

Electron entry in a Cu_A mutant of cytochrome *c* oxidase from *Paracoccus denitrificans*. Conclusive evidence on the initial electron entry metal center

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Abstract A cytochrome *c* oxidase subunit II C216S mutant from *Paracoccus denitrificans* in which the Cu_A site was changed by site-directed mutagenesis to a mononuclear copper site [Zickermann, V., Wittershagen, A., Kolbesen, B.O. and Ludwig, B. *Biochemistry* 36 (1997) 3232–3236] was investigated by stopped-flow spectroscopy. Contrary to the behavior of the wild type enzyme, in this mutant cytochrome *a* cannot be reduced by excess cytochrome *c* in the millisecond time scale in which cytochrome *c* oxidation is observed. The results conclusively identify and establish Cu_A as the initial electron entry site in cytochrome *c* oxidase. Partial rapid reduction (ca. 20%) of the modified Cu_A site suggests that the mononuclear copper ion has a redox potential ca. 100 mV lower than the wild type, and that internal electron transfer to cytochrome *a* is $\geq 10^3$ -fold slower than with the wild type enzyme.

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1. Introduction

The electron transfer processes between cytochrome *c* and cytochrome oxidase have been extensively studied [1]. The experiments have been carried out following either cytochrome *c* oxidation or cytochrome *a* reduction, often at high ionic strength (i.e. neutral 0.1 M phosphate buffer) since the reaction is very fast approaching the diffusion limit of 10^8 M⁻¹ s⁻¹ [2–8] at low ionic strength. Probing the ET kinetics at wavelengths monitoring Cu_A reduction proved more difficult owing to the low extinction changes accompanying Cu_A reduction in the near-infrared charge transfer absorption band centered at ca. 830 nm (1 mM⁻¹ cm⁻¹) [9], and known examples include pulse radiolysis and flash photolysis experiments [10,11]. The results of these investigations indicate that: (i) at all cytochrome *c* concentrations tested, the time courses of cytochrome *c* oxidation and cytochrome *a* reduction are synchronous; (ii) from absorbance recovery calculations, the electron equivalents transferred to cyanide-inhibited oxidase approaches 2 per aa₃ functional unit at high cytochrome *c* concentrations, as expected from stoichiometry.

In such a scenario it is possible that both cytochrome *a* and Cu_A accept electrons *directly* from cytochrome *c* and *independently* of each other, or that cytochrome *a* and Cu_A

communicate very rapidly with only one of the two sites accepting the electron directly from cytochrome *c*. Indeed, the monomolecular ET kinetics between Cu_A and cytochrome *a* is fast with relaxation times of tenths of microseconds [10,11], and in a site-directed subunit II M227I Cu_A mutant from *Paracoccus denitrificans* [12], this relaxation is significantly slower.

The now available three-dimensional structure of cytochrome oxidase from *Bos taurus* [13,14] and *P. denitrificans* [15,16] strongly suggests Cu_A as the initial and unique electron entry site, since this bimetallic center is closest to the aqueous medium and cytochrome *a* is deeply buried in the protein matrix of subunit I and topologically inside the membrane.

In this study we analyzed the subunit II C216S mutant and probed the ET kinetics from reduced cytochrome *c*. In this mutant the Cu_A site has drastically changed as it contains only one copper ion, it has lost the characteristic 830 nm charge transfer band and resembles a type I copper site as judged by EPR [17]. Our results show that cytochrome *a* is not reduced in a time scale (milliseconds) in which oxidation of cytochrome *c* takes place. The very slow reduction (tenths of seconds) of cytochrome *a* by Cu_A is consistent with the extremely low turnover activity of this mutant [17]. Therefore, we may exclude cytochrome *a* as the direct electron entry site in cytochrome oxidase, and conclusively support the mechanism of electron entry in cytochrome oxidase proposed by others [18–20] and supported by structural data [21].

2. Materials and methods

Site-directed mutagenesis, expression of C216S (on subunit II) mutant enzyme in a deletion strain of *P. denitrificans*, and membrane preparation were carried out as described earlier [17]. Wild type and C216S cytochrome oxidase were purified by streptavidin affinity chromatography with an engineered monoclonal antibody fragment (F_v) directed against an epitope on the back side of the periplasmic domain of subunit II [22]. The procedure yields a cytochrome oxidase-F_v complex used in the present work. The C216S mutant was undistinguishable from the WT protein from the spectroscopic point of view, except for the near-infrared absorption band at 830 nm which is lacking in the mutant and for the liquid nitrogen EPR spectrum [17]. Cyano-cytochrome oxidase was prepared by incubating the proteins in 0.02 M phosphate buffer pH 7.6, containing 0.02% β-dodecylmaltoside, 1 mM EDTA, 100 mM KCl and 3 mM KCN for at least 5 h at 4°C. Stopped-flow experiments were carried out at 20°C using a thermostatted Applied Photophysics DX.17MV stopped-flow (Leatherhead, UK) (1 cm light path, dead time 1.3 ms). Curve fitting, computations and graphical procedures were carried out using the Matlab (Math Works, South Natick, MA, USA) or Micromath Scientist (Micromath Scientific Software, Salt Lake City, UT, USA) software.

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3. Results

When subunit II C216, one of the Cu_A ligands, is mutated to Ser, the binuclear center becomes a mononuclear copper site, resembling type I blue copper proteins [17]. In this investigation the ET kinetics from reduced cytochrome *c* were investigated with the cyanide derivative, which blocks the cytochrome a_3 - Cu_B site; thus, electrons entering the oxidase will distribute in between Cu_A and cytochrome *a*, according to their redox potentials. The result of this experiment, depicted in Fig. 1, shows that there is no significant reduction of cytochrome *a* in the C216S mutant in the time scale (1 s) in which the wild type is significantly reduced. At 605 nm (Fig. 1A), where cytochrome *a* reduction is followed, absorbance recovery of wild type accounts for ca. 70% of the expected value (using an extinction coefficient of $9 \text{ mM}^{-1} \text{ cm}^{-1}$ [23]). In the mutant, reduction of cytochrome *a* takes place on a much longer time scale (Fig. 1C) and with a rate constant which is ca. 1500-fold smaller than that of the wild type protein (see figure legend). Nonetheless, in the C216S mutant, oxidation of cytochrome *c* takes place, as probed at 550 nm (Fig. 1B), suggesting that the mononuclear copper site must be partially reduced by cytochrome *c*. From the absorbance change at 550 nm, we calculate that approximately 20% of the expected electrons are delivered to the modified Cu_A in C216S. This in turn indicates that the redox potential of the mononuclear Cu_A site is approximately 100 mV lower than that of the wild type enzyme. Moreover, since the modified Cu_A site can still transfer electrons to cytochrome *a*, albeit at a very reduced rate (Fig. 1C), the introduced mutation must have drastically increased the ET activation barrier between Cu_A and cytochrome *a*, despite the increased driving force (see above). We conclude that cytochrome *a* is not significantly reduced by cytochrome *c* in the time scale (see Fig. 1) in which oxidation of cytochrome *c* by modified Cu_A has already oc-

curred. Therefore, a direct functional interaction between cytochrome *a* and cytochrome *c* may be excluded, and on this basis, we support the mechanism whereby electrons enter the oxidase exclusively via Cu_A . Since the mutant enzyme as purified (see Section 2) is obtained as a complex with an F_V fragment [22], we carried out control experiments with the wild type protein purified with an identical procedure, which yields a fully active enzyme. The result (Fig. 1) and additional data (not shown) indicate that the bimolecular rate constant for cytochrome *c* oxidation of the wild type enzyme complexed with the F_V fragment is, within experimental error, identical to that of the wild type enzyme purified by alternative procedures not involving the affinity technique used here [24]. Therefore the lack of efficient ET to cytochrome *a* in the C216S mutant cannot be explained by steric interference of the bound antibody fragment, in agreement with the location of the F_V fragment in the crystal structures of the 4- and 2-subunit enzymes [15,16].

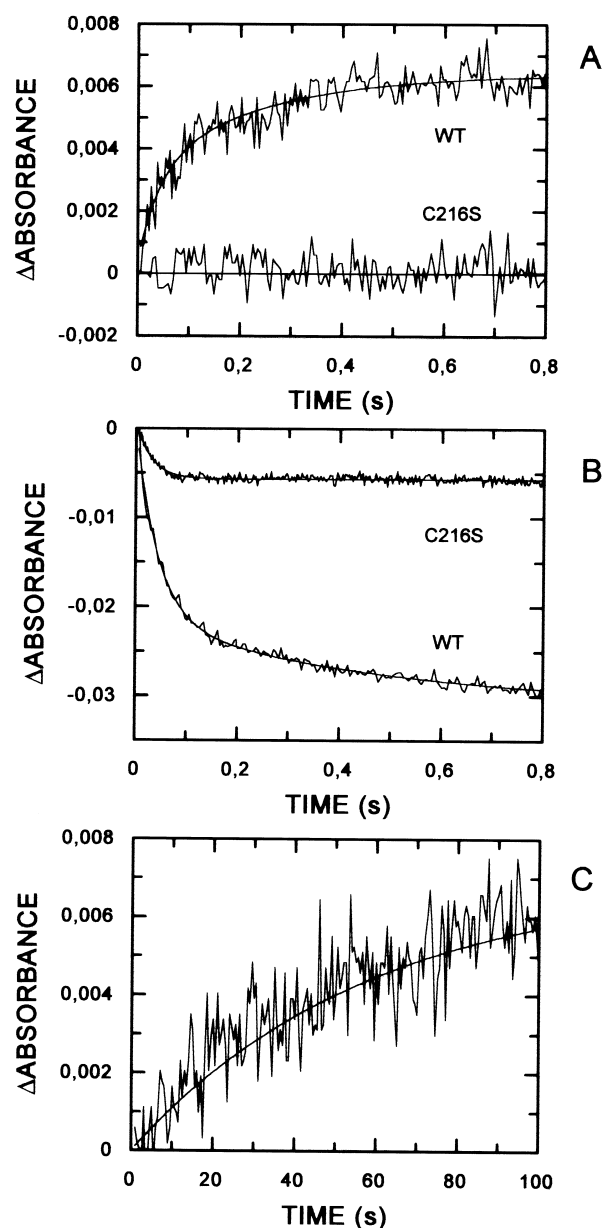
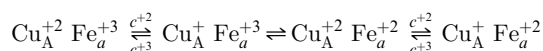


Fig. 1. Electron transfer from cytochrome *c* to wild type or mutant (C216S) cytochrome *c* oxidase. $2.4 \mu\text{M}$ wild type or C216S cytochrome *c* oxidase, in 20 mM phosphate buffer pH 7.6 containing 1 mM EDTA, 100 mM KCl, 0.02% β -dodecylmaltoside and complexed with 3 mM KCN, were mixed in the stopped-flow apparatus against $56.8 \mu\text{M}$ ferrocyanide *c*. The latter was prepared by reduction with 2 mM sodium dithionite for 10 min and subsequent passage through a G25 Sephadex gel filtration column to remove the excess reductant. The ET kinetics were probed at 605 nm (cytochrome *a* reduction, panels A and C) or 550 nm (cytochrome *c* oxidation, panel B) and time courses fitted to 2 (wild type) or 1 (C216S) exponential relaxations. The results of the fitting session, shown as solid lines, are as follows. Wild type at 605 nm: $k_{\text{FAST}} = 22.8 \pm 5.1 \text{ s}^{-1}$; $k_{\text{SLOW}} = 4.3 \pm 0.5 \text{ s}^{-1}$; amplitudes: $A_{\text{FAST}} = (2.8 \pm 0.4) \times 10^{-3}$; $A_{\text{SLOW}} = (3.1 \pm 0.4) \times 10^{-3}$. Wild type at 550 nm: $k_{\text{FAST}} = 22.9 \pm 0.5 \text{ s}^{-1}$; $k_{\text{SLOW}} = 2.20 \pm 0.05 \text{ s}^{-1}$; amplitudes: $A_{\text{FAST}} = (2.19 \pm 0.02) \times 10^{-2}$; $A_{\text{SLOW}} = (9.5 \pm 0.1) \times 10^{-3}$. C216S 605 nm: $k = (1.7 \pm 0.07) \times 10^{-2} \text{ s}^{-1}$; amplitude: $A = 7 \times 10^{-3} \pm 2 \times 10^{-4}$. C216S at 550 nm: $k = 33.3 \pm 1.6 \text{ s}^{-1}$; amplitude: $A = (6.3 \pm 0.2) \times 10^{-3}$. Note differences in the time scale in A-B and C. In all cases the shown traces represent the average of ≥ 4 independent experimental determinations. Parameters were first fitted using a simplex algorithm available in the MATLAB software (the 'fmins' function) and the obtained parameter values were used as entries for a MicroMath Scientist non-linear fitting routine based on a Levenberg-Marquardt algorithm (see Section 2). From the amplitudes at 550 nm we calculate that 1.38 and 0.28 electrons/mol oxidase are transferred from cytochrome *c* to wild type and C216S cytochrome oxidase in the burst reaction, respectively.

4. Discussion

In this work we have demonstrated that cytochrome *a* cannot receive electrons directly from cytochrome *c*, as probed by studying the ET kinetics to a Cu_A site mutant of *P. denitrificans* oxidase in which one of the two cysteine ligands (C216) is replaced by serine. Therefore, contrary to our hypothesis [25] and in agreement with others (see [26] for a review), electrons must enter the oxidase from the Cu_A site. The idea that cytochrome *a* may be a bona fide electron entry site in parallel with Cu_A became difficult to sustain once the three-dimensional structures of cytochrome oxidase from *P. denitrificans* [15,16] and *B. taurus* [13,14] had become available, and is now excluded by kinetic experiments.

The simplest mechanism which accounts for early ET kinetics from cytochrome *c* to the cyano-oxidase at relatively high ionic strength is the following:



i.e. two bimolecular reactions between cytochrome *c* and Cu_A coupled by an intramolecular ET reaction in which Cu_A and cytochrome *a* exchange an electron. Since the standard redox potentials of cytochrome *c* and Cu_A are very similar (with cytochrome *c* being a slightly better reductant), the bimolecular reactions are likely to have equilibrium constants close to 1 (although it is known from spectroelectrochemical studies that there is an anticooperative interaction between Cu_A and cytochrome *a* in the mixed valence CO adduct [27]). Therefore, the driving force for cytochrome *c* oxidation in cyano-oxidase must be provided by the higher stability of ferrocyanide *a* relative to cuprous Cu_A.

This mechanism is valid only in neutral 0.1 M phosphate buffer or at higher ionic strength values. At lower ionic strengths (e.g. below 10 mM) the reaction is more complex because of the formation of a stable 1:1 complex [28] than that isolated between beef heart cytochrome oxidase and cytochrome *c* both in the oxidized states. Under such conditions, cytochrome *c* binds to a site which, by definition, is referred to as the high affinity site [29], involving some clustered subunit II surface carboxylates (E126, D135, D159 and D178, in *P. denitrificans* numbering) which delimit a hydrophobic patch (containing the conserved residues W121 and Y122) in close proximity to Cu_A. We have shown [30] that each of these acidic residues contributes a small fraction of the overall binding free energy and that W121 is crucial for efficient electron transfer [31]. At low ionic strength complex formation is essentially driven by the surface electrostatic potential on subunit II and I which orients the positively charged cytochrome *c* to maximize all possible interactions. The resulting complex is stable; however, it is not in a configuration optimal for efficient ET, as suggested by the bell-shaped dependence of the ET activity on ionic strength [30,31] and by photo-induced ET experiments [32,33]. Indeed, optimal ET takes place at intermediate ionic strengths, which considerably weakens the electrostatically stabilized high affinity site complex prevailing at low ionic strength. The concept of a broad binding site is the best account for the whole set of observations which suggest Cu_A to be the only electron accepting site in the oxidase from cytochrome *c*.

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