cdot 10^5$, respectively. As the sum of the 13 cytochrome *c* oxidase subunits adds up to the lower number, the peaks could be identified as monomeric and dimeric forms of the enzyme. The two components had the same absorbance ratio $A_{280\,nm}/A_{405\,nm}$, which is in line with the conclusion that they only differ in aggregation state.

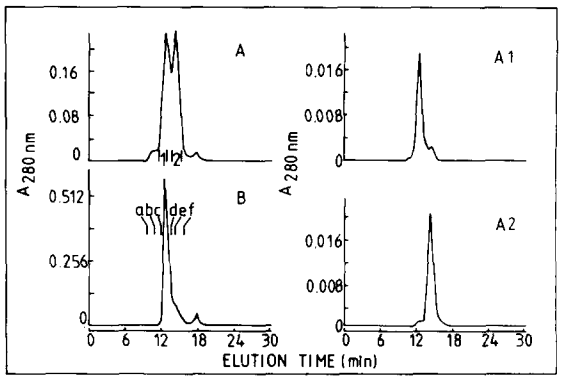

Fig. 1. Elution profile of HPLC size-exclusion chromatography of cytochrome *c* oxidase. The TSK SW3000 column (600 × 7.5 mm) was equilibrated with 100 mM Tris/acetate, 1 mM EDTA, 100 mM Na_2SO_4, 0.1% laurylmatoside, pH 7.5, $I = 388$ mM. Absorbance was measured at 280 nm at a flow rate of 1.0 ml/min at 3.7 mPa. In Fig. 1A cytochrome *c* oxidase (100 μl, 120 μM, partly dimerised, see Materials and Methods) was injected onto the column and fractionated as indicated: 1 = dimer, 2 = monomer. Rechromatography of the fractionated forms of cytochrome *c* oxidase is shown in the right-hand part of the figure. A1 represents the dimeric fraction and A2 represents the monomeric fraction. In Fig. 1B the elution of dimeric QH_2 : ferricytochrome *c* oxidoreductase is shown (M_r = 430000) along with the position of other molecular-weight standards: a = NADH : Q oxidoreductase 1 600 000, b = thyroglobulin 669000, c = ferritin 440000, d = F_1-ATPase 360000, e = catalase 235000, f = ceruloplasmin 130000.

It may be argued that the rather hydrophilic proteins used as markers could behave differently from the rather hydrophobic cytochrome *c* oxidase. Therefore, a sample of purified QH_2 : ferricytochrome *c* oxidoreductase (Complex III) from bovine heart was injected into our chromatographic system. In vivo this dimeric protein complex is embedded in the mitochondrial inner membrane, like cytochrome *c* oxidase. Fig. 1B shows that Complex III eluted just ahead of the oxidase dimer at a position corresponding to a mass of $4.3 \cdot 10^5$, close to the known value for the Complex III dimer. Thus, our selection of column, buffer and detergent gives comparable results for hydrophilic and hydrophobic proteins.

The fact that in Fig. 1A two distinct peaks are observed indicates that no rapid interconversion of oxidase monomers and dimers takes place. This conclusion is supported by rechromatography of samples from the two peaks of Fig. 1A shown in panels A1 and A2 of Fig. 1. The cytochrome *c*

oxidase monomers remained monomers and the dimers remained dimeric. Note that also the 10-fold dilution in the rerun did not induce monomerisation of the dimeric fraction. In our hands a Yonetani-type preparation of cytochrome *c* oxidase consisted of equal amounts of monomers and dimers under these conditions [13]. The ratio hardly changed when samples were injected that varied in concentration, for 1, 10 and 213 μM cytochrome *c* oxidase always 44% monomers were observed. Again, we see that no equilibrium was established, otherwise the strong dilution would have caused a shift to the monomeric side.

When the HPLC technique is applied to hydrophobic proteins, one should be aware of two potential dangers: the enzymic activity may decrease and the protein may stick to the column material or the in- or outlet frit. The results presented in Table I indicate that none of these problems occurred with bovine cytochrome *c* oxidase on a TSK SW3000 column equilibrated in buffers of high ionic strength and containing laurylmaltoside. Recovery of total protein and enzymic activity was close to 100%.

Monomers and dimers have identical spectra and subunit patterns

The subunit patterns of monomeric and dimeric cytochrome *c* oxidase compared to that of the parent preparation were obtained by polyacrylamide gel electrophoresis in the presence of SDS/urea. Within the limits of the resolution, no differences were visible (Fig. 2). The roman numerals denote subunits (I–IV) or groups of subunits (V–VIII) according to the nomenclature of Kadenbach [20,21]. It is clear that the rather

TABLE I

RECOVERY OF CYTOCHROME *c* OXIDASE IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

		Turnover number		
		determined at the cytochrome *c* concentrations:		
		14 μM	9 μM	5 μM
Applied	2.7 nmol	202	174	159
Recovered mono + dimer	2.7 nmol	189	175	168

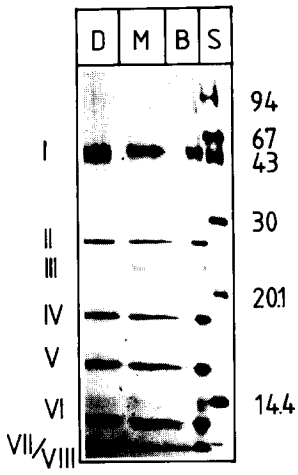

Fig. 2. Polypeptide composition of monomeric ($1 \times aa_3$) and dimeric ($2 \times aa_3$) cytochrome *c* oxidase. Monomeric and dimeric cytochrome *c* oxidase was fractionated as indicated in Fig. 1. Coomassie brilliant blue staining after polyacrylamide gel electrophoresis in the presence of SDS/urea as described in Materials and Methods: lane B, oxidase not subjected to size-exclusion chromatography; lane M, monomeric cytochrome *c* oxidase obtained after size-exclusion chromatography; lane D, dimeric cytochrome *c* oxidase obtained after size-exclusion chromatography. In all cases about 0.1 nmol cytochrome *c* oxidase was applied to the gel. Lane S are standard proteins with known molecular mass. Roman numerals indicate the positions of the oxidase subunits (I–IV) and groups of oxidase subunits (V–VIII). The subunit nomenclature of Kadenbach [21] is used.

loosely bound [11] subunit III was retained in both fractions. In the lane with the dimeric oxidase, the subunit III band was less intense than the band of the monomer, while the band of subunit I had broadened. This clearly shows dimerisation of subunit III in the dimeric oxidase. No dimers of subunit III were observed when the monomeric oxidase was dissociated with SDS under the same conditions.

The visible-light spectra of monomeric and dimeric cytochrome *c* oxidase proved to be identical and similar to those found for bovine heart cytochrome *c* oxidase. The same holds for the ultraviolet region where the protein part of the enzyme absorbs, in line with the evidence from gel electrophoresis. This indicates the presence of all subunits in both fractions.

Interconversion of monomers and dimers

As mentioned above, the cytochrome *c* oxidase

monomers and dimers can be subjected to an HPLC run at room temperature for 20 min without marked changes in the aggregation state. The interconversion became accelerated when the enzyme was under conditions of slow turnover (depicted in Fig. 3). Starting with an almost purely monomeric oxidase solution (70 μM, Fig. 3A), this was incubated with 70 μM cytochrome *c* and 2 mM ascorbate under occasional aeration. After 45 min, a sample was injected on top of the TSK SW 3000 column and analysed (Fig. 3B). After the turnover incubation, 60% of the oxidase had been converted to the dimeric state. The large peak eluting just before the total bed volume is cytochrome *c*, which is characterised by a high $A_{405\,nm}/A_{280\,nm}$ ratio. In a control experiment the monomeric oxidase was aerated at room temperature for 1 h without addition of cytochrome *c* and ascorbate. Some dimerisation took place (Fig. 3C), but very little compared to the turnover incubation. The dimerisation of cytochrome *c* oxidase under turnover conditions in the presence of ascorbate plus cytochrome *c* was fully blocked when oxygen was replaced by carbon monoxide. As shown in Fig. 3D, the mixture of monomers and dimers resulting from the turnover incubation could be separated equally well on a 25 cm GF-250 column (Zorbax Bioseries, Du Pont) as on a 60 cm TSK SW 3000 column. The seemingly inferior separation in Figs. 3A–C was due to deterioration of the column after extensive use.

The slow dimerisation of cytochrome *c* oxidase

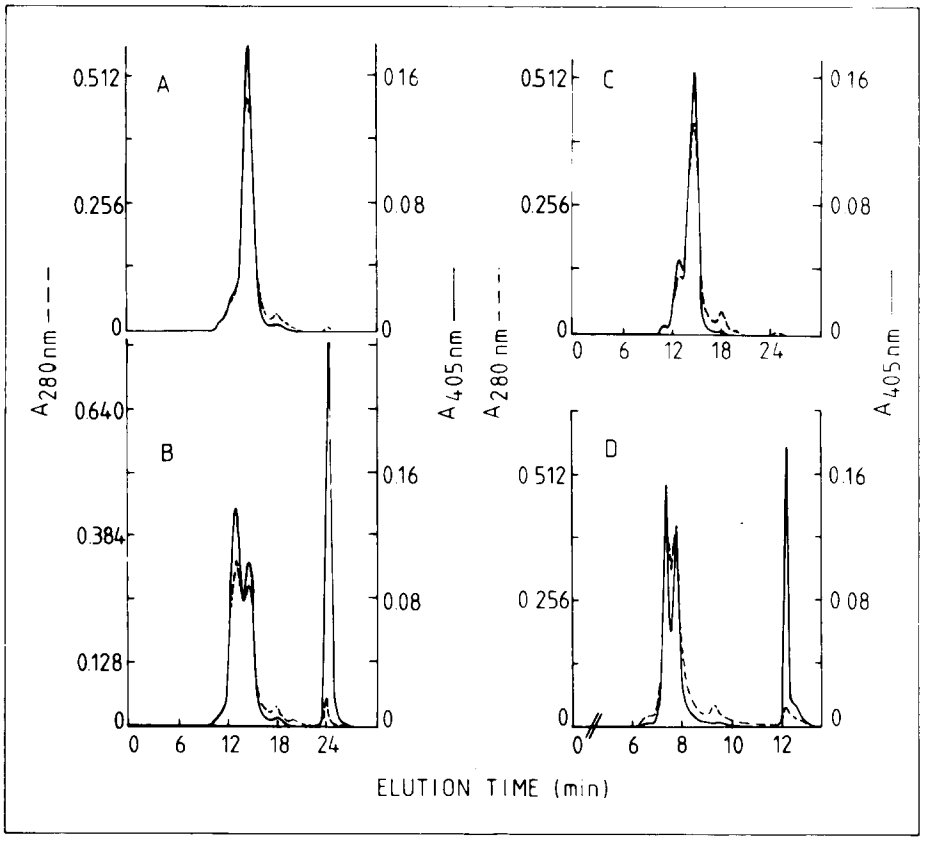

Fig. 3. Elution profile of HPLC size-exclusion chromatography of cytochrome *c* oxidase brought under turnover conditions. The TSK SW3000 column (600×7.5 mm) was equilibrated with 100 mM Tris/acetate, 1 mM EDTA, 100 mM Na_2SO_4, 0.1% laurylmaltoside (pH 7.5, $I = 388$ mM). Absorbance was measured at 280 nm (broken line) and 405 nm (solid line) at a flow rate of 1.0 ml/min (3.7 MPa). The oxidase was under turnover conditions as described in Materials and Methods. (A) Untreated monomeric cytochrome *c* oxidase (Fowler-type oxidase). (B) Cytochrome *c* oxidase after 45 min under turnover conditions. (C) A parallel incubation of cytochrome *c* oxidase in the absence of cytochrome *c* and ascorbate. (D) Identical conditions as under B, except that here a GF 250 Bioseries HPLC column was used.

monomers during turnover could also be demonstrated in the presence of other detergents than laurylmaltoside. In the experiment shown in Fig. 4 cholate was used. In this case, catalytic turnover was slower, since cholate inhibits the enzyme. From Fig. 4B it is clear that an appreciable number of dimers had been formed. In addition to dimers also oligomers (visible in Fig. 4B as a shoulder on the leading edge of the dimer peak) were present in cholate. The presence of oligomers is in agreement with the results of Bolli and coworkers [9]. In the presence of laurylmaltoside hardly any oligomers of the oxidase were formed (compare Fig. 4 with Figs. 1 and 3).

The results of the turnover experiment suggest a link between the redox reactions of the enzyme and the interconversion of monomers and dimers.

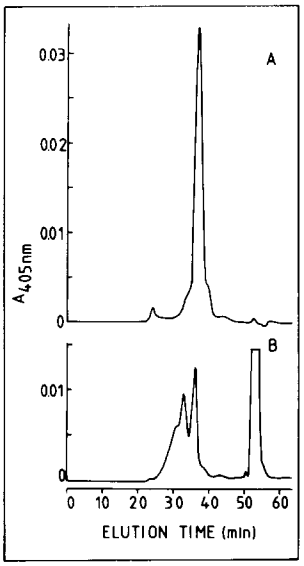

Fig. 4. Elution profile of HPLC size-exclusion chromatography of cytochrome *c* oxidase brought under turnover conditions in the presence of cholate. The TSK SW4000 column (600×7.5 mm) was equilibrated with 50 mM Tris/sulphate, 2% sodium cholate (pH 8.0; $I = 75$ mM). Absorbance was measured at 405 nm at a flow rate of 0.5 ml/min (1.3 MPa). Cytochrome *c* oxidase (3 μM) was incubated with cytochrome *c* (600 μM) and ascorbate (10 mM) in 50 mM Tris/sulphate, 2% sodiumcholate (pH 8.0). After 150 min incubation at room temperature, a sample was injected into the separation system. (A) Cytochrome *c* oxidase after 200 min at room temperature in the absence of ascorbate and cytochrome *c*. (B) Cytochrome *c* oxidase after 150 min at room temperature in the presence of ascorbate and cytochrome *c*. The protein eluting at 52 min is cytochrome *c*.

To investigate a possible involvement of molecular oxygen, reduced monomeric enzyme was reoxidised with ferricyanide under anaerobic conditions. Some dimers were still formed and the interconversion of monomers towards dimers was reversible. When a mixture of oxidised monomeric and dimeric cytochrome *c* oxidase was reduced with either dithionite or dithiothreitol, the equilibrium shifted more rapidly to the monomeric side than during the formation of dimers. We propose that it is the oxidation and reduction that counts, not a specific effect of oxygen or of reduced cytochrome *c*. Nevertheless, cytochrome *c* oxidase tends to be slightly more dimeric in the presence of oxidised cytochrome *c* than in its absence, even more so in the covalent cytochrome *c* oxidase/cytochrome *c* complex [13].

Monomeric cytochrome c oxidase showed biphasic steady-state kinetics at low ionic strength, like the dimeric form

We studied the catalytic activity of monomeric and dimeric cytochrome *c* oxidase purified with the aid of the HPLC technique. The two enzyme forms were separated in a buffer of high ionic strength (388 mM), then assayed for cytochrome *c* oxidase activity in a medium of lower ionic strength (43 mM). This ionic strength is low enough for biphasic kinetics to show up clearly if present. A wide range of concentrations of reduced cytochrome *c* was used (0.06–50 μM) in the

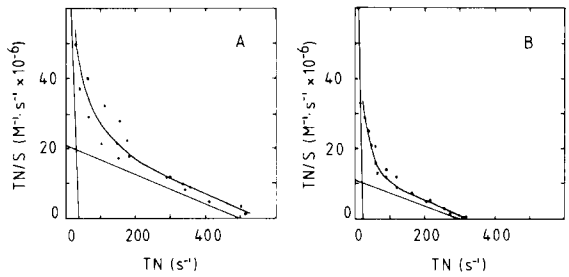

Fig. 5. Eadie-Hofstee plots of the kinetics of oxidation of cytochrome *c* by dimeric and monomeric cytochrome *c* oxidase. Monomeric and dimeric cytochrome *c* oxidase were fractionated as indicated in Fig. 1. The oxidation of ferrocytochrome *c* (0.05–50 μM) by monomeric (part A) and dimeric (part B) cytochrome *c* oxidase was measured in a spectrophotometric assay [22]. The activity was measured in 50 mM Tris/acetate, 1 mM EDTA, 0.1% laurylmaltoside (pH 7.5; $I = 43$ mM). *TN* = turnover number.

enzyme activity assay. Since for the low cytochrome *c* concentrations half-life times were in the order of seconds, a stopped-flow apparatus had to be used. For the high concentrations a conventional spectrophotometer was adequate.

The experimental results, as depicted in the Eadie-Hofstee plot of Fig. 5, clearly show that monomeric as well as dimeric cytochrome *c* oxidase displayed biphasic steady-state kinetics. The K_m values given by the negative slopes of the high-affinity and low-affinity lines are similar for monomeric and dimeric enzyme. The monomeric form appears to have somewhat higher maximal turnover numbers than the dimeric one.

Discussion

In the present paper we have shown that a mixture of monomeric and dimeric bovine cytochrome *c* oxidase could be separated. This implies that the two forms were relatively stable, at least under our experimental conditions in media with laurylmaltoside or cholate. This result is in line with the work of Azzi and coworkers [8–10]. In our hands, however, the monomeric form was the predominant form, while Azzi et al. claim that the monomeric enzyme is only present in buffers of very low ionic strength. Our separation of monomers and dimers by gel-permeation HPLC owes its success to the speed of a run (20 min). Classical gel-permeation chromatography takes many hours, thereby increasing the risk of equilibration of the two forms. The draw-back of the HPLC columns, however, is their small capacity compared to classical column chromatography used by other authors [6,7,9,10].

It is generally accepted that cytochrome *c* oxidase can display biphasic steady-state kinetics when asayed in media of low ionic strength. No consensus exists, however, on the underlying mechanism. Our measurement of the cytochrome *c* oxidase activity at a rather low ionic strength (43 mM) in the presence of laurylmaltoside clearly shows that both the monomeric and the dimeric enzyme forms display the usual biphasic steady-state kinetics. In this respect, our view differs from that of Azzi and coworkers [8–10] who concluded that biphasic kinetics are an intrinsic property of the oxidase dimers only, the monomers having monophasic kinetics. It is interesting to note that Suarez et al. [6] suggest that the dimeric oxidase with biphasic kinetics described by Azzi's group [8] is in fact monomeric. The K_m values of monomer and dimer are similar. The higher TN_{max} values seen with the monomeric oxidase compared to those observed in the dimeric cytochrome *c* oxidase are remarkable and can be explained in several ways, for instance, by dimerisation partly shielding off one of the reaction sites for cytochrome *c*. Detailed information should come from studies currently in progress in our laboratory.

Our finding of biphasic kinetics for monomeric oxidase in laurylmaltoside is in agreement with the results of Suarez et al. [6] and of Robinson and Talbert [7].

In order to reconcile the conflicting views we might speculate that monophasic and biphasic kinetics are not intrinsic properties of monomeric and dimeric cytochrome *c* oxidase, respectively, but functions of the phospholipid content of the enzyme preparations. The lipid-rich oxidase would then have biphasic kinetics, while the lipid-depleted enzyme would have nearly monophasic kinetics. An extreme case was reported by Vik et al. [23] who found loss of the so-called low-affinity site upon removal of the tightly bound cardiolipin from the oxidase, leading to monophasic kinetics associated with the presence of only a high-affinity site. The group of Azzi uses an oxidase preparation purified according to the method of Yu et al. [24], which may differ in lipid content from that used by us and by others.

Our finding that upon SDS dissociation of the dimeric oxidase the hydrophobic subunit III was partly dimerized, while in the SDS-dissociated monomeric oxidase subunit III was a monomer (Fig. 2) leads us to the speculation that subunit III is located at the interface between the monomers in the dimeric oxidase. This is in line with the proposal that subunit III stabilises the oxidase dimers [25].

In Figs. 3 and 4 we presented evidence for slow dimerisation of our monomeric oxidase upon incubation at room temperature under conditions where enzymic turnover was possible. The dimerisation was slower but still occurred when the detergent laurylmaltoside was replaced by cholate, an inhibitor of cytochrome *c* oxidase activity. The

basic reversibility, dimerisation after turnover and monomerisation upon reduction with sodium dithionite, together with the occurrence of the same two peaks when the detergent laurylmaltoside was replaced by cholate, supports our claim that we have true monomeric and dimeric oxidase. These findings of two fractions differing by a factor of 2 in apparent molecular mass cannot be explained with particles containing the same protein but differing in detergent and/or lipid contents. It should be noted that dimerisation is a slow process and does not interfere with the activity assays lasting from seconds to a couple of minutes. It has been shown that after reduction and reoxidation the resting enzyme does not return to the resting conformation but ends up in the 'pulsed' state [26], possibly via a transient '$g = 5$ conformation' leading to a mixture of the 'oxygenated' and '$g = 12$ conformations' [27]. Therefore we assume that the monomer/dimer conversion we observed during turnover incubations occurs when the enzyme is in such a transient state. Brudvig and coworkers [27] also concluded that the oxidase as isolated is a mixture of resting, $g = 12$ and oxygenated species, the ratios depending on the type of enzyme preparation. If we assume that these forms of the enzyme differ not just in ligandation of the prosthetic metal atoms but also in protein conformation, their preference for the monomeric or dimeric states may also differ. That would explain the differences in aggregation state and in monomer stability reported by the various authors in the field.

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