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Paracoccus denitrificans cytochrome *c* oxidase: a kinetic study on the two- and four-subunit complexes

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Abstract

Cytochrome *c* oxidase from *Paracoccus denitrificans* has been purified in two different forms differing in polypeptide composition. An enzyme containing polypeptides I–IV is obtained when the purification procedure is performed in β-D-dodecylmaltoside. If, however, Triton X-100 is used to purify the enzyme under otherwise identical conditions, an enzyme is obtained containing only subunits I–II. The two enzymes are undistinguishable by optical spectroscopy but show significant differences in the transient and steady-state time regimes, as studied by stopped-flow spectroscopy. The observed differences, however, are not due to removal of subunits III and IV, but rather to a specific effect of Triton X-100 which appears to affect cytochrome *c* binding. From these results it is not expected that subunits III and IV play any significant role in cytochrome *c* binding and, possibly, in the subsequent electron transfer processes. The results also suggest that both electrostatic and hydrophobic interactions may be important in the initial electron transfer process from cytochrome *c*.

Keywords: Cytochrome *c* oxidase; Electron transfer; Stopped-flow spectroscopy; Protein purification; Triton X-100

1. Introduction

The tertiary structure of cytochrome *c* oxidase from *Paracoccus denitrificans* has been resolved at 2.8 Å resolution [1]. This enzyme contains four different polypeptide subunits (I, II, III and IV) associated in the active complex by non-covalent interactions (see [2–7] for reviews on the structure and function). Subunits I, II and III are highly homologous to the corresponding subunits found in the much more complex eukaryotic enzyme from beef heart which contains 13 different polypeptides [8,9] and whose structure has also been determined by X-ray crystallography [10,11]. Subunit IV is not homologous in sequence to any known protein [12] and makes different contacts with the other subunits relative to the beef heart enzyme. More recently the

Abbreviations: DM, β-D-dodecylmaltoside; ET, electron transfer; PD_2 and PD_4, *Paracoccus denitrificans* cytochrome *c* oxidase containing subunits I, II and I, II, III, IV, respectively; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SVD, singular value decomposition; TMPD, *N*′,*N*′,*N*,*N*-tetramethyl-*p*-phenylenediamine; TX, Triton X-100

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three-dimensional structure of cytochrome oxidase containing only subunits I and II was determined at 2.7 Å resolution following purification using lauryl-*N*,*N*-dimethylamine-*N*-oxide [13].

P. denitrificans cytochrome oxidase, as originally purified by Ludwig and Schatz [14] using the non-ionic detergent Triton X-100, also contained only subunits I and II, with all the redox-active metals, i.e. the Cu_A centre (a homobinuclear site), cytochrome *a* and cytochrome a_3-Cu_B centre (a heterobinuclear centre, where O_2 and respiration inhibitors bind). Subsequently the gene for subunit III was reported [15] and an enzyme containing an additional subunit (III) isolated using β-D-dodecylmaltoside as the membrane-solubilizing detergent [16]. Later a fourth polypeptide was found in the complex [17,18] and sequenced [12]. The purification procedures reported here for the two- and four-subunit containing enzymes (PD_2 and PD_4, respectively) are based on previously published procedures [16,19–21] and are identical in all respects with the exception of the type and amount of non-ionic detergent used in the membrane-solubilizing process (Triton X-100 and β-D-dodecylmaltoside, respectively). This simple difference in the purification procedure has prompted a study of the transient and steady-state kinetics of electron transfer from cytochrome *c*. Comparison of all the data obtained with the PD_4 and PD_2 enzymes allows to conclude that the overall mechanism for cytochrome *c* oxidation, O_2 reduction [22], and presumably proton pumping [21], is similar for the two enzymes and comparable to the eukaryotic counterpart [2,23]. However, the early electron transfer kinetics to the Cu_A-cytochrome *a* sites are different (by a factor of 5–10 in 0.1 M phosphate buffer pH 7.6) in the PD_2 and PD_4 enzymes; the reduced electron transfer efficiency observed with PD_2 is shown to be related to the presence of Triton X-100 in the preparation and not to removal of subunits III/IV to any significant extent. From inspection of the crystal structure we find that the negative charge cluster present in subunit II, which is important for electrostatic interactions with cytochrome *c* [24], delimitates a water-exposed hydrophobic patch containing conserved aromatic residues which are important for the early ET events [25].

2. Materials and methods

2.1. Cell growth and membrane preparation

P. denitrificans strain ATCC 13543 was grown as described by Ludwig [19]. Cells were suspended in 0.2 M potassium phosphate pH 8, containing 2 mM EDTA and 12 mg/l of a protease inhibitor mixture (Pefabloc SC, from Biomol, Hamburg) and broken using a press operating at 550 atm for 45 min at 4°C. Following removal of cellular debris by centrifugation, membranes were prepared by overnight centrifugation in a Sorval RC5B plus GS3 rotor (8000 rpm). The resulting pellet was resuspended and homogenized in 20 mM potassium phosphate pH 8, containing 1 mM EDTA and ultracentrifuged (Beckmann L5.65, 45Ti rotor) for 1 h at 40 000 rpm. The final pellet containing purified membranes was suspended in the same buffer, assayed for protein concentration and stored at −80°C.

2.2. Protein purification

Preparation of the PD_4 and PD_2 enzymes was in all respects identical with the exception of the solubilizing detergent and is based on procedures developed previously [16,19–21]. All operations were performed at 4°C. Membranes were diluted to 10 mg/ml (membrane protein) in 20 mM potassium phosphate pH 8, containing 1 mM EDTA 12 mg/l Pefabloc SC. Membrane solubilization was achieved by slowly adding either β-D-dodecylmaltoside (1.5 mg/mg membrane protein) or Triton X-100 (4 mg/mg membrane protein) and stirred for 10 min. 0.1 M NaCl was added dropwise and stirred for 10 min. Solubilized membranes were then ultracentrifuged in a Beckmann 45Ti rotor (1 h at 40 000 rpm) and the supernatant loaded on a Pharmacia Biotech DEAE-Sepharose CL-6B column (5.5×20 cm, flow rate 1.1 ml/min) equilibrated in the above buffer (containing either 0.5 g/l β-D-dodecylmaltoside or 2 g/l Triton X-100). A linear NaCl gradient (100–600 mM) was then applied and fractions containing cytochrome oxidase with a haem *a*:protein ratio >6 pooled. Following approx. 10-fold concentration of the pooled fractions by ultrafiltration (30 000 cutoff Amicon membrane), the solution was applied to an

Ultrogel AcA 34 (IBF Biotechnics, France) gel filtration column (2.5×90 cm, flow rate ≤0.5 ml/min) equilibrated in the above buffer (containing either 0.2 g/l β-D-dodecylmaltoside or 2 g/l Triton X-100). Fractions with a haem *a*:protein ratio >10 were pooled, and applied to the first DEAE-Sepharose CL-6B column equilibrated in the same buffer and containing the same amounts of detergents. Cytochrome oxidase was eluted following application of a linear NaCl gradient (100–400 mM) and fractions with a haem *a*:protein ratio ≥14 (PD_4) or ≥20 (PD_2) were pooled, concentrated and stored at −80°C. Purity was assessed by spectroscopy and electrophoresis. Protein concentrations are expressed as the minimal functional ($a+a_3$) unit.

2.3. Stopped-flow kinetics

Stopped-flow experiments were performed using a thermostatted Applied Photophysics DX.17MV stopped-flow (Leatherhead, UK) (1 cm light path, dead time: 1.3 ms) or a rapid-scanning photodiode array spectrometer (Tracor Northern, model TN6500, Madison, WI, USA) adapted to a thermostatted Durrum-Gibson stopped-flow apparatus (2 cm light path, dead time: 10 ms). The rapid-scanning spectrometer acquires 1024 photodiode elements from 10 ms on and can store up to 80 spectra. In these experiments cytochrome oxidase was thoroughly degassed and N_2-exchanged in a gas-tight flask, transferred anaerobically to the stopped-flow syringe to which the desired amounts and types of reductants were added. Reduced samples were obtained within minutes but were used after approx. 30 min. O_2 solutions of known concentration were prepared by dilution of air-equilibrated buffer with anaerobic buffer. Cyanocytochrome oxidase was prepared by addition of KCN from a buffered stock solution and incubation for at least 5 h at 4°C. CO binding was performed by mixing a dithionite-reduced cytochrome oxidase solution against a buffer containing sodium dithionite and known amounts of CO taken from a 1 mM stock solution prepared in a tonometer. CO binding was followed at 445 nm.

2.4. Detergent exchange and treatment procedures

40 μM PD_2 cytochrome *c* oxidase was diluted 30-fold and incubated for 1 h at 4°C in 50 mM potassium phosphate pH 7.6 containing 0.1% DM. The enzyme was then loaded on a DEAE Sepharose column previously equilibrated in the above buffer. After exhaustive washing, the enzyme was step eluted with 0.3 M NaCl, dialysed overnight to lower the ionic strength and finally incubated with 3 mM potassium cyanide for 5 h at 4°C. PD_4 (20 μM) in 0.1 M phosphate buffer pH 7.4 was treated with 0.2% TX and incubated for several hours at 4°C. The sample was subsequently loaded on a BioSep Sec 4000 HPLC gel filtration column (Phenomenex USA, separation range 10^4–10^6 Da) equilibrated in the above buffer containing either 0.2% TX or 0.1% DM, flow rate 1 ml/min. Elution of cytochrome oxidase was followed at 420 nm and the pooled fractions were analysed by SDS-PAGE to assess the polypeptide composition.

2.5. Miscellaneous procedures

Protein concentration was determined by the method of Lowry et al. [26] using bovine serum albumin as standard. All reagents were of the best grade available. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to Laemmli [27]. Static spectra were recorded using both a Kontron Instruments Uvikon 941 or a Jasco V550 thermostatted spectrophotometer. Spectral analysis, curve fitting, computations and graphical procedures were performed using the Matlab (Math Works, South Natick, MA, USA) or Micromath Scientist (Micromath Scientific Software, Salt Lake City, UT, USA) software.

3. Results

3.1. Preparation of the two- and four-subunit containing enzymes

Cytochrome oxidase from *P. denitrificans* may be prepared in two forms which, depending on the detergent used to solubilize the membranes, have different polypeptide compositions. The two-subunit enzyme was originally purified using Triton X-100 by a different procedure [14]. In the present work the PD_4 and PD_2 enzymes have been prepared according

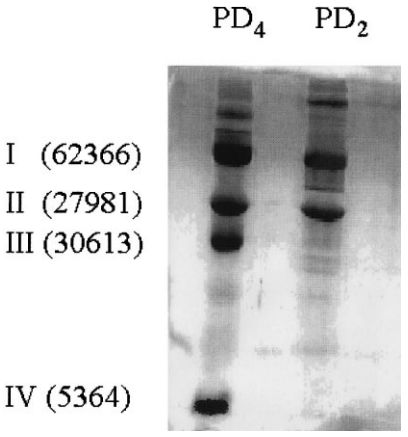

Fig. 1. SDS-PAGE of two- and four-subunit *P. denitrificans* cytochrome oxidase. The Coomassie blue R 250-stained 12% polyacrylamide gel was prepared and run according to Laemmli [17]. Each lane was loaded with approx. 5 μg of protein. The molecular weights of the subunits were obtained from the primary sequences and are shown beside each band (see text).

to procedures pioneered in different laboratories [16,19–21] which involve ion exchange and gel filtration chromatography using either DM or TX (see Section 2). Fig. 1 shows an SDS-PAGE gel demonstrating the polypeptide composition of the PD_2 and PD_4 enzymes. The molecular weights of the subunits, as obtained from the primary sequences [12,28,29], are shown beside each band. The optical spectra of PD_4 and PD_2 are indistinguishable (α and γ bands centred at 598 and 422 nm in the oxidized enzyme).

3.2. Burst kinetics

In these stopped-flow experiments horse heart ferrocytochrome *c* was mixed with cyanide-inhibited cytochrome oxidase to prevent turnover and cytochrome *c* oxidation or cytochrome *a* reduction probed at 550 and 605 nm, respectively, in 0.1 M phosphate buffer, pH 7.6. These experiments show that oxidation of cytochrome *c* or reduction of cytochrome *a* may be described with exponential processes in both enzymes, as also found with the beef heart enzyme. However, PD_2 is apparently 5–10-fold slower than PD_4. In Fig. 2A the observed pseudo-first-order rate constants for cytochrome *c* oxidation or cytochrome *a* reduction are plotted as a function of the initial concentration of ferrocytochrome *c*. From the slope of the linear pseudo-first-order plots we obtain the following values for the apparent second-order rate constant: $k_{PD2} = 2.0\times10^5 \pm 1\times10^4$ M^{-1} s^{-1} and $k_{PD4} = 1\times10^6 \pm 3\times10^4$ M^{-1} s^{-1} (see legend to Fig. 2), which compare with 5×10^6 M^{-1}

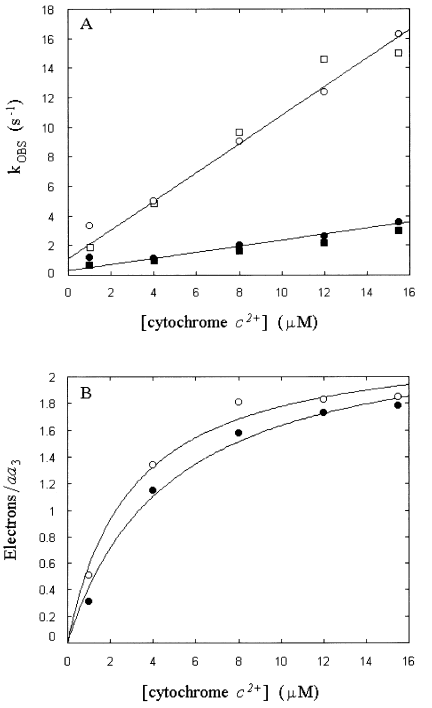

Fig. 2. Cytochrome *c* oxidation by cyano-PD_2 and cyano-PD_4. 3.2 μM cyano-PD_4 and 4.2 μM cyano-PD_2 in 0.1 M potassium phosphate buffer pH 7.6 containing 0.1% DM were 1:1 mixed in the stopped-flow apparatus with ferrocytochrome *c* (at the following concentrations: 2, 8, 16, 24, 31 μM).The cyano-enzymes were prepared by incubation with 3 mM KCN for 5 h at 4°C. The PD_2 sample contained also approx. 0.01% TX, since it is prepared in TX and the stock solution is diluted in the above buffer containing DM. Closed symbols: PD_2; open symbols: PD_4. (A) Cytochrome *c* oxidation and cytochrome *a* reduction was followed at 550 (circles) and 605 nm (squares), respectively, at 20°C. The time courses could be fitted at both wavelengths to a sum of two exponential relaxations. The fitted rate constants of the fast phase are plotted versus the initial ferrocytochrome *c* concentration. At 550 nm the average amplitude of the fast phase accounted for 80% (PD_4) and 53% (PD_2) of the total absorbance changes. The data were also fit to a simple exponential process which yielded, however, poorer fits: from the slope of the linear pseudo-first-order plots the apparent second-order rate constants obtained are 8×10^4 and 8×10^5 M^{-1} s^{-1} for PD_2 and PD_4, respectively. In all cases each data point represents an average of three independent experimental determinations. (B) The observed 550 nm absorbance amplitude normalized to cytochrome oxidase concentration is plotted as a function of the initial ferrocytochrome *c* concentration.

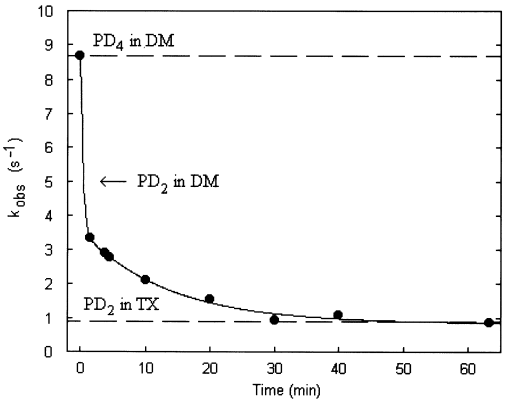

Fig. 3. Effect of Triton X-100 on cytochrome *c* oxidation by cyano-PD_2 and cyano-PD_4. 3 μM cyano-PD_4 was treated with 0.2% TX and mixed with 14 μM ferrocytochrome *c* in the stopped-flow apparatus. Ferrocytochrome *c* oxidation was probed at 550 nm as a function of time following TX addition. The dashed horizontal lines show the rate constants determined for PD_4 in 0.1% DM (top) and PD_2 in 0.2% TX (bottom). The arrow represents the observed rate constant obtained for the PD_2 sample after detergent exchange in 0.1% DM. Other conditions are as in Fig. 2.

s^{-1} for the beef heart enzyme under comparable conditions of ionic strength and pH [30]. The apparent bimolecular behaviour is a clear indication that complex formation between reduced cytochrome *c* and the oxidase rate-limits the ET process, and that in PD_2 the former is 5–10-fold slower. The total amplitude dependence on cytochrome *c* concentration is also depicted in Fig. 2B, where the data are reported as the amount of cytochrome *c* oxidized normalized to the enzyme concentration. It may be seen from Fig. 2B that in the burst reaction with cytochrome *c* both the PD_2 and PD_4 cyanide-inhibited enzymes accept two electrons which are shared by the cytochrome *a*-Cu_A sites, as also found for the mammalian enzyme [31,32].

In the above experiments the PD_2 sample still contained TX. Therefore, it is possible that the lower electron transfer activity found in the two-subunit enzyme could be due to direct interference from TX and not to subunit removal. This was tested in three experiments described below. In the first experiment cyanide-inhibited PD_4 (in DM) was mixed with 0.2% TX and the pseudo-first-order rate constant for cytochrome *c* oxidation determined as a function of time following addition of the detergent. As shown in Fig. 3 the observed rate constant decreases from approx. 9 s^{-1} (a value typical of PD_4 in DM) to approx. 0.9 s^{-1} (a value typical of PD_2 in TX) after 1 h incubation with TX (see top and bottom dashed horizontal lines in Fig. 3); in the first minute the observed rate constant drops by more than 50% of its initial value. Secondly, the PD_2 enzyme was exposed to an excess of DM, bound to an ion exchange column, extensively washed and eluted (see Section 2). When the detergent-exchanged PD_2 sample was treated with cyanide and mixed with cytochrome *c*, the observed rate constant (approx. 5 s^{-1}, see arrow in Fig. 3) was much higher than that observed in the presence of TX, indicating that TX and not subunit removal is the crucial parameter affecting the ET processes between cytochrome *c* and the oxidase. Complete reversibility with PD_2 could not be achieved because the detergent exchange procedure does not completely remove the TX; however, the observed partial recovery of ET rate efficiency with PD_2 is certainly independent of the presence of subunits III/IV. Finally, in order to exclude subunit dissociation induced by TX in the experiment shown in Fig. 3, PD_4 was treated with 0.2% TX and the sample loaded after several hours of incubation on a BioSep Sec 4000 HPLC gel filtration column (separation range 10^4–10^6 Da) equilibrated in either 0.2% TX- or 0.1% DM-containing phosphate buffers. The eluates were analysed by SDS-PAGE and no loss of subunits III and IV could be demonstrated (not shown).

3.3. Steady-state kinetics

These experiments were performed to substantiate the results described in the previous section which suggest that, in the presence of TX, PD_2 is a much weaker electron acceptor from cytochrome *c* than PD_4, and to explore on the kinetic efficiency of these enzymes during turnover. These experiments were performed by time resolved stopped-flow spectroscopy in the presence of a small stoichiometric excess of horse heart cytochrome *c* over cytochrome oxidase (1.5 equivalents) and the artificial reducing system ascorbate-TMPD to sustain O_2 consumption. The stock protein solutions are diluted in DM buffer and initially completely reduced and mixed in the stopped-flow apparatus against an O_2-containing buffer solution. Following exposure to dioxygen

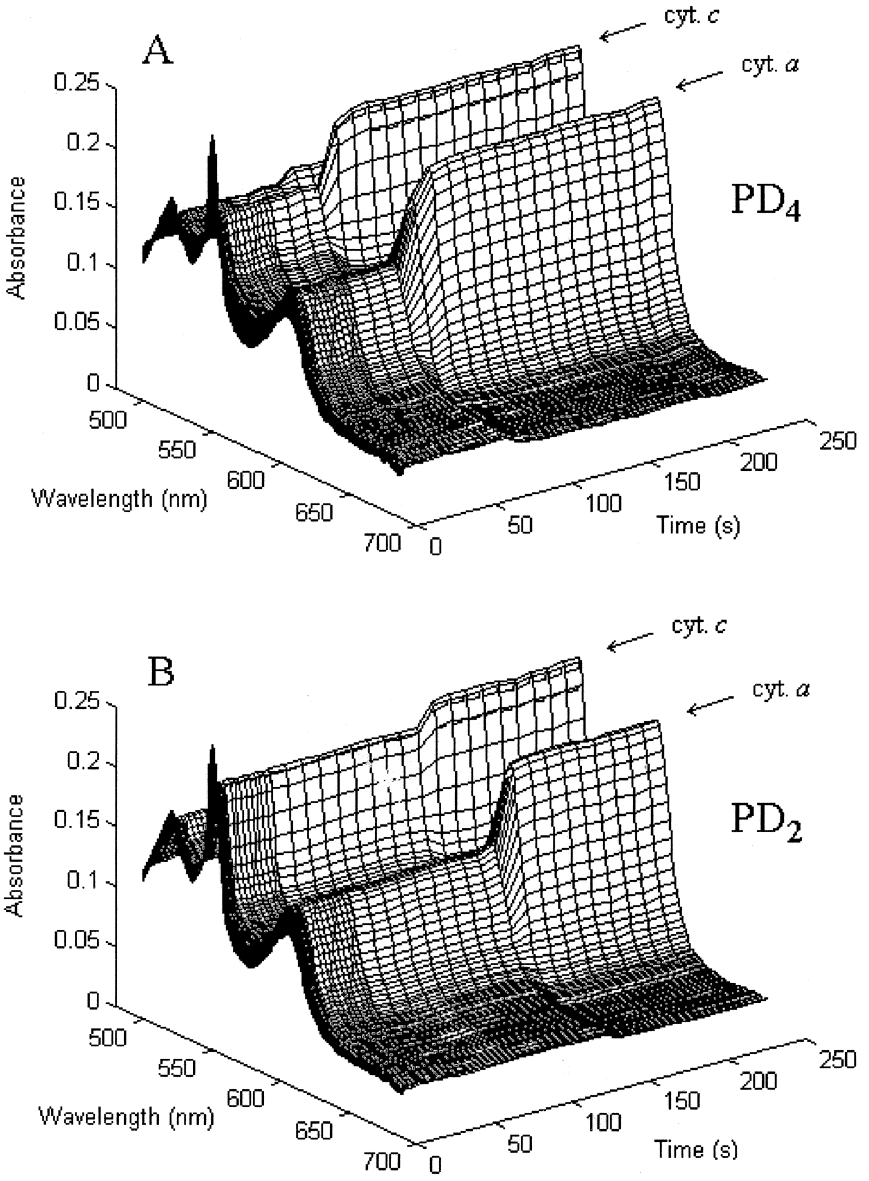

Fig. 4. Rapid-scan stopped-flow kinetics of PD_2 and PD_4 cytochrome oxidases. Fully reduced PD_4 (4 μM) and PD_2 (3.8 μM) cytochrome oxidase in the presence of 6 μM horse heart ferrocytochrome *c*, 2 mM sodium ascorbate and 0.1 mM TMPD were mixed in the photodiode array stopped-flow apparatus against a solution containing approx. 68, 136, or 270 μM O_2 (other conditions as in Fig. 2). All time-resolved spectra (70 spectra acquired in duplicate in the 387–688 nm range) were analysed by SVD and reconstructed with one U*S and V columns [44]. (A) Time-resolved spectra of PD_4 mixed with 136 μM O_2; (B) time-resolved spectra of PD_2 with 136 μM O_2. In both cases the spectra are presented in the 500–650 nm spectral region for clarity. (C,D) Time courses at 550 and 605 nm for PD_2 and PD_4 enzymes as obtained from the analysis of A and B. The percentage reduction levels were calculated from the total reduced minus oxidized spectral changes, the cytochrome *c* and cytochrome oxidase contributions being deconvoluted by SVD using fully oxidized and reduced static spectra of cytochrome *c* and cytochrome oxidase recorded in the stopped-flow apparatus. $T = 20$°C. The PD_2 sample contained approx. 0.01% TX.

(whose initial concentration was varied), the system approaches a steady state in which the enzyme is repeatedly cycling electrons from (ascorbate-TMPD) cytochrome *c* to O_2 and subsequently, when the latter has been completely reduced to water, all redox-active metals are re-reduced; one of these experiments is shown in Fig. 4. The results may be summarized as follows. (i) There is no clear

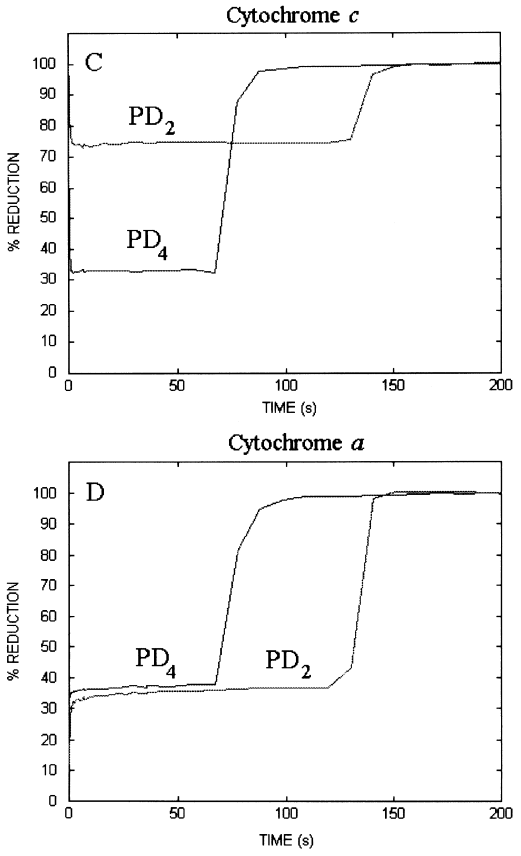

Fig. 4 (*continued*)

dependence of the steady-state reduction levels of cytochrome *c* and *a* on O_2 concentration in the range explored, while the steady-state length is approximately a linear function of O_2 concentration, as expected (not shown). (ii) Under the experimental conditions used (see legend to Fig. 4) the steady-state reduction level of cytochrome *a* is approx. 34% in the PD_4 enzyme and only slightly lower in PD_2, which is consistent with similar experiments obtained with the eukaryotic enzyme under comparable conditions. (iii) The steady-state reduction levels of cytochrome *c* are, instead, strikingly different in the PD_4 (approx. 32%) and PD_2 (approx. 76%) enzymes. Since under these experimental conditions the rate of reduction of cytochrome *c* by TMPD at steady state is approx. 1 s^{-1} [33], the data show that cytochrome *c* is oxidized at a much slower rate by PD_2 and therefore its steady-state reduction level will be higher as observed. The ratio of 5–10 in the apparent second-order rate constants for cytochrome *c* oxidation by the cyano-enzymes (see Fig. 2) is totally consistent with this finding. (iv) In these steady-state experiments the conditions are such (high ionic strength, low cytochrome *c* concentration) that the bimolecular processes between cytochrome *c* and the oxidase are rate-limiting with respect to the internal ET reactions between Cu_A and cytochrome *a* and the cytochrome *a* and cytochrome a_3-Cu_B sites, the latter process limiting turnover at high cytochrome *c* concentrations [34] and whose ET efficiency is critically dependent on the type and amount of detergent [35]. Consequently the steady-state lengths (Fig. 4C,D) are longer in PD_2 since cytochrome *c* oxidation by Cu_A is less efficient due to the presence of TX.

3.4. CO binding kinetics

In these experiments fully reduced cytochrome oxidase is mixed in the stopped-flow apparatus against a solution containing a known amount of CO in the presence of dithionite. The kinetic traces (not shown) were simple exponential processes for both enzymes and from the CO concentration dependence of the pseudo-first-order rates, the second-order rate constant was determined to be 3×10^4 M^{-1} s^{-1} for both enzymes which compares with 8×10^4 M^{-1} s^{-1} in the beef heart enzyme [36]. Thus, the reactivity of the reduced binuclear centre in *P. denitrificans* is very similar to beef heart cytochrome oxidase and the new results are totally consistent with previous flow-flash experiments [22].

4. Discussion

The experiments performed in this investigation were prompted by the possibility to prepare cytochrome oxidase from *P. denitrificans* in two forms differing in polypeptide composition (Fig. 1). In PD_4 all four subunits, seen in the crystal structure [1], are present. In PD_2, subunits III and IV have been removed as a result of the purification procedure which is otherwise identical to the PD_4 preparation but performed in the presence of Triton X-100.

Cytochrome oxidase from *P. denitrificans*, as originally prepared in TX [8], contained only subunits I and II. Subsequently the presence of a third and fourth polypeptide subunit could be demonstrated

[16,17]. The core structure of mammalian and bacterial cytochrome *c* oxidases contains subunits I, II and III which are virtually identical in folding, spatial relationships and the redox-active metal centres are also located at equivalent positions. Subunit III is the second largest polypeptide which co-purifies in the complex. It is important in the assembly of cytochrome oxidase as in vitro mutagenesis experiments show [37], displays a faster electrophoretic mobility on SDS-PAGE than expected (see Fig. 1), is easily removed from the enzyme [38] and has an unusual V-shaped tertiary structure [1]. Subunit IV is unique in primary structure [12] and location in the enzyme. In beef heart cytochrome *c* oxidase this subunit makes contacts with subunit I and also interacts with nuclear-coded subunits VIIb and VIII [6]. In *P. denitrificans*, instead, the N-terminal half of the single transmembrane helix interacts extensively with subunits I and III, while the C-terminal segment does not establish protein contacts and appears to be involved, among other subunits, in binding of a phospholipid molecule. On this basis we may dismiss this polypeptide from having a specific role in cytochrome *c* binding since its structure and location are not conserved. Moreover, this polypeptide does not appear to have a prominent role as an assembly factor or in the stabilization of the enzyme and is not required for cofactor insertion [12].

The results presented in this work strongly suggest that the mechanism of electron transfer in *P. denitrificans* cytochrome *c* oxidase is very similar to that of the mammalian enzyme both in the transient and steady-state regimes. This conclusion is supported by the X-ray structures of the bacterial [1,13] and beef heart [10,11] enzymes and by previous experiments on the O_2 reaction of the two-subunit *P. denitrificans* cytochrome *c* oxidase [22]. The X-ray structure of *P. denitrificans* oxidase strongly suggests Cu_A as being the initial electron acceptor from cytochrome *c*. Since the observed cytochrome *c* oxidation and cytochrome *a* reduction rates were, within the experimental error, identical (i.e. the time courses are synchronous), the internal electron transfer processes between Cu_A and cytochrome *a* must also be very rapid in the bacterial enzymes studied here. The steady-state and CO binding experiments also show that the mechanism of ET to the binuclear site and O_2 reduction (previous data on a two-subunit preparation, [22]) are comparable in the bacterial enzymes.

$$C + O \underset{}{\overset{1}{\rightleftharpoons}} (CO) \overset{2}{\rightleftharpoons} (C^{+}O^{-}) \longrightarrow \longrightarrow \text{Turnover}$$

Binding ET

Scheme 1.

We focus now on the early ET events to the oxidase to establish whether subunits III/IV do or do not play a role in the binding of cytochrome *c* to the oxidase and on the subsequent ET processes. Scheme 1 depicts a general mechanism for the early ET events in cytochrome oxidase, where C and O represent reduced cytochrome *c* and oxidized cytochrome oxidase and CO and C^+O^- are the collisional complexes before and after the ET process. The true ET process which takes place within the collisional complex (step 2) is monomolecular and therefore, by definition, cytochrome *c* concentration independent. This process is expected to be very rapid (rate $\gg 10^3$ s^{-1}), beyond the time resolution of the stopped-flow instrument (approx. 600 s^{-1}) and certainly much faster than the preceding bimolecular binding step (step 1). The linear dependence of the cytochrome *c* oxidation rate constants on cytochrome *c* concentration (Fig. 2) must therefore arise from complex formation which is rate-determining in the cytochrome *c* concentration range tested. It follows that the PD_4 and PD_2 enzymes must differ in the efficiency of the binding kinetics. In the absence of any additional information it may be suggested that subunits III/IV are mechanistically important for cytochrome *c* binding. However, an alternative interpretation relies on the experiments performed to establish the effect of TX. The results obtained following addition of TX to PD_4 or after its removal from PD_2 (Fig. 3) show that: (i) the cytochrome *c* oxidation rate constant of cyano-PD_4 decreases to a value typical of PD_2 after 1 h incubation in 0.2% TX; this is unlikely to be due to dissociation of subunits III/IV which requires (at least in beef heart cytochrome oxidase) alkaline pH and much higher TX concentrations [38–40]. (ii) There is a partial recovery in kinetic efficiency after (partial) detergent exchange of PD_2 for DM; since in this sample subunits III/IV are missing ab initio, the recovery in the ET activity must be due to partial TX removal.

These results suggest that in *P. denitrificans* cytochrome oxidase TX determines the lower apparent cytochrome *c* affinity for the PD_2 enzyme (different slopes in Fig. 2A). Due to limitation in the time resolution of the stopped-flow apparatus, we cannot establish whether TX is also affecting the ET step which follows the binding process. Within the limits of the present investigation we propose that subunits III/IV do not affect the binding dynamics (within a factor of 2) and, therefore, are not expected to contribute structurally to the cytochrome *c* binding site on the oxidase at relatively high ionic strength (0.1 M phosphate buffer, pH 7.6). The experiments reported in Figs. 2–4 were deliberately performed at high ionic strength because the binding process is primarily stabilized by strong electrostatic interactions and, at low ionic strength, using a zinc-cytochrome *c* fluorescence quenching assay [41], we detect very tight complexes with stoichiometries larger than one cytochrome *c* per aa_3 molecule (unpublished). These complexes are highly stable and can be isolated in vitro by gel filtration chromatography [33], and the ET kinetics are much slower due to rate-limiting thermal dissociative processes and/or electron gating [42].

We are left with the final consideration that the efficiencies of cytochrome *c* binding, and possibly ET to Cu_A, are determined not only by optimal ionic strength-dependent collisional configurations between the redox partners but also by hydrophobic interactions. From our results TX, at low concentrations (as low as 0.01% = approx. 0.15 mM), appears to affect cytochrome *c* binding as probed by cytochrome *c* oxidation by Cu_A. Both cytochrome *c* (from horse heart used here and *P. denitrificans*) and subunit II of cytochrome oxidase contain small hydrophobic surface patches surrounded by charge clusters close to haem *c* and Cu_A, respectively. In subunit II the patch contains a short and highly conserved sequence of aromatic residues located in the globular domain exposed to the periplasmic (*P. denitrificans*, W_{121}-Y_{122}-W_{123}) or intermembrane (*Bos taurus*, W_{104}-Y_{105}-W_{106}) space and just above the flat surface of subunit I (the third residue is actually buried). In *P. denitrificans* the peptide carbonyl oxygen of W_{121} is within 5 Å from the Cu_A site and is a crucial residue in early ET events [25]. TX is a non-ionic detergent with a bulky *iso*-octylphenolic tail bound to a long polyoxyethylene moiety, which in an extended conformation is longer than 20 Å. It is not impossible that the detergent may bind to these water-exposed hydrophobic patches (in addition to the membrane-spanning segments) and consequently hinder the approaching cytochrome *c* molecule yielding the apparently lower bimolecular rate constant observed in PD_2 or PD_4 treated with TX. In this respect TX appears to act as a competitive inhibitor in much the same way as polylysine, although the underlying mechanism of inhibition is clearly different. From the present experiments we cannot unequivocally establish whether the ET process is also contributed by hydrophobic interactions due to the time resolution of the stopped-flow apparatus. However, genetic experiments [43] show that the above aromatic residues are quite important for ET in yeast. The above discussion suggests that the mechanism of ET from cytochrome *c* is composite and both electrostatic (recognition and binding) [24] and hydrophobic interactions [25] are of some importance. The role of subunits III and IV in the initial (if any) and internal ET processes remains to be established.

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