ELECTRON PARAMAGNETIC RESONANCE- (EPR-) RESOLVED KINETICS OF CRYOGENIC NITRIC OXIDE RECOMBINATION TO CYTOCHROME *c* OXIDASE AND MYOGLOBIN

RUSSELL LOBRUTTO
Department of Physics, State University of New York at Albany, Albany, New York 12222

YAU-HUEI WEI AND SATOSHI YOSHIDA
Department of Chemistry and Laboratory for Bioenergetics, State University of New York at Albany, Albany, New York 12222

HARLAN L. VAN CAMP
Bruker Instruments, Inc., Billerica, Massachusetts 01821

CHARLES P. SCHOLES
Department of Physics and Center for Biological Macromolecules, State University of New York at Albany, Albany, New York 12222

TSOO E. KING
Center for Biological Macromolecules and the Department of Chemistry and Laboratory for Bioenergetics, State University of New York at Albany, Albany, New York 12222

ABSTRACT By the electron paramagnetic resonance (EPR) technique, recovery kinetics for nitric oxide (NO) to heme following cryogenic photolysis were studied for the nitrosylferrocytochrome a_3 center in cytochrome *c* oxidase and for myoglobin. The recovery was nonexponential, as has been observed in previous cryogenic CO and O_2 rebinding to heme systems. NO rebinding to heme a_3 started near a temperature of 50 K and was related to a distribution of thermal activation energies. At the peak of the distribution the activation energy was 3.1 kcal/mol, and the preexponential in the recovery rate was $10^{9.9}$ s^{-1}. For recovery of NO back to the a_3 heme, the activation energy was threefold less than that for CO where CO binds to nearby Cu_{a_3} following photolysis from heme a_3, but was larger than the activation energy for CO, O_2, and probably NO rebinding to myoglobin. NO ligand rebinding to myoglobin occurred at a temperature as low as 15 K and in a temperature regime where tunneling could occur. However, the rate of NO rebinding to myoglobin did increase with temperature in the 15–25 K range.

INTRODUCTION

Since the reaction mechanism of cytochrome oxidase is still not clear, all conceivable techniques must be employed in the attempt to clarify this problem. A very important aspect of oxidase function is the binding of molecular oxygen that is then reduced to H_2O. Electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) of the NO-ligated a_3 heme were recently used to explore the electronic structure of the reduced cytochrome a_3 ligand binding center and to provide comparisons with NO-myoglobin (Mb)(1). The purpose of this present study was to obtain information complementary to the EPR-ENDOR data.

CO recombines very slowly to cytochrome *c* oxidase below 160 K (2), whereas the CO recombination temperature is <50 K for other heme proteins. Following photolysis of CO from the a_3 heme, new infrared (IR) peaks occurred with the appropriate stretching frequencies for a copper-CO complex (3), likely with the Cu_{a_3}. The kinetics of CO

Dr. R. LoBrutto's current address is Johnson Research Foundation, G4, University of Pennsylvania, Philadelphia, PA 19104; Dr. Y-H Wei, Department of Biochemistry, National Yang-Ming Medical School, Shih-Pai, Taipei, Taiwan; Dr. H. L. Van Camp, Biomedical Physics Division, Cancer Research and Treatment Center, University of New Mexico, Albuquerque, NM 87131; Dr. S. Yoshida, Department of Biochemistry, Medical College of Oita, Hazama-cho, Oita, 870-56, Japan.

rebinding showed an activation energy at temperatures <215 K of ~10 kcal/mol and a preexponential factor of $2.5 \times 10^{10}\ s^{-1}$ (4). The oxygen complexes, which presumably possess the basic structure of a_3—O_2, have been studied (references 5 and 6 and references cited therein) to explore the mechanism of the reduction of diatomic oxygen. The intermediates of the oxygen complexes have also been extensively studied at temperatures as low as −125°C. The relatively low temperature enabled otherwise short-lived intermediates to be stabilized and spectroscopically characterized (7). The second-order rate constant for O_2 binding to the a_3 center had an activation energy of 9.9 kcal/mol.

Austin et al. (8) examined CO rebinding to Mb from liquid helium temperatures up to room temperature and over nine orders of magnitude in time. They discovered sequential processes in the ligand binding path from outside the protein to the heme binding site. In the 40–160 K region only a single process was observed for Mb and this was the final recombination over the innermost barrier near the heme, such as that shown in Fig. 4 *a*. In cases where significant ligand rebinding occurred below 20 K, it was found that through-the-barrier tunneling could be an important means of ligand recombination (9).

CO, O_2 (and NO as well) are about the same size, yet their recombination rates and energies differ markedly. As suggested by Doster et al. (10), the innermost recombination barrier may discriminate between ligands according to polarity, hydrogen bonding, or stereochemical restraint on metal-ligand bond angle. In establishing an overall second-order rate constant for ligand binding at room temperature, other prebinding steps establish a rapid preequilibrium, but the metal-ligand association rate may still be controlled by the important final binding step (10). The energetics of the final step can be probed in considerable detail at low temperatures by kinetic techniques, and spectroscopic techniques (e.g., EPR-ENDOR) may give information on the structures involved.

The work presented here was further prompted by the work of Yoshida et al. (11), who showed that the NO ligand recombines to the a_3 center only above 50 K following low temperature photolysis. In analyzing our data, we have followed the formalism of Austin et al. (8) so that our results can be compared with those obtained on other heme systems by their methods.

METHODS AND MATERIALS

Cytochrome *c* oxidase was prepared by methods described in references 12 and 13. Sperm whale metmyoglobin (sperm whale, type II; Sigma Chemical Co., St. Louis, MO) was chromatographically purified on DE-52 cellulose. NO complexes were prepared in a modified Thunberg reaction vessel. So that maximum photolyzing light could penetrate the sample, the heme concentration of these samples was several fold less than in the previous EPR-ENDOR work (1) and thus was in the 200 μM range. For oxidase, a system containing 50% glycerol (for glass formation), 0.5% sodium cholate, and 50 mM phosphate, pH 7.4, was used. Under strictly anaerobic conditions over ice, the sample was first reduced by an approximate 15-fold molar excess (0.5 mg/ml) of sodium dithionite (Hardman and Holden, Manchester, England) relative to heme A, and then the sample was incubated under ~200 mm (Hg) pressure of NO for 25 min. The NOMb sample was prepared after a similar fashion, except that sodium cholate was not used, and the phosphate buffer was pH 6.8. After the excess NO was removed by flushing several times with argon and evacuating, an EPR sample was tipped into the anaerobically attached 3-mm inside diameter EPR tube, frozen under liquid nitrogen, and the sample tube sealed off with a torch. In the aforementioned evacuating and flushing the oxidase concentration usually increased by 1.3 to 1.4 times.

Preliminary photolysis experiments were performed at Albany with our low temperature EPR-ENDOR apparatus.[1,2] At Albany, under conditions where recovery was slow enough, full EPR spectra were recorded to observe the recovery of the entire EPR signal as a function of time. The experiments involving precise temperature-controlled recovery of heme-bound NO EPR signals were performed at Bruker Instruments Inc. (Billerica, MA), where we used a spectrometer (model ER 200; Bruker Instruments, Inc.) and a double sample resonator (model ER 4105; Bruker Instruments, Inc.). The double sample resonator with ruby standard was used to monitor the EPR sensitivity over the course of the EPR experiment. Helium flow cryostat (model ESR-10; Oxford Instruments Inc., Columbia, MD) with a temperature controller (based on Au [Fe]/Chromel thermocouple) (model DTC-2; Oxford Instruments Inc.) was used to maintain and control the sample temperature. Prior to these experiments the temperature at the position of the sample was monitored by a carbon resistor (Allen-Bradley Co., Milwaukee, WI) frozen in an EPR tube. (The carbon resistor itself had previously been calibrated at 4.2 K [liquid He], 77 K [liquid N_2], and at intermediate temperatures vs. a platinum resistor [Rosemount Inc., Minneapolis, MN].) For experiments performed at Bruker Instruments Inc., the EPR signal was continuously monitored at the high field derivative minimum at $g \simeq 1.99$.

For photolysis, a 200-W mercury arc lamp (Oriel Corp. of America, Stamford, CT) was used whose output was filtered through a saturated copper sulfate solution to remove all but the visible radiation. The light was left on sufficiently long for the EPR signals to reach a steady state. Following a recombination run, the sample was warmed to 77 K so that the EPR signal fully recovered before the next run.

In Fig. 1 *a*, *b* we show $N(t)$, which is the fraction of recombined but potentially photolyzable (with our light source) molecules that remain at a time, t, after the lamp was extinguished. To obtain $N(t)$, we first found

[1]Preliminary reports of the photolysis work were presented at the Biophysical Society Meetings, Feb. 1981, Denver, Colorado, and the Denver Electron Spin Resonance Conference, August 1980. Since that time additional recombination studies have been done on the Bruker apparatus in 1981 and more sophisticated data analyses performed.

[2]This system, primarily designed for ENDOR use, has its EPR cavity enclosed in a set of glass double Dewar flasks. The Dewar flasks have light slots but the EPR cavity does not. Therefore, the photolysis at Albany was carried out within our helium-filled cryostat under liquid helium with the sample slightly above the cavity, rather than in the cavity as with the Bruker apparatus. The helium was then boiled away to below the cavity and the sample warmed to a desired temperature, which was controlled by the combination of a resistance thermometer and a heater both on the waveguide next to the cavity, a boil-off coil in the helium below the cavity, and a Linear Research (San Diego, CA) temperature controller. The thermal mass of this system was large, and because of the long time needed to attain and stablize temperatures near 65 K, it was not used for obtaining precise recovery kinetics. This sytem was also used to monitor EPR signals before and after photolysis at temperatures of 4.2 K and below. A recent superheterodyne modification has been employed in observing broad EPR signals in the absence of magnetic-field modulation.

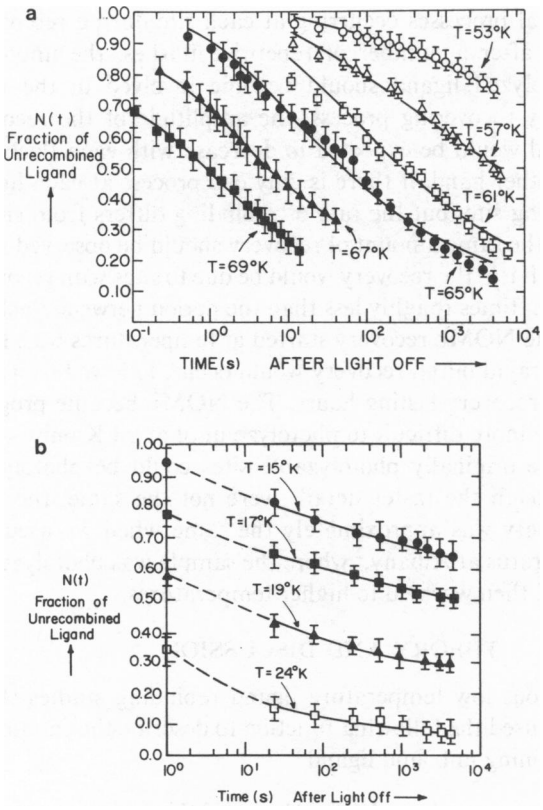

FIGURE 1 (*a*) Rebinding of NO ligand to ferrocytochrome a_3 in cytochrome oxidase as measured at temperatures above 50 K. The solid curves (—) are theoretical fits to the theory of Austin et al. (8). The errors in $N(t)$ were computed from errors in the signals $S(\infty)$, $S(t)$, and $S(u)$ that contribute to $N(t)$ as indicated in Eq. 1. Values of $N(t)$ for $T = 67$ and 69 K reflect the fact that our lamp did not yield complete photolysis of previously photolyzable sites at these temperatures. (*b*) Recombination kinetics for NO rebinding to Mb. The solid curves (—) simply connect data gathered at the same temperature, and these curves have no particular theoretical significance. Progressively lower values of $N(t)$ reflect that the photolysis that our lamp yielded diminished as the temperature was raised.

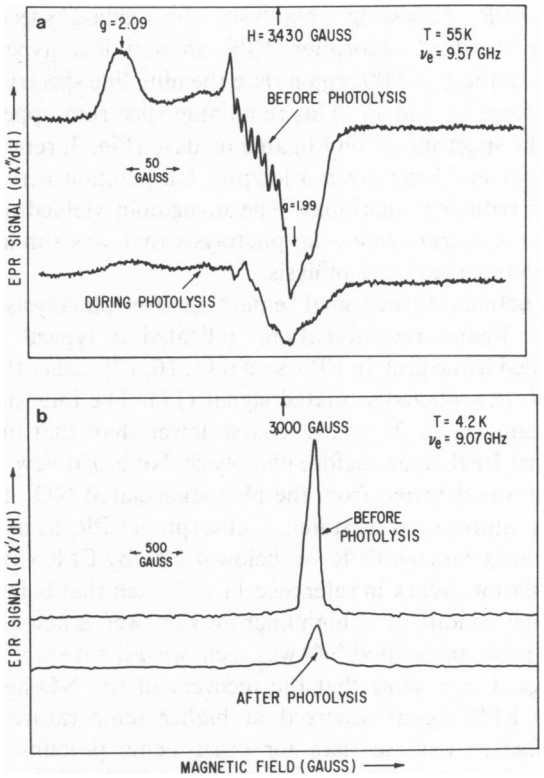

FIGURE 2 EPR spectra of nitrosyl ferrocytochrome a_3, photolyzed and unphotolyzed. (*a*) Absorption EPR. Comparison of standard absorption ($d\chi''/dH$) spectra taken at 55 K (Bruker apparatus; Bruker Instruments, Inc.) before photolysis and during sufficient photolysis that the minimal steady state signal was reached. Microwave power was ~5 mw, 100 kHz field modulation was 5 Gauss (G) peak-to-peak, experimental time constant was 0.1 s, a 500-G sweep range was used. (*b*) Compares the rapid passage dispersion signal at 4.2 K before and ~10 min after a 90 min photolysis at 4.2 K. Microwave power was ~100 μW, 100 kHz field modulation was ~3.8 G peak-to-peak, experimental time constant was 0.1 s, a 5,000 G sweep range was used.

the difference between the fully recombined signal, $S(\infty)$, and the signal $S(t)$ obtained at time t after the light was extinguished. This difference is proportional to the number of unrecombined sites. To convert this difference to a fraction whose maximum value at $t = 0$ would be unity, we divide this $[S(\infty) - S(t)]$ difference by the difference between the fully recombined signal, $S(\infty)$, and the minimal EPR signal, $S(u)$ that remains before ligand recombination occurs. $S(u)$ appears to be from photoinert, unphotolyzable sites that still give an EPR signal. Specifically,

$$N(t) = [S(\infty) - S(t)]/[S(\infty) - S(u)]. \qquad (1)$$

In these recombination studies it was the potentially photolyzable fraction of NO-ligated molecules that $N(t)$ represents. As shown in Fig. 2, oxidase contained about a 25% fraction of molecules that still gave an EPR signal after prolonged photolysis, even at 4.2 K. The Mb contained about a 40% fraction of such sites. For detailed analysis of our oxidase data we used a nonlinear least-squares fitting routine (14) as modified by Venable (15) for fitting functions such as those of Eqs. 2 or A5. By application of methods described in reference 14, errors in fitted parameters, n and t_0, were estimated from the root mean square (rms) error (fitted minus experimental value of $N[t]$) and from the matrix that was inverted in the least-squares fit.

RESULTS

Fig. 2 shows EPR signals from nitrosylferrocytochrome a_3. At 4.2 K the signal was taken before and after photolysis by rapid passage dispersion (χ') EPR, and at 55 K was taken before and during photolysis by standard absorption

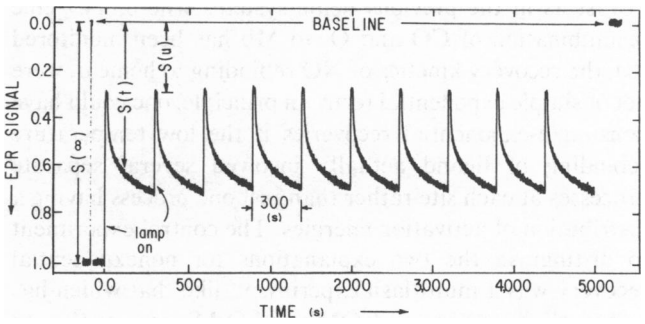

FIGURE 3 Results of a multiflash experiment obtained from the recording in time of the EPR signal from nitrosylferrocytochrome a_3 in cytochrome oxidase as monitored at $g = 1.99$ and $T = 65$ K.

(χ'') EPR. Following photolysis, the oxidase spectrum under standard absorption EPR showed less hyperfine detail in the $g = 2.00$ region than the nine-line spectrum of references 16 and 17. This remaining spectrum appeared like the spectrum of NO-ligated oxidase (Fig. 3, reference 18) that had been given a lengthy 1-h reaction with NO under reducing conditions. The myoglobin yielded a less intense spectrum following photolysis that was similar to its spectrum before photolysis.

In both cases the signal remaining after photolysis, but before ligand recombination, indicated a typical NO-liganded heme protein EPR signal (1, 16, 17) rather than a broad, new photodissociated signal (11). The intensity of the signal (Fig. 2) was of course lower than that of the original EPR signal before photolysis. No broad new EPR signal was detected from the photodissociated NO at any temperature either by standard absorption EPR, by dispersion rapid passage EPR, or, below 4.2 K, by EPR with no modulation. Work in reference 11 indicated that below 10 K under conditions of high microwave power, a new signal from photodissociated NO was seen; we did not see it.

Figs. 1 *a*, *b* show that the recovery of the NO-ligated heme EPR signal occurred at higher temperatures for NO-ligated oxidase than for other heme proteins. The recovery found here was approximately half complete within 4 min at 61 K, in accord with reference 11. Temperatures above 50 K were needed to observe any recovery at all. The recovery kinetics of the $g = 1.99$ and 2.09 signals, and hyperfine lines near $g = 2.03$, as followed with our apparatus at Albany, were identical. The recovery took hours at 50 K but only seconds at 69 K. At the higher temperatures of 67 and 69 K the recovery rate for some of the previously photolyzable sites was indeed so fast that our light source would not completely photolyze them. At 69 K only ~70% of the previously photolyzed sites were photolyzed. (This incomplete photolysis of previously photolyzable sites would not affect $S[u]$, as it is defined. However, the incomplete photolysis did increase $S[t = 0]$ so that $N[t = 0]$ was 0.70 at 69 K for the oxidase.) In analyzing data at the higher temperature, it was necessary to use the theory of Austin et al. (8) corrected, as indicated in the Appendix, for incomplete photolysis.

Just as in the previous heme systems where cryogenic recombination of CO and O_2 to Mb has been monitored (8), the recovery kinetics of NO rebinding to heme a_3 were not of simple exponential form. In principle, one could have nonsimple-exponential recoveries if the low temperature rebinding of ligand actually involved several separate processes at each site rather than just one process having a distribution of activation energies. The control experiment to distinguish the two explanations for nonexponential recovery was a multiflash experiment, like that which has previously been done on COMb and O_2Mb species (8). As shown in Fig. 3 for nitrosylferrocytochrome a_3, the amplitude of the recovery signal during the multiflash experiment was the same from one flash to the next. If there were several processes occurring at each site during rebinding, then after a number of repetitive flashes, the unbound, photolyzed ligand should become involved in the most slowly recovering process; the amplitude of the recovery signal would be expected to decrease with each flash. On the other hand, if there is only one process at each ligand binding site, but the rate of rebinding differs from site to site, the same amount of recovery should be observed after each flash; the recovery would be due to sites with recombination times roughly less than the period between flashes.

The NOMb recovery started at temperatures >12 K. A very rapid initial recovery would occur, followed by a very slow recovery lasting hours. The NOMb became progressively more difficult to photolyze until at 24 K only ~20% of the originally photolyzable sites could be photolyzed. Although the faster details were not the same, the slow recovery was approximately the same when we used our apparatus at Albany,[2] where the sample was photolyzed at 4.2 K then warmed to higher temperatures.

THEORY AND DISCUSSION

Previous low temperature ligand rebinding studies (8, 9) have used the following function to describe the fraction of remaining unbound ligand

$$N(t) = (1 + t/t_0)^{-n}. \tag{2}$$

This function was related to a frozen-in distribution of activation energies, $g(E_{ba})$, where E_{ba} is the activation energy of rebinding from the innermost unbound B state, shown in Fig. 4 *a*.

$$N(t) = \int_0^\infty dE_{ba}\, g(E_{ba}) \exp(-k_{ba}t). \tag{3}$$

k_{ba} is the rebinding rate corresponding to activation energy E_{ba} and $k_{ba} = A_{ba} \exp(-E_{ba}/RT)$, where A_{ba} is the preexponential factor. At the peak of the resultant activation energy distribution, it was shown that an effective Arrhenius relation exists where

$$\ln(n/t_0) = \ln(A_{ba}) - E_{ba}^{pk}/RT. \tag{4}$$

E_{ba}^{pk} is the activation energy at the peak of the distribution.

The solid lines in Fig. 1 *a* are the result of fitting the recovery data to Eq. 2, or for the two higher temperatures where photolysis was incomplete, to the related modified theory explained in the Appendix. In the temperature range 61–69K, values of n were found to increase from 0.20 to 0.30. The two lower temperatures of 53 and 57 K gave n values of 0.06 and 0.11, respectively. The major variation in n/t_0 was the variation in t_0. A plot of $\ln(n/t_0)$ vs. $1/T$ is given in Fig. 4 *b*, and a straightforward linear, least-squares fit to the data in that graph gave an activation energy of 3.1 kcal/mol and a preexponential factor of $10^{9.9}$ s^{-1}.

It appears inappropriate at this time to analyze the recovery of NO back to myoglobin in the 15–25 K range in

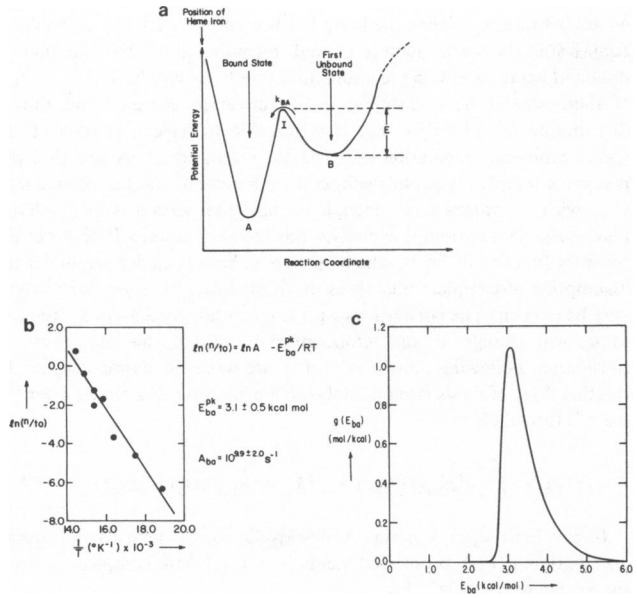

FIGURE 4 (*a*) Potential encountered by heme ligand molecule on binding. Reaction coordinate scheme used to explain low temperature rebinding kinetics of NO to heme. Well *A* is the potential well where covalently heme-bound NO resides, well *B* is the innermost potential well in the heme pocket for an unbound ligand, and an activation energy barrier separates *A* and *B*. Figure is similar to those shown in References 8 and 9. (*b*) Plot of $\ln(n/t_0)$ vs. $1/T$ to obtain peak activation energy and preexponential factor of NO rebinding to cytochrome oxidase. (*c*) Activation energy distribution of NO-ligated cytochrome oxidase. Plot of activation energy distribution, $g(E_{ba})$, based on theory of reference 8 and activation energy and preexponential factor determined for nitrosylferrocytochrome a_3 in fully reduced cytochrome *c* oxidase.

the detail for which the recovery of NO back to heme a_3 in oxidase was analyzed. For NO myoglobin we are in a temperature region where quantum mechanical tunneling has been strongly implicated as a recombination mechanism (9, 21). Yet, the temperature dependence of the recovery rules out simple temperature-independent tunneling from the vibrational ground state of well *B* (Fig. 4 *a*) to the heme iron at *A*. The overall recovery of NO to myoglobin was reminiscent of the CO recombination to carboxymethylated cytochrome *c* or heme *c* octapeptide, where in the same temperature regime a very fast, early exponential process occurred followed by a slower nonexponential process. (21). Fast optical techniques may be required to obtain explicit information on the fast initial recovery. Both processes occurred via quantum mechanical tunneling at cryogenic temperatures (21). Since ENDOR is a more effective tool then EPR in resolving separate ^{15}NO and ^{14}NO spectral features (1), an ENDOR experiment is in order to determine if there is a low temperature, tunneling-related, differential isotope effect on the recombination of NO ligand to myoglobin.

The EPR signals remaining immediately after photolysis, even at 4.2 K, both for NO-liganded oxidase and myoglobin were a factor perhaps not encountered by low-temperature photolysis studies of CO complexes. A study of NO hemoglobin complexes at 4.2 K revealed a nonphotolyzing fraction (19). For myoglobin the quantum yield of the ferrous NO complex is $\sim 10^{-3}$ the quantum yield of the highly photolabile CO complex (20). The quantum yield is sensitive to the relative levels of metal and ligand π orbitals and to protein structure (20). Given the already low quantum yield, it is not surprising that some NO heme sites should be frozen into conformations that do not photolyze even at 4.2 K.

We compare activation energies and recovery rates of nitrosylferrocytochrome a_3 with the energies and rates obtained from low temperature photolysis of other heme sytems. The 3.1 kcal/mol activation energy for recovery of NO back to heme a_3 is substantially less than that found for lower temperature CO rebinding to a_3, where CO-to-Cu_{a_3} binding is proposed (3). Thus, if interaction of NO with Cu_{a_3} does occur following photolysis from heme a_3, the interaction is considerably weaker than the interaction of CO with Cu_{a_3}. Unfortunately, we do not see direct spectroscopic EPR evidence for a $Cu_{a_3}-NO$ complex. Our evidence is for rebinding of NO to heme a_3 rather than breaking of a $Cu_{a_3}-NO$ bond.

Regardless of whether the observed rebinding of NO to Mb occurs by tunneling or by a mixture of tunneling and over-the-barrier motion, the fact that it occurs at a much lower temperature than for oxidase indicates that the barrier to NO recombination is much lower and/or narrower in Mb than in the oxidase. The 3.1 kcal/mol rebinding energy for NO to heme a_3 is larger than the corresponding low temperature activation energy for rebinding of CO or O_2 to Mb, which was ~2 kcal/mol (8). (The preexponential for CO and O_2 rebinding to Mb was $\sim 10^{8.5}$ s^{-1}.) At room temperature the pseudo–first-order rate constant for O_2 oxidation of heme a_3 is reported <900 s^{-1} (22). Although another process like electron transfer may be the rate limiting step, the present process seen for NO, if simply extrapolated to room temperature, would not appear to be rate limiting. However, in combination with preequilibria of several earlier binding steps (10), the present process could still have an effect on the binding or O_2 turnover rate.

ENDOR showed a unique proton that was associated with the cytochrome a_3 center but not with other heme-NO systems, and that may be part of a nearby protein side chain that perturbs an axial ligand like NO or O_2 (1). It is possible that such a nearby proton could influence the activation energy barrier and help steer the incoming ligand to its catalytically correct position. Nitrosylferrocytochrome a_3 gave better resolved EPR and ENDOR signals than Mb, and this implied more restriction on the available conformations of the ligand binding site. The following empirical correlations were noted between the kinetic results of this paper and the EPR-ENDOR results (1). The excellently resolved nitrosylferrocytochrome a_3 spectrum suggested fewer energetically available conformations in the bound *A* state, and the

kinetic results showed a higher barrier height necessary to arrive at these conformations. The overall more diffuse EPR-ENDOR spectra of NO-Mb implied a larger number of differing, energetically available conformations, and the present work showed a lower barrier height needed to arrive at these conformations in Mb. It is very likely because of this difference Mb acts only as an oxygen carrier, whereas cytochrome oxidase can participate in electron transport while the prosthetic group changes its oxidation state.

Subsequent to our preliminary reports (see footnote 1) and to completion in 1981 of our data gathering and analysis, the work of Boelens et al. (23) appeared on EPR of the photodissociation reactions of cytochrome *c* oxidase-nitric oxide complexes. This work treated NO complexes of fully reduced, a mixed valence, and oxidized forms of cytochrome *c* oxidase. The kinetics were analyzed by plotting half recovery times. The use of half recovery times will tend to gloss over the details of recovery kinetics. We believe that the comparison of recovery data is most meaningful when the more quantitative treatment introduced by Austin (8) and elaborated here is employed. Boelens et al. (23) observed half recovery of NO ligand to occur within 5 min at 45 K following photolysis, and their activation energy was obtained from a plot vs. $1/T$ of rates determined from half recovery times. The plot yielded an activation energy of 3.5 kJ/mol (<1 kcal/mol). The rate for their rebinding process, when extrapolated to room temperature, is ~5 orders of magnitude less than ours. The NO recombination above 50 K as observed here and in reference 11 (on a different preparation with no glycerol) points to a larger activation energy than that of Boelens et al. (23).

Note that the use of various detergents and/or various contents of phospholipid has a significant effect on oxidase activity. Boelens et al. (23) used Tween 80, whereas this work used cholate. Future experiments are indicated to probe the possibility of different conformational forms of oxidase with different kinetic and/or spectroscopic parameters. The existence of such forms could reflect, for example, different preparative methods; detergent and phospholipid content are variables that affect room temperatures activity. It is possible that differently prepared oxidases may be affected differently by cryogenic temperature. It will be important to delineate any such effects of temperature because important information on cytochrome oxidase has been determined from EPR, optical, kinetic (7), and x-ray absorption fine structure work (24) at cryogenic temperatures.

APPENDIX

Time Dependence of NO Rebinding Where Complete Photolysis Is Not Obtained

During photolysis the population of unbound molecules in well B (Fig. 4 *a*) will be affected by both the recombination rate, k_{ba}, and the photolysis rate, k_p. When the lamp has been on long enough to establish equilibrium between photolysis and recombination, the fraction of unbound heme sites with recombination rate k_{ba} would be $k_p/(k_p + k_{ba})$. If there should be a distribution of activation energies and thus a distribution of values for k_{ba}, it is possible to have a portion of the ligand-rebinding population that has k_{ba} values small enough that this portion is completely photolyzed; on the other hand, another portion may exist with k_{ba} values large enough so that that portion is incompletely photolyzed. If we attempt to analyze our recovery kinetics in terms of the recovery function of Eq. 2, which appears to have been derived under the assumption of complete photolysis of all photolyzable sites, our analysis may be in error. The populations with small or large values of k_{ba} relative to k_p will change as the temperature is varied; this fact must be considered. Following Austin et al. (8), we write the expression for the fraction $N(t)$ of dissociated photolyzable heme sites at a time t after the lamp is turned off as

$$N(t) = \int_0^\infty dE_{ba}\, g(E_{ba})\, k_p/(k_p + k_{ba}) \exp(-k_{ba}t). \quad \text{(A1)}$$

In the limit $k_p \gg k_{ba}$, Eq. A1 yields Eq. 3. So long as the overall distribution, $g(E_{ba})$, is one that yields $(1 + t/t_0)^{-n}$ for complete photolysis, we can rewrite Eq. A1 as

$$N(t) = k_p \int_0^\infty d\tau \int_0^\infty dE_{ba}\, g(E_{ba}) \cdot \exp(-k_p \tau - k_{ba} \tau - k_{ba} t) \quad \text{(A2)}$$

or

$$N(t) = t_0^n k_p \int_0^\infty (t_0 + t + \tau)^{-n} \exp(-k_p \tau) d\tau. \quad \text{(A3)}$$

We change the variable of integration to $y = k_p (t_0 + t + \tau)$ so that

$$N(t) = (k_p t_0)^n \exp(k_p t_0 + k_p t) \cdot \int_{k_p(t_0+t)}^\infty y^{-n} \exp(-y)\, dy. \quad \text{(A4)}$$

The incomplete gamma function (available on our Univac computer at Albany), $\Gamma(a, x)$, is defined as $\Gamma(a, x) = \int_x^\infty e^{-y} y^{a-1}\, dy$ (25). So, if we take $a = 1 - n$ and $x = k_p (t_0 + t)$, then $N(t)$ can be written as

$$N(t) = (k_p t_0)^n \exp(k_p t_0 + kt) \cdot \Gamma[(1 - n), k_p (t_0 + t)]. \quad \text{(A5)}$$

The equilibrium value $N(0)$ reached during photolysis is obtained from Eq. A5 by setting $t = 0$. For large values of $k_p (t_0 + t)$, the asymptotic expansion of the incomplete gamma function yields

$$N(t) = (1 + t/t_0)^{-n} [1 - n/(k_p t_0 + k_p t)]. \quad \text{(A6)}$$

Thus for long times or for nearly complete initial photolysis, $N(t)$ has the expected form of Eq. 2. So, in conclusion, we could fit our recovery data where photolysis was incomplete to Eq. A5 and k_p was an additional variable that was found to be $\sim 2\ s^{-1}$.

An alternative way of fitting the recovery data, which did not so explicitly depend on knowing what percentage of the potentially photolyzable sites were actually photolyzed or on a precise value of $S(u)$, was also used. In this method each experimental value of $N(t)$ was divided by the experimental value of $N(t = 0)$. With this method we fit our data to $N(t)/N(0)$ where both numerator and denominator could be computed from Eq. A5. Fitting data in this way gave values of n, t_0, and k_p little changed from those values obtained by the method of the previous paragraph.

Bruker Instruments Inc. was most helpful in donating spectrometer time on a new ER 200 EPR spectrometer. We are indebted to Dr. B.

Krawchuk for help in the least-squares analysis of our data and to M. Seaman for supplying starting material. We are grateful to Professor H. Frauenfelder for reading the manuscript and offering useful and stimulating ideas.

This work was supported by the National Institutes of Health grants GM 16767, AM 17884, and 2S07RR0712213. C. P. Scholes was the recipient of the National Institutes of Health Research Career Development Award No. AM00274.

Received for publication 14 December 1982 and in final form 9 August 1983.

REFERENCES

1. LoBrutto, R., Y. H. Wei, R. Mascarenhas, C. P. Scholes, and Tsoo E. King. 1983. Electron nuclear double resonance and electron paramagnetic resonance study on the structure of the NO-ligated heme a_3 in cytochrome *c* oxidase. *J. Biol. Chem.* 258:7437–7448.
2. Chance, B., B. Schoener, and T. Yonetani. 1965. The low temperature photodissociation of cytochrome $a_3^{+2} \cdot$ CO. *In* Oxidases and Related Redox Systems. T. E. King, H. S. Mason, and M. Morrison, editors. John Wiley and Sons, Inc., New York. 2:609–614.
3. Alben, J. O., P. P. Moh, F. G. Fiamingo, and R. A. Altschuld. 1981. Cytochrome oxidase (a_3) heme and copper observed by low-temperature Fourier transform infrared spectroscopy of the CO complex. *Proc. Natl. Acad. Sci. USA.* 78:234–237.
4. Sharrock, M., and T. Yonetani. 1977. Low-temperature flash photolysis studies of cytochrome oxidase and its environment. *Biochim. Biophys. Acta.* 462:718–730.
5. Orii, Y., and T. E. King. 1972. New species of the "oxygenated compound" of cytochrome oxidase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 21:199–202.
6. Orii, Y., and T. E. King. 1976. On the nature of the three intermediate species formed after reaction of reduced cytochrome oxidase with oxygen. *J. Biol. Chem.* 251:7487–7493.
7. Chance, B., C. Saronio, and J. S. Leigh, Jr. 1975. Functional intermediates in the reaction of membrane-bound cytochrome oxidase with oxygen. *J. Biol. Chem.* 250:9226–9237.
8. Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* 14:5355–5373.
9. Alben, J. O., D. Beece, S. F. Bowne, L. Eisenstein, H. Frauenfelder, D. Good, M. C. Marden, P. P. Moh, L. Reinisch, A. H. Reynolds, and K. T. Yue. 1980. Isotope effect in molecular tunneling. *Phys. Rev. Lett.* 44:1157–1160.
10. Doster, W., D. Beece, S. F. Bowne, E. E. Dilorio, L. Eisenstein, H. Frauenfelder, L. Reinisch, E. Shyamsunder, K. H. Winterhalter, and K. T. Yue. 1982. Control and pH dependence of ligand binding to heme proteins. *Biochemistry.* 21:4831–4839.
11. Yoshida, S., H. Hori, and Y. Orii. 1980. Photodissociation of cytochrome oxidase-nitric oxide at low temperatures. *J. Biochem. (Tokyo).* 88:1623–1627.
12. Kuboyama, M., F. C. Yong, and T. E. King. 1972. Studies on cytochrome oxidase VIII. Preparation and some properties of cardiac cytochrome oxidase. *J. Biol. Chem.* 247:6375–6383.
13. Yu, C. A., L. Yu, and T. E King. 1975. Studies on cytochrome oxidase. Interactions of the cytochrome oxidase protein with phospholipids and cytochrome *c*. *J. Biol. Chem.* 250:1383–1392.
14. Bevington, P. R. 1969. Data Reduction and Error Analysis for the Physical Sciences. McGraw-Hill, Inc., New York. 204–246.
15. Venable, J. H. 1965. Magnetic methods for protein single crystals: metal binding to insulin. Ph.D. Dissertation, Yale University, New Haven, CT.
16. Blokzijl-Homan, M. F. J., and B. F. Van Gelder. 1971. Biochemical and biophysical studies on cytochrome aa_3. III. The EPR spectrum of NO-ferrocytochrome a_3. *Biochim. Biophys. Acta.* 234:493–498.
17. Stevens, T. H., and S. I. Chan. 1981. Histidine is the axial ligand to cytochrome a_3 in cytochrome *c* oxidase. *J. Biol. Chem.* 256:1069–1071.
18. Morse, R. H., and S. I. Chan. 1980. Electron paramagnetic resonance studies of nitrosyl ferrous heme complexes, determination of an equilibrium between two conformations. *J. Biol. Chem.* 255:7876–7882.
19. Nagai, J., H. Hori, S. Yoshida, H. Sakamoto, and H. Morimoto. 1978. The effect of quarternary structure on the state of the α and β subunits within nitrosyl hemoglobin. Low temperature photodissociation and the ESR spectra. *Biochim. Biophys. Acta.* 532:17–28.
20. Hoffman, B. M., and Q. H. Gibson. 1978. On the photosensitivity of liganded hemoproteins and their metal substituated analogues. *Proc. Natl. Acad. Sci. USA.* 75:21–25.
21. Alberding, N., R. H. Austin, S. S. Chan, L. Eisenstein, H. Frauenfelder, D. Good, K. Kaufmann, M. Marden, T. M. Nordlund, L. Reinisch, A. H. Reynolds, L. B. Sorensen, G. C. Wagner, and K. T. Yue. 1978. Fast reactions in carbon monoxide binding to heme proteins. *Biophys. J.* 24:319–334.
22. Chance, B., and F. Schindler. 1965. The speed of the reaction of oxygen with cytochrome oxidase of intact cells, mitochondria, and the nonphosphorylating respiratory chain. *In* Oxidases and Related Redox Systems II. T. E. King, and M. Morrison, editors. John Wiley and Sons, Inc., New York. 2:921–929.
23. Boelens, R., H. Rademaker, R. Pel, and R. Wever. 1982. EPR studies of the photodissociation reactions of cytochrome *c* oxidase-nitric oxide complexes. *Biochim. Biophys. Acta.* 679:84–94.
24. Powers, L., B. Chance, Y. Ching, and P. Angiolillo. 1981. Structural features and the reaction mechanism of cytochrome oxidase. Iron and copper x-ray absorption fine structure. *Biophys. J.* 34:465–498.
25. Abramowitz, M., and I. A. Stegun. 1972. Handbook of Mathematical Functions with Formulas, Graphs, and Mathematical Tables. *Natl. Bur. Stand. Appl. Math. Ser.* 55:255–265.