THE INTERACTIONS OF COPPER AND a_3 IN CYTOCHROME OXIDASE

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SUMMARY

The absorption and circular dichroism difference spectra of cytochrome a_3, obtained in the form of $a_3^2\text{CO} - a_3^3\text{CN}$ by two different permutations, were not identical. The discrepancies were found by optical and EPR studies to be related to the oxidation state of copper in cytochrome oxidase. The results suggest that the copper moiety which interacts with cytochrome a_3 is most probably sandwiched between a and a_3.

Mammalian cytochrome oxidase is generally considered to be composed of two cytochrome entities, a and a_3, and copper (1-4). Cytochrome a_3 is not readily oxidized by ferricyanide when it is bound to carbon monoxide or reduced by dithionite when it is complexed with cyanide (3,5). Consequently, species such as $a^3 \cdot a_3^2\text{CO}$ and $a^2 \cdot a_3^3\text{CN}$ may be prepared. From the spectra of these species difference spectrum of cytochrome a may be computed if there is no interaction between cytochromes a and a_3 and the oxidation state of a_3 does not affect a. These postulates have been greatly strengthened by the experimental findings that the difference spectrum (light absorption (3,5) as well as circular dichroism (6)) of $a^2 - a^3$ obtained from Eq. 1 is identical with that from Eq. 2.

$$(1) \qquad a^2 \cdot a_3^2\text{CO} - a^3 \cdot a_3^2\text{CO} = a^2 - a^3$$

$$(2) \qquad a^2 \cdot a_3^3\text{CN} - a^3 \cdot a_3^3\text{CN} = a^2 - a^3$$

Likewise, analysis of the optical rotatory dispersion data of cytochrome oxidase also strongly suggests the lack of direct heme-heme interactions (7). However, functionally a paradoxical situation arises. How can electrons be transferred in cytochrome oxidase if there is no interaction between a and a_3?

Recently, we have studied the difference spectra of cytochrome a_3 obtained by a method based on similar reasoning used to obtain $a^2 - a^3$.

Results from light absorption spectrometry and circular dichroism (CD) suggest that the oxidation state of cytochrome $\underline{a}$ does affect $\underline{a}_3$, but this interaction is only indirect, apparently through an intermediate carrier which is most probably copper. This communication reports these facets.

EXPERIMENTAL

Cytochrome oxidase was isolated from the Keilin-Hartree preparation of beef heart (8). The purified oxidase preparations contained 10.4 to 11.2 nmoles heme $\underline{a}$ per mg protein with enzymatic activity in terms of turnover numbers ranging from 110 to 168 electron equivalents per sec per mole of heme. The apparent ratio of total heme $\underline{a}$ to $\underline{a}_3$ was 2.2 ± 0.1 calculated by the method of Vanneste (9).

Various forms of cytochrome oxidase were prepared as follows: $\underline{a}^2 \cdot \underline{a}_3^2\text{CO}$ -- by bubbling CO for 1 minute through an oxidase solution previously reduced with slight excess of dithionite; $\underline{a}^3 \cdot \underline{a}_3^2\text{CO}$ -- by adding a few ferricyanide crystals to the above solution; $\underline{a}^3 \cdot \underline{a}_3^3\text{CN}$ -- by mixing the oxidase solution with neutralized freshly prepared cyanide solution to give a final concentration of cyanide of 0.05 M; and $\underline{a}^2 \cdot \underline{a}_3^3\text{CN}$ -- by adding a slight excess of solid dithionite to the above solution which was then used within 20 minutes.

The difference spectra of cytochrome $\underline{a}_3$ in the form of $\underline{a}_3^2\text{CO} - \underline{a}_3^3\text{CN}$ were obtained by Eq. 3 or Eq. 4.

$$\underline{a}^2 \cdot \underline{a}_3^2\text{CO} - \underline{a}^2 \cdot \underline{a}_3^3\text{CN} = \underline{a}_3^2\text{CO} - \underline{a}_3^3\text{CN} \qquad \text{(Method A)} \tag{3}$$

$$\underline{a}^3 \cdot \underline{a}_3^2\text{CO} - \underline{a}^3 \cdot \underline{a}_3^3\text{CN} = \underline{a}_3^2\text{CO} - \underline{a}_3^3\text{CN} \qquad \text{(Method B)} \tag{4}$$

The basal buffer was 0.1 M phosphate, pH 7.4, containing 0.25% Emasol-1130. Absorption spectra were obtained from a Cary spectrophotometer, model 14; matched cells were used. CD measurements were made in a Jasco-ORD/CD/UV-5 spectropolarimeter. Electron spin resonance (EPR) experiments were conducted at liquid nitrogen temperatures in a Varian E-4 spectrometer.

RESULTS AND DISCUSSION

If there were no interaction between cytochromes a and a_3 and the oxidation state of a did not affect a_3 (an analogous case to the difference spectra of a^2 - a^3), then the difference spectra of cytochrome a_3 in the form of a_3^2CO - a_3^3CN obtained from Methods A and B should be identical.

Figure 1 shows the light absorption difference spectra of a_3^2CO - a_3^3CN obtained by Methods A (Curve A) and B (Curve B). It can be seen that these two sets of spectra were completely different. Curve A showed distinct maxima at 590 and 430 nm with small troughs at 450 and 415 nm (*cf*. Fig. 1). However, Curve B exhibited broad peaks at 604 and 436 nm and no troughs were discernable.

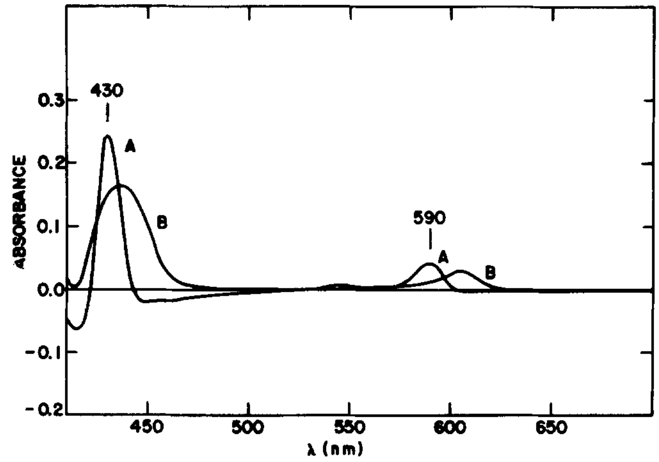

Fig. 1. Light absorption difference spectra of cytochrome a_3 in the form of a_3^2CO - a_3^3CN. Curve A, the carbon monoxide complex of cytochrome oxidase, ($a^2 \cdot a_3^2$CO) *minus* the "reduced" cyanide complex of cytochrome oxidase, ($a^2 \cdot a_3^3$CN). Curve B, the "oxidized" carbon monoxide complex of cytochrome oxidase, ($a^3 \cdot a_3^2$CO) *minus* the cyanide complex of cytochrome oxidase, ($a^3 \cdot a_3^3$CN). Enzyme concentration was 9.3 µM in terms of total heme a.

Likewise, as depicted in Fig. 2, the CD difference spectrum in the Soret region from Method A (Curve A) was not the same as that of Method B (Curve B). Curve A exhibited a distinct positive extremum at 435 nm flanked by two

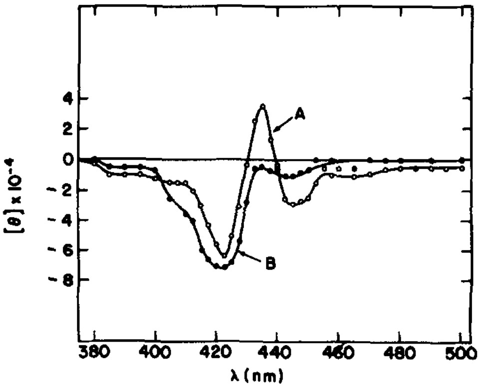

Fig. 2. Circular dichroic difference spectra of cytochrome $\underline{a}_3$ in the form of $\underline{a}_3^2CO$ - $\underline{a}_3^3CN$. Notations are the same as in Fig. 1. The ordinate is ellipticity in degrees-cm^2 per decimole of total heme $\underline{a}$.

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negative extrema at 445 and 423 nm, respectively, whereas Curve B showed only a broad negative extremum at 423 nm. However, the corresponding two difference spectra of cytochrome $\underline{a}$ ($\underline{a}^2$ - $\underline{a}^3$) computed from the same set of experimental data were similar, confirming the previously reported results (6).

The non-identity of the difference spectra observed in both the absorption and CD measurements in the visible region are probably related to variations in perturbations of the $\underline{a}_3$-heme group by the heme environment. The question is then, why should these differences appear in these two spectra ($\underline{a}_3^2CO$ - $\underline{a}_3^3CN$) but not in the difference spectra of cytochrome $\underline{a}$ ($\underline{a}^2$ - $\underline{a}^3$) obtained from Eqs. 1 and 2? We are well aware that the oxidation state of cytochrome $\underline{a}$ in the difference spectrum by Method A is different from that of Method B. But on the other hand, the oxidation state of cytochrome $\underline{a}_3$ is likewise different in Eqs. 1 and 2. This dilemma may be resolved if there is indirect communication between $\underline{a}$ and $\underline{a}_3$ through an intermediate carrier.

These considerations prompted us to examine the behavior of copper in cytochrome oxidase. It is known (10,11) that cytochrome oxidase in the oxidized form exhibits a broad absorption band in the region of 830 nm attributed to copper. Upon reduction this band disappears. We found that the cyanide complex of cytochrome oxidase ($\underline{a}^{3}\cdot\underline{a}_{3}^{3}\mathrm{CN}$) showed a copper band in the 830 nm region essentially the same as that in intact oxidase. Upon addition of dithionite, this band immediately disappeared although $\underline{a}_3$ remained in the oxidized form ($\underline{a}^{2}\cdot\underline{a}_{3}^{3}\mathrm{CN}$). In a similar experiment no copper band was observed in the carbon monoxide complex of cytochrome oxidase ($\underline{a}^{2}\cdot\underline{a}_{3}^{2}\mathrm{CO}$). Upon addition of a minute amount of ferricyanide ($\underline{a}^{3}\cdot\underline{a}_{3}^{2}\mathrm{CO}$), the band in the 830 nm region appeared.

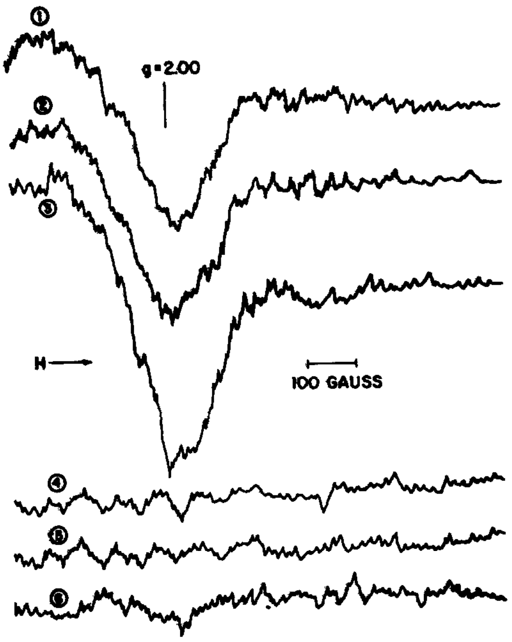

Fig. 3. The parallelism between the EPR signals of copper and the oxidation state of cytochrome $\underline{a}$ in cytochrome oxidase. (1) the oxidized oxidase, $\underline{a}^{3}\cdot\mathrm{Cu}^{2}\cdot\underline{a}_{3}^{3}$; (2) the cyanide complex, $\underline{a}^{3}\cdot\mathrm{Cu}^{2}\cdot\underline{a}_{3}^{3}\mathrm{CN}$; (3) the "oxidized" carbon monoxide complex, $\underline{a}^{3}\cdot\mathrm{Cu}^{2}\cdot\underline{a}_{3}^{2}\mathrm{CO}$ (the slightly higher amplitude could be due to a small amount of extraneous copper (I) in the oxidase preparation); (4) the reduced oxidase, $\underline{a}^{2}\cdot\mathrm{Cu}^{1}\cdot\underline{a}_{3}^{2}$; (5) the "reduced" cyanide complex, $\underline{a}^{2}\cdot\mathrm{Cu}^{1}\cdot\underline{a}_{3}^{3}\mathrm{CN}$; (6) the carbon monoxide complex, $\underline{a}^{2}\cdot\mathrm{Cu}^{1}\cdot\underline{a}_{3}^{2}\mathrm{CO}$. The conditions of EPR spectroscopy were: microwave powers, 50-75 mW; frequency, 9.06 GHz; modulation amplitude, 1 gauss; scanning rate, 250 gauss per min.; time constant, 3 sec.; and temperature, 77°K. Enzyme concentration was 130 μM (in terms of total heme $\underline{a}$) in 0.1 M phosphate, pH 7.4, containing 0.25% Emasol-1130.

EPR of cytochrome oxidase has been extensively studied and the signal at g = 2 conclusively assigned to Cu(II) (12). We examined the EPR behavior of the various complexes of cytochrome oxidase. Fig. 3 summarizes the results. The spectra of the oxidized and the reduced oxidase were practically the same as those reported by Beinert (12) and others. However, the addition of dithionite to the cyanide complex immediately abolished the signal at g = 2 whereas the appearance of the signal was observed by addition of ferricyanide to the carbon monoxide complex.

These results clearly indicate that the oxidation state of copper follows that of cytochrome *a* but not a_3. Therefore the observed differences in the so-called difference spectra of cytochrome a_3 obtained by Methods A and B may be reasonably explained by the effect of the oxidation state of copper. In other words, the conformation of cytochrome a_3 is not dictated by the oxidation state of *a* but by copper. That explains the identity of the difference spectra of cytochrome *a* obtained from Eqs. 1 and 2; it is because the oxidation state of copper is the same although that of a_3 is different. If these arguments are valid, then the difference spectra of cytochrome a_3 cannot be meaningfully obtained from Eqs. 3 and 4.

The role of copper in electron transfer in cytochrome oxidase has been implicated by various studies (11-15). Our results as presented here indicate that copper is most probably sandwiched between cytochromes *a* and a_3 within such geometrical distance so that not only electrons can be efficiently transferred from *a* to copper to a_3 but also the conformation of both *a* and a_3 may be affected by the copper moiety. This conclusion can afford to reconcile the divergent results reported in the literature (e.g. 3,5,6,11-15). We may thus revise Eqs. 1-4 as follows:

$$a^{2}\cdot\mathrm{Cu}^{1}\cdot a_{3}^{2}\mathrm{CO} - a^{3}\cdot\mathrm{Cu}^{2}\cdot a_{3}^{2}\mathrm{CO} = a^{2} - a^{3} \tag{1a}$$

$$a^{2}\cdot\mathrm{Cu}^{1}\cdot a_{3}^{3}\mathrm{CN} - a^{3}\cdot\mathrm{Cu}^{2}\cdot a_{3}^{3}\mathrm{CN} = a^{2} - a^{3} \tag{2a}$$

$$a^{2}\cdot\mathrm{Cu}^{1}\cdot a_{3}^{2}\mathrm{CO} - a^{2}\cdot\mathrm{Cu}^{1}\cdot a_{3}^{3}\mathrm{CN} = a_{3}^{2}\mathrm{CO} - a_{3}^{3}\mathrm{CN} \tag{3a}$$

$$a^{3}\cdot\mathrm{Cu}^{2}\cdot a_{3}^{2}\mathrm{CO} - a^{3}\cdot\mathrm{Cu}^{2}\cdot a_{3}^{3}\mathrm{CN} = a_{3}^{2}\mathrm{CO} - a_{3}^{3}\mathrm{CN} \tag{4a}$$

For convenience we have placed one copper atom on the electron path between cytochromes a and a_3. However, even the result of our experiments does not allow us to precisely decide whether one or two atoms of copper reside between the two cytochrome components.

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