REACTION OF CYTOCHROME *c* OXIDASE WITH CO: INVOLVEMENT OF THE INVISIBLE COPPER

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1. Introduction

Cytochrome *c* oxidase, the terminal complex of the mitochondrial respiratory chain, has been shown to contain two types of cytochrome, designated cytochromes a and a_3, and two atoms of intrinsic copper (see [1] for review). Classical studies on the spectral properties of the individual hemes of cytochromes a and a_3 have relied on trapping one of these components (usually cytochrome a_3) as an inhibitor complex (e.g. the cytochrome a_3^{2+}–CO compound) in an attempt to evaluate the relative contributions of these two species to the α and Soret bands of cytochrome oxidase. The application of a systematic potentiometric approach to the study of the respiratory chain carriers has permitted the a and a_3 hemes to be observed independently, in the presence or absence of added ligands on the basis of their differing midpoint potentials [2–5]. These studies have led to the further development of our understanding of cytochrome oxidase, particularly with respect to the interactions which are found to occur between the two heme a components [3–6].

A combination of the potentiometric technique in conjunction with EPR spectroscopy (or measurement of the 830 nm absorption band) has indicated that in pigeon heart mitochondria one atom of copper titrates with a midpoint of 245 mV. [6,7]. Recent evidence, using anaerobic coulometric titrations [8], suggests that four reducing equivalents are required for complete reduction of cytochrome oxidase, as does the earlier work of Van Gelder and Beinert [9], who performed quantitative titrations on anaerobic suspensions of the oxidase with accurately calibrated solutions of dithionite. The linearity of the appearance of the absorbance change at 605–630 nm as a function of reducing equivalents added, together with the knowledge that cytochromes a and a_3 contribute about equally to the 605–630 nm peak [3–5] permits the deduction that the 4th oxidation–reduction component, the 'invisible' copper, has a midpoint near that of cytochrome a_3. In general, however, the properties of the 'invisible' copper are poorly defined due to the absence of any known light absorption or EPR absorption attributable to this species.

In an earlier communication, we reported that the cytochrome a_3^{2+}–CO compound may be oxidised by ATP in the presence of ferricyanide and that this oxidation is readily reversed by uncouplers or oligomycin. This observation permitted a more precise investigation of the spectral and thermodynamic properties of the cytochrome a_3–CO species, leading to the surprising observation that, at saturating CO (1.2 mM), the course of oxidation–reduction of the cytochrome a_3–CO compound is a two electron process with an $E_{m7.2}$ of 340 ± 20 mV [10]. The anomalous redox properties of the cytochrome a_3–CO compound are further described in this report and discussed in terms

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of the involvement of the 'invisible' copper in the binding of CO and oxygen.

2. Materials and methods

Pigeon breast mitochondria, prepared essentially as described by Erecińska et al. [11], were stored frozen at approximately 50 mg/ml and thawed as required. Submitochondrial particles were prepared by diluting the mitochondrial suspension fourfold with 100 mM potassium phosphate buffer, pH 7.0 containing 1 mM EDTA. The material was passed through a French pressure cell (American Instrument Co., Silver Spring, Maryland) at a pressure of 4 000 p.s.i. Disrupted mitochondria were centrifuged at 105 000 *g* for 35 min to pellet the clear red membrane fraction. The membranes were washed once in the original buffer, centrifuged as before and finally suspended in medium containing 0.2 M mannitol, 0.05 M sucrose and 200 μM EDTA (MSE). Purified cytochrome oxidase was the generous gift of Dr. Tsoo E. King, State University of New York at Albany [12].

Simultaneous oxidation–reduction potential versus absorbance measurements were performed essentially as described by Dutton et al. [2,13]. In these experiments, a special design of cuvette was employed which enabled titrations to be performed under anaerobic conditions in the absence of a gas phase. All solutions were degassed by bubbling with argon (< 1 ppm O_2) and (O_2 < 1 ppm) stored in sealed containers containing a rubber septum. Oxidising equivalents were added using an anaerobic solution of 100 μM potassium ferricyanide and reducing equivalents by employing a dilute solution of sodium dithionite.

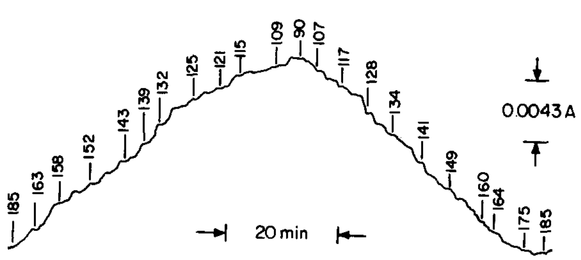


Fig. 1. The course of oxidation–reduction potential dependence of the cytochrome a_3 –CO compound in pigeon breast submitochondrial particles. Pigeon breast submitochondrial particles (2.5 mg protein/ml) were maintained under anaerobic conditions in medium containing 0.2 M mannitol, 0.05 M sucrose and 0.05 M Tris–HCl buffer, pH 7.8. Redox mediators employed were diaminodurene (80 μM), phenazine methosulfate (80 μM) and ferricyanide (500 μM). During reductive titrations diaminodurene was not added until the redox potential of the system was more negative than 290 mV. The spectrophotometer measuring wavelength pair was 590 nm minus 624 nm and the bandwidth was less than 0.5 nm. Both oxidative and reductive titrations were performed as described in Materials and methods. An upward deflection in the trace indicates formation of the cytochrome a_3 –CO compound and the numbers given are the measured potential in mV relative to a saturated KCl calomel reference electrode. The E_h values can be obtained by adding 245 mV. The CO concentration employed was 36 μM.

3. Results

A typical oxidation–reduction titration of the cytochrome a_3–CO compound in pigeon breast using the wavelength pair 590–624 nm is shown in fig. 1. After anaerobiosis, the potential of the system was adjusted to an E_h of 430 mV (185 mV relative to the calomel reference electrode used in the experiment) and a reductive titration was conducted in a stepwise manner with approx. 30 sec being allowed for equilibration at each stage. On complete reduction of the CO compound, an oxidative titration was performed in a similar fashion using a solution of 100 μM ferricyanide. Little or no hysteresis was evident in comparing oxidative and reductive titrations. Controls performed in the absence of CO gave little or no absorbance at these wavelengths. In addition, the total absorbance change observed during a complete titration was equivalent to that measured on addition of CO to the fully reduced suspension and is therefore due solely to the formation of the CO compound. It should be noted that most of the absorbance changes occur over a range of approx. 60 mV, suggesting that the course of oxidation–reduction of the CO compound is a two electron (n=2) rather than a one electron (n=1) process.

To facilitate observation of the course of oxidation–reduction of the a_3–CO compound, we have utilized the known pH dependence of the midpoint of cytochrome a_3 which becomes approximately 60 mV more negative as the pH is changed from 6.8 to 7.8 [3,4]. This allows experiments at various CO concentrations to be performed under anaerobic conditions employ-

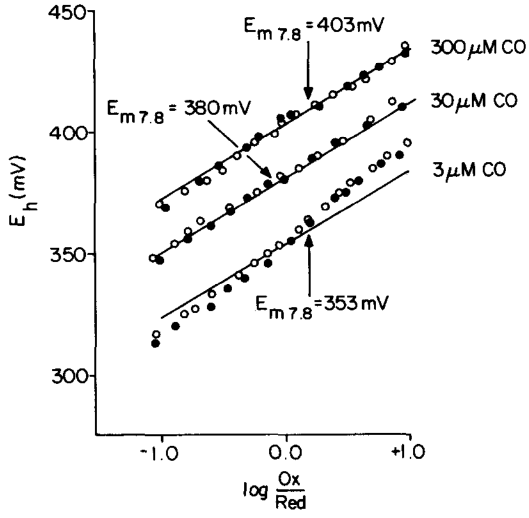


Fig. 2. Effect of CO concentration on the n value and midpoint potential of the cytochrome a_3–CO compound in pigeon breast particles. A series of oxidation–reduction titrations on pigeon breast submitochondrial particles were performed as described in fig. 1. CO was present at 3 µM, 30 µM or 300 µM concentrations. The data obtained was plotted as the logarithm of the oxidised to reduced forms vs oxidation–reduction potential of the system. Both oxidative (o–o–o) and reductive (•–•–•) titrations were conducted. In each case the cytochrome a_3–CO compound titrated as a single component. Theoretical curves for an n=2 acceptor are drawn through the experimental points.

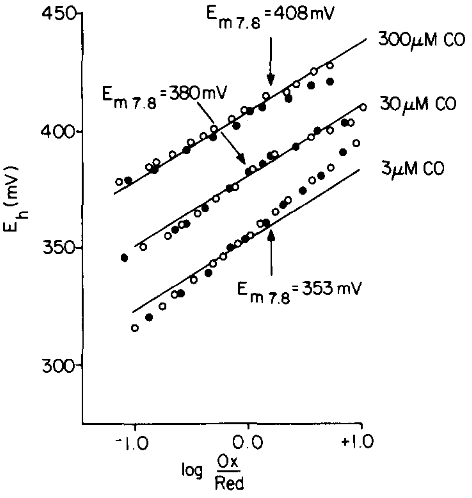


Fig. 3. Effect of CO concentration on the n value and midpoint potential of the cytochrome a_3–CO compound in purified cytochrome *c* oxidase. The experiment was carried out in an identical manner to fig. 2. Purified cytochrome *c* oxidase was present at 0.8 mg protein/ml (heme *a* concentration of 6 µM). The titration medium (MST) contained 0.3% (w/v) Emasol and 2 µM purified cytochrome *c* was added to facilitate mediation between the added dyes and the platinum-calomel electrode system. Both reductive (•–•–•) and oxidative (o–o–o) titrations were performed.

ing ferricyanide as an oxidant ($E_{m7.0}$=420 mV and pH independent).

Similar titrations were performed at several CO concentrations in pigeon breast submitochondrial particles (fig. 2) and in purified cytochrome oxidase (fig. 3). In each case the data was plotted as the logarithm of oxidised to reduced species versus the prevailing oxidation–reduction potential of the system. At all CO concentrations the total absorbance change due to the formation of the CO compound was found to be the same. At 30 µM and 300 µM CO, the CO compound titrated as a single species with an n value of 2 indicating that the oxidation–reduction of this species is a two-electron process. At low concentrations of CO (3 µM), significant deviations from n=2 were observed (n=1.5–1.8) in both the purified oxidase and pigeon breast particles. A possible basis of this effect will be mentioned later. From the data it is evident that the $E_{m7.8}$ of the CO compound is dependent on the CO concentration and in the range 3–300 µM CO increases by approx. 30 mV for each 10-fold increase in the CO concentration. The results obtained for purified cytochrome oxidase and pigeon breast particles were essentially identical and the midpoint potentials obtained at 3 µM CO, 30 µM CO and 300 µM were in the range 355 ± 10 mV, 385 ± 10 mV and 410 ± 10 mV respectively. At pH 7.8, the E_m value for cytochrome a_3 in the two preparations as determined by titration using the wavelength pair 605 minus 630 nm was 315 ± 10 mV, in agreement with earlier data [3,4].

Fig. 4 illustrates the midpoint potential of the CO compound in pigeon breast submitochondrial particles as a function of CO concentration. Titrations were performed at a number of CO concentrations as indicated on the figure. It is clear that in the region 3–300 µM CO the data points fit closely to a slope of 30 mV for each 10-fold increase in the concentration of CO.

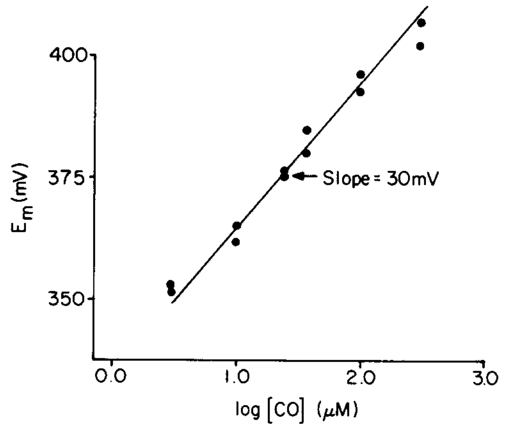


Fig. 4. Dependence of the midpoint potential of the cytochrome a_3–CO compound on the prevailing CO concentration. Titrations were carried out as described previously (fig. 1) [at a variety of CO concentrations] and the appropriate midpoint potentials calculated by plotting the data as shown in fig. 2.

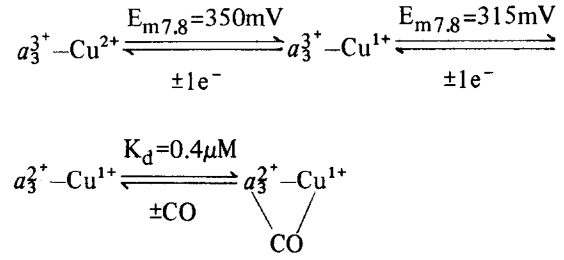
4. Discussion

Earlier communications from this laboratory [2,4,10,14] have dealt with the effects of ATP i.e. high (ATP)/(ADP)(Pi) on several components of the cytochrome *c* oxidase complex, namely cytochromes *a* and a_3, detectable copper and the cytochrome a_3–CO compound in pigeon heart mitochondria. Directly relevant to the present situation is that in the presence of ATP cytochrome a_3 is found to have a midpoint potential approx. 230 mV more negative ($E_{m7.2}$=155 ± 10 mV) than in the uncoupled system. The addition of saturating amounts of CO under these conditions results in the disappearence of the 155 mV-component and the appearance of the characteristic cytochrome a_3^{2+}–CO compound which titrates with an $E_{m7.2}$ of 350 ± 10 mV. Addition of uncouplers or oligomycin in this high potential region causes the immediate reduction of the cytochrome a_3–CO compound which is no longer readily oxidizable by ferricyanide. Thus it is concluded that ATP-induced alterations in the midpoint of the cytochrome a_3–CO compound occur in an analogous fashion to those observed on cytochrome a_3.

A surprising observation, however, is the fact that the a_3–CO compound, titrated in the presence *of ATP and saturating CO* (1.2 mM), always behaves as an n=2 (two-electron) acceptor in contrast to the commonly measured value of n=l for all a, b and c-type cytochromes in the mitochondrial respiratory chain (for reviews see [15,16]). The present data clearly confirm the n value of 2 in both pigeon breast particles and isolated cytochrome *c* oxidase, indicating that its anomalous redox properties are a function of CO binding and not related to the presence of ATP. Such titrations also clearly demonstrate that the E_m value of the cytochrome a_3–CO complex becomes 30 mV more positive for each 10-fold increase in CO concentration and is entirely consistent with CO binding to a reduced cytochrome a_3 – reduced copper complex rather than merely to reduced cytochrome a_3 heme.

In coupled pigeon heart mitochondria (ATP present) the midpoint potential of the cytochrome a_3–Cu–CO compound in the *range 150 mV to 300 mV* shifts by some 60 mV per 10-fold change in CO, suggesting that in this situation the formation of the CO compound is dependent only on reduction of the cytochrome a_3 heme [10].

Our present hypothesis is that both reduced cytochrome a_3 and the invisible copper participate in the CO binding, possibly with the CO forming a bridge between the two metal atoms. Schematically this can be expressed:



$$a_3^{3+}\text{–Cu}^{2+} \underset{\pm 1e^-}{\overset{E_{m7.8}=350\text{mV}}{\rightleftharpoons}} a_3^{3+}\text{–Cu}^{1+} \underset{\pm 1e^-}{\overset{E_{m7.8}=315\text{mV}}{\rightleftharpoons}}$$

$$a_3^{2+}\text{–Cu}^{1+} \underset{\pm\text{CO}}{\overset{K_d=0.4\mu\text{M}}{\rightleftharpoons}} \underbrace{a_3^{2+}\text{–Cu}^{1+}}_{\text{CO}}$$

where both cytochrome a_3 and the 'invisible' copper must be reduced before CO can bind with a high affinity (dissociation constant K_d, of 0.4 μM). In this scheme when the CO concentration is high, the E_m for the formation of the CO compound will be more positive than the E_m values for either cytochrome a_3 or copper. The formation of the CO compound will be a two-electron process (n=2.0) and essentially all of the reduced form is bound to CO. Thus a plot of

$$E_m \text{ vs log } \frac{\text{Total oxidase} - \text{CO compound}}{\text{CO compound}} \left(\log \frac{\text{ox}}{\text{red}}\right)$$

will show a strict two electron titration curve (30 mV per log unit). Under conditions for which the midpoint of the CO compound approaches the midpoint potential value for either cytochrome a_3 or the invisible copper (at low CO), or under conditions for which the cytochrome a_3 and Cu have greatly differing midpoints (e.g. in the presence of ATP) significant deviations from n=2 behavior may be expected. An example of the former case is seen in figs. 3 and 4 at 3 μM CO in which the n value of the titrations is in the region 1.5–1.8. Such anomalies have been utilized to permit an estimate of 350 ± 20 mV for the midpoint potential of the invisible copper at pH 7.8 in pigeon breast submitochondrial particles. A value of 350 mV for the $E_{m7.0}$ of a high potential copper of isolated cytochrome oxidase has recently been reported by Mackey et al. [17] using a coulometric titration method.

In the special case of coupled pigeon heart mitochondria, at high (ATP)/(ADP)(Pi), the midpoint potential of cytochrome a_3 becomes 220 mV more negative, and is probably much more electronegative than that of the invisible copper (assuming the latter to have an ATP-independent midpoint). Thus, under these conditions, at intermediate concentrations of CO, the cytochrome a_3–Cu–CO midpoint will be located between the E_m values of cytochrome a_3 and invisible copper. Formation of the CO compound in this potential range should be dependent solely on reduction of cytochrome a_3, giving an n value of 1.0 and a 60 mV change in midpoint potential per 10-fold change in CO, c.f. [18] in agreement with the experimental data [10]. A more detailed analysis of the n=1 to n=2 transition and a rigorous mathematical treatment of this situation will be presented in a later communication.

Carbon monoxide is a strictly competitive inhibitor of the reaction of oxygen with cytochrome *c* oxidase, suggesting that oxygen also binds to the reduced cytochrome a_3–reduced copper complex. The oxygen would then bind as a bridged compound between the two metal atoms. This would circumvent the thermodynamically unfavorable one-electron reduction of oxygen to O_2^- ($E_{m7.0}$=–0.45 V see [19]),leading instead to a two-electron reduction to a bridged peroxide compound. The peroxide intermediate could then be reduced to water in two one-electron steps or a single two-electron step.

Acknowledgements

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