The Binding of Carbon Monoxide by Cytochrome c Oxidase and the Ratio of the Cytochromes a and a₃

QUENTIN H. GIBSON, GRAHAM PALMER, AND DAVID C. WHARTON

From the Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and the Institute for Enzyme Research, 1710 University Avenue, Madison, Wisconsin 53706

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Although the affinity of cytochrome c oxidase for carbon monoxide has been the subject of several investigations, and has been determined both by equilibrium methods (e.g. Wald and Allen (1)) and as the ratio of velocity constants (Gibson and Greenwood (2)), few measurements of the amount of CO bound have been reported. Since, by definition, cytochrome a₃ binds CO while cytochrome a does not (Keilin and Hartree (3)), determination of the CO-binding capacity of the cytochrome oxidase should also establish the ratio of a₃ to a. This ratio has from time to time been the subject of speculation (cf. Lemberg (4)), and guesses have ranged from 1 a₃:1 a to 1 a₃:4 a. The first measurements of bound CO were reported by Gibson and Greenwood (2), who found that about one-third of the heme a of preparations made by the method of Yonetani (5) appeared to combine with CO. The CO-binding capacity of cytochrome oxidase has also been determined by Horie and Morrison, who concluded that in their preparation about one-fourth or one-fifth of the heme a was able to bind CO. More recently, Van Gelder and Muijser (6) have attempted to define the ratio by reductive titration of cytochrome oxidase with reduced diphosphopyridine nucleotide, using phenazine methosulfate as a mediator. Their results, which depend in part on the interpretation of the effect of metal-chelating agents, suggest a ratio of a₃ to a of 1:1 and so require a CO-binding capacity 1.5 times as great as that found by Horie and Morrison.

As the results of the work cited embrace the full range of ratios of a₃ to a previously covered by speculation, the problem has been re-examined by new and more precise methods which have been applied to the preparations of Griffiths and Wharton (7) and to an electron transport particle preparation from beef heart muscle (8).

EXPERIMENTAL PROCEDURE

Determination of Carbon Monoxide-binding Capacity

The chief difficulty in applying the method described in detail by Gibson and Greenwood (2) is that the quantities of cytochrome oxidase available even from a well furnished laboratory yield very small quantities of gas for final measurement, typically 4 to 6 μl, while blank determinations give about one-tenth of this amount. It is difficult, therefore, to investigate the reproducibility of the determinations and, especially, to determine accurately the size of the blanks and of the corrections required for CO in physical solution. The amount of CO present in the enzyme solutions has therefore been determined by measuring the radioactivity of the extracted gases after equilibration of the enzyme with ¹⁴CO. The procedure finally adopted was as follows.

Preparation and Storage of Sample—A sample of cytochrome oxidase (10 to 30 ml) about 2.5 to 3.5 × 10⁻⁴ M in heme a was placed in the top cup of a Van Slyke-Neill manometric apparatus, and 1 drop of Dow-Corning silicone 1107 was added. The liquid was drawn into the pipette and the top tap was sealed with mercury. The pipette was evacuated, the liquid shaken for 3 minutes, and the extracted gases (O₂ and N₂) were expelled. The extraction was repeated until no further gas was obtained. The top cup was dried with a tissue, and approximately 3 mg of ¹⁴CO (0.1 to 1 ml) was then drawn in, the ¹⁴CO tonometer was filled with mercury. After a 40-minute reduction period at room temperature, the tonometer with the enzyme solution could be transferred without contact with the air. After a 40-minute reduction period at room temperature, the tonometer with the enzyme was kept at 0°C until required for use.

Equilibration with ¹⁴CO—A dilution of ¹⁴CO (Volk Radiochemicals) in unlabeled CO sufficient to yield approximately 10⁸ counts sec⁻¹ ml⁻¹ (about 2 × 10⁴ disintegrations sec⁻¹ ml⁻¹ under our conditions) was prepared in a tonometer furnished with a 2-way stopcock at one end and a 3-way oblique stopcock with a capillary stem at the other. A sample of 3.5 ml of enzyme was drawn from the storage tonometer into the pipette of the Van Slyke apparatus and about 2 ml of mercury were poured into the top cup after removal of the enzyme storage tonometer. The capillary tip of the ¹⁴CO storage tonometer was filled with mercury from an auxiliary reservoir, fitted with a rubber tip, and seated in the cup of the Van Slyke apparatus. A suitable volume of ¹⁴CO (0.1 to 1 ml) was then drawn in, the ¹⁴CO tonometer was removed, and the top tap of the Van Slyke apparatus was sealed with mercury. The volume of ¹⁴CO was measured by expanding the sample to the 2-ml mark and recording the difference in pressure with and without the sample of ¹⁴CO in the chamber.
dimethyl benzyl ammonium hydroxide, Packard Instrument fluid (5 g of 2,5-diphenyloxazole + 0.3 g of 1,4-bis-2'-(5'-phenyl-
-Slyke apparatus, air was admitted to atmospheric pressure.
in the receiving tonometer through a 10-cm tube packed with
by the procedure already described for the removal of excess
shaking for 6 minutes (Procedure b).
4% (w/v) K,Fe(CN), in 1 N NaOH and extracting the gases for
distilled water, and 2 ml of enzyme were drawn in from the
enzyme was expelled into it together with a few drops of mercury.
The Van Slyke pipette was then washed out three times with
tubular pipette.

The determination was completed by either admitting 1 ml of
4% (w/v) K,Fe(CN), in 1 N NaOH and extracting the gases for
6 minutes (Procedure a) or admitting 1 ml of unlabeled CO from
a tonometer, lowering the mercury to the 50-ml mark, and
shaking for 6 minutes (Procedure b).
In either case the gases were collected in a vacuum tonometer
by the procedure already described for the removal of excess
14CO. After the tonometer had been detached from the Van
Slyke apparatus, air was admitted to atmospheric pressure.
The samples were prepared for counting by blowing the gas
in the receiving tonometer through a 10-cm tube packed with
copper turnings heated to 450° and through a wash bottle con-
taining methanol acidified with 0.001 N HCl; CO2 was collected
in 2 ml of Hyamine hydroxide (p-diisobutylcresoxyethoxyethyl)-
dimethyl benzyl ammonium hydroxide, Packard Instrument
Company). The samples were mixed with 10 ml of scintillation
fluid (5 g of 2, 5-diphenyloxazole + 0.3 g of 1, 4-bis-2'-(5'-phenyl-
oxazolyl)benzene in 1 liter of toluene), and thoroughly cooled
in the scintillation counter, and the time to give 2.0 × 106 counts
was determined in a Nuclear-Chicago model 703P scintillation
counter. A number of determinations were also made by the
method described in detail in Gibbon and Greenwood (2).

**Determination of CO Bound to Cytochrome Oxidase**

The CO found in any sample of cytochrome oxidase equilibrated
with a known pCO consists of two parts, CO in combination and
CO in physical solution. To determine the chemical binding
capacity of the enzyme, we assume that the amount of gas in
physical solution is proportional to the partial pressure of CO.
Thus, once the enzyme has been saturated, the total amount of
gas present in a sample will increase linearly with pCO. If a
graph of total CO against pCO is plotted, extrapolation of the
linear portion to zero pCO will give an estimate of the amount
of CO bound chemically.

In practice, three to five points have been determined in the
range 2 to 20 mm of pCO, and one or two additional points be-
tween 300 and 500 mm of pCO. Optical determinations of the
percentage saturation have shown that at pressures above 10
mm of CO the enzyme was more than 98% saturated; thus, the
increment in CO content between the points at 10 to 20 mm of
pCO and those around 400 mm of pCO measures the solubility
of CO in the enzyme sample.

With the solubility coefficient derived in this way, the amount
of gas in physical solution at each partial pressure was calculated
and subtracted from the gas content, giving the amount of gas
combined chemically with the enzyme. This was divided by the
proportional saturation of the sample obtained by spectropho-
tometry, and the amount of gas in physical solution was
added to the result. This procedure was repeated for each point,
and the results were plotted (Fig. 1).

The solubility of CO in these preparations is considerably less
that than in water. It varies from preparation to preparation
and appears to be correlated with the content of deoxycholate
and salt.

**Absorption spectra of cytochrome oxidase** were determined
routinely with a Cary model 14 spectrophotometer and the
0.4-mm cell already described. The concentration of cyto-
chrome \(a + a_3\) in the different preparations employed was
established initially with a Beckman model DU spectrophotom-
eter and cuvettes of 1-cm light path. For this determination,
an extinction coefficient of 16.5 mm-1 cm-1 was applied to the
\(\Delta E\) of the 605- and 630-nm wave length pair (7) of a dithionite-
reduced preparation of cytochrome oxidase. An extinction
coefficient of 100 mm-1 cm-1 was assigned to the absolute ab-
sorbance at 444 nm of a dithionite-reduced preparation and one
of 88 mm-1 cm-1 to the absolute absorbance at 430 nm of a
preparation containing the CO complex.

**Preparations**

Cytochrome c oxidase was prepared by the method of Griffiths
and Wharton (7). Hemoglobin (Grade C) was obtained from
the California Corporation for Biochemical Research. Solu-
tions of hemoglobin were prepared in 0.1 M phosphate buffer,
pH 7.4. Their concentration was determined by conversion to
methemoglobin cyanide and measurement of the absorbance at
540 nm adopting \(E = 11.3\) mm-1 cm-1 (9). An electron trans-
port particle preparation was made as described by Beyer (8).

![Fig. 1. Binding of carbon monoxide by the cytochrome oxidase prepa-
ration of Griffiths and Wharton (7). ](image)
RESULTS AND DISCUSSION

Cytochrome Oxidase Preparations Examined by Method of Gibson and Greenwood—The results obtained with five different Griffiths-Wharton (7) preparations by the method of Gibson and Greenwood (2) are summarized in Table I. They are quite similar to those reported (2) for Yonetani’s (5) preparation when freshly made. The similarity of the two preparations already reported (10) thus extends to their CO-binding capacity as measured by this particular procedure.

Combination of Cytochrome Oxidase with 14CO, and Correlation between Binding and Spectra—The procedure and results of one experiment are given in detail, and the supporting evidence derived from others is summarized in Table II.

1. Standardization procedure: 14CO from the storage tonometer was transferred to the Van Slyke apparatus, and the partial pressure was measured at 0.5-mL volume. The gas was transferred to a vacuo tonometer and oxidized as for a regular sample. Five determinations on volumes ranging from 13.0 to 151.6 f14CO at standard temperature and pressure gave a mean activity of 9.3 × 104 counts sec⁻¹ ml⁻¹ (range, 9.1 to 9.4 counts on a total of 50,000 counts for each sample).

2. About 30 mL of water were equilibrated with 290 mm of 14CO at 25°C, and portions of this transferred to tared tonometers, which were then weighed to determine the weight, and hence their volume, of water. The tonometers were attached to the oxidation train, and the CO was expelled by an air stream. The amount of CO recovered tallied closely with that calculated from solubility tables and was approximately proportional to the weight of solution taken within ±1 standard deviation based on the number of counts (four samples). Completeness of transfer from the tonometers to the Hyamine hydroxide was also checked by changing the receiving solution after 15 minutes (the standard time of running the gas train). The second receiver contained <0.5% of the total counts. When the change was made after 3 minutes, 18.5% of total counts appeared in the second receiver. Taken together, these experiments seem to establish the adequacy of the procedure for determining 14CO once this had reached the receiving tonometer.

3. As an over-all check, determinations were made subjecting hemoglobin to the same procedure as cytochrome oxidase. In one set of determinations, a solution of 1.33 × 10⁻⁴ M hemoglobin (in heme) was used. Duplicate determinations by Procedure a with ferrocyanide, with equilibration pressures of 9.3 mm of pCO and 9.8 mm of pCO, gave 5.90 ± 0.01 and 5.60 ± 0.09 p1 of CO, respectively, for samples of 2 mL when corrected for CO in physical solution, corresponding to 1.29 × 10⁻⁴ and 1.25 × 10⁻⁴ M, respectively. Application of Procedure b to the liquid remaining in the Van Slyke apparatus after Procedure a gave 0.02 p1 of CO as the amount left unextracted. The use of Procedure b with two further samples gave 1.33 and 1.31 × 10⁻⁴ M. Although the difference between the results by the two methods exceeds the calculated statistical error of counting, the agreement with one another and with the amount of hemoglobin used is regarded as satisfactory.

4. The determinations with the oxidase were made by the ferrocyanide method, using 2-mL portions of enzyme for the gasometric work. The results are plotted in Fig. 1, which shows the amount of CO present in samples equilibrated with various partial pressures of CO. These are corrected for background counts obtained on a portion of enzyme carried through the procedure without CO. The correction corresponded to 0.00 p1 of CO. In addition to the points plotted, one sample was equilibrated with 513 mm of pCO and yielded 21.4 p1 of CO. This point served to determine the slope of the line drawn in Fig. 1, since when the CO-binding power of the enzyme is satisfied, increments in CO content will be due to increase in the amount of gas in physical solution. The figure indicates that at pressures above about 10 mm of pCO, the enzyme was almost saturated with CO, and this impression was fortified by consideration of the spectra of portions of the samples recorded after equilibration.

The values for the percentage saturation calculated from the spectra of the CO compound, with 500 mm of pCO taken as giving full saturation, were next applied to each sample to yield the upper set of points through which a line has been drawn to intercept the ordinate at a value of 3.7 p1 or 8.3 × 10⁻⁵ M, the estimate of the gas-binding capacity of the sample. As the total heme a was 2.6 × 10⁻⁴ M, the ratio (a1 + a2):a2 was 3.1.
In addition to the spectrum of the CO compound in each sample, the spectrum of the enzyme was recorded 45 minutes after the addition of dithionite in the absence of CO. This spectrum showed an appreciable shoulder at 424 μm and gave a value of the ratio 444 μm:424 μm of 1.86.

Similar experiments have been performed on five other samples of cytochrome oxidase with the results shown in Table II.

**CO-binding Capacity of Electron Transport Particle Preparation**—The procedure was slightly modified by prolonging the equilibration period to 10 minutes to allow for possible difficulty due to the viscosity of the preparation. The results of these experiments are shown in Table III and one is illustrated in Fig. 2.

**Variability of Results in CO-binding Experiments**—The experiments of Tables I and II appear to establish with some precision the amounts of CO which are bound by the Griffiths-Wharton (7) preparation of the cytochrome oxidase, and by the electron transport particle preparation of Beyer (8) after treatment with dithionite. The oxidase preparation, when subjected to the procedure of Gibson and Greenwood (2), binds amounts of CO comparable with those earlier reported for Yonetani's preparation, with the use of the same experimental method. The amount of CO bound per heme a in these experiments is significantly greater than that reported by Horie and Morrison, since the application of a t test suggests that the difference observed would arise by chance less than once in 300 trials. Our results, like those of Horie and Morrison, show a marked scatter, which might be due to shortcomings in the experimental methods or to differences between preparations.

Detection of the values collected in Table I suggests that the variation between preparations exceeds that within preparations; although the numbers are too small for serious analysis, the variance between preparations is about 10 times that within preparations. The existence of real differences between preparations is further borne out by the results of experiments with 13CO. As the ratios (a + a3)/a2 are believed to be reproducible within ±5%, the differences shown in Table II should certainly be real.

Two possible causes of variation have been systematically investigated: (a) that heme a3′ was not saturated with CO under our conditions; (b) that not all heme a3′′ was reduced before equilibration with CO. As a measure of saturation, the extinctions at 430 μm and 444 μm were recorded after equilibration with low pCO, and compared with the values obtained after equilibration with pure CO, which were taken to represent 100% saturation of the a3′′ present in a given sample of enzyme. The values at these two wave lengths were also measured for reduced enzyme in the absence of CO, and the percentage of saturation was calculated by interpolation. The results showed that heme a3′′ was saturated at all pressures above about 10 mm of pCO within the precision of the spectrophotometric measurements (±2% saturation in the region near 100% saturation), and that failure of a3′′ to bind CO is not an important cause of variability in experiments with fresh Griffiths-Wharton preparations, although it may become so under other conditions (see Reference 2).

The reduction of a3′′ by dithionite was followed by injecting a portion of the stock enzyme solution into the short light path (0.4 mm) spectrophotometer cell and running spectra at intervals. In agreement with Lemberg et al. (11), it was found that changes at 444 μm continued for periods of up to 1 hour, and that the shape of the final absorption spectrum differed for different preparations. These results suggest that incomplete reduction of a3′′ may be an important cause of variability in CO binding; the slow changes would affect particularly the results in Table I by the method of Gibson and Greenwood (2) where each sample was reduced individually in the chamber of the Van Slyke, and was exposed to dithionite and CO for an aggregate period of the order of 10 minutes only. After the time-dependent effects had been observed, a period of at least 45 minutes was allowed for the action of dithionite; this applied to all the determinations in Table II, which, however, still show considerable variation in CO binding, so that slow reduction of a3′′ is not the only source of variability.

**TABLE III**

*Combination of carbon monoxide with electron transport particle preparation*

Temperature, 28°; electron transport particle preparation made by the method of Beyer (8). CO binding capacity determined by radioisotopic method described in text with samples of 2 ml reduced with dithionite.

<table>
<thead>
<tr>
<th>104 × heme a</th>
<th>103 × carbon monoxide capacity</th>
<th>Ratio (a + a3)/a3</th>
</tr>
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<tbody>
<tr>
<td>μ</td>
<td>μ</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>3.1</td>
<td>2.1</td>
</tr>
<tr>
<td>13.0</td>
<td>8.9</td>
<td>2.2</td>
</tr>
<tr>
<td>11.8</td>
<td>5.3</td>
<td>2.2</td>
</tr>
<tr>
<td>5.7</td>
<td>2.7</td>
<td>2.1</td>
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</tbody>
</table>

![Fig. 2. Binding of CO by an electron transport particle preparation (ETP) made by the method of Beyer (8).](http://www.jbc.org/)
When this result had been obtained, a retrospective examination of routine spectra recorded on over 50 preparations of cytochrome oxidase was made. The spectrum on reduction with dithionite differed significantly from sample to sample, and in some cases there was a marked shoulder on the short wavelength side of the Soret peak, at about 424 μm (e.g. Fig. 4). The ratio 444:424 μm ranged from 1.58 to 2.30 save for a single preparation, which had a ratio of 2.50 (and the unusually high ratio at 444:605 μm of 5.3). These findings would be explained if the preparations of cytochrome oxidase contained a variable amount of cytochrome a3 which could be reduced by dithionite. If so, preparations with a high ratio E 444:424 μm should bind more CO per heme than those with a lower value, and as Fig. 3 shows, this is indeed the case, the six values of the ratio (a + a3): a3 lying on a smooth curve when plotted against the corresponding values of the ratio 444:424 μm.

The proposal just made to explain the variability of CO binding by different preparations implies that only a3 is affected; all the a present is assumed to be normal. The data so far available do not seem to exclude the possibility that some a is affected as well as a3, although the first suggestion is favored by the increase in ΔE 444 μm/ΔE 605 μm as CO binding increases, as well as by Lemberg’s (11) kinetic observations. It is also possible that the ratio of a3 to a is not fixed, but there does not seem to be much reason why a low proportion of a3 in a preparation should be associated with the appearance of a shoulder at 424 μm in the spectrum of the reduced form.

Ratio of a3 to a—It is necessary, as a preliminary, to discuss the extinction coefficients used for determining the sum (a + a3) in the spectrum of the reduced form.

Van Gelder and Slater (12) that the extinction coefficients of all previous workers are some 20% too low.

With these coefficients, the values found in our best experiments for the ratio (a + a3):a3 lie between 2.3 and 4.0 by the 14CO method, while the mean of the five experiments by the simple method of gas analysis is 3.02. The spectrophotometric evidence in favor of the presence of oxidized a3 in our solutions even in the presence of dithionite suggests that the true ratio, if integral, is 2, i.e. that a and a3 would occur in equimolecular amounts in an ideal preparation. This conclusion is strengthened by the results of the measurements on the electron transport particle preparation, in which the ratio (a + a3):a3 in four experiments was very close to 2.

Although our final conclusion is widely different from that of Horie and Morrison, their results appear to fit readily into a common pattern with those reported here. Thus, they reduced their preparations with dithionite for only 3 minutes before equilibrating with carbon monoxide. Further, rough measurements of their spectra suggest values of 1.8 to 2.2 for the ratio 444:424 μm, which, according to Fig. 3, would indicate the presence of considerable amounts of unreduced cytochrome a3. Finally, it should be pointed out that measurements of the spectrum of the enzyme after equilibration with CO can give no assurance that all a3 in a preparation has been converted to the CO compound, since the band of the CO compound at 430 μm effectively masks the shoulder at 424 μm due to oxidized a3, and coexistence of large amounts of undetected oxidized a3 with the CO compound is possible. This point is illustrated in Fig. 4, which shows the spectrum of the CO compound of an enzyme preparation in which only one-half of the a3 was reducible by dithionite. The ratio 430:444 μm is 1.23, the same as that in enzyme preparations in which higher proportions of a3 were able to combine with CO. The spectra of Horie and Morrison are thus entirely compatible with their findings for the CO binding of their preparation.

**Fig. 3.** Relation of CO binding and the ratio of absorbance at 444 and 424 μm in preparations of reduced cytochrome oxidase. The enzyme was dissolved in 0.25 M sucrose containing 0.01 M phosphate buffer, pH 7.5, and was reduced by adding 2 mg per ml of sodium dithionite. The absorbance was measured after the solution had stood for 45 minutes at 28°C, and the CO-binding capacity was subsequently determined on portions of the same solution.

**Fig. 4.** Absorption spectrum of the Soret peak of reduced cytochrome oxidase and its CO compound. The enzyme, dissolved in 0.25 M sucrose containing 0.01 M phosphate, pH 7.5, was reduced by the addition of 1 mg per ml of sodium dithionite. The reaction was allowed to proceed for 45 minutes at 28°C before the spectrum was recorded. A portion of this reduced enzyme was equilibrated with 500 mm pCO to form the CO compound.

**Consequences of Presence of Inactive Component in Cytochrome Oxidase Preparations**—If the findings of the present paper are accepted, it follows that some revisions of interpretations in experiments with cytochrome oxidase will be required. Thus,
although the form of the various separated difference spectra for \(a_3\) and \(a\) should remain unchanged, the extinction coefficients for \(a_3\) require division by the proportion of active \(a_3\) in the preparation used, and in general, spectrophotometric experiments which yield anomalous results should be examined with the possibility of inactive enzyme in mind. For example, Horie and Morrison (13) have concluded that reasonable extinction coefficients for the carbon monoxide and cyanide compounds of cytochrome \(a_3\) are only attained if they suppose that about one-fourth of the heme \(a\) of their preparations combines with these reagents and infer from this that the ratio \(a_3:a\) is about 1:3. Their carbon monoxide determinations, however, suggest that about half the \(a_3\) was inactive, and if so, reasonable extinction coefficients would be obtained with a ratio of total \(a_3:a\) of 1:1. The ratio of active \(a_3\) to total heme \(a\), however, which is what such experiments really determine, would remain 1:3 or so, as they have stated.

On the other hand, the interpretation of kinetic experiments would be little affected by the presence of inactive material. Under many sets of experimental conditions, rates are measured as pseudo-first order constants with values and interpretations that are independent of inactive diluents. Difficulties are met with in complex reaction schemes, however, where in the reaction of reduced oxidase with oxygen, for example, some molecules of cytochrome \(a\) may donate electrons through “branching” mechanisms of the type discussed by Gibson and Greenwood (14).

**SUMMARY**

1. A method for the determination of small amounts of carbon monoxide is described.

2. The carbon monoxide-binding capacity of purified preparations of cytochrome oxidase and of an electron transport particle preparation has been determined.

3. Purified preparations of cytochrome oxidase contain variable and appreciable amounts of cytochrome \(a_3\) which cannot be reduced by dithionite.

4. The ratio of total cytochrome \(a_3\) to cytochrome \(a\) is 1:1 both in oxidase preparations and in electron transport particle preparations.

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**REFERENCES**

The Binding of Carbon Monoxide by Cytochrome c Oxidase and the Ratio of the Cytochromes \(a\) and \(a_3\)

Quentin H. Gibson, Graham Palmer and David C. Wharton