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The reduced *minus* oxidized difference spectra of cytochromes *a* and a_3

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Abstract

We have re-investigated the reduced *minus* oxidized difference spectra of the two heme centers of cytochrome oxidase, cytochromes *a* and a_3. In contrast to data obtained in an earlier study (Vanneste, W.H. (1966) Biochemistry 5, 838–848), we find that the spectrum for cytochrome a_3 agrees with that found with a 5-coordinate high-spin heme A model compound. Small but significant additional differences are noted for both heme centers.

Keywords: Cytochrome oxidase; Optical spectrum; High-spin; 5-Coordinate

1. Introduction

The separate contributions of cytochromes *a* and a_3 to the visible spectrum of cytochrome oxidase was first assessed quantitatively by Vanneste [1] following the lead of Horie and Morrison [2]. Vanneste combined difference absorbance changes obtained upon reduction in the absence and presence of inhibitors with data on the absolute spectrum of cytochrome a_3 determined photochemically. At first the results of this work were not universally accepted because of a belief that there might be heme–heme spectral interactions which could be modified upon addition of inhibitors or electrons but it is now generally believed that Vanneste's results are essentially correct.

A more persuasive reason for not completely accepting the conclusions of Vanneste's study is the form of the spectral changes observed in the alpha-band region. The data suggest that the difference spectrum of cytochrome a_3 should contain two absorbance maxima. However, from heme A model compounds it seemed that only one maximum should be present [3].

Part of the original strategy was to react the enzyme with cyanide and then obtain the reduced *minus* oxidized difference spectrum of this derivative; this difference spectrum was assigned to cytochrome *a*, an assignment predicated on the presumption that only this heme center was reduced easily in the cyanide-treated enzyme.

Unfortunately this strategy was marred by two circumstances. First, as spectra of cyanide-enzyme were recorded immediately after addition of 15 mM cyanide [1], it is highly unlikely that complete reaction of cyanide with the oxidized enzyme has occurred. Indeed as the enzyme preparation used had a Soret maximum at 421 nm it was likely to be a mixture of the fast and slow species of this enzyme [4] and the fraction of slow enzyme present would be essentially unreacted in the time it took to record the spectrum.

Second, the data were obtained with a conventional scanning spectrophotometer and thus the degree of reaction of the enzyme with cyanide will have varied with wavelength of measurement.

To clarify this discrepancy we have recently re-investigated these difference spectra, paying attention to the two potential technical errors by (1) utilizing enzyme that was completely fast in form and by reacting with cyanide for more than enough time to ensure complete conversion; and (2) recording data at 1-min intervals in a diode array spectrometer so that all wavelengths were recorded at the same moment and that the time at which the cytochrome *a* was fully reduced while cytochrome a_3 was still oxidized could be reliable assessed.

The difference spectra for cytochrome *a* and a_3 obtained in this way, are quantitatively similar to those to Vanneste [1] but reveal an important and interesting differ-

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ence which show that the original difference spectrum of a_3 is incorrect.

2. Materials and methods

2.1. Materials

The fast form of cytochrome oxidase [4] was prepared by the modification of the method of Hartzell and Beinert [5] developed in this laboratory [6].

2.2. Methods

Native cytochrome oxidase was converted to the cyanide derivative by reaction with 5 mM cyanide for 4 h at 0°C. Reduction was accomplished by addition of 1 mM dithionite which was dispensed from a burette into a Thunberg-type cuvette previously loaded with enzyme and made anaerobic. Optical spectra were recorded at 1-min intervals for 10 min, then at 2-min intervals for another 10 min and then at several intervals for a total 2 h. Spectra were recorded in a SLM 3000 diode array spectrometer using a 17-s integration time; points were recorded at 0.33-nm intervals.

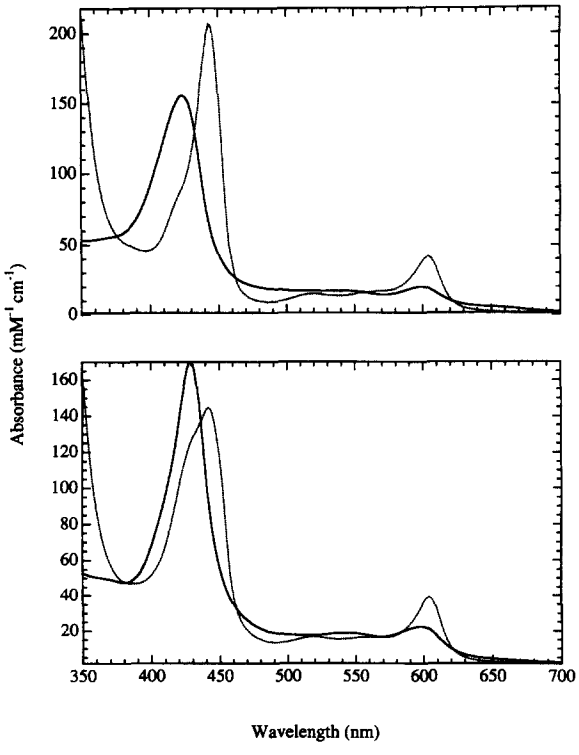

Fig. 1. Upper panel: the UV-vis spectrum of oxidized (unbroken line) and reduced (dotted line) spectra of native cytochrome oxidase. Lower panel: the oxidized (unbroken line) and reduced (dotted line) spectra of the cyanide complex of cytochrome oxidase. The enzyme concentration was 8.8 μM.

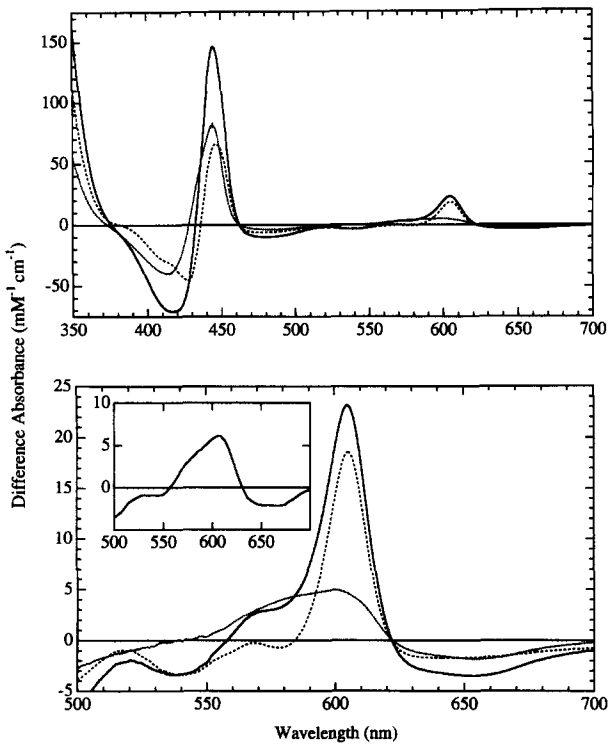

Fig. 2. The UV-vis difference spectra of cytochromes $a + a_3$ (unbroken line), cytochrome a (dashed line) and cytochrome a_3 (dotted line) obtained from the data of Fig. 1 as described in the text. Upper panel: the complete UV-vis spectra; Lower panel: the alpha band region on an expanded scale. The inset shows the corresponding data for the 1,2 dimethyl imidazole complex of heme A computed from the data of Ref. [3].

3. Results

Fig. 1 shows the absolute oxidized and reduced absorbance spectra of native and cyanide-reacted enzyme. The native, reduced enzyme spectrum was recorded after the Soret maximum of the reduced form had reached 1.34 × that of the oxidized form; from a survey of data both in the literature and repeated measurements in our laboratory, an increase of 1.34–1.35 seems to represent the end-point of reduction. The reduced cyanide enzyme was recorded 8 min after addition of dithionite; between 8–14 min there was essentially no change in the spectra but later data should a clear qualitative change in behavior in the α-band region which we interpret as reflecting reduction of a_3-CN.

We have constructed the following difference spectra: A: (reduced native enzyme − oxidized native enzyme) = $a_3^{2+} + a^{2+} - a_3^{3+} + a^{3+}$; B: (reduced cyanide enzyme − oxidized cyanide enzyme) = $a^{2+} - a^{3+}$; C = A − B = a_3^{2+}

Table 1
Selected absorbance indices for cytochrome *a*, cytochrome a_3 and cytochromes $a + a_3$

Species	Wavelength (nm)	Absorbance (mM^{-1} cm^{-1}) [a]
cyt. *a*	427.4	−45.4
	436	0
	446	66.4
	539	−3.4
	568	−0.23
	584	0
	605	18.6
	621.5	0
cyt. a_3	413	−39.8
	428	0
	444	82.3
	539	0.07
	568	2.8
	578.5	3.9
	600.5	4.9
	622	0
$a + a_3$	417	−71
	432.5	0
	444.8	146
	539	−3.3
	557.8	0
	604.7	23.2
	612.9	0

[a] Calculated assuming a value of 156 mM^{-1} cm^{-1} for the Soret absorbance of oxidized enzyme.
Full numeric data can be obtained from: http://www-bioc.rice.edu/~graham/CcO.html.

$- a_3^{3+}$. These difference spectra are shown in Fig. 2, and Table 1 summarizes some important features.

4. Discussion

The important striking difference is seen in the alpha band due to a_3; the data of Vanneste [1] show this to be a pair of peaks located at ca. 570 nm and at 603 nm. However, we find a single peak at 601 nm with a shoulder at 579 nm. Importantly our difference spectrum is strikingly similar to the reduced *minus* oxidized difference spectrum of the 1,2 dimethyl-imidazole complex of heme A (Fig. 2, inset) computed from the data of Carter and Palmer [3]. This heme A derivative is high-spin and 5-coordinate in both oxidation states and serves as a plausible model compound for cytochrome a_3. A second difference which appears from our measurements is that cytochrome *a* loses absorbance at wavelengths greater than 620 nm, in contrast to Vanneste's finding that there are no absorbance changes in the near-infra-red due to the reduction of cytochrome *a*. Finally the relative contribution of cytochrome *a* and a_3 to the changes in the alpha band are comparable to those reported earlier [1]; however, the differences in the Soret region are much less marked with *a* and a_3 contributing 45 and 55% respectively, compared to the values of 33 and 67% published previously. We have obtained similar data on cytochrome a_3 using enzyme prepared by the method of Soulimane et al. [7] (M. Fabian and G. Palmer, unpublished).

When we repeat the calculations of the difference spectra using data on the cyanide enzyme recorded after more than 16 min of reaction with dithionite, a double alpha band similar to that described by Vanneste appears in the spectrum of a_3. From this moment on it was also clear that the location of the alpha band had begun to shift to the blue; this shift increased monotonically with time with a corresponding appearance of peaks at 574 and 607 nm (trough at 592 nm) in the computed difference spectrum for a_3.

It is our belief that the differences in the results obtained in our experiments and those of Vanneste arise from (1) the presence of some native cytochrome a_3 in samples believed to be 100% $a - a_3CN$; and (2) the presence of some $a^{+2}a_3^{+2}CN$ in samples believed to be $a^{+2}a_3^{+3}CN$.

In conclusion, the reduced − oxidized difference spectrum redetermined in these experiments does not exhibit the atypical features reported earlier [1] and clearly resembles a high-spin heme A compound which serves as a model for cytochrome a_3.

Acknowledgements

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