The reduction of cytochrome *c* oxidase by carbon monoxide

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The rate of formation of the mixed-valence state of cytochrome *c* oxidase on incubation with carbon monoxide is strongly dependent on pH, supporting the concept that CO itself is the reducing agent. The reaction is biphasic due to the presence of two different enzyme forms in the resting oxidase. The kinetics of the reaction with the major enzyme form suggests an initial rapid binding of CO in a heme pocket of the oxidized enzyme and a subsequent slow intramolecular electron transfer to the cytochrome a_3-Cu_B site. Cytochrome *a* and Cu_A are not reduced even on prolonged incubation.

Cytochrome c *oxidase* *Cytochrome* a_3 *Carbon monoxide* *Reduction kinetics* *Heme pocket* *Mixed-valence state*

1. INTRODUCTION

Incubation of oxidized cytochrome *c* oxidase under an atmosphere of carbon monoxide, in the absence of dioxygen, results in the formation of the so-called mixed-valence state, a form of the enzyme in which the cytochrome a_3-Cu_B site has been reduced and bound CO, whereas cytochrome *a* and Cu_A remain oxidized [1]. The source of electrons in this 'autoreduction', as the process is generally called, has been thought to be an endogenous reducing agent. It is, however, known [2,3] that cytochrome oxidase can oxidize CO to CO_2 in the presence of O_2, suggesting that CO can donate electrons to the electron-transfer sites of the enzyme. Recently it has in fact been shown [4] that CO itself is the reducing agent when cytochrome oxidase as well as other dioxygen-binding heme proteins are incubated under CO.

In the process of studying the pH dependence of the electron-transfer kinetics in the mixed-valence oxidase, we discovered that the rate of autoreduction decreased drastically as the pH was lowered. This would be expected for a reaction in which CO reduced the binuclear cytochrome a_3-Cu_B site:

$$\begin{matrix} Fe_{a3}^{3+} \\ Cu_B^{2+} \end{matrix} + CO + 2OH^- \longrightarrow \begin{matrix} Fe_{a3}^{2+} \\ Cu_B^{+} \end{matrix} + CO_2 + H_2O \quad (1)$$

Furthermore, we found that the reduction kinetics is biphasic. The slow phase, which represents about 80% of the total change, is independent of the concentration of CO. This suggests that it involves an intramolecular electron transfer from CO which has become bound in a heme pocket of the cytochrome a_3-Cu_B site. The rapid phase, on the other hand, depends on the concentration of CO but its velocity, unlike that of the slow phase, does not decrease with decreasing pH. Thus, it is unlikely that this minor part (20%) of the reaction represents reduction by CO, and it is suggested that it involves a CO-driven electron transfer to cytochrome a_3 from Cu_A, which is reduced in about 20% of the molecules in the resting enzyme.

Bickar et al. [4] found CO to reduce not only cytochrome a_3-Cu_B but also cytochrome *a*. In our experiments, in contrast, cytochrome *a*, and also Cu_A, remained completely oxidized even after incubation under CO for an entire week. Conse-

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quently we would like to propose that reduction by CO is a specific property of the dioxygen-reducing site, whereas cytochrome *a* reduction can indeed be ascribed to a contaminating endogenous reducing agent present in some enzyme preparations.

2. MATERIALS AND METHODS

Cytochrome oxidase was prepared as described in [5]. The oxidase was dissolved in 0.05 M Hepes buffers, containing 0.167 M K_2SO_4 and 0.5% Tween 80, with different pH values.

The mixed-valence state cytochrome oxidase was prepared at room temperature by bubbling a mixture of CO and N_2 in different proportions for 10 min through an oxidase solution in a 1 cm cuvette provided with a side arm and a ground-glass stopcock.

Optical spectra were recorded in a Beckman Acta MIV spectrophotometer. For the flash photolysis experiments, a flashlamp-pumped dye laser (Phase-R model 2100-A with 1.5×10^{-4} M rhodamine 6 G) was used.

3. RESULTS

The spectral changes of cytochrome oxidase under 1 atm CO in the Soret and α-band regions are shown for 3 different pH values (6.0, 7.2 and 8.5) in fig.1. The development of a peak at 430 nm clearly shows the formation of cytochrome $a_3^{2+} \cdot CO$, whereas the shoulder remaining at 420 nm means that cytochrome *a* remains oxidized; the lack of reduction of cytochrome *a* is also seen from the lack of a peak at 605 nm. The spectra in fig.1A, B and C were recorded 15 min, 1 h and 4 h, respectively, after the start of the CO bubbling. It can be observed that at pH 8.5 the mixed-valence state has been formed to a considerable extent already after 15 min, while at this time there is less reaction at pH 7.2 and hardly any change at all at pH 6.0. The reaction at pH 8.5 is complete after 4 h, but the spectra at the lower pH values continue to change. After approx. 24 h all 3 solutions give essentially identical spectra. The spectrum of the solution with pH 7.2 did not change further on storage at 5°C for a period up to 1 week, demonstrating that cytochrome *a* remained oxidized. This was confirmed by EPR spectra,

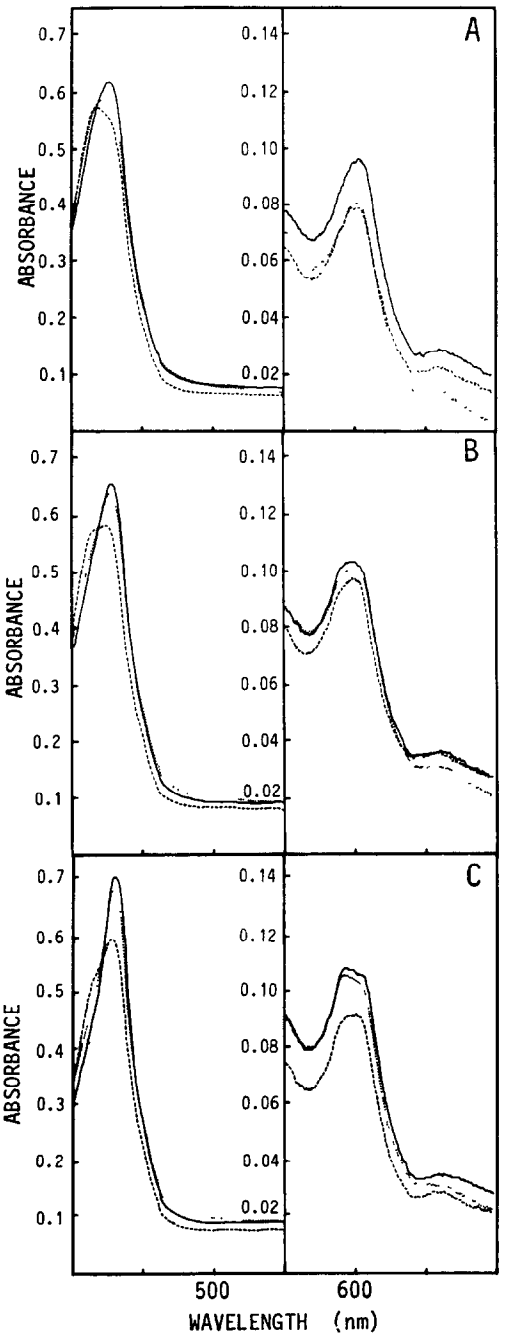

Fig.1. Spectral changes on reaction of cytochrome oxidase with carbon monoxide. Conditions: 3.3 μM cytochrome oxidase in 0.05 M Hepes with 0.167 M K_2SO_4 and 0.5% Tween 80; 1 atm CO; 20°C. (– – –) pH 6.0; (···) pH 7.2; (——) pH 8.5. (A) Spectra immediately after bubbling with CO; (B) after 1 h; (C) after 4 h.

which had full $g3$ and $g2$ resonances from cytochrome a^{3+} and Cu_A^{2+}, respectively.

The time course of the absorbance changes at 430 nm, shown in fig.2, demonstrates that the reaction is biphasic. About 20% of the total absorbance change occurs in an initial, fast phase which depends on the concentration of CO but is independent of pH. The second, slow phase, on the other hand, has a rate which changes with pH but is independent of the CO concentration. With 1 atm CO, the half-time of the rapid phase is approx. 7 min. The absorbance change in the slow phase is linear with time until it is close to completion with a rate of approx. 0.8 $\mu M \cdot h^{-1}$ at pH 7.2.

A flash photolysis experiment made at the end of the rapid phase, i.e. after approx. 20 min, showed absorbance changes corresponding to the dissociation and reformation of a cytochrome $a_3^{2+} \cdot CO$ complex.

The fast phase disappears if the oxidase is reduced with NADH and PMS (phenazine methosulfate) and then reoxidized with air prior to the CO bubbling. The difference spectrum between the reduced-reoxidized sample and the resting enzyme showed a small absorbance decrease at 415 nm and an increase in the near-infrared absorption, without any appreciable changes at other wavelengths.

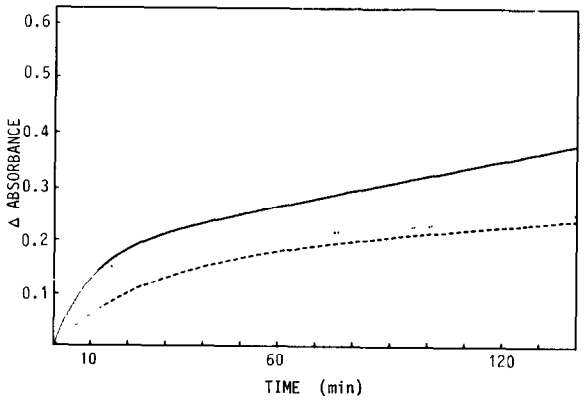

Fig.2. Time course of the absorbance change at 430 nm after bubbling with CO. The observations started 12 min after the beginning of bubbling but the curves have been extrapolated to time zero. Conditions: enzyme, buffer and temperature as in fig.1; pH and CO pressure (p_{CO}) varied. (——) pH 7.2, p_{CO} = 1 atm; (···) pH 6.4, p_{CO} = 1 atm; (– – –) pH 6.4, p_{CO} = 0.1 atm.

4. DISCUSSION

The spectra in fig.1 confirm the observation [4] that incubation of oxidized cytochrome oxidase under an atmosphere of CO leads to reduction of cytochrome a_3 and Cu_B, with subsequent coordination of CO to the reduced binuclear site. The results furthermore demonstrate that the rate of reduction decreases drastically if the pH of the solution is lowered from neutrality, whereas it increases when the pH is raised to 8.5. This observation is consistent with the reductive reaction given in eqn 1, first suggested by Bickar et al. [4], as 2 OH^- are consumed (or, equivalently, 2 H^+ are produced) in this process.

We found that the spectra at the longest time in fig.1 do not change on further prolonged incubation for a period up to 7 days. Thus, CO does not reduce cytochrome a and Cu_A. The presence of these components in their fully oxidized state was confirmed by EPR spectra. Our observations agree with the results of Greenwood et al. [1] but appear in conflict with those of Bicker et al. [4], who found reduction of cytochrome a as well, albeit at a much slower rate compared to cytochrome a_3. These findings suggest that cytochrome a reduction in the presence of CO only occurs with some preparations of the enzyme.

The reaction with CO is biphasic, as shown for pH 6.4 and 7.2 in fig.2. The slow, major phase, corresponding to about 80% of the total absorbance change, is essentially independent of CO concentrations. This suggests that the reduction process involves primary binding of CO to the oxidase, followed by a slow electron transfer in the complex. This requires the presence of a heme pocket with an affinity for CO even when cytochrome a_3 is oxidized. It has been observed [6] that CO changes the size and line shape of the high-spin EPR signal from cytochrome a_3^{3+}, establishing an interaction of CO with the oxidized site.

The rate of the rapid, minor phase is roughly proportional to the CO concentration, as shown in fig.2. It does not, however, represent a simple binding step, as flash photolysis at the end of this phase showed that it had led to the formation of a cytochrome $a_3^{2+} \cdot CO$ complex in about 20% of the oxidase molecules. As this phase is as rapid at low pH as at neutrality (fig.2), it is unlikely that CO is

the reducing agent. On the other hand, the rapid phase is eliminated if the enzyme is first reduced and then reoxidized in air (see section 3). In the resting enzyme only about 80% of Cu_A is detected by EPR but this amount increases to close to 100% on reduction and reoxidation [7]. Thus, we would like to suggest that Cu_A is reduced in 20% of the resting oxidase molecules, and that CO drives an electron transfer from Cu_A to cytochrome a_3. This interpretation is supported by the experiment with reduction and reoxidation, which leads to an increase in the near-infrared absorption associated with Cu_A^{2+}. The spectrum of the reoxidized sample also shows a small decrease in the heme absorption at 415 nm, without any concomitant increase at higher wavelengths. Thus, it does not represent heme reduction but rather a modulation of the heme absorption by Cu_A oxidation. This is consistent with the observation of Blair et al. [8] that reduction of cytochrome *a* and/or Cu_A causes an increase in the extinction at 415 nm associated with cytochrome a_3.

It is known that oxidized cytochrome oxidase exists in at least 3 conformations, the proportions of which depend on the method of preparation [9]. These conformations, in addition, differ with respect to ligand binding properties. It is noticeable that the minor fraction in our preparation reacts not only differently with CO but also with HCN compared to the majority of the oxidase molecules [10]. Thus, in 20% of the cyanide-inhibited molecules all sites except cytochrome a_3 are reduced, whereas in the rest of the molecules Cu_B remains oxidized. This is consistent with the presence of an additional reducing equivalent in the minor fraction.

The reduction potential for the reaction

$$CO_2 + 2e^- + 2H^+ \longrightarrow CO + H_2O \qquad (2)$$

has been calculated [11] to be -0.12 V at pH 0. At all pH values used in our experiments the potential would be more negative, whereas the lowest potential of the redox sites in the oxidase is $\sim +0.2$ V. From a thermodynamic point of view all metal centers should thus become reduced by CO. The fact that reduction of cytochrome a_3 and Cu_B only is observed suggests that the barrier to the reduction of cytochrome *a* and Cu_A is kinetic in nature. Probably the two-electron nature of the reaction (eqns 1 and 2) makes it rapid only with a two-electron acceptor, such as the binuclear cytochrome a_3-Cu_B site. A consequence of this conclusion is also that in those cases in which cytochrome *a* reduction is found, it is caused not by CO but by an endogenous reductant present as a contaminant in some oxidase preparations.

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