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Induction of photochemical auto-reduction of cytochrome-*c* oxidase by an organic peroxide

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Abstract

Cytochrome-*c* oxidase aa_3 (CcO) from *Paracoccus denitrificans* interacts with tertiary butyl hydroperoxide (t-Bu–O–O–H, TBHP) by forming an adduct as indicated by an absorption shift at 408/432 nm and the induction of photochemical auto-reduction. The adduct was stable at room temperature for several days even under aerobic conditions. Upon irradiation (413 nm) of the adduct, a photoproduct, similar to the oxygenated mixed valence species (607 nm form), was formed, as indicated by the 418/442 and 607 nm signals in the absorption-difference spectrum. It is concluded that the adduct formation changes the photochemical properties of heme a_3. A molecular model for the binding mechanism of TBHP to CcO and for the photochemistry of heme a_3–TBHP adduct is proposed.

Keywords: Cytochrome-*c* oxidase; Peroxide binding; Photochemistry

1. Introduction

In recent years, great progress has been achieved in understanding the structure and function of cytochrome-*c* oxidase (CcO) [1–3]. X-ray crystallographic models of the structure of the resting (ferric) form of CcO are available up to 2.4 Å resolution [4,5], and transport pathways for protons and oxygen have been identified (for reviews see J. Bioenerg. Biomembr., issue of February 1998). Many details of the mechanism of the oxygen reduction in the binuclear heme–copper center of CcO have been clarified by application of time-resolved resonance Raman (RR) spectroscopy to enzyme samples reacting with different oxygen isotopes [6,7].

CcO is known to undergo various photophysical and photochemical processes which can provide information about the electronic structure of the functionally crucial molecular devices. In general, either the apoprotein or the cofactors of CcO can undergo photochemical reactions. The first case, photo-reactions of the apoprotein [8], seems to be limited to UV irradiation (< 300 nm) which, in oxidized CcO under aerobic conditions, causes shifts of the heme absorption bands (difference signal at 414/438 nm and positive signals at 577 and 607 nm) indicating photochemical auto-reduction. UV radiation (from 230 to 255 nm) also leads to the reversible appearance of an RR band at 1654 cm^{-1} of yet unknown origin (Ma-

Abbreviations: CcO, cytochrome-*c* oxidase; RR, resonance Raman; TBHP, tertiary butyl hydroperoxide

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tysik et al., unpublished results). In the second case, the photo-reactions of the cofactors, a variety of different observations have been reported. It is well known that binding of carbon monoxide to the reduced heme can be cleaved photochemically. Photochemically-induced absorption spectral changes have been observed for reduced CcO at low temperature [9]. For some preparations of the oxidized enzyme, photochemical auto-reduction induced by blue laser radiation under anaerobic conditions has been reported previously [10–12]. These processes, indicated by absorption difference maxima at 413/445 and 605 nm, have been shown to be immediately reversed upon exposure to air, which has been interpreted in terms of a photochemically-induced turnover of the oxygen-reduction cycle [12]. Salmeen and Babcock observed that the photosensitivity of the oxidized enzyme depends 'for unknown reasons' on the buffer [13] and applied a flowing cell to avoid photochemical auto-reduction [14]. In other RR studies on oxidized CcO, using a rotating quartz cell, no photochemical auto-reduction has been observed [15].

One of the unresolved questions concerning the photochemistry of CcO refers to the structural differences controlling the photochemical behavior of heme a_3, which in some, but not in all, sample preparations lead to fast photochemical auto-reduction. In the present study, we provide data showing that interaction of tertiary butyl hydroperoxide (TBHP) with CcO is able to induce the photochemical auto-reduction during an RR experiment. A molecular model, explaining the principle of such a photochemical switch is discussed.

2. Materials and methods

CcO (heme aa_3) purified from *Paracoccus denitrificans* [16] was dissolved in dodecyl maltoside (DM) buffer (100 mM Kpi, 100 mM NaCl, 1 mM EDTA, 0.2 g/l DM, pH 8.0). The optical density at 424 nm was ca. 1.8 (1 cm pathlength) for both absorption and Raman spectroscopic experiments (15 μM enzyme concentration). TBHP solution (purum, ca. 70% in water) was purchased from Fluka. Twenty-five μl of 2% TBHP in water (v/v) was added to 100 μl enzyme solution (30 mM final concentration TBHP). Such CcO preparations have been shown to be photochemically inert under the conditions of an RR experiment [15].

The RR spectrum was measured with a double monochromator (Spex 1403) equipped with a Peltier cooled photomultiplier (Burley C31034/A02). The spectral resolution was 3.8 cm^{-1}. The laser excitation wavelength was 413 nm and the power at the sample was ca. 50 mW. Samples were replaced after 90 min exposure time. Total accumulation time was 6 h. The RR spectra were measured at ambient temperature using a rotating quartz cell (spinning frequency ca. 10 Hz). The absorption spectrum was recorded with a UV-visible spectrometer (Shimadzu).

3. Results

The absorption spectrum of the oxidized CcO shows maxima around 424, 600 and 812 nm (Fig. 1). In the dark, the presence of TBHP induces a small shift in the region of the Soret maximum from 424 to 426 nm on a time scale of minutes, which was clearly detectable by absorption-difference signals at ca. 408 and 432 nm (Fig. 2A). Interestingly, no change in the Q band region was observed (Fig. 2B). A small increase of background could be due to slight coagulation of protein. CcO was stable in this form for several days under aerobic conditions at room temperature.

Upon 413 nm irradiation in the RR experiment, differences in the absorption spectrum are noted in the Soret band region at 418 and 442 nm compared

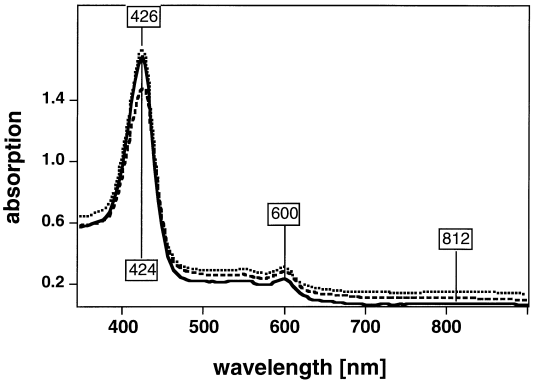

Fig. 1. Absorption spectra of the reaction products of TBHP with CcO: oxidized state without TBHP (solid line), adduct with TBHP (dotted line) and the sample after the RR experiment (dashed line).

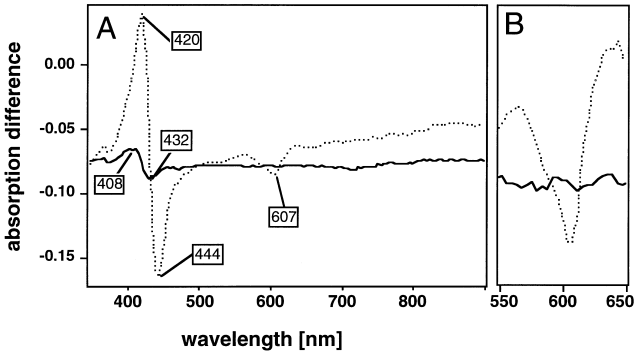

Fig. 2. (A) Absorption difference spectra of the reaction products of TBHP with CcO. Oxidized state without TBHP minus adduct with TBHP (solid line), and sample before minus after the RR experiment (dotted line). (B) Spectral region of the visible absorption-difference signals on an expanded scale (factor 6).

to the oxidized form (Fig. 2). In the visible spectral region, a significant difference signal at around 607 nm is detectable (Fig. 2B). The absolute position of the Soret maximum remains largely unchanged at ca. 426 nm (Fig. 1).

The RR spectrum (Fig. 3) shows that the position of the oxidation-state marker band ν_4 is at 1370.9 cm^{-1}. The RR spectrum looks similar to that of the oxidized state (6-coordinated a^{3+} and 6-coordinated a_3^{3+}) [15] and that of the oxygenated mixed-valence form (6-coordinated a^{3+} and 6-coordinated a_3^{3+} or a_3^{4+}) [17]. The oxidation state marker band is not split as known from the ligand-free mixed valence form (6-coordinated a^{3+} and 5-coordinated a_3^{2+}) [17], implying that heme a_3 is not reduced. On the other hand, it can neither be ruled out nor confirmed whether heme a_3 is further oxidized. A careful inspection of the RR spectrum reveals no indication that the structures of heme a and a_3 are affected by interaction with TBHP.

4. Discussion

Samples of oxidized CcO, which were studied under exactly the same experimental conditions but without addition of TBHP, do not show photochemical auto-reduction during RR measurement [15]. Hence, the interaction with TBHP modifies the photochemical properties of CcO.

CcO reduces H_2O_2 to water with H_2O_2 acting both as substrate and as reducing agent. At low concentrations of H_2O_2, the 607 nm species is mainly observed. At high concentrations, the subsequent reaction intermediate, characterized by an absorption difference band at 580 cm^{-1}, prevails (for review see [2]). Interaction of beef heart CcO with ethyl peroxide has been investigated by Wrigglesworth et al. [18,19] who found the 607 nm species, which did not proceed to further intermediates. On the other hand, Vygodina et al. observed a turnover of beef heart CcO induced by organic hydroperoxides including TBHP [20,21], presumably due to metal-catalyzed H_2O_2 formation. Our results for the interaction of bacterial CcO with TBHP in the dark show neither formation of the 607 nm form nor a turnover, but a spectral shift in the Soret region and the induction of photochemical auto-reductivity. This form of interaction is well distinguished from the reactions of peroxidases and myoglobin with TBHP (Matysik and Hildebrandt, unpublished results).

Direct binding of TBHP to the heme a_3 iron is unlikely as it would be reflected by the RR spectrum. Also, the formyl substituents of both heme a and heme a_3 are not affected since the corresponding stretching modes at 1647 cm^{-1} (heme a) and 1670 cm^{-1} (heme a_3) are unchanged. These modes are known to be sensitive indicators for changes of the heme environment [15]. On the other hand, interaction with TBHP alters the electronic properties of heme a_3. Binding of TBHP to Cu_B could explain these observations. Due to its hydrophobic properties, it is likely that TBHP approaches the heme–copper binuclear center via the oxygen channel connecting the binuclear heme–copper center to the membrane phase (Fig. 4A). It has been shown that

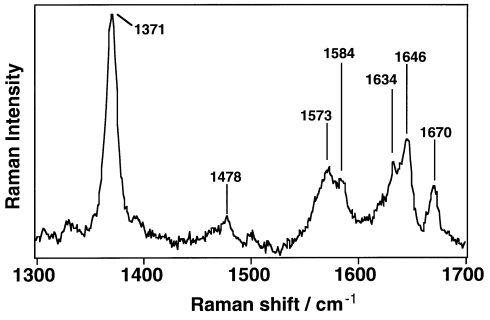

Fig. 3. High-frequency RR spectrum of the compound formed by the reaction of TBHP with CcO.

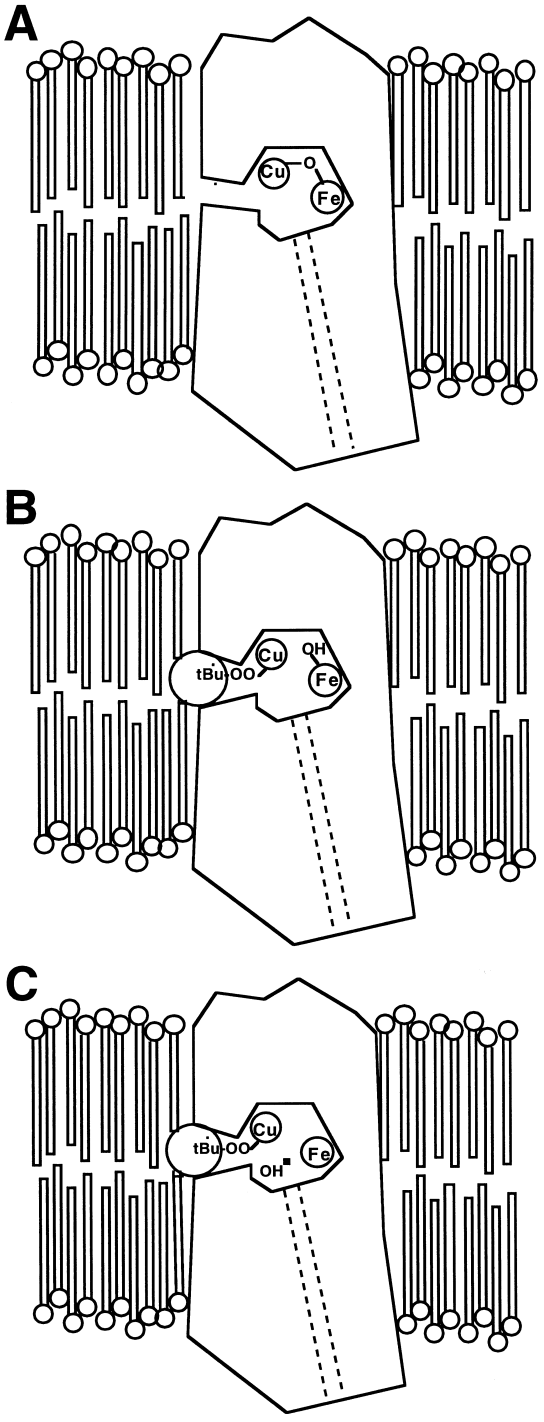

Fig. 4. Schematic representation of the binuclear center of CcO during TBHP binding. The oxygen channel links the membrane phase with the binuclear heme–copper center. (A) In the resting state, a μ-hydroxo ligand bridges heme-a_3 iron and copper Cu_B. In this state, photochemically formed hydroxy radicals (OH•) are not released. The dashed lines show proton channel K, which may help to remove chemically formed water from the binuclear center. (B) TBHP binds to copper Cu_B. TBHP binding disables the formation of a ligand bridge (e.g. by pulling away Cu_B from the heme iron). (C) In this state, heme a_3 may become photochemically reduced since OH• can diffuse away.

CO [22] and O_2 [23] first interact with Cu_B before reaching heme a_3. Molecular oxygen is rapidly channelled from the oxygen-rich membrane phase into the binuclear heme–copper center. Binding to CcO is described by a second order rate constant of 10^8 M^{-1} s^{-1}. It is at least 10 times faster than O_2 binding in most other heme proteins [24], suggesting a diffusion-controlled transport pathway with low restriction by protein residues. These findings are in line with the structural model of the oxygen channel [25]. Starting from the hydrophobic pore A [4], formed by transmembrane helices II to VI, a cleft between the two helix bundles II and VI opens a hydrophobic channel leading to Cu_B. The distance from the center of the cleft to Cu_B is ca. 10 Å. Between Phe-100 (helix II) and Phe-274 (helix VI), the cleft has an opening of at least 5 Å. Hence, the cleft provides access for a TBHP molecule, which has a maximum diameter of ca. 5 Å. Indeed, only the side chains of Glu-278 and Val-279 may cause some steric constriction. Hence, it is reasonable to assume that TBHP reaches the binuclear heme–copper center by the same transport path and binds to Cu_B (Fig. 4B). Due to its steric requirements, TBHP binding to Cu_B may block the oxygen channel.

When TBHP binds to Cu_B, it may replace the hydroxyl ligand, bridging the copper and the heme. Since the formation of a μ-hydroxo bridge is structurally restricted, it is possible that Cu_B is just slightly displaced from the iron. A structural flexibility of the Cu_B center has also been discussed in terms of crystallographic data [4,26] and, furthermore, recent Fourier transform infrared spectroscopic results revealed two different Cu(II)–CO stretching frequencies [27]. The conformational flexibility of Cu_B may be related to its different tasks during the reaction cycle. Large Fe–Cu distance may facilitate breaking of the O–O bond and short Fe–Cu distance may prevent formation of OH• radicals in the resting state. The broad feature in the absorption spectrum induced by the interaction of TBHP with CcO may

represent a charge-transfer band of the axial ligand–iron system, which is affected by weakening of the interaction between Cu_B and the axial ligand. Following this idea, it would be understandable why there is no effect in the red spectral region and on the geometry of heme a_3. Under these conditions, photochemically formed OH• radicals are not fixed anymore and can diffuse away from the reduced heme (Fig. 4C).

The photochemical auto-reduction of the heme in CcO may be based on the same mechanism as the formation of ferrous iron porphyrin and OH• radicals from Fe(III) porphyrins upon blue irradiation in the absence of O_2 [28]. This mechanism includes an intramolecular electron transfer from the axial ligand (a hydroxo group) to the heme iron [29]. For this reaction, a quantum yield ranging from 10^{-5} to 10^{-2} is reported, depending on (i) the efficiency of the charge separation in competition with radiationless deactivation channels and (ii) the diffusion rate of the OH• radicals from the heme. In the case of CcO, the OH• diffusion away from the heme is only possible if there is no interaction of the heme bound hydroxy group with Cu_B. This implies that the probability for the heme–ligand-induced photochemical auto-reduction depends on the strength of the interaction of the axial ligand (OH^-) with Cu_B and, therefore, it may also depend on the sample preparation.

It is still an open question, which molecule causes the observed electron density between the heme–iron and copper of the binuclear center. This electron density can be due to a bridging μ-hydroxo group [30], as also suggested for ba_3 oxidase from *Th. thermophilus* by RR studies [31]. Alternatively, a peroxy species [26] or two hydrogen-bridged ligands, an Fe–OH_2 together with a Cu–OH [32], have been suggested. The RR data of the reaction sequence show an isotope-sensitive mode at 356 cm^{-1}, which has been assigned to a bridged Fe–O–Cu species, followed by a mode at 450 cm^{-1} assigned to a Fe^{3+}–OH intermediate [1]. The unusually low frequency (compared to [33]) of the final hydroxy intermediate can be due to interaction of the hydroxy oxygen with Cu_B [34]. Therefore, the final species of the turnover, observed by RR spectroscopy, may have a bridged Fe–OH–Cu structure. It may already be identical with the resting enzyme, while still carrying the isotope label before the hydroxy group exchanges with bulk water.

Furthermore, analogous photochemically-induced one-electron transfer reactions from an axial ligand to the ferric iron have also been reported for various alcohols, carboxylic acids and amines [35,36]. Therefore, the mechanism proposed here for the photochemical auto-reduction of heme a_3 does not exclusively depend on, whether or not, the bridging ligand in the binuclear center is a μ-hydroxy group. Also other species bridging to Cu_B or hydrogen bonded to the distal side would be compatible with the proposed mechanism.

This interpretation of the photochemical auto-reduction mechanism of CcO also raises the question whether photo-induced effects were involved in previous RR experiments on the oxygen reduction by CcO. Photolability has indeed been observed in the earlier stage of the reaction course [37]. The 'enigmatic' [34] intermediate involving the oxygen sensitive band at 356 cm^{-1} has only been observed in some RR studies [7] and also no analogue and no branching of the reaction course have been reported by absorption spectroscopic studies [38]. It may arise from a stretching of a bridged Fe^{3+}–O^{2-}–Cu^{2+} entity [1]. The formation of this intermediate may be caused by a photochemically-induced increase of electron density in the anti-bonding π* orbitals (d_{xz}, d_{yz}), enhancing Lewis basicity of the ferryl and facilitating oxygen donation. Therefore, this species may originate from a laser-induced photochemical by-reaction and only occur in RR spectroscopic studies of some preparations.

5. Conclusions

CcO forms an adduct with TBHP, as indicated by an absorption shift in the blue spectral region and by the induction of photochemical auto-reduction upon 413 nm irradiation. This phenomenon is interpreted in terms of peroxide binding to Cu_B, substituting the μ-hydroxy group bridging Cu_B and the a_3 iron. The hydroxy group, remaining as terminal axial ligand on the iron, reduces heme a_3 by a photochemically-induced one-electron transfer.

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