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Assignment of Soret MLCT band of reduced form of copper binuclear cluster in cytochrome c oxidase film

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Abstract **Low concentration of dithionite results in the reduction of Cu-Cu binuclear and heme a active sites of the cytochrome c oxidase thin solid film immersed in the acidic phosphate buffer, but Fe-Cu binuclear center keeps in the oxidation state. It manifests as a negative peak at 426 nm and a positive one at ~408 nm in the difference spectra induced by dithionite. The former implies decrease of the oxidized form of heme a center, that is, Fe_a^{3+} Fe_a^{2+}. And the latter results from the contribution of metal-ligand charge transfer (MLCT) transition in the reduced binuclear Cu-Cu cluster, rather than from that of heme a center. This stronger Soret MLCT band must be helpful to overcoming the difficulty in distinguishing the weaker copper sign from the stronger one of iron when studying copper-iron protein.**

Keywords: metal-ligand charge transfer transition, reduced copper binuclear center, thin solid film, cytochrome c oxidase.

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Cytochrome c oxidase is a better candidate for study of molecular electrics and design of nano-devices [1]. There are many metal centers with electron-transfer active in it, including one Cu-Cu binuclear, one heme a, and a Fe-Cu binuclear centers[2,3]. This Cu-Cu center is a [2Cu-2S] cluster, which is input of electron into the oxidase. It receives the electron from cytochrome c and transfers to heme a center, then to Fe-Cu center for reduction of oxygen into water. This oxidase functions as an electron driving proton pump as well[4,5]. Spectroscopy is a conventional method for studying it and metal active centers in it[6,7]. These copper or iron components have respective characteristic absorption. But difficulty lies in studying copper component. The weaker copper sign is often covered by that of iron, which is stronger, so that is difficult to distinguish one from than other, or to be assigned. It is accepted that the unusual EPR signal and absorptive band at ~830 nm is the characteristic absorption of oxidized Cu-Cu center[8], but it is to weaker, only 2 cm^2/mmol. Furthermore, possible contribution from the Fe-Cu binuclear center has not been excluded perfectly[7]. No characteristic absorptive peak for reduced Cu-Cu binuclear center is available. It is a hindrance to studying further the electron transfer in the copper binuclear center.

Superposition of two or more bands from solution often forms an envelope, which is difficult to be assigned. The difference technique and thin solid film instead of the solution of the oxidase offer some advantages for assignment of the characteristic peak of respective metal active centers in cytochrome c oxidase, especially for assignment of the characteristic peak of copper. For the film of the cytochrome c oxidase from mitochodria of pig heart muscle, there is a peak at ~408 nm occurring in the difference spectra induced by low concentration of dithionite as electron donor. It is contribution mainly from MLCT transition of reduced form of double copper binuclear center (Cu^+-Cu^+). This discovery is of benefit to studying electron access into and out of the copper binuclear cluster by using cytochrome c oxidase film and difference technique.

1 Materials and methods

Cytochrome c oxidase was purified from pig heart muscle according to the method mentioned in refs. [10—12]. The purified oxidase was stored at 4℃ in a sealed tube filled with N_2. The thin solid film was formed on a plastic sheet by centrifugation or on the inner wall of the quartz cell by spreading the oxidase suspension[10]. and then dried under N_2 gas flow. Following this, it was washed with the buffer to remove the trace of detergent and then dried again under N_2 gas flow for two or three times. Finally, the film was kept in the N_2 atmosphere and stored at room temperature. The absorption measurement was carried out with a 1601 spectrophotometer.

2 Results and discussion

(1) Absorption spectra of the oxidase immersing in the phosphate buffer (50 mmol/L, pH5.8) are shown in Fig. 1. Only one peak at 419 nm occurs in the range of 350—500 nm if there is no dithionite in the buffer. The peak shifts blue induced by a small amount of dithionite, to 411 nm if the adding dithionite is increased up to 286 mmol/L. However, high amount (~10 or 20 mol/L) of dithionite changes this single peak into double peaks at 423 nm and 435 nm, respectively. These changes imply that a small amount of dithionite results mainly in reduction of both Cu binuclear and heme a centers, but Fe-Cu binuclear center is still in the oxidation state. While over-amount dithionite turnovers their redox states. The Fe-Cu center is reduced, but Cu-Cu and heme a centers returns to oxidation states. This turnover is more apparent in the difference spectra, as shown in Fig. 2. A negative peak at 426 nm and a positive one at 408 nm occur in the difference spectra induced by low concentration (286 mmol/L) of dithionte. The negative implies decrease of oxidized form of heme a center[9,14,15]. And the positive shifts blue, until to 403 nm when dithionite is up to 286 mol/L. To convenient statement, here this peak is still called the 408 nm peak. If dithionite is over-amount, the negative at ~411 nm and the positive at 442 nm with a shoulder at

423 nm are instead of the peaks at 426 nm and 408 nm. These characteristics mentioned above mean that low concentration of dithionite results in reduction of the Cu-Cu and heme a centers, but Fe-Cu binuclear center is still in the oxidation state.

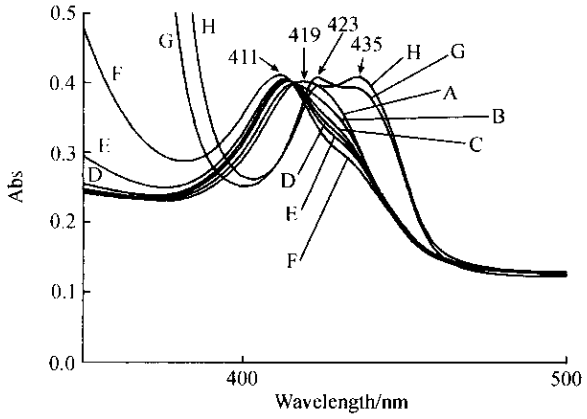

Fig. 1. Spectra of cytochrome c oxidase film immersing in 50 mmol/L sodium phosphate buffer, pH 5.8, (A), with 22.8 (B), 91 (C), 143 (D), 211 (E), and 286 mmol/L (F), as well as 10 (G) and 20 mol/L (H) dithionite, respectively.

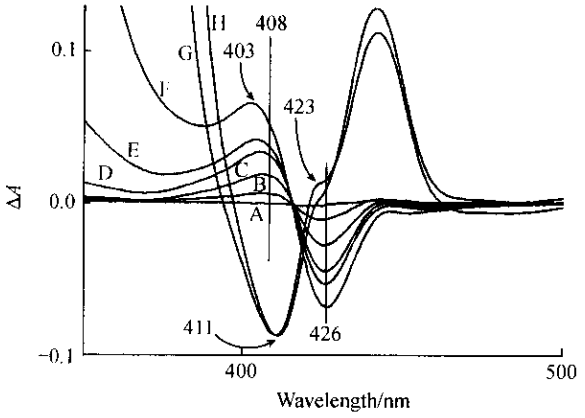

Fig. 2. Dithionite-induced difference spectra of cytochrome c oxidase film immersing in 50 mmol/L phosphate buffer, pH 5.8. The adding amount of dithionite is 9.9 (A), 47.6 (B), 143 (C), 143 (D), 211 (E), and 286 mmol/L (F), as well as 10 (G) and 20 mol/L (H), respectively.

(2) Figs. 3 and 4 show the dependence curves on the adding amount of dithionite. Change in absorption at 350 nm (ΔA_{350}) denotes the change of free dithionite and ΔA_{426} denotes the change in the amount of Fe^{3+} in the heme a center. As shown in Fig. 3, change in ΔA_{350} curve is in three phases. For extremely low amount of dithionite (4 mmol/L), only trace is oxidized. But for the lower amount (10—100 mmol/L), dithionite is almost oxidized. However, the free dithionite rapid accumulates in the solution if the amount is 200 mmol/L. In a whole, little of free dithionite is there in the solution when the amount is 100 mmol/L. In this range, the oxidized amount of dithionite, that is, number of electron donated by or released from dithionite is proportional to the adding amount of dithionite. Under these conditions, the Fe-Cu center is in the oxidation state, changes in absorption at 350, 408 and 426 nm, respectively, are almost proportional to the logarithm of the adding amount of dithionite, as shown in Fig. 4. The three curves cross the abscissa at 0 (the origin point), 0.6 and 0.9 mmol/L, respectively. These suggest that the change of the 408-nm species is earlier than that of the 426-nm one, but later than that of the 350-nm one. It is impossible that for the 408-nm and the 426-nm species, one is as reactant and the other product in the same reaction. In other words, the 408-nm species cannot be assigned to heme a center, but so is 426-nm species. More precisely speaking, the change in the 408-nm peak is mainly the change in the amount of the reduced Cu-Cu center.

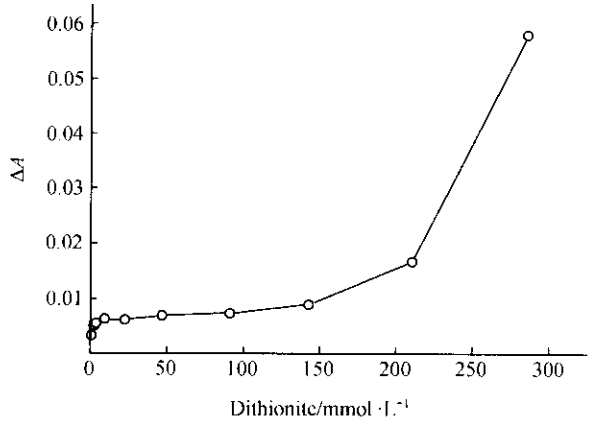

Fig. 3. Plot of ΔA at 350 nm against the adding amount of dithionite.

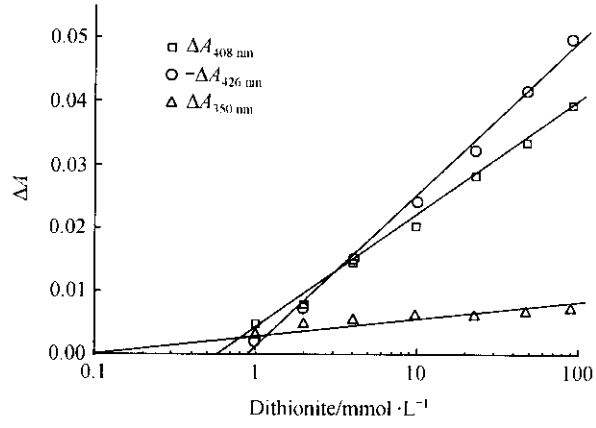

Fig. 4. Plots of change in absorption at 426 nm ( ), 408 nm (□), and 350 nm ( ), respectively, against the adding amount of dithionite.

(3) Resolution of crystal structure suggests that for

cytochrome c oxidase from bovine heart muscle[2] and from the soil bacterium *Paracoccus denitrificans*[3], the structure of Cu-Cu center is similar to that of [2Fe-2S] cluster. Two copper atoms are bridged by two cystein thiolates. The copper and the sulphur atoms lie in one plane. The ligands for each Cu atom form a distorted tetrahedron. The Cu_1 atom has N (from His) and S (from Met) as two other ligands and Cu_2 has N (from His) and O (from carbonyl of Glu) in addition to two S atoms. This [2Cu-2S] structure results in stabling of oxidation state with one-electron-delocalized oxidation. That is, the oxidation state of [2Cu-2S] in the copper binuclear center is $[Cu^{1.5}$-$Cu^{1.5}]$, similar to N_2O reductase. This structure becomes $[Cu^{+}$-$Cu^{+}]$ if it receives one electron. It is early notice that luminescence can be detected for the reduced copper center in protein, in which the excitation wavelength is within the MLCT transition band at ~400 nm[16]. Chemistry study of biological copper has shown that both oxidized and reduced forms share tetrahedral coordination[17]. So, it is reasonable to believe that the reduced Cu-Cu center has an absorption band at ~408 nm, so, the 408-nm band is assigned to LMCT transition of reduced form of Cu-Cu binuclear center.

(4) For the copper binuclear structure, two S bridges and Cu_1-N bond are stronger, while axial Met S-Cu_1 and carbonyl O-Cu_1 are weaker. However, they play an important role in regulating the redox potential of copper[17]. So, it has been proposed that in the cytochrome c oxidase, the ligand Met is hydrogen bonded to Trp121, which is within 3.2 Å of S in Met and might be involved in electron transfer from cytochrome c to the Cu-Cu center. A histindine might be involved in electron transfer from the Cu-Cu center to heme a center. It is one of ligands of Cu_2 atom, and can also donate a hydrogen to the carbonyl oxygen of an arginine[3], which is nearby the propionate group of heme a. It is convenient to study the electron transfer in enzyme like the oxidase if the MLCT band at ~408 nm is confirmed further.

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