Investigation of the electron-transfer properties of cytochrome *c* oxidase covalently cross-linked to Fe- or Zn-containing cytochrome *c*

Trevor A. ALLEYNE,*§ Michael T. WILSON,*¶ Giovanni ANTONINI,† Francesco MALATESTA,† Beatrice VALLONE,‡ Paolo SARTI‡¶ and Maurizio BRUNORI‡

*Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, Essex, U.K.,

†Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Rome, Italy,

and ‡Department of Biochemical Sciences and CNR, Centre of Molecular Biology, University of Rome 'La Sapienza', I-00185 Rome, Italy.

Complexes of cytochrome *c* oxidase and cytochrome *c* (Fe- or Zn-containing) have been prepared by 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide (EDC) cross-linking. The site to which the cytochrome *c* covalently binds has been identified as being the same, or close to, the site occupied by cytochrome *c* in the electrostatic complex which may be formed between the proteins. Stopped-flow experiments, monitored either at a single wavelength or through a rapid wavelength-scan facility, showed that covalently bound Fe-containing cytochrome *c* cannot donate electrons to cytochrome *a*. Free Fe-containing cytochrome *c* was, however, able to transfer electrons to cytochrome *a* in covalent complexes containing either Fe- or Zn-containing cytochrome *c*. Turnover experiments showed that the complexed enzyme remains catalytically competent but with decreased (40–80 %) activity. The steady-state levels of reduction of both free cytochrome *c* and cytochrome *a* in the covalent complex were higher than found in the control (uncomplexed) enzyme. These results are discussed with reference to the structure of the covalent complex and lead us to conclude that cytochrome *a* may accept electrons directly from free cytochrome *c* and that cross-linking impairs the redox properties of the Cu_A site.

INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1) catalyses the reduction of oxygen to water. In so doing it oxidizes its other substrate, ferrocytochrome *c*, to ferricytochrome *c*. This reaction requires, among other things, coupling of electron transfer from a single electron donor, cytochrome *c*, to the four-electron acceptor dioxygen. The enzyme accomplishes this task via a complex set of electron-transfer and protonation reactions, carried out by its four redox centres, cytochrome *a*, Cu_A and the binuclear cytochrome *a*₃/Cu_B site [1]. The complexity of these reactions is apparent in both the transient and steady-state kinetic behaviour of the enzyme [2]. For example, polarographic assays of the enzyme at low ionic strength (25 mM-Tris/HCl, pH 7.8) yield biphasic Eadie–Hofstee plots which have variously been interpreted to reflect the presence of two kinetically significant binding sites on the enzyme, a 'high-affinity' and a 'low-affinity' site [3,4], or alternatively the presence of a conformational change accompanying turnover [5].

A tight one-to-one electrostatic complex of ferricytochrome *c* and oxidized cytochrome *c* oxidase may be isolated under conditions of low ionic strength where the enzyme exhibits the high-affinity kinetic site, and it is therefore likely that under these conditions ferricytochrome *c* occupies this kinetically discerned site in the isolated complex. This site is thought to be close to the Cu_A centre in the enzyme [6] and thus it is of considerable interest to investigate the properties of this complex with respect to electron entry from free externally added cytochrome *c* as this may throw light on the problem of the primary electron-entry site(s), whether cytochrome *a* or Cu_A (or both). Experiments of

this kind have been carried out by Veerman *et al.* [7]. These authors found that, under conditions where free cytochrome *c* was in excess over the electrostatic cytochrome *c*–cytochrome oxidase complex, electron entry into the enzyme was very rapid and involved dissociation of the electrostatic complex by incoming positively charged ferrocytochrome *c*.

In the experiments we report here, the 1:1 electrostatic complex has been stabilized by cross-linking either the native (Fe) or the redox-inactive analogue (Zn) cytochrome *c* into the high-affinity site. We present evidence to show that electrons may still enter the enzyme rapidly, though more slowly than for the uncross-linked protein, a finding which bears on the problem of the primary electron-accepting site (whether cytochrome *a* and/or Cu_A). By transient spectroscopy, we show that, with the cross-linked complex, steady-state levels of reduction of cytochrome *a* and of externally added free cytochrome *c* are higher than those of the control experiment. We interpret these data to mean that electrons may directly enter cytochrome *a*, thus making it unlikely that *in vivo* Cu_A is the unique electron-entry site. The steady-state redox level is discussed in terms of modification of the electron-transfer pathway from cytochrome *c* to the oxygen-binding site, possibly related to a perturbation of Cu_A induced by the chemical modification and/or by blocking the high-affinity site on the enzyme.

MATERIALS AND METHODS

Cytochrome *c* oxidase was prepared from bovine heart by the method of Yonetani [8] and passed through a Sepharose 6B column (50 cm × 1.5 cm) equilibrated with 5 mM-sodium

Abbreviations used: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

§ Present address: Department of Biochemistry, University of the West Indies, Champs Fleurs, Trinidad and Tobago.

¶ Present address: Institute of Biochemistry, University of Cagliari, Cagliari, Italy.

¶ To whom correspondence and reprint requests should be sent.

phosphate buffer, pH 7.0, containing 1% Tween 80. Zn-containing cytochrome *c* was prepared as described by Vanderkooi *et al.* [9].

Cross-linking

Cytochrome *c* [either native Fe(III) or the Zn-containing derivative] (100 μM) was incubated for 3 h at room temperature in the dark with oxidized cytochrome oxidase (80 μM) in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide (EDC; 6.5 mM). The reaction medium was 5 mM-sodium phosphate, pH 7.0, containing 1% Tween 80. Excess unbound cytochrome *c* was separated from the resulting covalent complex by passage through a Sephadex G-100 column which had been equilibrated with 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside and 300 mM-NaCl. NaCl was removed by dialysis against the same 'salt-free' buffer. The spectrum of the complex was recorded in the visible region and its stoichiometry determined by use of the following absorption coefficients: cytochrome *c* (Fe^{2+}) $\lambda = 550 \text{ nm}$, $27600 \text{ M}^{-1} \cdot \text{cm}^{-1}$; cytochrome *c* (Zn) $\lambda = 422 \text{ nm}$, $243000 \text{ M}^{-1} \cdot \text{cm}^{-1}$; cytochrome *c* oxidase (dithionite-reduced) $\lambda = 605 \text{ nm}$, $21000 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

These absorption coefficients appeared to change little on complex-formation and cross-linking as the spectrum of the mixture did not change significantly during incubation. This view is consistent with previous results noting only very small spectral perturbations on complex-formation [10].

SDS/PAGE

SDS/PAGE was performed by using the discontinuous system of Laemmli [11]. The gels were prepared and electrophoresis conducted as described by Kadenbach *et al.* [12].

Stopped-flow experiments

Kinetic measurements were performed on a Durrum-Gibson-type stopped-flow apparatus.

Rapid wavelength-scan experiments were conducted using a similar instrument equipped with a diode array, yielding 1024 data points per 200 nm, interfaced to a rapid a/d converter and memory (Tracor Northern Ltd., Middleton, WI, U.S.A.).

Equivalence of cross-linked and electrostatic complex

Cross-linked complexes of cytochrome oxidase and either Fe- or Zn-cytochrome *c* having a range of partner stoichiometries were incubated at low ionic strength (10 mM-Hepes, pH 7.4, 0.5% Tween 80), with a 4-fold excess of native (Fe) cytochrome *c* for 30 min. Excess cytochrome *c* was removed by passage through a Sephadex G-75 gel-filtration column equilibrated with the same low-ionic-strength buffer. The spectrum of the complex was recorded in the visible region and the concentration of electrostatically bound cytochrome *c* determined by use of the absorption coefficients given above.

RESULTS

In agreement with others [13,14], we find that at low ionic strengths cytochrome *c* oxidase makes a tight 1:1 electrostatic complex with its substrate cytochrome *c*; this complex may be isolated by gel-filtration chromatography and may be dissociated into its separate components at higher ionic strengths ($\geq 0.1 \text{ M}$) [13]. Addition of EDC to the low-ionic-strength electrostatic complex is known to yield a covalent complex which does not dissociate on addition of salt [15]. In our hands, the stoichiometry of this complex approached 1 cytochrome *c* to 1 functional unit of oxidase, but the yield was found to be strongly dependent on experimental conditions and on enzyme preparation. Moreover, the freshness of the reagent EDC had an effect, and generally

with freshly purchased EDC higher cytochrome *c* to oxidase ratios could be obtained.

We have prepared a number of such complexes between cytochrome *c* oxidase and either native (Fe) cytochrome *c* or the redox-inactive form, (Zn) cytochrome *c*.

The site on cytochrome *c* oxidase occupied through electrostatic interactions by cytochrome *c* at low ionic strength and that to which cytochrome *c* may be cross-linked appear to be closely similar and are probably the same. This has been demonstrated by incubating cross-linked complexes of various partner stoichiometries with native cytochrome *c* at low ionic strength and, after passage through a gel-filtration column, monitoring the total cytochrome *c* associated with cytochrome oxidase. As shown in Fig. 1, irrespective of the value of the initial stoichiometry of the cross-linked complex, the final cytochrome *c* oxidase to cytochrome *c* ratio always approached unity. Thus the electrostatic and the covalent complexes are complementary, indicating that cytochrome *c* cross-links into the high-affinity site and thus cross-linking at this site prevents further association via electrostatic interactions.

SDS/PAGE revealed that, on covalent complex-formation, subunit II was largely depleted and a new band appeared with an apparent molecular mass consistent with the sum of the molecular masses of subunit II and cytochrome *c* (Fig. 2). Benzidine staining showed that this new band contained cytochrome *c*, the haem of which was not lost on SDS/PAGE, as it is covalently linked to the protein. However, some small fraction of subunit V was also cross-linked to cytochrome *c*. Thus, in agreement with others, we note heterogeneity in the cross-linking reaction, with subunit II being the major cross-linking site for cytochrome *c* [15], a portion of subunit V possibly being close by this site. As, however, the majority of the cytochrome *c* is covalently bound to subunit II, we conclude that it is to the complex between subunit II and cytochrome *c* that we may ascribe the changes described below in kinetic behaviour compared with that of the non-cross-linked enzyme.

Stopped-flow experiments showed that cytochrome *c* cross-linked to its oxidase was reduced by ascorbate at a rate some 5–10-fold lower than free cytochrome *c*, but this difference vanished in the presence of *NNN'*-tetramethyl-*p*-phenylenediamine (TMPD; 0.1 mM).

Stopped-flow experiments were also carried out in order to investigate the effect of covalently bound cytochrome *c* on both the fast electron-entry steps and the overall enzyme activity. Measurements of the enzyme activity, determined for several different preparations containing either Fe- or Zn-cytochrome *c*, indicated that the activity was impaired but not abolished. Different preparations exhibited activities which ranged from 40% to greater than 80% of that of the control enzyme, and no systematic difference was noticed between the Fe- and Zn-cytochrome *c* covalent complexes nor with the extent of cross-linking.

Stopped-flow experiments in which covalent complexes were rapidly mixed with reduced free cytochrome *c* showed that electron transfer to cytochrome *a* (see Fig. 3) was rapid and (not shown here) synchronous with oxidation of free cytochrome *c*. At low ionic strength the rate of electron transfer from free cytochrome *c* to cytochrome *a* was clearly lower in the cross-linked enzyme. Similar experiments carried out at $I = 0.3 \text{ mol/l}$ confirmed that the rate of electron entry into cytochrome *a* was lower than the control enzyme by a factor approaching 10-fold, and tended towards a cytochrome *c*-independent rate at high concentrations ($> 100 \mu\text{M}$) of free cytochrome *c*. Essentially identical results were obtained starting with the cyanide-inhibited enzyme or with the fully oxidized resting enzyme in air.

From these experiments we may conclude that the cross-linked

Fig. 1. Occupancy of the cytochrome *c*-binding site on cytochrome oxidase demonstrating that covalently and electrostatically bound cytochrome *c* complement each other

The histogram shows the ratio of cytochrome *c* to cytochrome *c* oxidase in the various complexes. ■, Contribution to this ratio made by cross-linked cytochrome *c*; ▨, contribution of electrostatically bound cytochrome *c*; □, overall ratio, which approaches unity irrespective of the initial ratio. Zn.X and Fe.X refer to covalent complexes between either Zn- or Fe-containing cytochrome *c* and cytochrome *c* oxidase. The subscripted numbers refer to different preparations of these covalent complexes. For further details see the Materials and methods section.

Fig. 2. SDS/PAGE of cytochrome *c* oxidase

The gel shows the four largest subunits, numbered I to IV, of a sample of cytochrome *c* oxidase (track A) and of cytochrome *c* oxidase cross-linked to ferricytochrome *c* (track B). In the latter, subunit II has disappeared and a new band, IIc, running at a position consistent with it comprising subunit II and ferricytochrome *c* has appeared.

enzyme may still accept electrons from free cytochrome *c*. However, the time-courses of the 'burst' phase of reduction of cytochrome *a* (Fig. 3) and of oxidation of exogenously added cytochrome *c* by the covalent cytochrome *c* complex at low ionic strength are similar to the time-courses of reduction of cytochrome *a* and oxidation of ferrocytochrome *c* by native oxidase at high ionic strength (see Fig. 3). This result indicates that covalently bound cytochrome *c* affects the kinetics of electron transfer to the oxidase from exogenously added cytochrome *c*²⁺. This effect is the result of at least two processes: (1) steric hindrance and (2) electrostatic repulsion. If the covalently bound

Fig. 3. Kinetics of electron entry into native and Zn-containing cytochrome *c* cross-linked to cytochrome *c* oxidase

Ferrocytochrome *c* (35 μ M after mixing) was mixed aerobically with either native cytochrome *c* oxidase (4.5 μ M after mixing) at low or high ionic strength (*a* and *b* respectively) or with Zn-cytochrome *c* cross-linked to cytochrome *c* oxidase at low ionic strength (*a*). Reduction of cytochrome *a* (monitored at 604 nm) is denoted by a downward deflection and reoxidation by excess oxygen by an upward deflection. The time per division is given for each trace. The buffer was 20 mM-Hepes, pH 7.4, containing 0.5% Tween 80. The ionic strength was altered by addition of NaCl. The temperature was 20 °C. (*a*) At low ionic strength (10 mM) the electron-entry 'burst' phase for the control enzyme is, as expected, lost in the dead time. Electron entry is seen to have occurred by monitoring reoxidation of cytochrome *a* (rising progress curve). The 'burst' phase is, however, observed under these conditions for the Zn-cytochrome *c*-cross-linked enzyme (Zn.X). (*b*) At high ionic strength (300 mM) the 'burst' is clearly discerned for the control enzyme in the ms time range.

cytochrome *c* were located at a site which partially overlaps the 'kinetically relevant' electron-transfer site on the oxidase, it would act partially to inhibit oxidation of externally added cytochrome *c*, without affecting the turnover number of the enzyme. Although we are unable to decide the relative roles of steric and electrostatic factors in this behaviour, we conclude that the site occupied by the covalently linked cytochrome *c* is close to that to which free cytochrome *c* donates electrons.

Parallel experiments on the same enzyme/complex preparation as used in Fig. 3, but monitored at 550 nm, showed that the half-time for oxidation of the total ferrocytochromes present (35 μ M) was increased by 15% by cross-linking, indicating that in this preparation the turnover activity of the enzyme was largely unmodified.

A series of experiments designed to monitor the redox state of the components in the approach to and during the steady state was performed using rapid-scan stopped-flow spectrophotometry. These experiments were carried out with the enzyme cross-linked to either Fe- or Zn-cytochrome *c* using ascorbate/TMPD as electron donors and oxygen as electron acceptor. In the absence of added free cytochrome *c*, both free and cross-linked enzymes turn over slowly, as the TMPD/ascorbate system delivers electrons with low efficiency. Under these conditions the cross-linked Fe-cytochrome *c* is fully reduced during the steady state, as shown unequivocally by the spectra recorded in the region around 550 nm where cytochrome *c* makes the major contribution (results not shown). Mixing fully reduced ferrocytochrome *c*-oxidase cross-linked complex with oxygen leads to oxidation of both cytochrome *a* and cytochrome

Fig. 4. Rapid-scan stopped-flow spectrophotometry of the overall oxidation of free cytochrome *c* by oxygen catalysed by free or cross-linked cytochrome *c* oxidase

Cytochrome *c* oxidase, either free or cross-linked to Fe- or Zn-cytochrome *c*, was mixed with excess free ferrocyanochrome *c* in the presence of sodium ascorbate (10 mM), TMPD (0.1 mM) and oxygen (250 μM). (a) and (d) show a set of 62 absorption spectra taken from time $t = 10$ ms to $t = 100$ s and over a wavelength range from 500 to 630 nm. The arrowheads indicate wavelengths, 550 nm and 605 nm, which predominantly report redox changes in cytochrome *c* and cytochrome *a* respectively. For clarity of presentation, the scaling of amplitudes was chosen arbitrarily; the time amplitudes may be seen by reference to panels below. (b) and (c) report the time-courses of the fractional reduction of free cytochrome *c* (550 nm) and cytochrome *a* (605 nm). The ΔA scale is also shown. These panels also compare the approach to length and collapse of the steady state which occurs on exhaustion of oxygen for the control (O) and Fe-cytochrome *c*-cross-linked cytochrome *c* oxidase (X) (0.9:1). The time-course at 550 nm shows only the free cytochrome *c* participating in the reaction. (e) and (f) show the time-courses for control (O) and Zn-cytochrome *c* cross-linked enzyme (X). Differences between the control profiles (e, c and f) reflect some differences in enzyme concentration.

Fig. 5. Kinetic difference spectrum (a_3^{2+} CO minus a_3^{2+}) for native and Fe-cytochrome *c*-cross-linked cytochrome *c* oxidase

The enzyme concentration was $4.4 \mu\text{M}$ (haem) dissolved in 10 mM-Hepes buffer, pH 7.4, containing 0.5% Tween 80. The enzyme was reduced by a small excess of solid sodium dithionite and equilibrated with 0.5 mM-CO. ●, control enzyme; ○, covalent complex with ferricytochrome *c*. The temperature was 20°C .

a_3 while leaving Fe-cytochrome *c* reduced, confirms that cross-linked Fe-cytochrome *c* cannot donate electrons to cytochrome *a*.

In the presence of free cytochrome *c* (1 or 2 electron equivalents), the enzyme turns over more rapidly, enters a steady state in which the components are partially reduced and, on exhaustion of oxygen, both the enzyme and free cytochrome *c* become fully reduced. Analyses of the time-courses from 10 ms to 100 s at 550 nm and 605 nm are shown in Fig. 4. The general behaviour of enzyme cross-linked with cytochrome *c* (Zn or Fe) is similar to that of the control enzyme, but the steady-state levels of both cytochrome *c* and cytochrome *a* differ from those of the control. As seen in Fig. 4, the steady-state levels of cytochrome *c* and cytochrome *a* in the cytochrome *c* cross-linked enzyme are more reduced; in particular, cytochrome *a* is 50–70% reduced in the steady state, whereas in the native enzyme this value is close to 10%. This observation is independent of the nature of the cross-linked cytochrome *c*, whether Zn or Fe. Spectral analysis of Fig. 4(a) shows that, in agreement with the results reported above, cross-linked Fe-cytochrome *c* is always fully reduced in the steady state. These observations confirm that cross-linked Fe-cytochrome *c*, though fully reduced, is not capable of delivering electrons to the enzyme.

The spectral and kinetic properties of cytochrome a_3 in the covalent complexes were probed by investigating the reaction of this centre with CO. The fully reduced CO derivative of the enzyme covalently bound to either Fe- or Zn-cytochrome *c* was exposed to a brief intense pulse of white light which fully dissociates CO. The rate constant ($7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) at which CO recombined with the enzyme and the spectral distribution of this reaction were found to be identical with those of the control enzyme, indicating that the ligand-binding site was unimpaired by the cross-linking procedure (see Fig. 5).

DISCUSSION

Structural information obtained from the 1:1 electrostatic complex of cytochrome *c* with cytochrome *c* oxidase led to the suggestion that cytochrome *c* binds to subunit II close to the invariant residues comprising the Cu_A binding site [6,15–18]. On

this basis it was suggested that this site, which is part of a hydrophilic domain protruding outside the bilayer, is the primary electron-entry site, thus refuting the earlier proposal [19] that cytochrome *a* directly receives electrons from cytochrome *c*.

Since the 1:1 electrostatic complex may be correlated with the high-affinity site observed (at low ionic strength) in steady-state assays [4], it was of interest to characterize the transient kinetics of this complex once stabilized by covalent cross-linking.

This covalent complex has been formed using either Fe-containing or Zn-containing cytochrome *c*, to ascertain whether the redox properties of the bound cytochrome *c* play an active role in electron transfer. All the kinetic experiments reported above were obtained using preparations with cytochrome *c* to oxidase ratios ranging from 0.8:1 to 1.1:1.

In agreement with others, we have found that cross-linking cytochrome *c* to cytochrome *c* oxidase by using carbodi-imides does not lead to a unique chemically homogeneous product [20]. Depending on conditions, the stoichiometry of cytochrome *c* covalently bound to the oxidase may vary. Competition experiments (Fig. 1) in which the complexes with a low cytochrome *c* to oxidase stoichiometry were incubated with an excess of free cytochrome *c* showed that there is only one high-affinity site, which may be occupied by either cross-linked or electrostatically bound cytochrome *c*.

As discussed above, the high-affinity site is thought to involve a region on subunit II close to the Cu_A binding site. Amino-acid-sequence analysis has shown that four invariant carboxylates (E126, D178, D193, E218) of subunit II are close to the Cu_A binding site and likely to be involved in electrostatic interactions with invariant lysines on cytochrome *c*. In agreement with this conclusion, Millett *et al.* [6] have shown that E126 and E218 on

Fig. 6. Schematic diagram of subunits I and II of cytochrome *c* oxidase

The approximate distribution of the metal centres is shown together with the likely site of cross-linking of cytochrome *c*. This scheme proposes that free cytochrome *c* may deliver electrons into cytochrome *c* oxidase even when this is cross-linked to a molecule of cytochrome *c*. The electron-entry site is, however, close to the covalently bound cytochrome *c* and this latter perturbs the kinetics of electron entry. It is also suggested that cross-linking may perturb the Cu_A site such that its electron-transfer properties (broken lines) are altered so that electron entry to the binuclear centre, and hence activity, is impaired.

subunit II are protected against reaction with a water-soluble carbodi-imide when cytochrome *c* is electrostatically bound to the oxidase.

Since we have shown that cytochrome *c* occupies the same site in the electrostatic and covalent complex, we presume that the cross-linking may involve a reaction between the invariant aspartates D178 and/or D193 with critical lysines in cytochrome *c*. Other studies have shown that bound cytochrome *c* also protects portions of subunit I from chemical modifications, thus indicating that cytochrome *c* makes contacts with both subunits I and II. We have confirmed these findings; our gels (see one example in Fig. 2) showed that subunit II contains the major site to which cytochrome *c* cross-links, but other subunits participate to a minor extent in the cross-linking reaction. We indicate schematically in Fig. 6 the contact between cytochrome *c* and cytochrome *c* oxidase in the major product.

Two important conclusions may be drawn from the transient spectroscopy stopped-flow experiments reported here. First, since electrons rapidly enter cytochrome *a* in the cross-linked enzyme, Cu_A cannot be the unique electron-entry site of cytochrome *c* oxidase. It is possible and even likely that *in vivo* both cytochrome *a* and Cu_A may accept electrons from ferrocycytochrome *c*. Secondly, covalently bound ferrocycytochrome *c* cannot donate electrons to cytochrome *c* oxidase, or does so only at a very low rate. Since the site of covalent reaction with carbodi-imides overlaps with the cytochrome *c* high-affinity site and because this is near Cu_A, this finding is surprising. Thus Cu_A cannot accept electrons from the cross-linked Fe-cytochrome *c*, either because the latter is cross-linked to the enzyme in an orientation unfavourable for electron transfer or, given that possible reaction sites for EDC are close to the putative Cu_A ligands (H181, C216, C220, H224), because the cross-linking reaction had modified the Cu_A site. Chemical modification of Cu_A ligands [21] (F. Malatesta, unpublished work) drastically lowers the redox potential of Cu_A rendering it inactive towards cytochrome *c*.

If inactivation of Cu_A is the reason why covalently formed cytochrome *c* does not donate electrons to the enzyme, then this may also account for the higher level of reduction of cytochrome *a* at steady state (Fig. 4). Thus modification would impair electron transfer between cytochrome *a* and Cu_A, which normally occurs at a high rate [1] and thereby lead to an increase in reduced cytochrome *a* at steady state. The cross-linked enzyme can nevertheless accept electrons from external ferrocycytochrome *c* into cytochrome *a* and reduce the binuclear centre, but without the necessary participation of Cu_A. The proposition that cytochrome *c* oxidase is active in reducing oxygen without the involvement of Cu_A is in agreement with the finding of Numata *et al.* [22], who reported that cytochrome *c* oxidase from *Nitrosomonas europaea* may be obtained in an active form even without Cu_A. Moreover, chemical modification of Cu_A by mercuric salts (*p*-hydroxymercuribenzoic acid) leads to an enzyme

in which Cu_A is redox inactive but which nevertheless retains partial activity and accepts electrons rapidly from cytochrome *c* into cytochrome *a* (F. Malatesta, unpublished work). The higher steady-state reduction level of cytochrome *a* in the cross-linked enzyme and the slower turnover number may be due to impairment of electron-transfer efficiency between the electron-entry sites and the binuclear centre by removal of the electron-transfer route via Cu_A.

It remains to be seen whether the proposed impaired electron transfer to Cu_A in the cross-linked enzyme is induced directly by the chemical modification or whether it results from covalently linking non-physiological partners. It should be remembered that the complex investigated here and by others is formed between oxidized cytochrome *c* oxidase and oxidized cytochrome *c*, whereas the natural partner is reduced cytochrome *c*.

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REFERENCES

1. Brunori, M., Antonini, G., Malatesta, F., Sarti, P. & Wilson, M. T. (1988) *Adv. Inorg. Biochem.* **7**, 93–153
2. Cooper, C. (1990) *Biochim. Biophys. Acta* **1017**, 187–203
3. Nicholls, P. (1974) *Biochim. Biophys. Acta* **346**, 261–310
4. Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1976) *J. Biol. Chem.* **251**, 1104–1115
5. Brzezinski, P. & Malmström, B. G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4282–4286
6. Millett, F., de Jony, C., Poulson, L. & Capaldi, R. (1983) *Biochemistry* **22**, 546–552
7. Veerman, E. C., Van Leeuwen, J. W., Van Buuren, K. S. & Van Gelder, B. F. (1982) *Biochim. Biophys. Acta* **680**, 134–141
8. Yonetani, T. (1961) *J. Biol. Chem.* **236**, 1680–1688
9. Vanderkooi, J. M., Adar, F. & Erecinska, M. (1976) *Eur. J. Biochem.* **64**, 381–387
10. Alleyne, T. & Wilson, M. T. (1987) *Cytochrome Systems; Molecular Biology and Bioenergetics* (Papa, S., Chance, B. & Ernster, L. eds.), pp. 713–720, Plenum Press, New York
11. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
12. Kadenbach, R., Jarausch, J., Hartmann, N. & Herle, P. (1983) *Anal. Biochem.* **129**, 517–521
13. Hill, B. C. & Nicholls, P. (1980) *Biochem. J.* **187**, 809–818
14. Alleyne, T. & Wilson, M. T. (1987) *Biochem. J.* **247**, 475–484
15. Millett, F., Darley-Usmar, V. & Capaldi, R. A. (1982) *Biochemistry* **21**, 3857–3862
16. Holm, L., Saraste, M. & Wikström, M. (1987) *EMBO J.* **6**, 2819–2823
17. Capaldi, R. A. (1990) *Arch. Biochem. Biophys.* **280**, 252–262
18. Bisson, R., Jacobs, B. & Capaldi, R. A. (1980) *Biochemistry* **19**, 4173–4178
19. Wilson, M. T., Greenwood, C., Brunori, M. & Antonini, A. (1975) *Biochem. J.* **147**, 145–153
20. Mauk, M. & Mauk, A. (1989) *Eur. J. Biochem.* **186**, 473–486
21. Gelles, J. & Chan, S. I. (1985) *Biochemistry* **24**, 3963–3972
22. Numata, M., Yamazaki, T., Fukumori, Y. & Yamanaka, T. (1989) *J. Biochem. (Tokyo)* **105**, 245–248