Biochimica et Biophysica Acta, 462 (1977) 718–730

BBA 47399

LOW-TEMPERATURE FLASH PHOTOLYSIS STUDIES OF CYTOCHROME OXIDASE AND ITS ENVIRONMENT

M. SHARROCK* and T. YONETANI

Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pa. 19174, (U.S.A.)

(Received April 21st, 1977)

SUMMARY

The CO-binding kinetics of cytochrome a_3 in isolated, detergent-solubilized cytochrome oxidase have been studied by flash photolysis over wide ranges of CO concentration and temperature. The results strongly suggest that CO has an intermediate bound state in its path to the final bound state at the heme iron. In the temperature range 230–273 K in frozen aqueous solutions, the recombination rates depend upon CO concentration; at low CO concentrations the kinetics are biphasic. The rate of the faster process depends upon the detergent concentration, that of the slower process upon the salt concentration. In addition, the faster process depends upon the amount of CO photodissociated. It is concluded that the cytochrome oxidase molecules are aggregated in regions that contain detergent and possibly some lipids. These regions retain considerable fluid character well below the macroscopic freezing point of the solution. The faster phase of the recombination is interpreted as the rebinding of CO molecules that remain in the fluid region after photodissociation. The slower phase would then be due to the migration of some dissociated CO out into the surrounding frozen solvent. The non-Arrhenius behavior of both phases probably represents partial melting of the medium; preliminary NMR measurements of mobile protons support this hypothesis. Many of the kinetic features described here are also seen in mitochondria; thus the detergent-solubilized cytochrome oxidase may be a useful model system for the ligand-binding behavior of the enzyme in the mitochondrial membrane.

INTRODUCTION

Cytochrome oxidase has been the subject of many studies [1–3] because of its unique role as the donor of electrons to molecular oxygen in the respiratory chain. In particular, reactions of this enzyme with oxygen have been probed by low-temperature experiments aimed at elucidating the features of ligand-binding

* To whom correspondence should be addressed. Present address: Department of Physics, Gustavus Adolphus College, St. Peter, MN 56082, U.S.A.

and electron transport [4, 5]. The binding of CO to the cytochrome a_3 of cytochrome oxidase is very probably analogous to the binding of oxygen; in addition this inhibitor molecule is easily and reversibly photodissociable. For these reasons, flash photolysis of the cytochrome a_3-CO complex is a valuable probe of the active site.

The use of low temperatures greatly enhances the information available from the flash photolysis method. In many heme proteins, for example myoglobin, the binding of CO involves a number of separate steps that can only be resolved by the use of low temperatures [6]. The simpler behavior of cytochrome oxidase [7, 8] is strong evidence that the heme site of cytochrome a_3 differs in some fundamental way from those of myoglobin and hemoglobin. The unusual nature of the cytochrome a_3 site was distinguished in early photodissociation studies by an extremely slow recombination with CO at temperatures below 160 K, in contrast to easily observable rebinding well below 50 K by other heme proteins [6, 9, 10, 11].

A second advantage of low-temperature photolysis studies is the ability to confine the reaction to the immediate vicinity of the heme site, by the freezing of the surrounding medium. Thus, one can separate those aspects of the kinetics that depend upon the nature of the heme site from those that depend upon the solvent or membrane environment. A preliminary account described some observations on the effect of the cytochrome oxidase environment on its CO-binding [12]; here we discuss in detail the data and its significance.

METHODS

Cytochrome oxidase was isolated from beef-heart by published procedures [13] and stored under liquid nitrogen until used. It was then dissolved at 10 μM in an aqueous potassium phosphate buffer (pH 7, 0.1 M unless stated otherwise) with the addition of Tween 20 detergent (Sigma Chemical Co.) at a concentration of 1 % by vol. unless stated otherwise. The solution was equilibrated with the desired gas mixture at room temperature and reduced with a small amount of sodium dithionite. At least 15 min were allowed for the reduction. Pure CO gas was obtained from the Matheson Company; 1 % CO/99 % N_2 and 10 % CO/90 % N_2 mixtures were supplied by Airco (Riverton N.J.). CO partial pressures smaller than 0.01 atm were obtained by diluting the 1 % mixture with nitrogen. For experiments below 273 K, the sample was injected into a special cuvette that had been cooled in liquid nitrogen and flushed with the gas mixture with which the sample had been equilibrated. It is difficult to assure rigorous control of the CO concentration in a freezing aqueous solution because of changes of solubility and possible solute migration. Therefore, the concentrations referred to for frozen samples must be considered as approximations. A thermocouple of gold-cobalt alloy and copper inside the cuvette was used for temperature measurement.

Rebinding of CO to the heme iron was detected by observing the light absorption at 448 nm. The measuring beam was provided by a tungsten lamp and a monochromator; the intensity was not sufficient to appreciably perturb the measured kinetics. The transmitted light was monitored by an end-window photomultiplier tube of one-inch diameter (EMI Electronics, Ltd.) protected from the flash by glass filters (Corning CS 5-58 and CS 4-96). Photolysis was achieved in most experiments by the use of a pulsed dye laser (Model DL-1000, Phase-R Co., New Durham,

N.H.) using a solution of Rhodamine 6G in ethanol. The maximum flash energy was about 0.2 J at 590 nm. Experiments involving partial photolysis were done by reducing the voltage on the laser and by the use of neutral density filters in the beam. The laser beam was filtered with Wratten 16 filters to avoid perturbing the photomultiplier. Some experiments were done using an electronic flash (Kako 822) fitted with Wratten 9 and 15 filters. The electronic flash (approximately 1 ms duration) and the laser flash (approximately 1 μs duration) produce the same recombination rates, for a given amount of initial photodissociation. The flash, from either source, entered the cuvette from the same side as did the measuring beam.

The signal from the photomultiplier was processed by a computer-interfaced apparatus [14]; final data are displayed on a logarithmic time scale with 24 points per decade. The system optimizes the signal-to-noise ratio by applying progressively more signal-smoothing as time elapses after the flash.

The sample cuvette was placed in a quartz dewar and cooled by boil-off gas from liquid nitrogen. The temperature was adjusted with the aid of an electric heater. After flash photolysis at very low temperatures, the sample was warmed sufficiently to allow complete recombination of the CO complex before the next experiment.

NMR measurements were made with a Varian 220 MHz spectrometer fitted with a special low-temperature attachment.

RESULTS AND ANALYSIS

Fig. 1 shows some experimental curves for the recombination of cytochrome a_3-CO after photodissociation in a sample equilibrated with 0.002 atm CO*. It can be seen that a slower phase develops as temperature is increased. Fig. 2 shows the dependence of the biphasic kinetics on CO concentration, at a fixed temperature.

The two phases do not represent different forms of cytochrome a_3, since the biphasic nature of the rebinding disappears at CO concentrations corresponding to

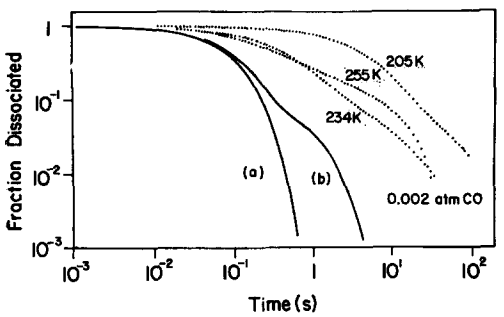

Fig. 1. Plot of photodissociated cytochrome a_3-CO vs. time, using logarithmic axes. The points shown are experimental values. With increasing temperature, the faster phase is accelerated, while a slower phase grows in magnitude. The solid lines are calculated curves included for comparison. Curve (a) is the exponential e^{-kt} where k is 10 s^{-1}. Curve (b) is biphasic; it is given by $0.9\,e^{-k_1 t}+0.1\,e^{-k_2 t}$ where $k_1 = 10\,s^{-1}$ and $k_2 = 1\,s^{-1}$.

* Unless otherwise stated, the data in this report were obtained by photodissociating, with a single flash, virtually all of the cytochrome a_3-CO complexes in the sample.

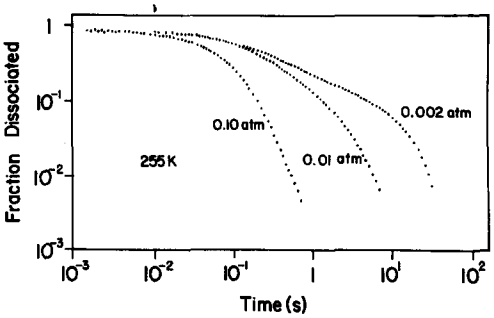

Fig. 2. Plot of photodissociated cytochrome a_3-CO vs. time, using logarithmic axes. The data shows the dependence of the two recombination processes upon CO concentration at a fixed temperature, 255 K. Before freezing, the samples were equilibrated with gas mixtures having the CO partial pressures shown.

partial pressures of 0.1 atm or more (Fig. 2). Furthermore, if a second flash is applied very soon after the initial flash, so that only the fast phase of recombination has proceeded appreciably, the subsequent kinetics show a larger proportion of the slow process than would be seen after a single flash. Thus, it appears that at least some of the hemes have the ability to recombine with CO by either the fast or the slow process. It should be stressed, however, that repeated flashes sufficiently separated in time to allow complete recombination give reproducible kinetics. That is, the photodissociation process causes no irreversible change in the sample.

The biphasic data were fit by the method of least squares to the expression

$$f(t) = (1-x)\,(1+t/t_1)^{-m}+x(1+t/t_2)^{-n} \tag{1}$$

where f (t) is the fraction of the photodissociated heme sites not yet recombined at time t after the flash. Such "power-law" expressions have been used to analyze the low-temperature recombination of myoglobin-CO [6] and the monophasic recombination of cytochrome a_3-CO studied earlier [8]. The parameter x is the fraction recombining by the slower process.

We will use as rate constants for the fast and slow processes, respectively,

$$k_f = m/t_1 \tag{2a}$$

and

$$k_s = n/t_2{}^{\star} \tag{2b}$$

As discussed earler [8], a "power-law" decay approaches an exponential decay for large values of m or n. In the fits to the data for recombining cytochrome a_3-CO at low CO concentrations, m is in the approximate range 1–2 while n is generally greater by one or more orders of magnitude. The significance of the low values of m will be discussed below. The large values of n indicate that the slow phase is approximately exponential.

Fig. 3 shows a plot of k_f and k_s as functions of inverse temperature. Below

---

* Rate constants defined in this way give the initial rate of change of the decaying quantity, just as k does for the exponential decay $f(t) = e^{-kt}$.

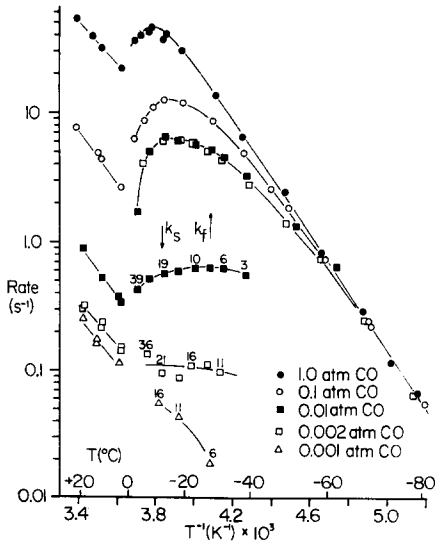

Fig. 3. Dependence of recombination rates upon inverse temperature, for various CO concentrations. The short, straight lines shown for temperatures above 273 K (0 °C) represent the monophasic kinetics seen in liquid samples. For frozen samples (below 273 K), the rates k_f and k_s (if observed) are shown; the numbers associated with the points for k_s give the approximate percent of the recombination occurring by the slower process. The values of k_f for 0.001 atm CO are not shown, since they do not differ significantly from those for 0.002 atm CO.

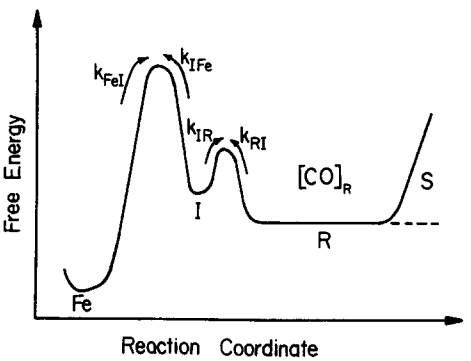

Fig. 4. Model for the cytochrome a_3 heme site and its environment in soluble cytochrome oxidase. S represents the frozen aqueous solvent; the broken line suggests the change that occurs upon melting.

about 215 K, the slow process (k_s) is not observed ; k_f follows an Arrhenius relationship with temperature and is independent of the CO concentration. At higher temperatures, k_f decreases with decreasing CO concentration. At about 0.01 atm CO, however, k_f reaches a minimum value and the slower phase appears. This slower process increases in magnitude with increasing temperature; its rate k_s is approximately proportional to the CO concentration in solution. Both k_f and k_s tend, with increasing temperature, toward the rate observed in liquid solution (above 273 K). This observation suggests that some partial melting takes place near the heme site, even at temperatures well below the macroscopic freezing point of the aqueous solution.

Above the freezing point, only a single process is observed. It obeys second-order kinetics and follows an Arrhenius relationship with temperature. The activation energy is about 35 kJ/mol (8.4 kcal/mol).

The dependence of k_f upon the CO concentration is not a simple proportionality; the rate increases only up to a limit given by the Arrhenius line extrapolated from low temperatures. A model that explains these features is shown schematically in Fig. 4. It postulates a region R and an intermediate state I between which CO can move. From I the CO can bind to the heme iron; it is then in the potential well designated Fe. We define rate constants for the various processes; for example, k_{IR} for the transition from I to R*. These are all first-order rate constants with the exception of k_{RI}, which is second-order.

As in the earlier report [8], we explain the loss of concentration dependence at low temperatures by assuming that only one CO molecule can be in state I at a time, for each heme site. Data presented here, measured using very low CO concentrations, help to verify that this is in fact a good assumption. It is also assumed, since k_f departs from the Arrhenius relationship with temperature but is not multiphasic, that $k_{IFe} \ll k_{IR}$**. Then, an equilibrium is established between I and R so that I is occupied a fraction y of the time between photodissociation and recombination. The average rate of transfer of CO from R to I will be $(1-y)k_{RI}[CO]_R$ where $[CO]_R$ is the concentration in R after photodissociation with I unoccupied. (There can be no transfer from R, according to the model, if I is already occupied.) The rate for motion from I to R is yk_{IR}. Equating the two rates:

$$yk_{IR} = (1-y)k_{RI}\,[CO]_R \tag{3}$$

and

$$k_f = yk_{IFe} = k_{IFe}(1+k_{IR}/k_{RI}[CO]_R)^{-1} \tag{4}$$

In the previous report [8], the quantity corresponding to $[CO]_R$ was influenced by the presence of an additional region; this refinement will not be needed here. Eqn. 4 accounts for the temperature and concentration dependences of k_f (Fig. 3), provided that $k_{IR}/k_{RI}[CO]_R$ increases with increasing temperature. At low temperatures (below 215 K),

$$k_{IR}/k_{RI}[CO]_R \ll 1 \tag{5}$$

and

$$k_f \simeq k_{IFe} = Ae^{-E/RT} \tag{6}$$

where $A = 2.5 \cdot 10^{10}\,s^{-1}$ and $E = 43$ kJ/mol (10.3 kcal/mol). For convenience, we define the empirical parameters F and P by

$$k_f = Fk_{IFe} \tag{7}$$

---

* The notations Fe, I, R are used in place of the A, B, C of the earlier report [8] in order to avoid confusion with features of myoglobin [6] and with the intermediate oxygen complexes of cytochrome oxidase [4, 5].

** The CO dissociation rate for the cytochrome a_3 heme is very small ($k_{dissoc} = 0.02\ s^{-1}$ at 293 K, ref. 15); k_{FeI} will be neglected in all calculations.

and

$$k_f = k_{IFe}/(1+1/P) \tag{8}$$

where k_f is measured experimentally and k_{IFe} is extrapolated from low temperatures. If the faster process (k_f) were a simple second-order bimolecular reaction, the parameter F would be proportional to the CO concentration in the immediate environment of the heme iron after photodissociation. However, if Eqn. 4 is a good representation of the process (that is, if the intermediate I with the assumed properties exists), then the parameter P will be equal to $k_{RI}[CO]_R/k_{IR}$ and is expected to be proportional to the CO concentration. Fig. 5A shows that P is in fact linearly related to the CO partial pressure with which the sample was equilibrated before freezing, while F follows a non-linear plot. These results are in accord with the model.

The plots in Fig. 5A do not pass through the origin, in the limit of very low CO concentrations. The lower limit of P corresponds to the one free CO molecule per cytochrome a_3 heme made available by photodissociation. The upper value of P in Fig. 5A is about 27 times the lower limit. It can be inferred that in a sample equilibrated with 1 atm CO about 26 CO molecules per cytochrome a_3 heme are trapped in R and I after freezing. A sample equilibrated with 0.01 atm CO or less has virtually no free CO in these regions (until photodissociation takes place). These observations, combined with the fact that the recombination rates observed below 215 K (where $k_f \cong k_{IFe}$) are independent of CO concentration from 0.002 atm to 1.0 atm (Fig. 3), show that the intermediate state I can hold only one CO molecule, as assumed.

Fig. 5B shows that at a temperature where the fast phase depends upon CO concentration, k_f also depends upon the fraction of the cytochrome a_3-CO sites photodissociated. Therefore, a number of heme sites must share a common reservoir of free CO; more complete photodissociation gives rise to a larger transient concentration of CO in this reservoir. The region R in Fig. 4 should then be considered as common to several cytochrome a_3 sites, although each site presumably has its own I.

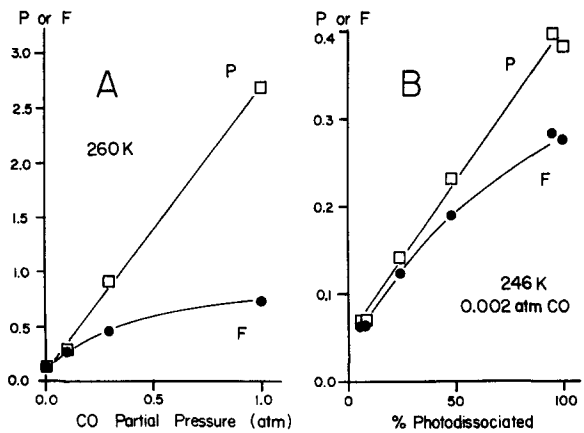

Fig. 5. (A) Plot of the kinetic parameters P and F, defined in the text, vs. the CO partial pressure of the gas mixture with which samples were equilibrated before freezing. The temperature is the same (260 K) for all points. (B) Plot of P and F vs. the percent of the cytochrome a_3-CO that was photodissociated by the laser flash. The temperature and CO concentration are the same for all points.

As in Fig. 5A, the linearity of the plot of P, in contrast to the behavior of F, in Fig. 5B is evidence for the existence of the intermediate state I.

The data in Fig. 5B were obtained from a sample that had been equilibrated with 0.002 atm CO; the results mentioned above indicate that in such a sample virtually all the free CO molecules in I and R are those released by photodissociation. Thus, a comparison of the upper limit of P (100 % dissociation) with the lower limit (extrapolated to 0 %) in Fig. 5B suggests that the average number of cytochrome a_3 hemes having access to each reservoir of free CO is at least eight (the transient CO concentration after complete dissociation is about eight times that after minimal dissociation).

If the temperature is sufficiently high, and the CO concentration sufficiently low, to make $k_{IR}/k_{RI}[CO]_R$ appreciably greater than unity, Eqn. 4 takes the approximate form

$$k_f \cong k_{IFe}k_{RI}[CO]_R/k_{IR} = k'[CO]_R \tag{9}$$

If the CO concentration corresponds to 0.01 atm or less, then $[CO]_R$ is essentially proportional to the number of cytochrome a_3-CO complexes photodissociated. Thus,

$$\frac{dFe_t}{dt} \cong -k'[CO]_R\, Fe_t \cong -k''Fe_t^2 \tag{10}$$

where Fe_t is the number of cytochrome a_3 heme sites not having a bound CO molecule at time t. Eqn. 9 can be integrated to give

$$Fe_t = Fe_{t=0}(1+k''Fe_{t=0}t)^{-1} \tag{11}$$

Eqn. 11 is a "power-law" decay and accounts for the values of m in Eqn. 1 being approx. 1, as mentioned above.

It was stated earlier that the quantity $k_{IR}/k_{RI}[CO]_R$ in Eqn. 4 must increase with temperature, in order to account for the observed rates (Fig. 3). In the earlier report [8], this increase was explained by differences of enthalpy between various regions in or near the heme site. However, the existence of a common reservoir of free CO, available to several cytochrome a_3 heme sites, suggests strongly that region R is a fluid volume in the frozen samples. The increase of this volume, due to local melting, could be sufficient to cause the increase of $k_{IR}/k_{RI}[CO]_R$ through the dilution of trapped CO. Some direct evidence for an increase of fluid volume with temperature, well below the macroscopic freezing point of the solution, is provided by proton nuclear magnetic resonance (NMR). This method detects those protons that are somewhat mobile, since those frozen into crystalline ice have an extremely broad resonance [16]. Fig. 6 shows that the NMR-detectable protons increase with temperature in a manner roughly proportional to the increase in volume needed to account for the temperature dependence of k_f. These mobile protons may be those of the detergent, or of lipids [17] or water [16, 18] associated with the protein. The NMR data are preliminary; more measurements would be needed to study in detail a quantitative relationship. It is not possible, at this point, to distinguish unambiguously between enthalpic effects and melting effects in the increase of $k_{IR}/k_{RI}[CO]_R$ with temperature.

Figs. 1, 2, and 3 show that a second phase (rate k_s) occurs in the CO-binding

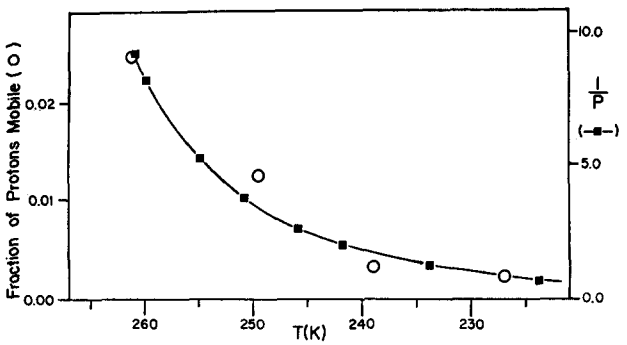

Fig. 6. Plot, vs. temperature, of the kinetic parameter $1/P$ and the fraction of the protons having sufficient mobility to be readily detected by 220 MHz NMR. The quantity $1/P$ (see Eqns. 4 and 8) is expected from the model to be proportional to the volume available to the free CO molecules near the heme iron; these experimental values were obtained using 0.002 atm CO. The mobile fraction of the protons was obtained by comparing the integrated NMR absorption (width approx. 300 Hz) of the frozen sample to that of a liquid sample (width approx. 20 Hz), correcting for the effect of inverse temperature. The samples used for both low-temperature kinetic measurements and NMR had 0.1 M potassium phosphate, 1 % Tween 20, and 10 μM cytochrome oxidase.

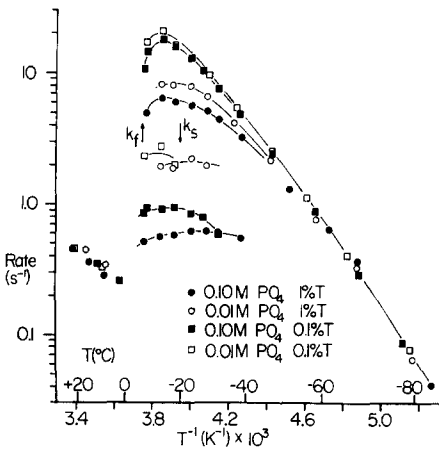

Fig. 7. Dependence of the recombination rates upon the concentration of potassium phosphate buffer (PO_4) and Tween 20 detergent (T). All samples were equilibrated with 0.01 atm CO before freezing. Above the melting point of the aqueous solutions, little or no dependence upon buffer or detergent concentration is seen. The fraction of the heme sites that rebind CO by the slower process is difficult to determine precisely; however, it does not appear to depend strongly upon the Tween or phosphate concentration.

kinetics at temperatures above about 230 K in samples prepared with low CO concentrations (0.01 atm or less). This process can be interpreted as resulting from the diffusion of some of the free CO molecules out of R and I and into the surrounding solvent (S in Fig. 4) after photodissociation has taken place. Complete recombination then requires diffusion of CO (not necessarily those molecules originally present in R and I) back from S to the active site. It is not clear whether the slow process proceeds via R or by an alternate pathway.

The slower phase is not seen in samples prepared with CO partial pressures of 0.1 atm or more; at such concentrations there are enough free CO molecules in region R to assure that recombination takes place by the faster process (rate k_f). Biphasic kinetics are also not seen in liquid samples (above 273 K) because, presumably, the distinction between R and S vanishes when the sample melts.

The slower phase appears to obey second-order kinetics. Fig. 3 shows that k_s is approximately proportional to the CO partial pressure used in the sample preparation. At the lowest concentrations used (0.001 and 0.002 atm), the photodissociated CO molecules make an appreciable contribution to the total free ligand population in the vicinity of the cytochrome oxidase molecule. This is seen from the increase of k_s with the amplitude of the slower process (especially for 0.001 atm).

Austin *et al.* [6] have used a system of linear differential equations to represent the transfer of CO between various proposed regions of the myoglobin heme site. Owing to the second-order form of the fast phase (Eqn. 4 or 10), such a system would be non-linear for the case of cytochrome a_3 and hence more difficult to solve.

The fast rate k_f and the slow rate k_s depend upon the constituents of the aqueous solution in quite different ways. Fig. 7 shows rate plots for various concentrations of potassium phosphate buffer and Tween 20 detergent. It is clear that k_f above about 230 K is increased by decreasing the detergent concentration, but has much less dependence on the phosphate. The slower rate k_s, in contrast, depends primarily on the phosphate concentration and secondarily on the detergent. The rates at low temperatures (below about 215 K) and in liquid samples (above 273 K) are not appreciably affected by changing the salt or detergent concentration.

DISCUSSION

The kinetics of CO rebinding, studied over wide ranges of temperature and CO concentration, give strong evidence for the existence of an intermediate state in the binding of CO by cytochrome a_3 in solubilized cytochrome oxidase. This intermediate complex corresponds to a CO molecule being in state I of Fig. 4. The evidence consists of the observation that the binding rate k_f is not proportional to the effective concentration of free CO near the heme site after photodissociation. The rate of the process corresponding to a transfer from R to I (characterized by the parameter P in Fig. 5), however, does show this proportionality.

The model proposed earlier [8] and refined here implies that the rate k_f depends upon the fraction of the time between photodissociation and recombination that I is occupied. At low temperatures, I is essentially always occupied by a CO molecule and $k_f \cong k_{IFe}$ (the linear region below 215 K in Figs. 3 and 7). As the temperature is raised, the quantity $k_{IR}/k_{RI}[CO]_R$ in Eqn. 4 increases and k_f becomes smaller than k_{IFe} (the curved regions in Figs. 3 and 7). The dependence of k_f upon CO concentration shows that I can hold at most one CO molecule. At temperatures below 215 K, where k_f depends only on k_{IFe}, samples having many free CO molecules in R and I (e.g., those equilibrated with 1.0 atm CO) have the same recombination rates as those having only the photodissociated CO (0.01 atm or less).

The physical nature of I cannot be specified with certainty. It might be simply envisioned as a very small pocket adjacent to the heme iron. However, the region R must have a relatively large volume in order to exchange free CO with a number

of cytochrome oxidase molecules (Fig. 5B). If I were only a pocket that can hold one CO molecule, the volume ratio of R to I would make the value of k_f in the range 240–260 K fall several orders of magnitude below the value of k_{IFe} (extrapolated from low temperatures). Since this is clearly not the case, I must have some ability to bind CO. It may correspond to some feature of the heme site, such as a copper atom or a group comparable to the distal histidine of hemoglobin [19, 20], that can form a bond with the CO molecule.

The detection of only one intermediate state in the CO-binding of cytochrome a_3, as compared with at least three in that of myoglobin [6], is somewhat surprising. Possibly the relatively large activation enthalpy of the innermost observed barrier in cytochrome a_3 (43 kJ/mol, as compared with 10 kJ/mol in myoglobin [6]), prevents the detection of other barriers and the corresponding intermediate states.

The concept of intermediate states in the binding of CO to heme has been previously used by Gibson and Kamen [21] and Cusanovich and Gibson [22] to explain the complex kinetics and equilibrium behavior of cytochrome c' at physiological temperatures.

It is observed (Fig. 5B) that a number of cytochrome a_3 sites can share a common pool of free CO molecules. (In other words, exchange of CO between heme sites can occur.) This, together with the NMR data on mobile protons (Fig. 6), suggests that R is a fluid region containing CO molecules that have been trapped by the freezing of the bulk solution. As temperature is increased, local melting of detergent, lipids, or water takes place and the volume of R increases. The value of $[CO]_R$ decreases and the rate k_f is diminished according to Eqn. 4.

The character and volume of region R appear to be largely determined by the detergent, although residual lipids in the purified protein preparation [17] may also be involved. The fluid nature of R is evident at 246 K (Fig. 5B), well below the eutectic point (256 K) of potassium phosphate buffer solutions [23]. Furthermore, as shown in Fig. 7, the rate k_f is decreased by increasing the Tween 20 concentration but is not much affected by changing the potassium phosphate concentration. Thus, increasing the amount of detergent increases the volume of R.

It should be pointed out that the CO concentration in regions of detergent, lipid, or protein could be much higher than that in the aqueous solution, since CO is considerably more soluble in most organic liquids than it is in water [24].

Chance and co-workers have found that the rates of oxygen binding by cytochrome oxidase depend upon the O_2 concentration at temperatures as low as 170 K [5, 25]. It was concluded [25] that there is a pocket, capable of holding mobile oxygen, near the heme even when the medium is completely frozen. This pocket may be similar or identical to the region of mobile CO described here and earlier [8]; however comparison is difficult since the observed binding rates for oxygen are much higher than those for CO and since the oxygen-binding experiments were done using ethylene glycol/water solvent systems.

The second phase of CO binding, characterized by the rate k_s (Figs. 1–3, 7), can be interpreted as resulting from the escape of some of the free CO in I and R into the surrounding environment. This slower phase is not seen at CO concentrations sufficient to provide appreciable free CO in region R prior to photodissociation; it is also absent below about 230 K. A likely interpretation is that the surrounding environment (S in Fig. 4) is aqueous and at least partly frozen. Thus, the slower

process appears and increases with temperature because of increasing permeability of S to the free CO molecules.

Fig. 7 shows that k_s is sensitive to the salt concentration in the solution, and has a somewhat weaker dependence upon the amount of detergent present. It has been found [26] that ice is made more permeable to gas molecules by the presence of salts. Thus an increase in buffer concentration may have the effect of increasing the volume in S that is accessible to the free CO and thereby lowering the value of k_s.

The biphasic nature of the recombination disappears above the melting point (273 K) of the aqueous solution. There are two possible interpretations for this observation. First, region R may in effect be expanded to include the entire solution volume as the ice lattice melts. Second, R may remain as a distinct region of detergent (and possibly lipids) near the cytochrome oxidase molecules but the CO transfer across the R–S boundary becomes very fast. In either case, CO molecules not bound to the heme iron or in the intermediate state I have access to a large volume; recombination is monophasic and much slower than k_{IFe} (extrapolated from low temperatures).

In summary, an explanation of low-temperature CO binding by soluble cytochrome oxidase requires three features: an intermediate bound state (I) for the CO molecule, a detergent-sensitive region (R) near the protein in which CO has sufficient mobility to be exchanged between heme sites at low temperatures, and a salt-sensitive outer environment (S). These separate aspects are not resolved above the freezing point of the solvent (see Figs. 3 and 7). Below the freezing point, the progressive decrease in fluidity or permeability of the medium with decreasing temperature allows the study of kinetic processes that are progressively nearer to the heme iron site.

CO-binding by mitochrondria in frozen aqueous suspensions shows many of the kinetic features described here, including the biphasic recombination and the dependence of rates upon the extent of photodissociation (unpublished results). These similarities suggest that the region R in soluble preparations of cytochrome oxidase corresponds to the membrane environment of the enzyme in mitochondria. Thus, the sensitivity of the low-temperature ligand-binding kinetics to various aspects of the heme site and its environment may be a useful probe of protein-membrane interaction. A preliminary study (Ref. 27 and further work, to be published.) has found that membrane energization by ATP affects the CO capacity of a region near the heme site of cytochrome a_3. A similar explanation probably accounts for the effect of ATP on the rates at which cytochrome oxidase binds molecular oxygen [27, 28].

ACKNOWLEDGEMENTS

The authors express their thanks to Dr. B. Chance and Dr. H. J. Harmon for helpful discussions, to Dr. G. McDonald and Mr. T. McGurk for assistance with the NMR measurements, and to Mr. Elliot Kolodner and Mr. William Phillips for valuable technical assistance. This work was supported by research grant HL 14508 from the National Institutes of Health and PCM 77-00811 from the National Science Foundation. M. S. was the recipient of a National Institutes of Health Postdoctoral Fellowship (HL 03335).

REFERENCES

1 Yonetani, T. (1963) in The Enzymes (Boyer, P. D., Lardy, H. and Myrbach, K., eds.) Vol. 8, pp. 41–79, Academic Press, New York
2 Lemberg, M. R. (1969) Physiol. Rev. 49, 48–121
3 Malmström, B. G. (1974) Quart. Rev. Biophys. 6, 389–431
4 Chance, B., Saronio, C. and Leigh, Jr., J. S. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1635–1640
5 Chance, B., Saronio, C. and Leigh, Jr., J. S. (1975) J. Biol. Chem. 250, 9226–9237
6 Austin, R. H., Beeson, K., Eisenstein, L., Frauenfelder, H. and Gunsalus, I. C. (1975) Biochemistry 14, 5355–5373
7 Yonetani, T. (1965) in Oxidases and Related Redox Systems I (King, T. E., Mason, H. S., and Morrison, M., eds.) Vol. 2, pp. 614–619, Wiley and Sons, New York
8 Sharrock, M. and Yonetani, T. (1976) Biochim. Biophys. Acta 434, 333–344
9 Chance, B., Schoener, B. and Yonetani, T. (1965) in Oxidases and Related Redox Systems I (King, T. E., Mason, H. S. and Morrison, M., eds.) Vol. 2, pp. 609–614, Wiley and Sons, New York
10 Yonetani, T., Iizuka, T., Yamamoto, H. and Chance, B. (1973) in Oxidases and Related Redox Systems II (King, T. E., Mason, H. S. and Morrison, M., eds.) Vol. 1, pp. 401–405, University Park Press, Baltimore
11 Iizuka, T., Yamamoto, H., Kotani, M. and Yonetani, T. (1974) Biochim. Biophys. Acta 371, 126–139
12 Sharrock, M. and Yonetani, T. (1977) Biophys. J. 17, 241a
13 Yonetani, T. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.) Vol. X, pp. 332–335, Academic Press, New York
14 Sharrock, M. (1977) Rev. Sci. Instrum. 48, 1202–1206
15 Gibson, Q. H., Greenwood, C., Wharton, D. C. and Palmer, G. (1965) in Oxidases and Related Redox Systems I (King, T. E., Mason, H. S., and Morrison, M., eds.) Vol. 2, pp. 591–603, Wiley and Sons, New York
16 Kuntz, I. D. and Kauzmann, W. (1974) in Advances in Protein Chemistry (Anfinsen, C. B., Edsall, J. T. and Richards, F. M., eds.) Vol. 28, pp. 239–345, Academic Press, New York
17 Robinson, N. C. and Capaldi, R. A. (1977) Biochemistry 16, 375–380
18 Cooke, R. and Kuntz, I. D. (1974) in Annual Review of Biophysics and Bioengineering (Mullins, L. J., Hagins, W. A., Stryer, L. and Newton, C., eds.) Vol. 3, pp. 95–126, Annual Reviews, Inc. Palo Alto, Calif.
19 Pauling, L. (1964) Nature 203, 182–183
20 Yonetani, T., Yamamoto, H. and Iizuka, T. (1974) J. Biol. Chem. 249, 2168–2174
21 Gibson, Q. H. and Kamen, M. D. (1966) J. Biol. Chem. 241, 1969–1976
22 Cusanovich, M. A. and Gibson, Q. H. (1973) J. Biol. Chem. 248, 822–834
23 Van den Berg, L. and Rose, D. (1959) Arch. Biochem. Biophys. 81, 319–329
24 International Critical Tables of Numerical Data, Physics, Chemistry, and Technology (1928) (Washburn, E. W., West, C. J., Dorsey, N. E., Bichowsky, F. R., and Klemenc, A., eds.), Vol. III, pp. 260, 265
25 Chance, B. (1977) Biophys. J. 17, 241a
26 Gosink, T. A., Pearson, J. G. and Kelley, J. J. (1976) Nature 263, 41–42
27 Harmon, H. J. and Sharrock, M. (1977) Biophys. J. 17, 241a
28 Harmon, H. J., Chance, B. and Wikström, M. K. F. (1976) Biophys. J. 16, 135a