Probing the high-affinity site of beef heart cytochrome *c* oxidase by cross-linking

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A covalent complex between cytochrome *c* oxidase and *Saccharomyces cerevisiae* iso-1-cytochrome *c* (called *caa*₃) has been prepared at low ionic strength. Subunit III Cys-115 of beef heart cytochrome *c* oxidase cross-links by disulphide bond formation to thionitrobenzoate-modified yeast cytochrome *c*, a derivative shown to bind into the high-affinity site for substrate [Fuller, Darley-Usmar and Capaldi (1981) *Biochemistry* **20**, 7046–7053]. Stopped-flow experiments show that (1) covalently bound yeast cytochrome *c* cannot donate electrons to cytochrome oxidase,

whereas oxidation of exogenously added cytochrome *c* and electron transfer to cytochrome *a* are only slightly affected; (2) the steady-state reduction levels of cytochrome *c* and cytochrome *a* in the covalent complex *caa*₃ are higher than those found in the native *aa*₃ enzyme. However, (3) *K*_m and *V*_{max} values obtained from the non-linear Eadie–Hofstee plots are very similar in both *caa*₃ and *aa*₃. The results imply that cytochrome *c* bound to the high-affinity site is not in a configuration optimal for electron transfer.

INTRODUCTION

Cytochrome *c* oxidase is a member of a wide family of so-called terminal oxidases [1–3]. These ubiquitously found membrane proteins transfer electrons from a substrate (either a *c*-type cytochrome or a quinol) to molecular oxygen with formation of water and build up of a proton electrochemical gradient. Recently the X-ray structure of two of these proteins has been obtained from beef heart mitochondria [4] and *Paracoccus denitrificans* [5]. Terminal oxidases contain at least one low-potential and one high-potential haem (of types *a*, *b* or *o*); in all cases the high-potential haem is in close proximity (approx. 5 Å) to a copper ion termed Cu_B to form the heterobinuclear O₂-binding site where the O₂-reductive chemistry and inhibitor binding take place. Much of this has been unravelled by extensive kinetic and spectroscopic measurements. All the known eukaryotic terminal oxidases, and also some prokaryotic oxidases, are oligomeric proteins and utilize cytochrome *c* as substrate and carry an additional prosthetic group which contains copper called Cu_A. This site has been extensively studied by EPR and related techniques and is currently thought to be a half-met homobinuclear copper site [6]. Cu_A is bound to subunit II, the third largest subunit in cytochrome oxidase, in a protein fold which recalls that of cupredoxins [7]; in contrast the other metal sites are all bound to subunit I [8]. On the basis of several chemical modification experiments, it has been proposed that the cytochrome *c*-binding site on cytochrome oxidase is a cleft flanked by subunits I and II from one monomer and subunit III provided by the vicinal monomer [9].

At low ionic strength (< 10 mM) it is possible to isolate a stable and tight (*K*_D = 0.01 μM) one-to-one electrostatic complex between ferricytochrome *c* and cytochrome *c* oxidase [10,11]. The site to which cytochrome *c* binds, called the high-affinity site, has been generally assumed to coincide with the high-affinity site detected under steady-state turnover conditions. When formation

of the complex is allowed to occur with horse heart cytochrome *c* specifically modified at Lys-13 by a photoactive arylazido group [12] or in the presence of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodi-imide (EDC) [13,14] an irreversible cross-linking reaction takes place between cytochrome *c* and subunit II at a location in the amino acid sequence which provides some of the Cu_A ligands.

Subunit III of beef heart cytochrome *c* oxidase (*aa*₃) contains two cysteine residues [15], one of which (Cys-115) is reactive to water-soluble sulphydryl reagents such as iodoacetamide [16] and 5,5'-dithiobis-(2-nitrobenzoate) (DTNB). 5-Thio-2-nitrobenzoate (TNB)-modified yeast cytochrome *c* (TNBc) has been demonstrated to bind to the high-affinity site [16–18] and cross-links to subunit III via this cysteine residue by a disulphide exchange reaction.

In the present work we have prepared this covalent complex (as shown by SDS/PAGE) and studied, by stopped-flow spectroscopy, the transient and steady-state kinetics. The results indicate that electron transfer (ET) from cytochrome *c* to oxidase in the *caa*₃ covalent complex is inefficient, supporting and extending previous results obtained with a covalent complex where cytochrome *c* was cross-linked by EDC [13]. However, cytochrome *a* is still capable of accepting electrons from exogenously added cytochrome *c* and the ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenyldiamine (TMPD) ET efficiency is enhanced. Moreover, non-linear Eadie–Hofstee plots obtained with the *caa*₃ complex are very similar to the native *aa*₃, an indication that (i) the rate-limiting step in both enzymes is unchanged, and (ii) the previously proposed two-site model for cytochrome oxidase is difficult to maintain [19].

MATERIALS AND METHODS

Beef heart cytochrome *c* oxidase (EC 1.9.3.1) was purified according to Yonetani [20]. Cytochrome oxidase concentration,

Abbreviations used: *aa*₃, beef heart cytochrome *c* oxidase; *caa*₃, yeast iso-1-cytochrome *c*–cytochrome *c* oxidase covalent complex linked by a disulphide bond between yeast Cys-107 and subunit III Cys-115; DTNB, 5,5'-dithiobis-(2-nitrobenzoate), Ellman's reagent; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide; ET, electron transfer; SVD, singular value decomposition; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenyldiamine; TNBc, thionitrobenzoate-activated yeast iso-1-cytochrome *c* at Cys-107; TNB, 5-thio-2-nitrobenzoate.

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expressed as functional units, was calculated using the absorption coefficient $\Delta\epsilon = 22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 605 nm for the reduced-minus-oxidized transition [21]. Cytochrome *c* from horse heart and *Saccharomyces cerevisiae* were from Sigma and were used without further purification. Static spectra were recorded at 20 °C in either an OLIS-converted Cary 14 spectrometer or a Hewlett Packard 8452A photodiode array spectrometer.

TNBc preparation

TNB-activated yeast iso-1-cytochrome *c* (TNBc) was prepared according to the procedure of Birchmeier et al. [18]. The TNB-to-cytochrome *c* molar ratio was typically in the range of 0.7–0.9, as measured at 450 nm following addition of 2-mercaptoethanol to the TNBc preparation (absorption coefficient $\Delta\epsilon = 7.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

Reaction of beef heart cytochrome *c* oxidase with TNBc

This derivative was prepared essentially according to the method of Malatesta and Capaldi [16] at 20 °C to minimize protein denaturation. Briefly, oxidized aa_3 (20 μM) was mixed with TNBc (400 μM) and incubated for 30 min in 5 mM sodium phosphate, pH 7.6, containing 2% sodium cholate. Excess TNBc was separated from the covalent complex (caa_3) by passage through a Sephadex G-100 column equilibrated in 0.1 M sodium phosphate, pH 7.6, containing 0.2 M NaCl and 0.5% Tween 80. The cross-linking yield was somewhat aa_3 -preparation dependent, with the only common factor being that oxidase preparations more suitable for reconstitution into liposomes also gave high cross-linking stoichiometries (> 0.7). The ionic strength was lowered by overnight dialysis at 4 °C against a 1000-fold excess of 5 mM sodium phosphate, pH 7.6, containing 0.5% Tween 80. Cyano- aa_3 and - caa_3 were prepared by overnight dialysis against 5 mM sodium phosphate, pH 7.6, containing 0.5% Tween 80 and 5 mM KCN. All caa_3 preparations were used immediately.

PAGE

SDS/PAGE was carried out according to the method of Laemmli [22] and gels were stained and destained as described by Downer et al. [23] or by the haem stain procedure of Thomas et al. [24]. In the latter case it was found that several subunits were stained, making the assignment of the yeast cytochrome *c*-subunit III cross-link more difficult. This observation is probably due to the binding of haem *a* to the hydrophobic polypeptides of cytochrome oxidase following denaturation. This problem was partially solved by treatment of the samples with 1 mM iodoacetamide which dramatically reduced the number of haem-positive polypeptide bands.

Transient and steady-state stopped-flow experiments

Stopped-flow experiments were carried out by using a thermostatically controlled Applied Photophysics DX.17MV (Leatherhead, U.K.) stopped-flow (1 cm light path; dead time, 1.3 ms) or a rapid-scanning photo-diode array spectrometer (Tracor Northern, model TN6500; Madison, WI, U.S.A.) adapted to a thermostatically controlled Durrum-Gibson stopped-flow apparatus (2 cm light path; dead time, 10 ms). The rapid-scanning spectrometer acquires 1024 photo-diode elements in 10 ms and can store up to 80 spectra. The data were downloaded to a 486 66 Hz computer operating in the MS-DOS environment. Spectral analysis, curve fitting, computations and graphical procedures were carried out either by using the

MATLAB (Math Works, South Natick, MA, U.S.A.) software or programs written in Turbo Pascal (Borland, Version 7).

Polarographic measurements

Oxygraphic determinations were carried out using a 600- μl magnetic-bar-stirred thermostatically controlled cell containing a Clark-type O_2 electrode. The 0–100% O_2 span was calibrated according to the method of Misra and Fridovich [25] using sodium dithionite to determine the 0% O_2 baseline.

RESULTS

Preparation and characterization of caa_3

The extent of cross-linking yeast cytochrome *c* to the high-affinity site obtained by the method of Birchmeier et al. [18] was checked by SDS/PAGE. One such experiment is shown in Figure 1 where the gel was stained for protein (Figure 1A) or for haem (Figure 1B). In the caa_3 sample (Figure 1A, lane 1) a protein band ($R_F = 0.28$) migrating between subunits I and II is present (see arrow); this band is absent in the control (Figure 1A, lane 2). Also the intensity of subunit III in the caa_3 sample is greatly reduced, in favour of the $R_F = 0.28$ band. Treatment with 2-mercaptoethanol leads to loss of the band migrating between subunits I and II with recovery of subunit III at the appropriate position (Figure 1A, lane 4); this experiment is consistent with the presence of a disulphide bond between subunit III and cytochrome *c*. The mobility of band III-*c* was affected in the same way by other disulphide-reducing agents such as dithiothreitol and sodium dithionite but not by sodium ascorbate, TMPD or cyanide.

When the SDS/PAGE gel is stained by the haem-staining procedure [24], see Figure 1(B) (lane 1'), an $R_F = 0.27$ haem-positive band is detected (arrow), demonstrating that the new band intermediate between subunits I and II indeed contains cytochrome *c* and subunit III. By treatment with 2-mercaptoethanol (lane 4') the haem-positive band migrates at a position ($R_F = 0.56$) consistent with the molecular mass of cytochrome *c*. Such a band is absent in lane 1', an indication that in the caa_3 sample all of the cytochrome *c* present is covalently bound to subunit III. The other haem-positive bands seen in Figure 1(B) are of artifactual nature. In lane 2', which contains an aa_3 sample (i.e. no cytochrome *c* is present), several haem-

Figure 1 SDS/PAGE of native and cytochrome *c* cross-linked cytochrome oxidase

(A) Coomassie Blue-stained gel and (B) haem-stained gel. Lanes 1 and 1', caa_3 ; lanes 2 and 2', aa_3 ; lanes 3 and 3', 2-mercaptoethanol-treated aa_3 ; lanes 4 and 4', 2-mercaptoethanol-treated caa_3 . All samples were treated with 1 mM iodoacetamide to prevent aspecific haem staining (see the Materials and methods section) and each lane contained approx. 50 μg of total protein.

positive bands are detected. However, these are quenched in the presence of 2-mercaptoethanol (Figure 1B, lane 3'). It is interesting to note, however, that 2-mercaptoethanol does not quench the cytochrome *c* band ($R_f = 0.56$, lane 4'), an indication that the artifactual haem-positive bands observed in lane 2' may come from the peroxidatic activity of haem *a* bound to the most hydrophobic subunits of cytochrome oxidase. Moreover, in the SDS/PAGE experiment of Figure 1, all lanes were loaded with the same amount of protein, yet, as seen by comparing lanes 1' and 4', the intensity of the subunit III-cytochrome *c* and cytochrome *c* bands are exceedingly different, though both bands contain the same amount of type *c* haem. The stoichiometry of the covalent complex was therefore also evaluated in a different way.

Spectroscopic evaluation of the yeast cytochrome *c*-cytochrome oxidase cross-linking stoichiometry

Following the reaction of aa_3 with TNBc, excess TNBc is easily removed by gel-filtration chromatography at high ionic strength. If the experiment is carried out with a non-modified yeast cytochrome *c* or with horse heart cytochrome *c* (which does not contain a DTNB-reactive cysteine residue), no spectroscopic signature of cytochrome *c* can be recovered in the cytochrome oxidase eluate. This demonstrates that in the caa_3 sample the equilibrium interaction between cytochrome *c* and oxidase is not electrostatic but covalent, as was also consistently suggested by the SDS/PAGE experiments described above. The stoichiometry of the covalent caa_3 complex was also evaluated spectroscopically. The optical spectrum of each caa_3 preparation was recorded and fitted to a linear combination of two reference spectra (aa_3 and cytochrome *c*) recorded separately. The stoichiometry was calculated by applying the Lambert-Beer law to the experimental spectrum:

$$\mathbf{d} = \mathbf{R} \cdot \mathbf{x} \quad (1)$$

where, in matrix notation, $\mathbf{d}$ is a column vector containing the experimental static spectrum of caa_3 , $\mathbf{R}$ is a matrix containing the reference spectra (cytochrome oxidase and cytochrome *c*) expressed in $\text{mM}^{-1} \cdot \text{cm}^{-1}$, as determined either directly or according to the literature [26,27], and $\mathbf{x}$ is a column vector containing the unknowns, i.e. the coefficients of the linear combination which yields the concentrations of the components and hence the stoichiometry. Since the system is overdetermined (more equations than unknowns) the $\mathbf{x}$ vector was approximated by using the singular value decomposition (SVD) algorithm according to the following: $\mathbf{R}$ was decomposed by SVD into the product $\mathbf{R} = \mathbf{U} \cdot \mathbf{S} \cdot \mathbf{V}^T$ (see Henry and Hofrichter [28], for details) and the pseudoinverse $\mathbf{R}_i$ calculated by the inverse transformation $\mathbf{R}_i = \mathbf{V} \cdot \mathbf{S}^{-1} \cdot \mathbf{U}^T$. The $\mathbf{x}$ vector was then obtained by left multiplication of eqn. (1) by $\mathbf{R}_i$:

$$\mathbf{x} = \mathbf{R}_i \cdot \mathbf{d} = \mathbf{V} \cdot \mathbf{S}^{-1} \cdot \mathbf{U}^T \cdot \mathbf{d} \quad (2)$$

The stoichiometry was calculated by appropriate division of the $\mathbf{x}$ vector elements. Typical values of the stoichiometry obtained in several (> 10) independent preparations using five different oxidase preparations ranged from 0.7 to 1.1 mol of cytochrome *c*/mol of oxidase. Figure 2 shows an example of a caa_3 preparation in the reduced state (spectrum a) with the calculated component spectra (b and c), as obtained by the procedure described above, and the residuals (spectrum d).

The complementation test

In order to ascertain that the site of cross-linking is indeed the high-affinity site of the oxidase, cross-linked complexes, with

Figure 2 Determination of the cytochrome *c*/cytochrome oxidase stoichiometry in caa_3

Spectra in 0.1 M sodium phosphate buffer, pH 7.6, containing 0.5% Tween 80, 5 mM sodium ascorbate and 0.1 mM TMPD. Light path: 1 cm; $T = 20^\circ \text{C}$. Spectrum a: experimental data ($\circ$) and best fit of data to eqns. (1) and (2) (continuous line) using the following $\mathbf{x}$ vector elements: $\mathbf{x}(1) = 5.0 \mu\text{M}$ cytochrome *c* and $\mathbf{x}(2) = 5.4 \mu\text{M}$ cytochrome oxidase, yielding a stoichiometry of 0.92. Spectra b and c: component spectra of cytochrome *c* and cytochrome oxidase; spectrum d: residuals (offset by -0.1 absorbance unit for clarity).

stoichiometries ranging from 0.3 to 0.8, were prepared and exposed to excess native cytochrome *c* at low ionic strength. After Sephadex G-100 removal of the excess cytochrome *c*, the total cytochrome *c* associated with oxidase (i.e. cross-linked and electrostatically bound) was determined. As shown previously [13] it is found that irrespective of the value of the initial stoichiometry of the covalent complex, the final cytochrome *c*-to-oxidase ratio always approached unity. Thus the electrostatic and covalent complexes are complementary [13,17], indicating that cytochrome *c* cross-links into the high-affinity site and cross-linking prevents further association via electrostatic interactions.

Transient stopped-flow experiments

The experiments were carried out by mixing aa_3 and caa_3 in the stopped-flow apparatus against an air-equilibrated solution of yeast ferrous cytochrome *c*. The reaction was followed at 550 and 605 nm (Figure 3A) which report respectively cytochrome *c* oxidation and cytochrome *a* reduction. At 605 nm and in the burst phase of the reaction, i.e. the first 10 ms, 25 and 31 % of the expected amplitude for cytochrome *a* reduction is observed for aa_3 and caa_3 respectively. The corresponding rates are 780 and 600 s^{-1} . These results demonstrate that, although the high-affinity site is covalently blocked, cytochrome *a* in caa_3 is very quickly reduced by externally added cytochrome *c*. From the cytochrome *c* concentration-dependence of the observed rates (not shown) it is possible to evaluate the apparent second-order rate constant for cytochrome *a* reduction by cytochrome *c*: 2.1×10^7 and $1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively for aa_3 and caa_3 , which are in agreement with previous findings [29]. Thus in caa_3 the covalently bound cytochrome *c* does not appear to hinder the incoming cytochrome *c* molecule.

Similar results are obtained when monitoring the oxidation of cytochrome *c* at 550 nm (Figure 3A, bottom traces) both in the burst rates and amplitudes and in the subsequent turnover phase, shown in Figure 3(B), which proceeds at 0.49 and 0.48 s^{-1} respectively for caa_3 and aa_3 . The latter result suggests that the

Figure 3 Kinetics of electron entry into native and cytochrome *c*-cross-linked cytochrome oxidase

7.4 μ M aa₃ (open circles) or 7.8 μ M caa₃ (closed circles) in 5 mM sodium phosphate buffer, pH 7.6, containing 0.5% Tween 80 were mixed in the stopped-flow apparatus against a solution of 89 μ M yeast cytochrome *c*. (A) Burst phase of the reaction followed at 605 nm (top traces) or 550 nm (bottom traces). The 605 nm data were corrected for cytochrome *c* contribution (see the text). (B) Turnover phase of oxidation of cytochrome *c* as observed at 550 nm. T = 20 °C. Solid lines through the data points are best fits. See the text for details.

rate-limiting step of the cytochrome oxidase reaction is not affected by the cross-linking procedure.

Steady-state experiments

The experiments described below were similar to those of the preceding section except that TMPD and sodium ascorbate were always present to sustain steady state. Five different samples were examined: (1) aa₃, (2) aa₃ + 1*c*, (3) aa₃ + 2*c*, (4) caa₃, and (5) caa₃ + 1*c*; the results of some of these experiments are shown in Figure 4. In all cases three phases are distinguished: (a) a pre-steady-state phase in which the initially reduced enzyme is rapidly oxidized and 'relaxes' to steady state; (b) the steady-state phase; and (c) the reduction by the excess reductants which takes place following consumption of O₂. Figure 4(A, B) shows a three-dimensional view of the time-dependent optical spectra collected in such experiments (relating absorbance to wavelength to time). Figure 4(C) depicts the time courses at selected wavelengths (550 and 605 nm, where cytochromes *c* and *a* dominate), as obtained following decomposition and recon-

Figure 4 Rapid-scan stopped-flow kinetics of native and cytochrome *c*-cross-linked cytochrome oxidase

Reduced caa₃ or aa₃ in the presence of 10 mM sodium ascorbate and 0.1 mM TMPD were mixed in the stopped-flow apparatus against a solution containing approx. 65 μ M O₂. Other conditions were as in Figure 3. All time-resolved spectra (500–650 nm range) were analysed by SVD and reconstructed with one **U**'s and **V** columns. The experiments, corresponding to numbers 2 and 4 in Table 1, are: (A) aa₃ 11.7 μ M in the presence of 12 μ M yeast cytochrome *c*: 80 spectra recorded from 10.5 ms at time intervals of 105.4 ms; (B) caa₃ 10.8 μ M (stoichiometry 0.92) with no externally added cytochrome *c*: 60 absolute spectra recorded from 10.5 ms at time intervals of 316.2 ms; (C) time courses at 550 and 605 nm obtained either for the aa₃ + 1*c* (continuous lines) or for the caa₃ sample (broken lines). The percentage reduction levels were calculated from the total reduced-minus-oxidized spectral excursion, the cytochrome *c* and *a* + a₃ contributions being deconvoluted by SVD using eqns. (1) and (2). Arrows: see the text.

Table 1 Some steady-state parameters of cytochromes *c* and *a* in aa_3 and caa_3

The steady-state reduction levels were calculated using eqns. (1) and (2) from the total reduced-minus-oxidized spectral changes observed.

Experiment number	Percentage reduction at steady state		Steady-state length (s)
	Cyt. <i>c</i>	Cyt. <i>a</i>	
1 aa_3	—	12	100 ^a
2 $aa_3 + 1c$	27	40	8
3 $aa_3 + 2c$	36	43	7
4 caa_3	100	65	7
5 $caa_3 + 1c$	88 ^b	80	3

^a This value may be overestimated due to O_2 leak in the stopped-flow apparatus.

^b This number refers to the steady-state reduction level of the exogenous cytochrome *c*, the cross-linked cytochrome *c* being completely reduced at steady state (see experiment 4 and text).

struction of the data by SVD. The steady-state reduction levels of cytochromes *c* and *a*, together with steady-state lifetimes in the five experimental conditions, are summarized in Table 1. From Figure 4 and Table 1 it is apparent that in the presence of ascorbate/TMPD, caa_3 is catalytically more efficient than aa_3 in the absence of exogenously added cytochrome *c* (compare experiments 1 and 4 in Table 1), since the steady-state lifetime decreases from approx. 100 s to 7 s with cytochrome *c* cross-linked to the high-affinity site of the oxidase. This finding may be explained if part of the cross-linked cytochrome *c* were uncomplexed, but this is excluded by the SDS/PAGE experiment (see Figure 1), the G-100 chromatography experiments at high ionic strength used to purify the caa_3 sample (see the Materials and methods section), and the steady-state reduction level of bound cytochrome *c* which is very close to 100 %. The kinetic behaviour of aa_3 in the absence of free cytochrome *c* (Table 1, experiment 1) is consistent with the poor kinetic efficiency of the ascorbate/TMPD reducing system, despite the relatively large driving force (compare the redox potentials of cytochrome *a* and ascorbate, 280 and 58 mV respectively [30–32]). It is also consistent with the rate-limiting process, i.e. ET from the cytochrome $a-Cu_A$ to the cytochrome a_3-Cu_B sites [33,34], being much more efficient than the bimolecular reduction by ascorbate/TMPD [35] (the bimolecular rate constant for reduction of cytochrome *a* by TMPD is $2500\text{ M}^{-1}\cdot\text{s}^{-1}$, see Figure 6). In the case of caa_3 , the increased steady-state reduction level of cytochrome *a* and particularly of covalently bound cytochrome *c* (see experiment 4 in Table 1) requires an alternative explanation, especially in view of the results of the pre-steady-state experiments (see Figure 3) which indicate that the burst phase of the ET from cytochrome *c* to aa_3 or caa_3 is not significantly different. One possible explanation is that in the caa_3 complex the cross-linked cytochrome *c* is delivering electrons to the target metal on the oxidase as efficiently as in the case of the native oxidase in the presence of externally added cytochrome *c*. This possibility, however, may be excluded by several lines of evidence. (i) The turnover number of caa_3 , determined independently at high cytochrome *c* concentrations (see below), is, within the errors, identical to that of aa_3 ; therefore the rate of oxidation of cytochrome *a* by O_2 is unaffected. (ii) The steady-state reduction level of the cross-linked cytochrome *c* is close to 100 %, as shown unequivocally from analysis of the spectra in Figure 4 (panel B). (iii) Stopped-flow experiments carried out as in Figure 4, albeit with no TMPD present, indicate that in the first spectrum obtained after mixing of the fully reduced enzymes with O_2 (i.e. after 10.5 ms)

Figure 5 Eadie-Hofstee plots of native and cytochrome *c*-cross-linked cytochrome oxidase

Cytochrome oxidase activity was determined polarographically under the following conditions: 10 mM sodium ascorbate, 0.1 mM TMPD in 5 mM sodium phosphate buffer, pH 7.6, containing 0.1% Tween 80, $T = 20^\circ\text{C}$. Cytochrome *c* concentration was varied from 0.5 to $128\text{ }\mu\text{M}$ in geometric progression. Baselines were recorded in the presence of ascorbate, TMPD and cytochrome *c* and the assay started by addition of $0.27\text{ }\mu\text{M}$ aa_3 (open circles) or $0.27\text{ }\mu\text{M}$ caa_3 (closed circles). Turnover numbers are expressed in mol of O_2 /mol of oxidase per s. The steady-state parameters obtained for aa_3 are: $K_{m1} = 0.1$ and $K_{m2} = 7\text{ }\mu\text{M}$, $V_{max1} = 0.7$ and $V_{max2} = 2.3\text{ s}^{-1}$; and for caa_3 : $K_{m1} = 0.2$ and $K_{m2} = 4.3\text{ }\mu\text{M}$, $V_{max1} = 0.8$ and $V_{max2} = 1.8\text{ s}^{-1}$.

cytochrome *c* is more than 20 % oxidized in the caa_3 sample as opposed to 80 % in the $aa_3 + 1c$ experiment; therefore immediate oxidation of the cross-linked cytochrome *c* is considerably less efficient in caa_3 . This finding is also demonstrated by inspection of Figure 4(C) (see arrows), which in addition reveals that the approach to the steady state of caa_3 takes place reductively both for cytochromes *c* and *a*, whereas it occurs oxidatively in the $aa_3 + 1c$ sample. Therefore in the cross-linked enzyme cytochrome *c* is poorly oxidized by the oxidase and cytochrome *a* is better reduced by the reductants. (iv) The rate of reduction by ascorbate/TMPD of cytochrome *c* either cross-linked, electrostatically bound to the oxidase, or alone was determined to be the same (in the two former cases the cyanide derivatives of cytochrome oxidase were used); in all cases cytochrome *c*, irrespective of its state (cross-linked, electrostatically bound, or free), was reduced with a rate constant of $2 \times 10^4\text{ M}^{-1}\cdot\text{s}^{-1}$, in agreement with independent data [36]. This result indicates that the 100 % steady-state reduction level of cytochrome *c* in caa_3 (Table 1, experiment 4) is not due to a dramatic increase in the rate of reduction of bound cytochrome *c* by TMPD.

By adding 1 equivalent of cytochrome *c* to the aa_3 sample (experiment 2 in Table 1) the steady-state length is very similar to that of caa_3 (experiment 4), although no externally added cytochrome *c* was present in the latter case. Thus, in terms of catalytic efficiency these two samples are very similar, though in the case of aa_3 both the cytochrome *c* and *a* steady-state reduction levels are consistently more oxidized than in caa_3 . This finding indicates that some rate parameter is significantly different in the two enzymes.

Steady-state polarographic measurements were also carried out with the aa_3 and caa_3 samples by varying the yeast cytochrome *c* concentration. In both cases very similar biphasic Eadie–

Hofstee plots were obtained (Figure 5) with apparent K_m and V_{max} values in agreement with published values [37]. The V_{max} values are also consistent with other data obtained under these experimental conditions, i.e. temperature, ionic strength, pH and detergent type [34].

From the results discussed so far it is clear that the increased steady-state reduction levels of cytochrome *a* in caa_3 compared with aa_3 can not be explained on the basis of a reduced catalytic efficiency (see Figures 3B, 4 and 5) of the enzyme, as was observed instead for the EDC-treated cytochrome oxidase [13]. The steady-state reduction levels of cytochrome *a* suggest that this site is more efficiently reduced by TMPD in caa_3 than aa_3 . This was shown to be the case by mixing the cyanide derivatives of the two enzymes against a solution containing sodium ascorbate and TMPD. Reduction of cytochrome *a* (monitored at 605 nm) follows a simple exponential behaviour; the dependence of the pseudo-first-order rate constant on TMPD concentration, reported in Figure 6, yields the following values of the second-order rate constants: $k = 2.5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ for aa_3 and $k = 1.6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for caa_3 . Thus, reduction of cytochrome *a* by TMPD is 6.4-fold more efficient in caa_3 .

The increased steady-state reduction levels of cytochrome *a* in caa_3 as compared with aa_3 and the apparent higher catalytic efficiency of the former, especially as observed in the absence of externally added cytochrome *c*, may be partially explained on the basis of the following kinetic (Scheme 1):

Scheme 1

where E represents both aa_3 and caa_3 in various oxidation states, TMPD_{red} and TMPD_{oxi} indicate reduced and oxidized TMPD respectively, and A and dA represent ascorbate and dehydro-ascorbate respectively. In E the first and second digits represent cytochromes *a* and *a₃* respectively (0 = oxidized, 1 = reduced). Here a two-electron turnover scheme has been assumed for simplicity, incorporating Cu_A and Cu_B into the first and second digit. Extensive simulations have been carried out by numerical integration of the above mechanism. In all these simulations k_2 , i.e. the monomolecular rate constant for ET between the cytochrome *a*- Cu_A and cytochrome *a₃*- Cu_B sites, was varied in absolute value but kept identical for aa_3 and caa_3 , as suggested from the polarographic steady-state experiments (see Figure 5). The bimolecular rate constant for reduction of cytochrome *a* (k_1) by TMPD was in all cases higher (by 6.4-fold) for caa_3 (see Figure 6). In all simulations it was assumed that cross-linked cytochrome *c* would not transfer electrons to the enzyme (as implied from the data discussed above). The simulations show

Figure 6 Kinetics of reduction of cytochrome *a* by TMPD in the cyano derivatives of native and cytochrome *c*-cross-linked cytochrome oxidase

Cyano- caa_3 (10 μM , c/aa_3 stoichiometry = 1.0) or cyano- aa_3 (7 μM) in 5 mM sodium phosphate buffer, pH 7.6, containing 0.5% Tween 80 and 5 mM potassium cyanide were mixed in the stopped-flow apparatus against an anaerobic solution of 10 mM sodium ascorbate and TMPD at different concentrations (20, 80, 140 and 233 μM) in the same buffer. $T = 20^\circ\text{C}$.

that a 6.4-fold increase in k_1 significantly increases the steady-state reduction level of cytochrome *a* and decreases the steady-state lifetime in agreement with the experiments. The effect of increasing k_2 is, as expected, to decrease both the steady-state reduction levels of cytochrome *a* and the steady-state lifetime. Under all conditions explored cytochrome *a* is always more reduced in caa_3 at steady state and its lifetime shorter.

DISCUSSION

We have characterized the covalent complex between beef heart cytochrome *c* oxidase and yeast iso-1-cytochrome *c*, obtained starting from the 1:1 electrostatic complex. This is known to be stabilized by charge interactions between cytochrome *c* amine cations and cytochrome oxidase carboxylate anions present (and conserved through evolution) in subunit II, close to the Cu_A -binding site. In a previous study cytochrome *c* containing Fe or Zn was cross-linked to cytochrome oxidase using a water-soluble carbodiimide (EDC) [13]. In this reaction carboxyl groups of subunit II react with the carbodiimide to form a highly reactive O-acylisourea intermediate [38], which condenses with amine nucleophiles present in the lysine cluster of the cytochrome *c* front face. Time-resolved stopped-flow experiments showed that the covalently bound cytochrome *c*, though reduced, cannot donate electrons to cytochrome oxidase [13]. If the cross-linking procedure were to impair the ET pathway linking cytochrome *c* to the oxidase, i.e. residues condensed in the cross-linking procedure, this result would have a trivial explanation. Since the result of that study was important, but questionable, it was of interest to prepare and characterize a covalent adduct of cytochrome oxidase and cytochrome *c* linked via a portion of cytochrome *c* well separated from the front face which is thought to be involved in the recognition-binding and ET processes. Yeast iso-1-cytochrome *c* contains a cysteine residue Cys-107 which is positioned on the back side near the C-terminus. This cysteine was modified with DTNB to yield TNBc according to the procedure of Birchmeier et al. [18]. When TNBc is mixed at

low ionic strength with beef heart cytochrome oxidase an electrostatic complex is readily formed and a subsequent disulphide-exchange reaction takes place with Cys-115 of subunit III, which in dimeric oxidase is known to be part of the cytochrome *c*-binding site [9]. Covalent complex formation is prevented if the latter residue is alkylated by iodoacetamide [16].

The results of the experiments carried out on this *caa*₃ covalent complex are shown schematically in Scheme 2 and may be summarized as follows.

Scheme 2

(1) Reduction of cytochrome *a* in *caa*₃ by exogenous cytochrome *c* follows bimolecular kinetics [3,39] with a second-order rate constant ($1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) not significantly different from that of *aa*₃ ($2.1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) (see Figure 3A). Therefore in *caa*₃ the incoming exogenously added cytochrome *c* is not hindered by covalently bound cytochrome *c*, a result which is at variance with the *caa*₃ derivative obtained using EDC [13]. This finding also implies that since exogenous cytochrome *c* can donate electrons to cytochrome *a*, the ET pathway either is direct or occurs via *Cu*_A in spite of the cross-linked cytochrome *c* at the high-affinity site.

(2) The *caa*₃ and *aa*₃ turnover rates determined from the exponential oxidation of free cytochrome *c* are identical (see Figure 3B). This indicates that the cross-linking procedure does not affect the rate-limiting step of the cytochrome oxidase reaction, which we assign [34] to the monomolecular ET from cytochrome *a*-*Cu*_A to cytochrome *a*₃-*Cu*_B when the latter site is in the oxidized state. This result is also at variance with the EDC-treated enzyme in which 40–80% inhibition of the catalytic efficiency was reported, accounting for an increase in the cytochrome *a* steady-state reduction level. Inhibition may have arisen from modification of some of the numerous carboxylate groups present in beef heart cytochrome oxidase (66 aspartates and 72 glutamates [15]), or alternatively by intrasubunit cross-linking. Enzyme inhibition was not observed in the present experiments, a result most likely related to the fact that the cross-linking procedure employed here involves only one sulphhydryl residue (on subunit III) being modified.

(3) From the steady-state experiments carried out in the presence of the artificial TMPD/ascorbate reducing system it is clear that cross-linked cytochrome *c* is not capable of efficiently delivering electrons to the oxidase, since: (i) the steady-state reduction level of bound cytochrome *c* is always close to 100%; (ii) immediately after exposure to excess *O*₂ the cytochrome is not significantly oxidized, as also concluded by Alleyne et al. [13], whereas, under comparable conditions, free cytochrome *c* added to *aa*₃ is considerably more oxidized initially and at steady state (see Figure 4 and Table 1); (iii) cytochrome *c* irrespective of its state (cross-linked, bound or free) is reduced at the same rate by the ascorbate/TMPD system. These findings also suggest that the cross-linking procedure is irrelevant to the ability of covalently bound cytochrome *c* to release electrons to the oxidase, as both the EDC-modified and the disulphide-linked *caa*₃ complexes behave similarly. We are forced to conclude that (in so far as cross-linking is not crucial) the configuration of cytochrome *c* proper of the electrostatic complex is not competent to transfer electrons efficiently to *Cu*_A, in spite of the fact that (i) cytochrome *c* is bound very close to the *Cu*_A-binding site [12]; (ii) previous kinetic studies have identified *Cu*_A as the primary target [40,41] with an intracomplex ET rate of 2600 s^{-1} [42]; and (iii) chemical modification experiments have identified four carboxylate groups on subunit II that are involved in cytochrome *c* binding [43], the peptide carbonyl of Glu-198 being one of the *Cu*_A ligands [4].

(4) The increased steady-state reduction levels of cytochrome *a* in *caa*₃ are difficult to explain on the basis of an impaired rate of cytochrome *a* oxidation (as turnover rates are unaffected by cross-linking; Figure 3B and below), or of an increased ET efficiency from exogenously added cytochrome *c* (bimolecular rate constants almost identical). This finding requires an alternative explanation, based on the observation that the catalytic efficiency of *caa*₃ in the absence of exogenously added cytochrome *c* is much higher than that of the control *aa*₃ (see Figure 4 and steady-state lifetimes in Table 1). From the data shown in Figure 6 we calculate that reduction of cytochrome *a* by TMPD is 6.4 times more efficient in *caa*₃ than in *aa*₃, a factor which, according to simulations, is sufficient to account for the higher steady-state reduction levels of cytochrome *a* and the decrease in the steady-state lifetime which is observed experimentally.

(5) The non-linear Eadie–Hofstee plots shown in Figure 5 are very similar for the two enzyme samples. An interpretation proposed in several related models [19,44,45] for such a non-linear behaviour assumes two independent cytochrome *c*-binding sites, with different dissociation (*K*_m) constants with only one site being competent in ET [19]. If one of these two were to be 'covalently' occupied as a consequence of cross-linking, then a simple hyperbolic Michaelis–Menten behaviour would be expected with a *K*_m value corresponding to the site with the greater dissociation constant. This is clearly not the case. Thus, we conclude that the two-site model of cytochrome oxidase is very unlikely; also in view of results from another laboratory [46,47] which show that cytochrome *c* binding to the oxidase (either dimeric or monomeric) may be described by a simple binding isotherm. The biphasic Michaelis–Menten behaviour may reflect the presence of two different conformations of cytochrome oxidase characterized by different affinities for the substrate [48], the behaviour being consistent with expectations for the proton pumping properties of cytochrome oxidase.

The above findings may suggest that binding of cytochrome *c* may well occur to a site on cytochrome oxidase quite close to *Cu*_A, without, however, the expected ET process to this metal centre. For ET to occur, a structural rearrangement should take place allowing for optimal orientation in the redox pair. A pair of metalloproteins can form multiple complexes in solution and

an orientation optimal for recognition and binding need not be optimal for ET. Known examples include the complexes between cytochrome *c* and plastocyanin [49] and between cytochrome *c* and cytochrome *b₂* in yeast lactate dehydrogenase [50] in which, however, the configurational changes accompanying ET are rate-limiting. In such processes (called electron gating, conformational gating, or diffusive electron transfer) the well known dependence of ET rate on driving force [51] would not hold. Evidence for electron gating in cytochrome oxidase has been recently put forward. Since the interaction between cytochrome *c* and oxidase is electrostatic, it is expected that ET efficiency were to be impaired at higher ionic strengths. On the contrary, Hazzard et al. [52] find that formation of a strong electrostatic complex at low ionic strength is not coincident with the fastest ET rate which was observed at ionic strengths (0.11 M) at which complex formation is considerably weakened, a result which is consistent with the steady-state kinetics of cytochrome *c* oxidation as a function of ionic strength [53]. In the dynamic pathway involved in optimal ET efficiency between cytochrome *c* and the oxidase, the role of hydrophobic interactions and/or water molecule displacement at the contact surface may have been overlooked and needs to be reassessed.

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