THE a_3 : TOTAL HEME a RATIO IN CYTOCHROME aa_3 OF HEART MUSCLE

Walter H. Vanneste*

Department of Biochemistry, University of Oregon Medical School, Portland

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The binding of CO has proven to be useful in the determination of the a_3 content of purified cytochrome c oxidase. Gibson and Greenwood (1963) studied a Yonetani preparation (Yonetani, 1960, 1961) and reported that 1 out of every 3 hemes a was able to combine with CO. Upon repetition of the experiments with Enzyme Institute preparations, Gibson (1965) recently concluded that the CO-binding varied greatly from one preparation to the other with extremes of 1 : 2.6 and 1 : 5 for the a_3 : total heme a ratio. The author was inclined to assume a 1 : 2 ratio for the native oxidase. Morrison and Horie (1964) studied the CO-binding capacity of their preparation (Horie and Morrison, 1963) and found that in the average 1 CO binding heme occurred per total of 3.8. A different approach to the problem was made by Van Gelder and Muijsers (1964) who as a result of redox titrations suggested that the a_3 : total heme a ratio was strictly 1:2.

In view of the existing controversy we thought it useful to investigate the CO-binding capacity of both freshly prepared mitochondrial particles and cytochrome c oxidase preparations with a sensitive radiochemical technique. Results of these experiments are reported in this communication.

Methods and Materials. Keilin-Hartree particles were prepared from beef heart muscle mince serially washed with 0.1 M and 0.02 M phosphate buffer pH 7.4. The homogenate freed of nuclei and cell debris was centrifuged, washed with 0.1 M phosphate buffer pH 7.4 and recentrifuged to yield a preparation which was virtually free of myoglobin or hemoglobin contamination (see below). The final suspension medium was 0.25 M sucrose.

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

Cytochrome c oxidase activity was assayed polarographically. The conditions of the assay matched those described by Smith and Camerino (1963, a and b) for obtaining consistent maximal turnover rates. Cytochrome c was Sigma type V. Its concentration was determined with 21.1 $mM^{-1}cm^{-1}$ as extinction coefficient for the difference in OD at 550 mμ between a reduced and an oxidized sample (Van Gelder and Slater, 1962).

Purified cytochrome c oxidase was either a gift from Dr. M. Morrison or was prepared by the procedure of Fowler, Richardson and Hatefi (1962). However, Keilin-Hartree particles instead of mitochondria were successfully used as starting material for this preparation.

Heme a in cytochrome c oxidase preparations was analyzed as pyridine hemochromogen according to the method of Williams (1964). An extinction coefficient of 12 $mM^{-1}cm^{-1}$ for the difference in OD at 605 mμ between the reduced and oxidized preparation (Van Gelder and Slater, 1963) was also used for the same purpose. The hemochromogen procedure when applied to Keilin-Hartree particles apparently overestimates the heme a content (Williams, 1964). We therefore relied more on a method described by the same author, however, with some minor modifications, to determine the content of a type cytochrome in mitochondrial particles.

CO was determined by a radiochemical method. The material under study was equilibrated under both (a) reduced and (b) oxidized form with a same pressure of a $C^{14}O$, CO, N_2 mixture. The CO from (a) (i.e. bound + dissolved) and (b) (i.e. dissolved) was released by the action of alkaline ferricyanide in a nitrogen atmosphere. The radioactivity of the gas phases so obtained were counted and the difference in counting rate was converted in micromolarity of CO in chemical combination by reference to the data for a hemoglobin standard run simultaneously.

Results and Discussion. Our experiments on the CO binding of Keilin-Hartree particles gave the results shown in Table 1. The identification of all CO in chemical combination with the a_3 content requires the proof

that no other CO binding pigment besides cytochrome a_3 is present.

Table 1. CO binding to Keilin-Hartree particles.

Heme a from pyridine hemochromogen method	a type cytochrome from modified Williams procedure	Carbon monoxide in chemical combination	a_3 : total heme a *
62 μM (1)**	56.3 μM (7)	29.6 μM (4)	1:2.09 1:1.90
	(52.9 μM-59.1 μM)	(27.9 μM-31.4 μM)***	

* The first and second ratio were obtained with the results of the hemochromogen procedure and of Williams' method for simultaneous estimation of the cytochromes, respectively.
** The values given are the means of the number of observations indicated between brackets.
*** Extremes.

Myoglobin is the most likely contaminant of heart muscle preparations (Colpa-Boonstra and Minnaert, 1959). The figure of merit described below indicates both myoglobin and hemoglobin contamination. The ratio $\Delta OD_{428.5 - 445} / \Delta OD_{428.5 - 422}$ in the CO difference spectrum of the heart muscle preparation (Fig. 1) is very sensitive to traces of myoglobin (or hemoglobin) which will increase it appreciably. The value of this ratio is about 5.0 in purified cytochrome c oxidase. It can be calculated that the presence of myoglobin in a concentration equal to 5% of that of the CO bound to the heart muscle preparation of Table 1 increases the ratio to 5.9. We found with this preparation ratios of 5.4 with succinate and 5.9 with dithionite as reducing agent, which indicates a contamination too small, however, to seriously affect our a_3 : heme a ratio.

On the basis of the identity between the CO in chemical combination and the a_3 content, we calculated extinction coefficients for the peak to trough differences in both Soret (428.5 - 445 mμ) and visible region (590 - 605 mμ) of the CO difference spectrum of the heart muscle preparation. We found 136 $mM^{-1}cm^{-1}$ and 10.1 $mM^{-1}cm^{-1}$, respectively.

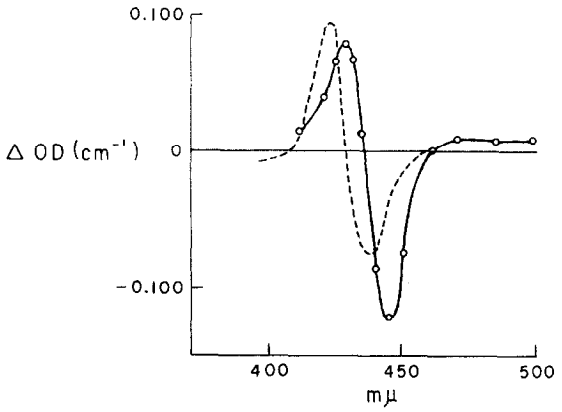

Fig. 1. Carbon monoxide difference spectra of the 20 x diluted heart muscle preparation and 0.001 mM myoglobin. The full and dashed lines represent the differences in absorption between the CO derivative and the reduced form of cytochrome a_3 and myoglobin, respectively. The Cary scattered transmission attachment and high intensity light source were used if necessary to allow observations on turbid samples. The Keilin- Hartree particles were suspended in 0.1 M phosphate buffer pH 7.4. Reduction was accomplished by adding 1-2 mg dithionite to both cells. A baseline correction was done as described by Chance (1953 a).

These values are higher than those reported by Chance (1953 b) which were obtained from the photochemical action spectrum and an assumed analogy with lactoperoxidase.

Turnover number of cytochrome a_3 has been defined by Smith and Camerino (1963 b) as O_2 uptake in μM per second x 4 / μM cytochrome a_3. Using the concentration of a_3 obtained from the CO binding experiments, we found this TN to be 475 sec^{-1} at infinite cytochrome c concentration compared to 330 - 390 sec^{-1} reported by Smith et al. (1963 b) The reason for this difference is that their extinction coefficient (23 $mM^{-1}cm^{-1}$ for the 605 - 630 difference in the reduced minus oxidized spectrum of the heart muscle preparation) seems to overestimate the a_3 content by about 10%. Furthermore, we used a slightly higher extinction coefficient, resulting in a lower concentration for cytochrome c.

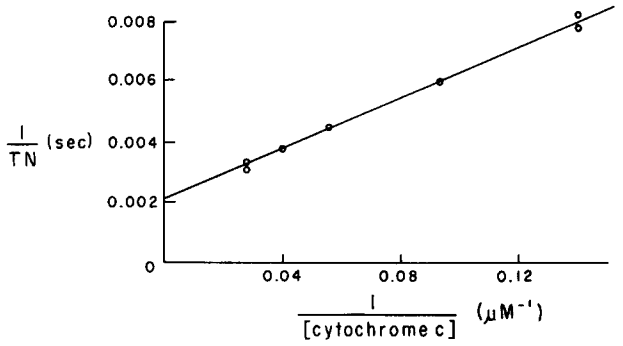

Fig. 2. Plot of reciprocal of cytochrome c oxidase activity of Keilin-Hartree particles expressed as turnover number of a_3 versus reciprocal of cytochrome c concentration. Oxygen uptake was measured with the GME oxygraph. The medium was 0.05 M phosphate buffer pH 7.0, 50 mM ascorbate, 0.1 mM EDTA. Cytochrome c concentration was varied between 7.1 and 35.8 μM. Particles were treated with deoxycholate before assay (Smith and Camerino, 1963 b).

Table 2. CO binding capacity of cytochrome c oxidase preparations.

Preparation	Heme a from pyridine hemochromogen	Heme a from Δ CO $_{red-ox}^{605}$	Carbon monoxide in chemical combination	a_3 : total heme a *
modified Fowler et al.	77.1 μM (2)**	76.7 μM (2)	31.7 μM (8) (27.4 μM-36.9 μM)***	1:2.43, 1:2.42
Morrison	75.7 μM (2)		30.1 μM (2)	1:2.51
	72.7 μM (2)		30.7 μM (2)	1:2.37

*, **, ***, cf. Table 1.

The CO binding was also studied on purified cytochrome c oxidase preparations (Table 2). The relevant extinction coefficients in the CO difference spectrum were calculated again taking the CO in chemical combination as an exact measure of a_3 (Table 3).

Table 3. Extinction coefficients for the CO difference spectrum of cytochrome c oxidase preparations.

Preparation	peak (428.5) to trough (445) in Soret region ($mM^{-1}cm^{-1}$)	peak (590) to trough (605-608) in visible region ($mM^{-1}cm^{-1}$)
Modified Fowler et al.	148	10.1
Morrison	149	9.7

The ratios now deviate consistently from 1 : 2 but there is general agreement with Gibson's result. This may indicate that some capacity for binding CO has been lost during the preparation. Our evidence is not completely conclusive, however, since two different methods of heme analysis were used.

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