REDOX REACTIONS IN MIXED-VALENCE CYTOCHROME *c* OXIDASE

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1. Introduction

Cytochrome *c* oxidase catalyses the electron transfer from the one-electron ($1e^-$) donor cytochrome *c* to the $4e^-$ acceptor dioxygen. The way in which the 4 redox centres in the enzyme participate in the electron transfer to the dioxygen molecule is not yet fully clarified. Anaerobic reductive titrations in the presence of CO indicate that both cytochrome a_3 and Cu_B need to be reduced for binding to occur [1–3]. This suggests that when the oxygen molecule is bound to cytochrome a_3, it is reduced in a $2e^-$ step.

As shown [1,4], a flow of electrons to cytochrome a_3 is observed when CO combines with cytochrome a_3 in the partially reduced enzyme. A reverse electron flow can be induced upon photo-dissociation of the mixed-valence CO compound both in the absence and presence of ligands for oxidized cytochrome a_3. In the presence of azide and formate as ligands for cytochrome a_3 photo-dissociation of cytochrome $a_3^{2+} \cdot CO$ in the mixed-valence CO oxidase causes an extensive electron flow from cytochrome a_3 to cytochrome *a* [4,5]. Similarly, EPR experiments showed that under these conditions not only cytochrome *a* is reduced, but also the EPR-detectable copper (Cu_A).

It is generally agreed that the weak absorption band at 830 nm in the spectrum of oxidized cytochrome *c* oxidase is in large part due to Cu_A, since a good correlation of the band was found with the intensity of the EPR-detectable copper signal [6,7]. It was, therefore, of interest to determine whether upon photo-dissociation of the mixed-valence CO compound in the presence of formate and upon association of CO with cytochrome a_3 in the dark, the intensity of the 830 nm band was affected. Indeed, as shown here, the band decreases when cytochrome *a* accepts an electron as a consequence of the light-induced oxidation of cytochrome a_3. In the dark, when CO combines with cytochrome a_3, the intensity of the band at 830 nm increases, concomitant with oxidation of cytochrome *a*. In line with [1,5] this shows, that both cytochrome *a* and Cu_A donate electrons when the cytochrome $a_3^{2+} \cdot CO$ compound is formed.

2. Materials and methods

Beef-heart cytochrome *c* oxidase was prepared as in [8,9]. The absorption coefficient (reduced minus oxidized) of cytochrome *c* oxidase was 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [10]. Chemicals were analar grade, mainly obtained from British Drug Houses. Carbon monoxide containing 10 ppm oxygen (Matheson Gas Products) was freed from oxygen by passing it through an adsorption column filled with Oxisorb (Messer, Griesheim).

The samples were made anaerobic in Thunberg cuvettes by repeated evacuation and flushing with helium (<1 ppm oxygen, Hoek Loos, Amsterdam). The mixed-valence CO compound was prepared by incubation of oxidized cytochrome *c* oxidase under CO (60 kPa) at 20°C for 3 h. Formate was added to the mixed-valence CO enzyme from a side-arm of the cuvette. Optical spectra were obtained on a Cary-17 recording spectrophotometer. Samples were photodissociated by illumination with white light of a Xenon lamp (150 W).

3. Results and discussion

When cytochrome *c* oxidase is incubated anaerobically with CO, the mixed-valence CO compound is formed, in which cytochrome *a* is oxidized and cyto-

chrome a_3 is reduced and combined with CO. Both the EPR copper signal at $g = 2$ and the 830 nm band show the same intensity [11–13] as those in the fully oxidized enzyme. Thus,this copper component (Cu_A), like the heme iron in cytochrome a, is in an oxidized state. The redox state of the copper component associated with cytochrome a_3 (Cu_B) is more difficult to ascertain. Reductive titration data in the presence of CO suggest that Cu_B is in the reduced state [1–3].

When formate (1 M) is added to the mixed-valence CO enzyme, the redox state of the redox centres is not affected, since the affinity of this ligand towards cytochrome a_3 is lower than that of CO in the dark [5]. Upon illumination of cytochrome $a_3^{2+} \cdot CO$ the optical spectrum in the Soret region and the EPR spectrum of the enzyme demonstrate that CO dissociates from the heme iron and formate binds to oxidized cytochrome a_3 with a concomitant transfer of electrons to cytochrome a and Cu_A [5].

Fig.1 illustrates the changes in the α-band and near-infrared region of the spectrum of cytochrome c oxidase. Upon illumination the band at 590 nm decreases in intensity caused by dissociation of cytochrome $a_3^{2+} \cdot CO$ while the band at 605 nm increases due to formation of reduced cytochrome a. The near-infrared region of the spectrum also shows a light-induced change. It is obvious that the band at 830 nm decreases in intensity, corresponding with reduction of Cu_A.

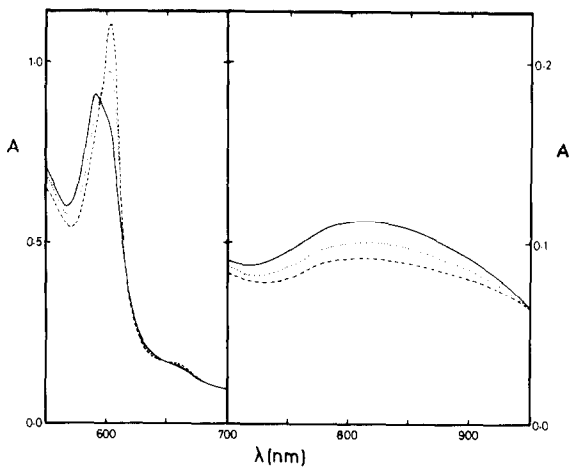

Fig.1. Photo-dissociation of the mixed-valence CO compound in the presence of formate. Cytochrome c oxidase was 37.6 μM in 1 M sodium formate, 0.1 M potassium phosphate (pH 7.4) and 1% Tween 80. (——) Before illumination; (– – –) after 1 min illumination; (. . .) after 15 min in the dark; pCO, 60.5 kPa.

When the illumination is ceased, formate is slowly expelled from its binding site and CO rebinding occurs. As fig.1 shows, this process is accompanied by both a decrease of the band at 605 nm and an increase of the band at 830 nm corresponding to oxidation of cytochrome a and Cu_A, respectively. Thus, these experiments demonstrate that both cytochrome a and Cu_A participate in redox reactions, not only upon photodissociation of the cytochrome $a_3^{2+} \cdot CO$ compound, but also when this compound is formed.

In principle it is possible to calculate from the absorbance changes in the optical spectra how many reducing equivalents are transferred in these light-induced reactions, since the absorption coefficients of the various redox centres are known. This approach requires, however, that the absorption coefficients of the various redox centres are not affected by the redox state of the enzyme, i.e., the proposed spectral interaction [14,15] between the heme groups of cytochrome c oxidase should be absent. As suggested and discussed by several authors [3,16–18] an interaction between the redox potentials of the redox centres is more likely. In that case the band at 605 nm can be used to calculate the change in the valence state of cytochrome a since the spectrum $a^{3+}a_3^{2+} \cdot CO - a^{3+}a_3^{3+}$ is isosbestic at 605 nm and the absorbance of $a^{3+}a_3^{3+}$ at this wavelength is nearly equal to that of $a^{3+}a_3^{3+}$ HCOOH. Addition of CO to fully reduced cytochrome c oxidase decreases the intensity of the α-band at 605 nm in the reduced minus oxidized difference spectrum ($a^{2+}a_3^{2+} \cdot CO - a^{3+}a_3^{3+}$) to 83% of that observed in the absence of CO ($a^{2+}a_3^{2+} - a^{3+}a_3^{3+}$). Thus the absorption coefficient of cytochrome a (reduced minus oxidized) at 605 nm is 20 $mM^{-1} \cdot cm^{-1}$, which is in line with [19–21]. For the determination of the valence state of Cu_A the band at 830 nm can be used. CO has no effect on this band, neither in the fully oxidized nor in the fully reduced state of cytochrome c oxidase, and cytochrome a_3 does not make a significant contribution to the absorbance [1,13]. The value of 2.3 $mM^{-1} \cdot cm^{-1}$ for the absorption coefficient (reduced minus oxidized) of this band found by us is similar to that reported elsewhere [1,22].

The change in the redox state of cytochrome $a_3^{2+} \cdot CO$ is more difficult to determine. However, the spectrum of the mixed-valence carboxy cytochrome c oxidase treated with ferricyanide ($a^{3+}a_3^{2+} \cdot CO$) minus that of

the fully reduced carboxy cytochrome *c* oxidase ($a^{2+}a_3^{2+} \cdot CO$) has an isosbestic point at 586.4 nm (not shown). Thus at this wavelength cytochrome *a* does not contribute to the reduced minus oxidized spectrum, whereas cytochrome a_3 still has a significant contribution. Consequently, the change in redox state of cytochrome a_3 can be calculated from the absorbance changes at this wavelength. For the spectrum $a^{3+}a_3^{2+} \cdot CO - a^{3+}a_3^{3+}$ at 586.4 nm, an absorption coefficient of 8.1 $mM^{-1} \cdot cm^{-1}$ is found. However, since the formate compound of cytochrome a_3 (a_3^{3+} HCOOH) is formed instead of unliganded cytochrome a_3^{3+}, this absorption coefficient should be corrected by the contribution of $a_3^{3+} - a_3^{3+}$ HCOOH in the difference spectrum, which is 1.9 $mM^{-1} \cdot cm^{-1}$ [5].

With the absorption coefficients of 20 $mM^{-1} \cdot cm^{-1}$ at 605 nm, 2.3 $mM^{-1} \cdot cm^{-1}$ at 830 nm, and 10 $mM^{-1} \cdot cm^{-1}$ at 586.4 nm for cytochrome *a*, Cu_A and cytochrome a_3, respectively, it is now possible to calculate the number of reducing equivalents involved in the light-induced redox changes. It is found from the experiments shown in fig.1 that upon going from the dark state ($a^{3+}a_3^{2+} \cdot CO$) to the illuminated state ($a^{2+}a_3$ HCOOH), Cu_A and cytochrome *a* accept 9.2 and 15.2 μM reducing equivalents, respectively, while cytochrome a_3 donates 13.5 μM. Vice versa, for the slow changes in the dark after illumination it was found that Cu_A and cytochrome *a* donate twice the number of reducing equivalents that can be accounted for by cytochrome $a_3^{2+} \cdot CO$ alone. Therefore we have to conclude that during oxidation and reduction of cytochrome a_3 in the presence of CO, the cytochrome $a_3^{2+} \cdot CO$ compound acts as a $2e^-$ donor and acceptor, respectively.

Our experiments demonstrate clearly that during photo-dissociation of cytochrome $a_3^{2+} \cdot CO$, followed by binding of formate to oxidized cytochrome a_3 and upon binding of CO to partially reduced enzyme, both Cu_A and cytochrome *a* participate in electron-transfer reactions. Quantitation of the corresponding optical changes shows, in line with [1–3], that the CO compound of cytochrome a_3 acts as a $2e^-$ acceptor. This suggests that also the EPR-undetectable copper (Cu_B) is involved in these light-induced oxidation–reduction reactions. Since CO and O_2 bind at the same site (cytochrome a_3) this indicates that dioxygen is reduced in a $2e^-$ step when it reacts with cytochrome a_3. However, it should be borne in mind that our results are obtained on the basis of the assumption that the spectral interaction between the heme iron groups of cytochrome *a* and a_3 is weak or absent and individual absorption coefficients can be obtained.

Recently it was proposed on the basis of X-ray data [23] that the 830 nm band was in fact partly due to Cu_B, which is in disagreement with the generally accepted view that the near-infrared band is mainly due to Cu_A. However, it was found (fig.1) that the optical absorption spectrum of the mixed-valence CO compound in the wavelength region 800–900 nm was identical to that of the fully oxidized enzyme. Since in the mixed-valence CO compound Cu_B seems to be cuprous, whereas it is cupric in the oxidized enzyme, at least a spectral difference between the two enzyme species in this wavelength region is expected. This is not observed and this shows that, if Cu_B contributes, its contribution is small. Furthermore, in our experiments both copper atoms change their valence state going from cupric to cuprous and from cuprous to cupric, respectively. Since it is not likely that both copper ions absorb at the same wavelength, one would expect that the 830 nm band behaves inhomogeneously. However, this is not the case, since fig.1 clearly shows that the intensity of the whole band from 800–950 nm is affected. We have to conclude, therefore, that Cu_B does not contribute significantly to the absorbance in this wavelength region.

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