NEW SPECIES OF THE "OXYGENATED COMPOUND" OF CYTOCHROME OXIDASE

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1. Introduction

Okunuki and coworkers [1, 2] have proposed the oxygenated complex of cytochrome oxidase as an intermediate of the reaction between molecular oxygen and oxidase. This hypothesis has attracted many workers in the study of the complex but unfortunately severe disparities still exist among them (e.g. [3–9]). These conflicts, we think, may well be due to the possible involvement of at least 3 species, heretofore unsuspected, of the oxygenated complex of cytochrome oxidase which we wish to report in this communication.

2. Experimental

Cytochrome oxidase was purified from the Keilin-Hartree beef heart muscle preparation according to Kuboyama [10] with the modifications to be published. Absorption spectra were obtained on either a Cary Model 14 or Model 16 recording spectrophotometer. The rapid reactions were followed on a Durrum-Gibson stopped-flow apparatus equipped with a 20 mm cell. The absorbance of the reaction product of the reduced cytochrome oxidase with oxygen could be determined at approx. 5 msec "dead time" after the mixing. Cytochrome oxidase of about 15 μM concentration was usually reduced with an excess of solid sodium dithionite or DPNH in the presence of phenazine methosulfate (PMS) (the molar ratio of PMS/heme $\simeq$ 0.2).

Alternatively, cytochrome oxidase was reduced under anaerobic conditions by titration with a stoichiometric amount of DPNH, with PMS/heme $\simeq$ 0.1. The oxygenated compound was prepared by shaking the solution of the reduced oxidase on a Vortex mixer for 30 sec at low speed, or by a Sephadex method [11].

3. Results and discussion

Fig. 1 shows the spectral changes of the oxygenated compound of cytochrome oxidase against the oxidized enzyme. The absorption maximum at 605 nm decreased first followed by a decrease of the peak at 578 nm. However, the absorption spectrum of the reaction product did not revert back to that of the oxidized oxidase even after several hours. The spectral behavior clearly indicates that the reaction of the reduced cytochrome oxidase with oxygen involves at least 3 species as the intermediates, which will be referred to as comp. I, II and III according to the order of their appearance. The half times of the decay of comp. I and II, on the average, were found to be 2.75 min and 15 min, respectively. Spectral changes in the Soret region also paralleled those in the visible region, but spectral separation of each intermediate was not as clear as the α band.

From the difference spectrum against the oxidized oxidase, comp. I was characterized by a positive peak at 435 nm and a negative trough at 412 nm, and comp. III by a 442-3 nm peak and 418 nm trough.

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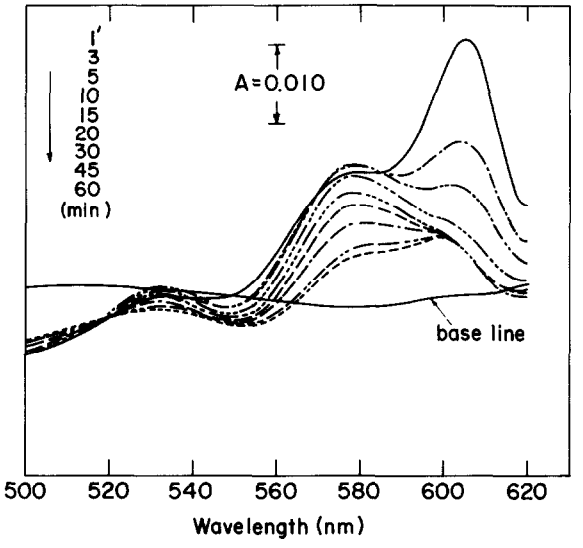

Fig. 1. Changes in the difference spectrum of the oxygenated compound of cytochrome oxidase. The sample cuvette contained the "oxygenated compound" prepared by the shaking method, and the reference was the oxidized oxidase. The concentration was 16 μM in terms of heme *a*. The spectra were run from 620 nm to 500 nm at a scanning speed of 80 nm/min. Numbers in the figure are the times in minutes of the start of recording at 620 nm.

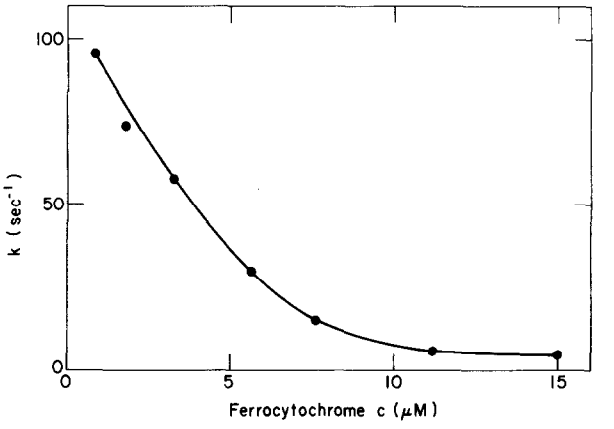

Fig. 2. Effect of ferrocytochrome *c* on the decay of comp. I. The absorbance decrease at 605 nm of comp. I was followed in a stopped-flow apparatus. The final concentration of cytochrome oxidase in the observation cell was 8.25 μM in terms of heme *a*, pH 7.4, temp. 22°. The reduced cytochrome oxidase was prepared by the titration method in the presence of 1.5 μM PMS and 16.5 μM of cytochrome oxidase in terms of heme *a*.

The occurrence of comp. II was reflected by a heterogeneous change of the spectrum during the transition from comp. I to III. These species must be considered under the generic name of the "oxygenated compound" by following the concept advanced by Okunuki [12] although he originally developed it on the basis of a single entity. Previous workers considered these multiple intermediates as a statistically single entity apparently because of the short lives of comp. I and comp. II.

The difference spectrum of the reaction product at 20 msec after mixing the reduced oxidase with oxygen against the reduced enzyme reference in the stopped-flow apparatus showed a deep trough at 445 nm and a positive peak at 420. If the reaction product were the oxidized enzyme, then the trough would be expected to appear at 412–414 nm (cf. also [8]). Obviously the product is neither the reduced nor the oxidized form of cytochrome oxidase. Chance and Pring [9] have shown that the spectrum formed at 0.25 msec is indistinguishable from that at 50 msec. The spectrum we found at 20 msec is evidently the same as the ones reported by Chance and Pring [9] for pigeon breast muscle and also by Gilmour et al. [8] for the isolated oxidase. In the absence of excess reducing agent, a slow decrease of the absorbance at the peak position of the reaction product occurred after a rapid change. The slow change followed first order kinetics with a rate constant of about 5×10^{-3} sec^{-1} which was in agreement with the result obtained by a different method described at the beginning of the paper for the measurement of the decay of comp. I. The initial product of the reaction of cytochrome oxidase with oxygen in a time scale of 20 msec, or even down to 0.250 msec, is evidently comp. I. In the absence of cytochrome *c*, comp. II was rapidly formed from the conversion.

In the presence of ferrocytochrome *c* the disappearance of comp. I differed dramatically than when it was absent. The reaction usually proceeded biphasically. The rapid phase followed a first order kinetics with respect to comp. I concentration, and

Table 1
Effect of ferrocytochrome *c* on the disappearance of comp. I.

Concentration of ferrocytochrome *c* (μM)	Absorbance at 605 nm at 5 msec	at 5 min	ΔA	$\Delta\epsilon_{mM}$
0.87	0.200	0.149	0.051	3.09
1.81	0.194	0.147	0.047	2.85
3.24	0.205	0.149	0.056	3.39
5.65	0.207	0.152	0.055	3.33
7.60	0.213	0.158	0.055	3.33
11.2	0.219	0.164	0.055	3.33
15.0	0.232	0.176	0.056	3.39

The experimental conditions were the same as those described in the legend of fig. 2. Cytochrome oxidase was 8.25 μM and the optical path of the observation window 20 nm.

the rate constant decreased with the increase of ferrocytochrome *c* as shown in fig. 2. Likewise, ferrocytochrome *c* accelerated the decay of comp. II, so that in the presence of a moderate concentration of *c* the clear distinction between the behavior of comp. I and II became obscure. However, the absorption spectrum of the reaction product reverted to that of the original oxidized cytochrome oxidase. The first order constant for the disappearance of comp. I in the system of cytochrome *c* to cytochrome oxidase ratio of approx. 0.1 was found to be at least 20,000 times greater than that in the absence of cytochrome *c*. Presumably, the increase would be even higher when cytochrome *c* is structured as it is in the mitochondrial inner membrane.

Table 1 summarizes the absorbance and other values of the systems of reduced cytochrome oxidase and the air-saturated medium which contained ferrocytochrome *c* at 5 msec and 5 min after rapid mixing. It can be clearly seen that the difference in absorbance between 5 msec and 5 min durations is almost the same in all cases tested independent of the ferrocytochrome *c* concentration. This result can be simply explained if we assume that the oxidation of ferrocytochrome *c* occurs solely during the dead time of the stopped-flow apparatus, and that the inflow of electron(s) from ferrocytochrome *c* to comp. I, if this happens, does not affect the absorbance at 605 nm of this compound.

All the cytochrome *c* effects described could be shown only for the reduced form. Therefore, the inhibition at high concentrations of cytochrome *c* we observed is not of the classical type of Smith and Conrad (see [13]). The kinetics of the formation of comp. I was not measurable in the equipment used since the reaction was completed in the "dead time", 5 msec or less.

These data presented suggest that comp. I is possibly involved in the cytochrome oxidase reaction as an active species, and that for the "normal" action of cytochrome oxidase ferrocytochrome *c* is required. In other words, the reduction of molecular oxygen occurs in successive steps (see for example [14]) in each of which ferrocytochrome *c* may be essential. These results also show that the measurement of a decrease at 605 nm of the α band or 445 nm of the Soret band cannot be equated as the oxidation of the reduced to the oxidized cytochrome oxidase. The reliable criterion must correlate the decrease of absorbance at these wave lengths with the reduction of molecular oxygen and reappearance of the oxidized oxidase. Without the latter, all results must be considered provisional.

Acknowledgements

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