ABSOLUTE AND DIFFERENCE SPECTRA OF CYTOCHROMES a AND a_3

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Received February 3, 1965

Yonetani (1960) obtained a number of difference spectra of the components a and a_3 of cytochrome c oxidase, using the principle that cyanide delays the reduction of ferric iron in cytochrome a_3. On the same basis, Horie and Morrison (1963) deduced absolute spectra of their preparation with components in different oxidation-reduction states. Lemberg and associates (1964), however, concluded that the results from Yonetani's method could be only approximations. An attempt to obtain absolute spectra of the entities a and a_3 in cytochrome c oxidase was recently made by Horie (1964 a and b). It seemed to us that the assumption adopted by this author was lacking evidence, and we thought it valuable to introduce a different approach.

We have reported on the a_3 content of cytochrome c oxidase preparations (Vanneste, 1965). This study opened the way to a calculation of extinction coefficients in the difference spectra. Moreover, it allowed us to make a simple and successful computation of the absolute spectra of the components a and a_3. We are presenting here in a preliminary way the results obtained by this approach.

Materials and Methods: Cytochrome c oxidase was prepared from washed Keilin-Hartree particles (Fowler, Richardson and Hatefi, 1962). The preparation was dissolved in 0.1 M Tris-HCl buffer, pH 8.0, containing 1% deoxycholate. The a_3 content was derived from CO-binding capacity determined with fresh preparation (Vanneste, 1965). All spectrophotometric observations were made within a few hours after preparation of the oxidase, to avoid changes during storage.

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* Fulbright grantee, on leave from the University of Ghent, Belgium.

Whereas other workers obtained difference spectra by direct recording, we have chosen to record only absolute spectra, since this shortened the time required for observations and also avoided slit-widening with accompanying decrease of resulution. Absolute spectra were afterwards combined algebraically to yield the desired difference spectra*:

$$(\underline{a}^{++} - \underline{a}^{+++}) = \underline{a}^{++}\,\underline{a_3}^{+++}\,CN - \underline{a}^{+++}\,\underline{a_3}^{+++}\,CN$$

$$(\underline{a_3}^{++} - \underline{a_3}^{+++}\,CN) = \underline{a}^{++}\,\underline{a_3}^{++} - \underline{a}^{++}\,\underline{a_3}^{+++}\,CN$$

$$(\underline{a_3}^{+++}\,CN - \underline{a_3}^{+++}) = \underline{a}^{+++}\,\underline{a_3}^{+++}\,CN - \underline{a}^{+++}\,\underline{a_3}^{+++}$$

$$(\underline{a_3}^{++} - \underline{a_3}^{+++}) = (\underline{a_3}^{++} - \underline{a_3}^{+++}\,CN) + (\underline{a_3}^{+++}\,CN - \underline{a_3}^{+++})$$

$$(\underline{a_3}^{++}\,CO - \underline{a_3}^{++}) = \underline{a}^{++}\,\underline{a_3}^{++}\,CO - \underline{a}^{++}\,\underline{a_3}^{++}$$

$$(\underline{a_3}^{++}\,CN - \underline{a_3}^{++}) = \underline{a}^{++}\,\underline{a_3}^{++}\,CN - \underline{a}^{++}\,\underline{a_3}^{++}$$

Values for the photochemical action spectrum of baker's yeast cytochrome c oxidase were taken from the figure and table in a discussion comment by Chance (1961). We combined these data with the determined $\underline{a}_3$ content to obtain the absorption spectrum of $\underline{a_3}^{++}\,CO$ in the preparation. Since the difference spectra of the components in the preparation were available, we were able to calculate the absorption spectra of $\underline{a_3}^{++}$, $\underline{a_3}^{+++}$, $\underline{a}^{++}$, $\underline{a}^{+++}$, $\underline{a_3}^{++}\,CN$, and $\underline{a_3}^{+++}\,CN$. The procedure went as follows:

$$\underline{a_3}^{++} = \underline{a_3}^{++}\,CO - (\underline{a_3}^{++}\,CO - \underline{a_3}^{++})$$

$$\underline{a_3}^{+++} = \underline{a_3}^{++} - (\underline{a_3}^{++} - \underline{a_3}^{+++})$$

$$\underline{a}^{++} = \underline{a}^{++}\,\underline{a_3}^{++} - \underline{a_3}^{++}$$

$$\underline{a}^{+++} = \underline{a}^{+++}\,\underline{a_3}^{+++} - \underline{a_3}^{+++}$$

$$\underline{a_3}^{++}\,CN = \underline{a}^{++}\,\underline{a_3}^{++}\,CN - \underline{a}^{++}$$

$$\underline{a_3}^{+++}\,CN = \underline{a}^{+++}\,\underline{a_3}^{+++}\,CN - \underline{a}^{+++}$$

Results and Discussion: The difference spectra obtained by our method

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* The notations and conditions used are those suggested by Horie and Morrison (1963). The symbols do not imply a 1 : 1 stoichiometry but refer to the spectra of the components $\underline{a}$ and $\underline{a}_3$ in the molecular proportion characteristic of the preparation.

are in satisfactory agreement with those reported previously (especially Lemberg, 1964). Some of our data are presented in Table 1.

Table 1. Some difference spectra of the components a and a_3 in cytochrome c oxidase.

Spectrum	Wavelength or wavelength pairs of maxima or minima; the calculated extinction coefficients ($mM^{-1}cm^{-1}$) appear between brackets				
$a_3^{++} - a_3^{+++}$	603 (4.8)	465*	444 (112)	428	411 (-50)
a_3^{++} CO $- a_3^{++}$	590-605 (10.1)		428.5-445(148)		
a_3^{++} CN $- a_3^{++}$	590-610 (14.4)				
$a^{++} - a^{+++}$	605 (20)	461	445 (57.2)	435.5	425 (-41)

* Isosbestic points

In contrast to a_3, the concentration of cytochrome a could not be determined by a direct method. An assumption had to be made in order to derive extinction coefficients for a, for the possibility exists that not all of the non-CO binding heme a belongs to cytochrome a*. In our calculations we have taken a = 1/2 total heme a, thus assuming that the rest of heme a was accounted for by a_3 and a non-CO binding degradation product different from a.

* Our evidence (see Vanneste, 1965) seems to point to a loss of CO-binding capacity during the preparation procedure. As to the composition of the final preparation, three extreme situations appear to be possible.

1. Some a_3 was removed from its counterpart a or some a_3 has been converted into a. Both cases result in
   (a) = (total heme a) - (CO binding heme a)
2. Some a_3 has been altered to the extent that it lost the ability to bind CO. The degradation product, present in the preparation, is spectrally different from a. This would give
   (a) = (1/2 total heme a) (the 1/2 is derived from our results with the particulate enzyme)
3. An equal amount of a was altered (in a spectral sense) simultaneously with a_3. The preparation in this case would be seriously contaminated. We would have
   (a) = (a_3)

The second situation is somewhat intermediate and seems to be the most likely one at the present time.

Table 2 describes the absolute spectra of components a and a_3. Although the γ/α ratio in the difference spectrum (reduced minus oxidized) of cytochrome a_3 is as high as 23, it appears that this ratio is only 13-14 in the absolute spectrum of reduced a_3 which is reasonably close to known values for hemoglobins and bacterial cytochromoid hemoproteins. The corresponding ratio for cytochrome a is 2.9. The shift in Soret maximum upon reduction was found to be 27 mμ for a_3 and only 18 mμ for a. This information together with the obtained γ/α ratios constitutes better experimental background for Williams' (1961) suggestion that a is the more low spin, and a_3 the more high spin compound.

Table 2. Positions of most pronounced maxima and extinction coefficients ($mM^{-1}cm^{-1}$) in absolute spectra of cytochromes a and a_3.

Compound	Soret	Visible	
a_3^{++}	442 mμ (125)	565 mμ (6)	602-603 mμ (9)
a_3^{+++}	415 mμ (81)	560 mμ (5)	600 mμ (4)
a^{++}*	444 mμ (113)	520 mμ (18.5)	604-605 mμ (39)
a^{+++}*	426 mμ (120)	545 mμ	595 mμ (18.5)
a_3^{+++} CN	broad double band	560 mμ (5.5)	595 mμ (6)
a_3^{++} CN	441 mμ (96)		595 mμ (16.5)

* Extinction coefficients are derived on the basis that a = 1/2 heme a.

The limitations inherent in this approach are:

1. The difference spectra obtained by Yonetani's method are of an approximate nature (Lemberg, 1964).

2. We used the photochemical action spectrum of baker's yeast. This approximation is reasonable, since the spectral properties of at least the CO-derivative of yeast and muscle cytochrome a_3 are very nearly identical (Chance, 1953).

3. An error may be introduced by presence of a non CO-binding hemoprotein, different from both a and a_3.

4. The assumption is made that the spectral intensities of a and a_3 in the same particle are independent of one another.

Acknowledgements. The authors are greatly indebted to Dr. H. S. Mason for his advice during this work.

This study was supported by grants from the American Cancer Society and the United States Public Health Service, to whom we are indebted.

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