Dynamics of carbon monoxide recombination to fully reduced cytochrome *c* oxidase in plant mitochondria after low-temperature flash photolysis

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(Received 6 April 1982/Accepted 14 May 1982)

Rebinding of CO to reduced cytochrome *c* oxidase in plant mitochondria has been monitored optically at 590–630 nm after flash photolysis at low temperature from 160 to 200 K. (1) Under 100% CO saturation, CO rebinding exhibits a four-step mechanism. The thermodynamic parameters of the first phase have been determined; its activation energy, E_{a1} , is $38.9 \text{ kJ} \cdot \text{mol}^{-1}$ and its enthalpy, $\Delta H_1^\ddagger$, and entropy, $\Delta S_1^\ddagger$, of activation are respectively $37.5 \text{ kJ} \cdot \text{mol}^{-1}$ and $-75.8 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. (2) When the CO concentration is decreased to 0.2%, rebinding still occurs according to a four-step mechanism. The rate constant of the first phase is CO-concentration-independent. Under non-saturating conditions there is only one CO molecule per occupied site. The rebinding mechanism does not require additional CO molecules to be present in the haem pocket. (3) Dual-wavelength scanning experiments failed to detect optical forms correlated with the resolved phases. (4) Results are discussed with respect to previous work related to CO rebinding to mammalian cytochrome *c* oxidase and myoglobin.

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal oxidase of the electron-transport system of all higher organisms, contains, as a minimum functioning unit, two haem groups and two copper atoms, Cu_A being associated with haem *a* and Cu_B haem *a*₃. An extensive review on this complex, which catalyses the reduction of dioxygen to water, has been published by Malmström (1979). Cytochrome *c* oxidase is also involved in ATP synthesis (see review by Wikström & Krab, 1979). This interaction between electron transport and energy transfer made this enzyme the object of intensive studies, carried out essentially with the mammalian system, over many years. Though some optical differences were known for plant cytochrome *c* oxidase (Bendall & Bonner, 1966), the plant system was thought to behave and function like the mammalian one (Storey, 1970). The seven subunits resolved in sodium dodecyl sulphate/polyacrylamide gel with the yeast cytochrome *c* oxidase (Mason & Schatz, 1973) suggest an analogy with the mammalian system (Ludwig *et al.*, 1979) rather than with the bacterial cytochrome *c* oxidase, where only two subunits have been resolved (Ludwig & Schatz, 1980).

After the development of the low-temperature flash-photolysis technique of Chance *et al.* (1975*a*), intermediate compounds of the reaction with O_2 have been investigated in plant mitochondria from

the mixed-valence state (Denis & Bonner, 1978; Denis & Clore, 1981). The formation of the *c*-type species (Chance *et al.*, 1975*b*) which was resolved into two forms, C_{606} and C_{610} , by Denis (1981) in ox heart mitochondria, yielded only one component in potato mitochondria (Denis & Bonner, 1978) with distinct optical properties, a split α -band in particular. These findings have been confirmed in a kinetic study by Denis & Clore (1981) involving rapid-scan spectrometry (Denis & Ducet, 1975). The observed differences do not seem to affect the general process; the redox state assigned to the final stage of the reaction is identical with the one attributed to the mammalian enzyme, i.e. $\text{a}^{3+} \text{Cu}_A^{2+} \text{a}_3^{2+} \text{Cu}_B^{2+} \text{O}_2^-$ (Denis, 1977). The split α -bands of the intermediate compounds observed in potato mitochondria suggested the possibility of different environments for haem *a*₃ (Denis & Bonner, 1978). To check this hypothesis, liganding studies at the active site offer an opportunity to explore the binding site and its surroundings with no interference from the complex electron-transfer mechanism. Kinetic studies of CO rebinding to the mammalian reduced haem *a*₃ revealed an essentially monophasic process (Sharrock & Yonetani, 1976; De Fonseka & Chance, 1978). However, a controversial intermediate step has been reported by Clore & Chance (1978).

We present here an investigation on CO rebinding to reduced cytochrome *c* oxidase in potato mitochondria after flash photolysis in the low-temperature range 160–200 K. Four steps have been resolved in the rebinding process, which was found to be CO-concentration-independent. The thermodynamic parameters of the first phase have been determined. Experiments with CO concentration down to one-quarter the cytochrome *c* oxidase concentration showed that the overall process can be simplified to one CO molecule binding one haem iron atom with no requirement for other surrounding CO molecules. Results are also discussed with respect to CO rebinding to myoglobin, with which four distinct processes were observed (Austin *et al.*, 1975). Rebinding mechanisms are suggested in the light of recent far-i.r. investigations by Alben *et al.* (1981) and Caughey *et al.* (1981). Our findings unveil new properties of cytochrome *c* oxidase and demonstrate that the plant system deserves more attention.

Experimental

Biochemical methods

Mitochondria were extracted from potato (*Solanum tuberosum* L.) tubers as described by Ducet *et al.* (1970a) and purified by centrifugation on a discontinuous sucrose gradient. Purified mitochondria were suspended at 273 K in a medium comprising 0.1 M-mannitol, 50 mM-potassium phosphate buffer, pH 7.2, and 5 mM-succinate. Ethylene glycol was added (final concn. 30%, v/v) and the preparation was left for 10 min (i.e. until anaerobiosis was established), then saturated with CO. The concentration of CO in the CO-saturated preparation was 1.2 mM (Chance, 1978). The concentration of mitochondria was 40 mg of protein/ml, which contains 8.8 μ M-cytochrome *c* oxidase [calculated from $\Delta\epsilon_{\text{red./ox.}} = 24.0 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 605 nm (Van Gelder, 1963)]. The CO-saturated preparations were stored in an airtight syringe at 273 K until further use.

In experiments with CO-saturated samples, mitochondria were transferred into 2 mm-optical-path-length cuvettes at 253 K and previously de-aerated with CO for optical studies. Then the sample was promptly frozen in liquid N₂. In order to vary CO concentration, appropriate amounts of reduced mitochondria were stored in an airtight syringe before bubbling with CO. Mixture of CO-bound and unbound mitochondria was performed at 253 K inside 2 mm-optical-pathlength cuvettes under N₂ before being frozen in liquid N₂. This procedure prevents loss of the liganded form. Although unnecessary for CO-saturated experiments, ethylene glycol was used to maintain identical conditions in all assays and to make comparisons reliable.

Spectrometric techniques

Dual-wavelength spectrometry (Chance & Graham, 1971) was carried out with a Johnson Foundation spectrometer built from two Bausch and Lomb 250 mm grating monochromators. The recording conditions correspond to a 4 nm bandwidth. The transmitted light was monitored by using a fused-silica-window photomultiplier (EMI 9558QB) encompassing all the visible range. A microprocessor-controlled unit performed the data acquisition, either in a scanning mode with automatic baseline correction, or by kinetic observations at a fixed wavelength pair. Absorbance variations were plotted respectively on an *xy* recorder (Sefram, TGM 164) or on a single-trace recorder (Sefram, Servotrace PE) with a 0.3 s time constant. The fixed reference wavelength employed was 630 nm.

Data recordings

The CO-inhibited system was placed in the Dewar flask of the spectrometer through which thermo-regulated N₂ (Air Liquide, Philips) was flowing at the desired temperature in the range 120–220 K and equilibrated for 10 min in the dark. The temperature of the sample was controlled by a K-type thermocouple inserted in its holder. Deviations from the indicated values were less than 1 K. The sample was photolysed with a 500 J xenon flash lamp (Cunow) with a pulse width of 1 ms. A single flash ensured better than 95% CO dissociation. For experiments at a single wavelength pair, the absorbance level before photolysis was taken as reference. In the spectra-recording procedure, the CO-bound state was recorded and stored in the digital memory for use as the baseline after photolysis. CO recombination was monitored in a repetitive scanning mode at a rate of 40 s per scan with a 100 nm interval. The output was the difference spectrum between the reaction state and the fully reduced CO-bound state stored before photolysis. This low-temperature trapping technique was derived from the one described by Chance *et al.* (1975a). Linear regressions were performed with a desk calculator.

Results

Our investigations were performed in the temperature range 160–200 K, where the rates of CO recombination were compatible with the ones of our recording system. Absorbance changes were monitored essentially in the α -region. The wavelength pair for kinetic recordings was set at 590–630 nm, taking into account the optical properties of the CO compound difference spectrum (Ducet *et al.*, 1970b) and the band shift due to low temperature. Absorbance changes recorded after photolysis (ΔA) are referred to the absorbance level of the CO-bound state and therefore express the amount of the

mitochondrial unbound cytochrome *c* oxidase at time *t* after the flash. Fig. 1 shows a typical chart recording of CO rebinding at 192 K after a single flash photolysis.

CO-saturated mitochondria

A series of experiments were carried out with mitochondrial cytochrome *c* oxidase saturated with CO. Results are presented in the classical way by $\ln(\Delta A/\Delta A_0)$ versus time, where ΔA_0 is the maximum absorbance change resulting from the flash photolysis. Fig. 2 illustrates such kinetics of CO recombination monitored at 192 and 173 K after a single flash. The rebinding of CO to the fully reduced cytochrome *c* oxidase in this plant system exhibits clearly a multiphasic process. In all experiments performed over the indicated temperature range (160–200 K), four steps have been resolved. Attention is drawn to the difference in the time scale between Fig. 2(a) and Fig. 2(b), which makes the two first steps less distinct in Fig. 2(b).

Thermodynamic characterization of the first step

For each recorded kinetic curve, the rate constant k_{+1} of the first observed step was calculated after correction from the contributions brought by the other phases. Thus, at 192 K, $k_{+1} = 25.2 \times 10^{-3} \pm 0.8 \times 10^{-3} \text{ s}^{-1}$. The temperature-dependence of k_{+1} is illustrated by the Arrhenius plot shown in Fig. 3. It corresponds to an activation energy for the first phase of $E_{a1} = 38.9 \pm 1.6 \text{ kJ} \cdot \text{mol}^{-1}$. Representing the same data as $\ln(k_{+1}/T)$ versus $1/T$ (not shown) yielded directly the values of the enthalpy, $\Delta H^\ddagger$, and entropy $\Delta S^\ddagger$, of activation, i.e. $\Delta H^\ddagger = 37.5 \pm 1.6 \text{ kJ} \cdot \text{mol}^{-1}$, $\Delta S^\ddagger = -75.8 \pm 9.1 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. This allows one to calculate the free

Fig. 2. log-linear plot for the 590–630 nm trace at 192 K (a) and 173 K (b) under 100% CO saturation. The experimental points from the 590–630 nm traces illustrate the absorbance change (ΔA) of the unbound species with respect to the overall deviation (ΔA_0) produced by the flash. Straight lines correspond to the best linear fit in the associated time interval and reveal four distinct steps in the rebinding process. The samples were as in Fig. 1. Note the different time scales between (a) and (b).

Fig. 1. Chart recording of CO rebinding to fully reduced cytochrome *c* oxidase in plant mitochondria after a single flash photolysis at 192 K under 100% CO saturation. Absorbance changes were monitored at 590–630 nm. The sample, containing $8.8 \mu\text{M}$ -cytochrome *c* oxidase, was prepared as described in the Experimental section.

energy of activation of the first step, $\Delta G^\ddagger$, at a given temperature, from the relationship:

$$\Delta G^\ddagger = \Delta H^\ddagger - T \Delta S^\ddagger$$

For instance, at 177 K, a central value in the investigated range, $\Delta G^\ddagger = 50.9 \pm 3.2 \text{ kJ} \cdot \text{mol}^{-1}$.

Effect of CO concentration

CO concentration was varied from 1.2 mM (corresponding to 100% CO saturation; Chance, 1978) to $2.4 \mu\text{M}$ (0.2% CO saturation). At whatever CO concentration over this range, the rebinding process remained multiphasic, with four distinct steps (Fig. 4). As shown in Fig. 5 for a fixed temperature of 196 K, the rate constant of the first step is not sensitive to changes in CO concentration. We must emphasize that data include a CO concentration significantly lower than that of cytochrome *c* oxidase ($8.8 \mu\text{M}$). As expected, when CO concentration was

Fig. 3. Temperature-dependence of the rate constant k_{+1} of the first phase observed under 100% CO saturation. The Arrhenius plot of k_{+1} over the investigated temperature range exhibits a straight-line behaviour corresponding to an activation energy, E_{a1} , of $38.9 \pm 1.6 \text{ kJ} \cdot \text{mol}^{-1}$.

lower than that of the enzyme, the maximum absorbance change on photolysis, ΔA_0 , decreased correspondingly. Within the experimental precision, the ratio of $\Delta A_0/\Delta A_0$ (100% CO) was identical with the ratio of $[\text{CO}]/[\text{cytochrome } c \text{ oxidase}]$. This means that under non-saturating conditions there is only one CO molecule per occupied site. However, accumulation of several CO molecules in the vicinity of one binding site cannot be excluded under saturating conditions. We must point out that such an accumulation, if it occurs, has no apparent effect on the rebinding process. Data in Fig. 5 also demonstrate the lack of a diffusion process in the rebinding mechanism. This could be considered as a direct proof for the existence of a pocket including the active binding site of haem a_3 (Chance, 1977; Denis & Clore, 1979).

Optical properties

It was important to know if the observed multiphasic CO rebinding process was generating different spectral forms. With this in mind, kinetics have been monitored in a wavelength-scanning mode

Fig. 4. Log-linear plot for the 590–630 nm trace at 196 K (a) and 167 K (b) in presence of 0.2% CO. At low CO concentration, CO rebinding still follows a four-step mechanism with better resolved phases than under 100% CO saturation, as illustrated by Fig. 2. Other-than-specified conditions are as defined in the Experimental section.

Fig. 5. CO-concentration-dependence of $\ln k_{+1}$ at 196 K. Within the experimental error, k_{+1} is not affected by changes in CO concentration over the investigated temperature range. Note that two different scales are used to illustrate the CO concentration variations expressed in terms of percentage of CO saturation.

as described in the Experimental section. Fig. 6 shows such a set of spectra recorded at 183 K from 650 to 550 nm, with 630 nm as reference wavelength. The reference spectrum is the one of the CO-bound species recorded before flash photolysis. The indicated time intervals do not include the 40 s of

Fig. 6. CO rebinding at 183 K under 10% CO concentration followed by dual-wavelength scanning spectrometry

The reference spectrum is the one of the CO-bound form memorized before the flash. The reference wavelength is taken at 630 nm. Spectra 1–8 were recorded in a repetitive mode, then time intervals were added as follows: spectrum 9, +0.5 min; 10, +1 min; 11, +1 min; 12, +1.5 min; 13, +2 min; 14, +3 min; 15, +5 min; 16, +5 min. The given values do not include the 40 s required for scanning between 650 and 550 nm. No significant optical feature can be correlated with the resolved phases.

scanning time. The optical features remain unchanged all through CO rebinding. Observations in the Soret region (not shown) also failed to give evidence of distinct optical forms correlated with the four steps of the CO rebinding process. From continuous recordings at 590–630 nm at the same temperature it was checked that the first spectrum belongs to the first stage, so that all the resolved phases are optically present in the set of spectra recorded in Fig. 6.

Discussion

Semilogarithmic plots of CO rebinding to fully reduced cytochrome *c* oxidase in plant mitochondria have resolved a four-step process in the investigated temperature range (160–200 K) as illustrated by Figs. 2 and 4. No spectral form has been resolved in correlation with the observed four phases (Fig. 6). In a kinetic study of the reaction of cytochrome *c* oxidase with oxygen in the same plant mitochondria, Denis & Clore (1981) did not observe multiphasicity in O₂ binding (compound A₂ formation). We can therefore exclude hetero-

geneity in the protein preparation to account for the observed complex CO-rebinding mechanism.

The good fit of the linear regressions identifies step 2 as a real one, and one which is not produced by a hypothetical overlap of the first and third phases. Though numerical analysis will be necessary to assess the real number of stages which can be resolved from the rebinding kinetics, the present data show that CO rebinding follows a multiphasic mechanism in this plant system. To our knowledge this is the first significantly multiphasic process reported for CO rebinding to reduced cytochrome *c* oxidase after low-temperature flash photolysis. Indeed, studies related to the mammalian system (ox heart cytochrome *c* oxidase) revealed an essentially monophasic process over temperature ranges overlapping the one used in the present work (Sharrock & Yonetani, 1976; De Fonseka & Chance, 1978, 1979). Sharrock & Yonetani (1976) reported in addition preliminary results detecting two phases when a 1% CO concentration was used.

Though CO was still in excess with respect to haem a₃, the two rate constants observed were smaller than the one of the monophasic process under CO saturation. (These preliminary findings are still waiting to be confirmed.) Dealing with cytochrome *c* oxidase in ox heart mitochondria, Clore & Chance (1978) derived a two-step mechanism from numerical analysis (see also Clore, 1981). Their conclusion is not supported by De Fonseka & Chance (1978, 1979), arguing that correcting the data from the photodissociation produced by the measuring beam leads unambiguously to a monophasic CO-rebinding process. The magnitude of the correction for photodissociation was insufficient to be evident in the semilogarithmic representation of the data. For the plant cytochrome *c* oxidase studied here, the multiple decays cannot be accounted for by a photodissociation effect, given the magnitudes and time scales involved.

Some homologies can be drawn with CO rebinding to sperm-whale myoglobin (Austin *et al.*, 1975). Investigating over a large temperature range (40–350 K), these authors found up to four distinct processes, depending on the temperature. A single step was observed below about 180 K, and successively between 180 and 280 K up to four processes could be seen, the last one being proportional to ligand concentration. In the present study, the number of steps was not temperature-dependent.

Our results do not involve a diffusion mechanism (no CO concentration effect). We should therefore expect to detect one at higher temperature, close to the melting point of the sample, as suggested by Sharrock & Yonetani (1976). As for the mammalian system, CO recombines extremely slowly at temperatures below 160 K (Chance *et al.*, 1965; Denis & Clore, 1979). By revealing no CO diffusion

into the solvent, the low-CO-concentration experiments provide good evidence for the existence of a haem pocket previously suggested by Chance (1977) and Denis & Clore (1979) from low-temperature flash-photolysis studies.

The first step of the observed multiphasic CO recombination has an apparent energy of activation, E_{a1} , of $38.9 \pm 1.6 \text{ kJ} \cdot \text{mol}^{-1}$, a value close to that of the monophasic mammalian system, namely $35 \text{ kJ} \cdot \text{mol}^{-1}$ (Erecinska & Chance, 1972), $36 \text{ kJ} \cdot \text{mol}^{-1}$ (Yonetani, 1965), $42 \text{ kJ} \cdot \text{mol}^{-1}$ (Sharrock & Yonetani, 1976), or to the value of $40.9 \text{ kJ} \cdot \text{mol}^{-1}$ associated with the first phase described by Clore & Chance (1978). For comparison, the apparent energy of activation of process I in CO rebinding to myoglobin is only $10 \text{ kJ} \cdot \text{mol}^{-1}$ (Austin *et al.*, 1975). However, the entropy changes are of comparable extent, $-82 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ for myoglobin and $-75.8 \pm 9.1 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ for the present first step. The overall decrease in entropy observed for CO rebinding to myoglobin was interpreted by Austin *et al.* (1975) by the bridge $\text{Fe}-\text{CO}-\text{N}_{\epsilon 2}$ linking the haem group and the distal histidine residue (His-64) and stabilizing a major part of the protein. No such bridge function can be assigned to haem a_3 -bound CO. Indeed, in a recent study by low-temperature Fourier-transform i.r. spectroscopy of CO complexes, Alben *et al.* (1981) demonstrated that CO photolysed from haem a_3 is bound reversibly to Cu_B and ruled out the possibility for bridging CO complexes. The presence of Cu_B in close vicinity of the active site of haem a_3 is also supported independently by Denis (1981) from the study of the C-type compounds.

To account for their multiprocess CO rebinding to myoglobin, Austin *et al.* (1975) postulated a model involving four barriers in sequence and, on the basis of the known structure of myoglobin, assigned tentatively these barriers. At the present stage of our study it is difficult to go into such details, since the tertiary structure of cytochrome *c* oxidase is still to be determined. However, we are left with a more simple situation. From the low-CO-concentration experiments, we can say that the multiphasic process of CO rebinding to haem a_3 has to be explained in terms of one CO binding one iron a_3 and does not need to involve additional CO molecules trapped inside the pocket. The general scheme could be the following: the flash photolysis would reject CO to one of four possible binding sites within the pocket, one of them being Cu_B (Alben *et al.*, 1981). Then CO would bind haem a_3 either directly or getting closer and closer through the intermediate binding positions, the last one being Cu_B . Recent evidence for four discrete rapidly interconverting conformers at the CO-binding site of bovine heart myoglobin (Caughey *et al.*, 1981) makes reasonable a similar situation in cytochrome *c* oxidase. Whatever the

model to be selected, it must include Cu_B as the binding position preceding iron liganding in the recombination process.

Numerical analysis of the observed kinetics will probably help in discriminating between the above models. The present findings show that cytochrome *c* oxidase in higher-plant mitochondria is a very interesting system for investigating the properties of this complex owing to its differences and its specificities with respect to the mammalian one.

We are indebted to Professor G. Ducet and Dr. D. Job for many stimulating discussions. This work was supported by the Centre National de la Recherche Scientifique.

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