Spectroscopic evidence for a heme–heme binuclear center in the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*

(Fourier transform infrared spectroscopy/flash photolysis/photoselection/heme pocket/CO stretching vibration)

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ABSTRACT The cytochrome *bd* complex is a ubiquinol oxidase, which is part of the aerobic respiratory chain of *Escherichia coli*. This enzyme is structurally unrelated to the heme–Cu oxidases such as cytochrome *c* oxidase. While the cytochrome *bd* complex contains no copper, it does have three heme prosthetic groups: heme *b*₅₅₈, heme *b*₅₉₅, and heme *d* (a chlorin). Heme *b*₅₅₈ appears to be involved in the oxidation of quinol, and heme *d* is known to be the site where oxygen binds and is reduced to water. The role of heme *b*₅₉₅, which is high spin, is not known. In this paper, CO is used to probe the oxygen-binding site by use of Fourier transform infrared spectroscopy to monitor the stretching frequency of CO bound to the enzyme. Photodissociation at low temperature (e.g., 20 K) of the CO–heme *d* adduct results in CO associated with the protein within the heme binding pocket. This photodissociated CO can subsequently relax to form a kinetically trapped CO–heme *b*₅₉₅ adduct. The data clearly show that heme *d* and heme *b*₅₉₅ must reside within a common binding pocket in the enzyme. The catalytic active site where oxygen is reduced to water is, thus, properly considered to be a heme *d*–heme *b*₅₉₅ binuclear center. This is analogous to the heme *a*₃–Cu_B binuclear center in the heme–Cu oxidases. Heme *b*₅₉₅ may play roles analogous to those proposed for the Cu_B component of cytochrome *c* oxidase.

The aerobic respiratory chain of *Escherichia coli* contains two terminal oxidases, the cytochrome *bd* complex (*bd*-type oxidase) and the cytochrome *bo* complex (*bo*-type oxidase) (1, 2). Each of these enzymes functions as a quinol oxidase and reduces molecular oxygen to water (3). Under conditions in which the oxygen tension is high in the growth medium, the cytochrome *bo* complex predominates. The cytochrome *bd* complex, which has a high affinity for molecular oxygen [$K_m = 0.024$ mM (4)], is present at high levels only when oxygen becomes limited or under anaerobic growth conditions (5–7). Unlike the cytochrome *bo* oxidase and the *aa*₃-type cytochrome *c* oxidases, the cytochrome *bd* oxidase does not contain copper and, therefore, does not contain a heme–copper binuclear center at the site where oxygen is reduced. The cytochrome *bd* complex contains three heme components: a six-coordinate, low-spin heme *b*₅₅₈, a high-spin heme *b*₅₉₅, and a high-spin heme *d*. Heme *d* has been identified as a chlorin (8, 9). It now appears that the ratio heme *b*₅₅₈/heme *b*₅₉₅/heme *d* is 1:1:1 (10, 11), although other studies have indicated two heme *d* moieties per complex (12–14). The enzyme is a 1:1 complex of two integral membrane polypeptides: subunit I (58 kDa) and subunit II (43 kDa) (15). Subunit I contains the heme *b*₅₅₈ component and is likely the site of ubiquinol oxidation (16–18). Both heme *b*₅₉₅ and heme *d* have been reported to bind exogenous ligands (11, 13, 19), and it has been suggested that these two hemes interact coopera-

tively in the oxidase reaction (20). Other studies have proposed that the function of heme *b*₅₉₅ is direct electron donation to heme *d* or to oxygenated intermediates of the chlorin (21, 22).

The cytochrome *bd* complex has some unusual properties for an oxidase. In the one-electron reduced state, O₂ forms a stable adduct with ferrous heme *d* without undergoing rapid autooxidation, unlike most high-spin heme proteins (23, 24). The stability of the O₂ complex (oxy form) is comparable to that of the O₂ adduct with myoglobin, with a dissociation rate constant, k_{off} , of 20 sec^{−1} at room temperature (R. J. Rohlfs, J. J. H., R. B. G., and C. R. Hille, unpublished data). Recently, it has been shown by resonance Raman spectroscopy that the cytochrome *bd* complex at room temperature also forms a remarkably stable oxoferryl adduct (25). In contrast, the enzyme has a surprisingly low affinity for anionic heme ligands such as CN[−] and N₃[−] (26, 27).

In this report, low-temperature Fourier transform infrared (FTIR) spectroscopy is used to study CO adducts of the cytochrome *bd* complex. CO, which has a strong IR absorption and a high affinity for hemes, is used in this study as an IR probe of the heme-pocket environment. The absorption band shape and center frequency (ν_{CO}) are very sensitive to the coordination state and surrounding solvation shell of the CO molecule (28–30).

Photodissociation of CO from heme *d* at low temperatures results in an adduct in which CO is associated with the protein within the heme pocket. However, both heme *b*₅₉₅ and heme *d* are accessible to the photodissociated CO molecule upon relaxation. This demonstrates that the two heme irons are close together in the oxidase and essentially can be considered to form a heme–heme binuclear center where oxygen is reduced to water. FTIR spectra provide evidence that there is only one heme *d* per complex and that the protein environment surrounding the two high-spin hemes is very hydrophobic. This is consistent with an environment that can stabilize the oxy (O₂) and oxoferryl adducts as well as impede the binding of anionic ligands.

MATERIALS AND METHODS

All reagents used were scientific grade. ¹²C¹⁶O was purchased from Matheson as 99.5% pure, and ¹³C¹⁶O (enriched to 90% ¹³C) was purchased from Monsanto. Glycerol was purchased from Aldrich and was anhydrous grade. The sample spacer material was obtained from the foil wrapper of a Pop Tart package made by Kellogg.

E. coli strain GO102/pFH101 was grown aerobically at 37°C in LB medium with 0.3% lactate and in the presence of ampicillin (100 µg/ml), streptomycin (50 µg/ml), and kanamycin (25 µg/ml). Cells were grown to late-exponential phase and then harvested and disrupted by two passes through a

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Abbreviation: FTIR, Fourier transform infrared.

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microfluidizer cell disrupter (Microfluidics, Newton, MA). The remaining protocol for cytoplasmic membrane preparation was as reported (31).

The CO adducts were prepared by adding 2 ml of the membrane pellet to 20 ml of 50 mM phosphate buffer with 1 mM lactate (pH 7.00) (at 4°C) in a screw-cap, 60-Ti centrifuge tube (Beckman). The tube was sealed with a rubber septum and made anaerobic by an argon purge in conjunction with respiration by the aerobic respiratory system in the membranes using the lactate as reducing substrate. After 1 h of argon purge, a 3-ml aliquot (at room temperature and pressure) of ^{13}CO gas was added to the sample by syringe. The septum was replaced with the standard 60-Ti screw cap under a flow of argon. The membranes were pelleted at $160,000 \times g$ for 2 h at 4°C. The supernatant was decanted under a flow of argon and the pellet was overlaid with ^{12}CO -saturated glycerol to further concentrate the membranes and to remove excess water. Some of the ^{12}CO in the glycerol exchanges with the ^{13}CO during the extraction process to generate the mixed isotope sample. The sample was extracted for 6–8 h at 4°C.

A portion of the glycerol-treated sample was placed between two 3×25 mm CaF_2 windows (Janos Technology, Townshend, VT) and pressed to the thickness of the foil spacer, which was $38 \mu\text{m}$ as measured by interference fringes (28). IR spectra were obtained with a Mattson Sirius 100 Fourier transform interferometer at 0.5 cm^{-1} resolution. A liquid nitrogen-cooled indium antimonide detector was used to observe the spectra in the $1750\text{--}3000 \text{ cm}^{-1}$ range, which permitted observation of both the iron-bound and photodissociated CO. Seventeen-volt peak/peak interferograms were recorded in both directions of mirror movement through a 16-bit ($\pm 10 \text{ V}$) A/D converter and signal averaged into 32-bit words. These interferograms were used for all further computations. The number of scans chosen for data collection was dependent on signal size, sample, and type of experiment. Typically, a peak/peak noise level of $\approx 20 \mu\text{A}$ was obtained with 512 scans. Full, double-sided interferograms were triangularly apodized, with the real part of the FT yielding the single-beam spectrum. Spectra are presented as absorbance difference spectra, for which the spectrum before photolysis (dark) is subtracted from the spectrum obtained during or after photolysis (light). Photodissociation was achieved with a 500-W tungsten lamp focused through a slide projector and optically filtered through glass and water to remove UV and IR radiation. Subtractions of the least-squares fits of a cubic polynomial to the baseline regions of the spectra were used for baseline correction. There was no further averaging, smoothing, or other correction of the spectra.

Low temperatures were maintained and measured with a Lake Shore Cryotronics helium refrigerator (model LTS-21-D70C).

Visible spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer.

RESULTS

In this study, cytoplasmic membrane preparations from *E. coli* strain GO102(*cyo cyd*)/pFH101 were used. This strain overproduces the cytochrome *bd* complex and does not contain any other membrane-bound CO-binding components. Lactate, via the aerobic respiratory system in the cytoplasmic membrane, was used to reduce the cytochrome *bd* complex prior to formation of the CO adduct. This method of reduction successfully scavenges molecular oxygen in the system without deleterious side reactions, as can occur with dithionite as a reductant (32). The iron carbonyl is readily photodissociated at low temperatures, and comparison of the FTIR spectrum before and after photodissociation provides

a nearly ideal sample and reference. This results in a difference spectrum with a flat baseline, where the only troughs and peaks seen correspond to the changes in environment of the CO in going from the iron-bound state to the surface of the heme pocket. All spectra presented in this report were recorded with one sample, but different samples have yielded the same results.

Fig. 1 shows visible absorbance spectra of the CO adduct of the complex at 12 K. The "light" spectrum shows the heme *d* absorbance peak at 628 nm, representing the unligated state. The "dark" spectrum shows the heme *d* peak at 634 nm, reflecting the CO-ligated state. The absorbance difference spectrum (not shown) prominently displays this shifted peak but no other significant features. The absorbance difference between the dark and light spectra, therefore, suggests that only heme *d* binds CO in the complex. This is consistent with previously published solution spectra taken at room temperature (12).

Fig. 2 shows the FTIR absorbance difference spectrum (continuous photolysis minus dark) of the membrane sample at 15.5 K. The sample in this study was enriched with $^{13}\text{C}^{16}\text{O}$ but also contained $^{12}\text{C}^{16}\text{O}$, so the spectrum contains a pair of absorbance bands for CO, both in the dark and upon photolysis. The two major bands pointing downward are due to CO bound to the iron in the dark spectrum, and those that point upward are due to CO following photolysis. The two bands from iron-bound CO centered at 1984 cm^{-1} (for $^{12}\text{C}^{16}\text{O}$) and 1939 cm^{-1} (for $^{13}\text{C}^{16}\text{O}$) both have a bandwidth at one-half peak height ($\Delta\nu_{1/2}$) of 3.9 cm^{-1} . The two bands in the photodissociated state, centered at 2133 cm^{-1} ($^{12}\text{C}^{16}\text{O}$) and 2088 cm^{-1} ($^{13}\text{C}^{16}\text{O}$), have $\Delta\nu_{1/2}$ of 2.0 cm^{-1} . The features of the spectrum of $^{12}\text{C}^{16}\text{O}$ bound to the complex have been reported (33). The carbon isotope shifts are those expected for a simple harmonic oscillator. The ratio of frequencies $1939/1984 = 0.9773$, whereas $\mu^*/\mu = 0.9777$, where μ and μ^* are the reduced masses of $^{12}\text{C}^{16}\text{O}$ and $^{13}\text{C}^{16}\text{O}$, respectively. The carbon isotope shifts demonstrate that all absorption bands discussed in this report are due to CO complexes with the protein. The narrow bandwidth indicates a highly ordered, nonpolar environment for CO when bound to heme *d* (29). The photolyzed ^{12}CO band center frequency is only 10 cm^{-1} below the vibrational frequency of the free gas and is the same frequency found for CO in a benzene matrix at 30 K (34). This indicates that, following photolysis, there are only noncovalent, weak, dipole-induced dipole interactions

Fig. 1. Visible absorbance spectra of membranes containing the CO adduct of the cytochrome *bd* complex at 12 K shown before (Dark) and after (Light) photolysis with white, visible light. The absorbance peak centered at 634 nm in the dark spectrum corresponds to the CO-heme *d* adduct, and the absorbance peak centered at 628 nm in the light spectrum corresponds to the unligated, photodissociated state.

FIG. 2. IR absorbance spectrum (continuous photolysis minus dark) of the CO adduct of the cytochrome *bd* complex at 15.5 K. The two Fe–CO absorbance bands centered at 1939 cm^{-1} and 1984 cm^{-1} correspond to $^{13}\text{C}^{16}\text{O}$ and $^{12}\text{C}^{16}\text{O}$, respectively, of the CO–heme *d* adduct. The two absorbance bands centered at 2088 cm^{-1} and 2133 cm^{-1} correspond to $^{13}\text{C}^{16}\text{O}$ and $^{12}\text{C}^{16}\text{O}$, respectively, of the photodissociated state.

between CO and the molecular groups at the surface of the heme pocket.

Fig. 3 shows the difference spectrum of the sample at 20 K 23.9 h after a 10-s pulse of white visible light, referenced to the original dark spectrum. The most striking feature of this spectrum is the presence of new bands at 1974 cm^{-1} (for ^{12}CO) and 1929 cm^{-1} (for ^{13}CO) pointing upward. This spectrum corresponds to a time point during the relaxation of CO from the surface of the heme pocket and, therefore, represents a partial relaxation. The new bands at 1974 cm^{-1} and 1929 cm^{-1} are formed by CO bound to a second heme site. As the relaxation process proceeds, the 1974 cm^{-1} and 1929 cm^{-1} bands increase in size while the bands corresponding to CO bound to the pocket surface (2088 cm^{-1} and 2133 cm^{-1}) decrease in size. The negative bands at 1939 cm^{-1} and 1984 cm^{-1} correspond to CO photodissociated from heme *d* but not yet rebound to that site. They decrease in size during the relaxation process since CO rebinds to both heme *d* and the second heme site. The endpoint of this relaxation of CO from the pocket surface always generates equal areas between the positive bands at 1974 cm^{-1} and 1929 cm^{-1} and their corresponding negative bands at 1939 cm^{-1} and 1984 cm^{-1} (data not shown) and therefore suggests that all the CO

FIG. 3. IR absorbance spectrum of the CO adduct of the cytochrome *bd* complex at 20 K, 23.9 h after a 10-s pulse of white, visible light and referenced to the original dark spectrum. The two Fe–CO absorbance bands pointing upward and centered at 1929 cm^{-1} and 1974 cm^{-1} correspond to the CO–heme *b*₅₉₅ adduct $^{13}\text{C}^{16}\text{O}$ and $^{12}\text{C}^{16}\text{O}$, respectively. The absorbance bands centered at 1939 cm^{-1} , 1984 cm^{-1} , 2088 cm^{-1} , and 2133 cm^{-1} are the same as in Fig. 2.

that did not relax back to heme *d* is bound to the second heme site. These observations are most simply interpreted by a second heme site accessible within the heme *d* pocket. The $\Delta\nu_{1/2}$ of 3.5 cm^{-1} indicates that the environment of CO bound to the second heme is similar to the environment of CO when bound to heme *d*. The assignment of the 1974 cm^{-1} band as being due to a second CO–heme adduct is demonstrated by the observed bleaching of this band by illuminating the sample with white light at 16 K (data not shown). Hence, CO in this state can be photodissociated by visible light and must be ligated to a heme.

Since heme *b*₅₉₅, like heme *d*, is high spin it is reasonable to speculate that heme *b*₅₉₅ is the site of the second iron–CO adduct. Alternatively, the second heme could be a second heme *d*, suggested to exist in earlier studies (11–13), or even a modified form of heme *d* resulting from the photolysis. To test this possibility, the photolysis light was filtered through a series of longpass, cut-off filters that absorb visible light with $\lambda < 700$ nm. Light of $\lambda > 700$ nm has been shown to photolyze CO–heme *d* adducts and not CO–heme *b* adducts (35). The expectation is that under these conditions of continuous photolysis the CO will be “pumped” from heme *d* to the pocket surface, with subsequent recombination of a portion of this population to heme *b*₅₉₅, where it will stick and not be photolyzed, as is the case when white light is used for photolysis. During this process some of the CO will relax back to heme *d*, where it will be rephotolyzed. The results of this experiment are shown in Fig. 4. The spectrum was taken at 50 K, 20.6 h after continuous photolysis with the 700-nm cut-off filters in place. Qualitatively this spectrum is similar to Fig. 3. This experiment shows that the second heme is not an additional heme *d*, which would be subject to effective photolysis with long-wavelength light. The second heme is concluded to be heme *b*₅₉₅. Spectra recorded at early time points during the continuous photolysis with $\lambda > 700$ nm show no evidence for direct, rapid formation of the CO–heme *b*₅₉₅ adduct (data not shown).

Fig. 5 is a schematic depiction of the heme *d*–heme *b*₅₉₅ pocket within the cytochrome *bd* complex based on the results described above. The drawings depict the two heme planes tilted with respect to each other, as suggested by *in situ* electron paramagnetic resonance data (14). At low temperatures, a single CO molecule in this pocket has been characterized spectroscopically in the three distinct states shown.

DISCUSSION

Most of the results of this paper can be summarized by the schematic in Fig. 5. The three drawings in the schematic

FIG. 4. IR absorbance spectrum of the CO adduct of the cytochrome *bd* complex at 50 K, 20.6 h after continuous photolysis with the 700-nm-long wavelength pass filters in place and referenced to the original dark spectrum. All absorbance bands are centered at the same frequencies as those in Figs. 2 and 3.

FIG. 5. Schematic of the three states of CO in the heme-heme binding pocket of the cytochrome *bd* oxidase. The two hemes are indicated as "b" (heme *b*₅₉₅) and "d" (heme *d*). Shaded region represents the protein surrounding the two hemes, forming a hydrophobic medium sensed by CO.

represent the three states of CO in the complex. State 1 represents the initial state of CO bound to the heme *d* component of the enzyme complex. CO has access to either heme *b*₅₉₅ or heme *d* but has a strong preference for binding to heme *d*. This is the ground state for CO at room temperature as well as at the low temperatures used in this study. After photolysis of the CO-heme *d* adduct, the CO interacts with the surface of the heme pocket (state 2). The 2133 cm^{-1} center frequency (¹²CO) of this photolyzed state (Fig. 2) confirms that CO is not bound covalently but is very weakly associated with the surface of the pocket. The narrow $\Delta\nu_{1/2}$ of 2.0 cm^{-1} suggests that the CO is not free to rotate but is constrained to a unique configuration (34).

State 3 represents the CO-heme *b*₅₉₅ adduct, which is generated by slow relaxation of the CO from state 2 (the pocket surface). We see no evidence for direct and rapid formation of the CO-heme *b*₅₉₅ adduct after photolysis of CO from heme *d* at temperatures < 100 K. Slow formation of a CO-heme adduct is not unusual for heme proteins at low temperatures (36). Many studies conducted on myoglobin at low temperatures have indicated that the rate-limiting steps in the formation of the CO-heme adduct are due to the slow undoming of the five-coordinate, high-spin heme to the more planar low-spin CO-heme adduct (37, 38). Furthermore, this rate of formation is very similar to the time scale we observe for relaxation of CO to both hemes *b*₅₉₅ and *d* (unpublished data). The fact that CO in state 3 can itself be photodissociated by white light demonstrates that CO in this state must be bound to a heme. It is important to note that after generation of either state 2 or 3, CO completely relaxes back to heme *d* exclusively when the sample is warmed to 180 K or above. This observation may be explained by thermal dissociation of the CO from heme *b*₅₉₅, occurring at a much greater rate at the higher temperature. Thermal dissociation under these circumstances is unusual for heme carbonyls, and suggests that the CO-heme *b*₅₉₅ complex may be sterically destabilized. The equilibrium partitioning favors the CO-heme *d* adduct over the CO adduct with heme *b*₅₉₅. At lower temperatures, the dissociation rate of CO from heme *b*₅₉₅ is so slow that the CO is effectively trapped on this heme. Therefore, a significant population of this state accumulates only at very low temperatures. These observations are consistent with CO preferring the heme *d* site exclusively under physiological conditions.

As stated above, relaxation processes allow the photodissociated CO to rebind to either heme *d* or to heme *b*₅₉₅, with the majority of the CO returning to heme *d*. At the time points

when the relaxation spectra were recorded, the area of the CO-heme *b*₅₉₅ absorbance band is $\approx 6\%$ of the total area associated with the CO-heme *d* band, based on the relative areas for the 1974 cm^{-1} bands in Figs. 3 and 4 compared with the 1984 cm^{-1} band in Fig. 2. This ratio of populations may be as high as 15%, as observed by relaxation studies at a variety of temperatures below 100 K. The method of selective photolysis (i.e., $\lambda > 700 \text{ nm}$) of CO from heme *d* to heme *b*₅₉₅ should, in principle, generate an amount of the CO-heme *b*₅₉₅ adduct equal to that of the originating CO-heme *d* population. However, experimentally the observed yield of the CO-heme *b*₅₉₅ adduct is maximally 15% of the initial CO-heme *d* population. Among the possible explanations are that the CO-heme *b*₅₉₅ adduct may also be photodissociated to some extent by the 700-nm filtered light, and the protein is frozen into a conformation that sterically hinders access to heme *b*₅₉₅.

The most important conclusion of this work is that heme *d* and heme *b*₅₉₅ occupy a common binding pocket in the oxidase, as evidenced by the ability of CO to transfer from one heme to the other at low temperatures. Previous studies of mutants of the cytochrome *bd* complex have suggested that these two hemes may be bound to a common region in the protein (39, 40). Electron paramagnetic resonance studies have also indicated some conformational coupling between the two hemes (10, 11). It is clear that heme *d* is directly involved in binding oxygen and in reduction of oxygen via an oxoferryl intermediate (25) to water. However, the role of heme *b*₅₉₅ is not known. The current work demonstrates that heme *b*₅₉₅ is physically close to the oxygen-binding site and that heme *b*₅₉₅ can bind exogenous ligands, at least transiently. Hence, heme *b*₅₉₅ may be in a position not only for mediating rapid electron transfer to facilitate the reduction of oxygen, but it could possibly be considered to bind oxygen transiently or to stabilize oxygen intermediates during catalytic turnover. The site that catalyzes the redox chemistry of oxygen should thus be properly considered to be a heme-heme binuclear center.

In this context, the cytochrome *bd* complex can be compared to the superfamily of heme-Cu oxidases, which includes the *aa*₃-type cytochrome *c* oxidases as well as the *bo*-type ubiquinol oxidase (41, 42). In heme-Cu oxidases, oxygen is reduced at a binuclear center consisting of a heme (e.g., heme *a*₃) and a nearby Cu (called Cu_B). Electrons are transferred to the heme-Cu center via a low-spin heme component (e.g., heme *a*), which plays a role analogous to that of heme *b*₅₅₈ in the *bd*-type oxidase. At the heme *a*₃-Cu_B binuclear center, Cu_B binds CO with much less affinity than does heme *a*₃. However, CO can be kinetically trapped on Cu_B at low temperatures (31, 43), just as in the case of *b*₅₉₅ in the *bd*-type oxidase. It is reasonable to suppose that these two accessory metal centers (Cu_B and heme *b*₅₉₅) have similar functions in their respective oxidases, which could include controlling access by oxygen to the oxygen-binding site (heme *d* or heme *a*₃), rapid electron transfer to facilitate catalytic turnover, and direct interaction with oxygenated intermediates during catalysis (42, 44). In the case of Cu_B, a potential role in proton pumping has been suggested (42), which is not likely for heme *b*₅₉₅ since the *bd*-type oxidase does not pump protons (1). It is worth noting that in ascorbate oxidase (45) the type 2 Cu may be considered to be somewhat analogous to Cu_B and heme *b*₅₉₅. In ascorbate oxidase, however, the primary oxygen-binding site is not a heme but a type 3 Cu-Cu center.

Besides the conclusion that the oxygen chemistry catalyzed by the cytochrome *bd* complex occurs at a heme-heme binuclear center, the data presented in this work support several other significant conclusions. The presence of single, narrow, absorbance bands for the Fe-CO and photolyzed states suggests the following.

(i) The narrow $\Delta\nu_{1/2}$ of the bands suggests that the carbonyls are in a nonpolar environment with a slightly polarizable solvation shell about the CO (29, 30). Furthermore, the distance between a CO bound to either of the hemes or the pocket surface, and an adjacent heme iron, is greater than the van der Waals contact distance so that no polarization of the CO by this adjacent heme iron occurs. This type of polarization would manifest itself as broader absorbance bands than are observed. The hydrophobic nature of the heme pocket is consistent with the stability of the oxoferryl intermediate that has been characterized (25) and with the high affinity for oxygen (4).

(ii) The single narrow IR absorbance bands associated with states 1 and 2 suggest that there is only one heme *d* in the complex that can bind CO and not two as had previously been suggested (12, 13, 15). Two CO-heme *d* adducts would generate at least two different FTIR absorbance bands for each of the heme-bound and photodissociated states, unless the adducts are in virtually identical environments, as found with the CO adducts of hemoglobin. The hemoglobin α and β subunits have an $\approx 1\text{ cm}^{-1}$ separation of the heme-CO center frequencies, and the two absorbance bands are not resolved (J.O.A. and K. A. Powell, unpublished data). The cytochrome *bd* complex is a dimer of two different subunits (15), and the sequences of the two subunits do not reveal any repeating structural motifs (46–48). Therefore, it is very unlikely that two heme *d* moieties with identical environments would be present in this enzyme. The FTIR spectra strongly indicate a single CO-binding heme *d* in the complex. This conclusion is consistent with recent results (10, 11) showing by quantitative electron paramagnetic resonance analysis that the ratio of heme *b*₅₉₅ to heme *d* is 1:1.

(iii) Single, narrow absorption bands for the iron-bound and photodissociated states of CO suggest that at low temperatures the complex is frozen into only one major conformational substate. This corresponds to tier 0 of the reaction energy landscape scheme at low temperatures described by Steinbach *et al.* (37). The presence of a single substate is quite unique among heme proteins that have been studied at low temperatures (36–38, 43, 49, 50). The dynamic interactions of the CO complex at low temperatures, in an effort to describe the relaxation processes and to characterize the conformational features of the protein, remain to be studied.

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1. Puustinen, A., Finel, M., Haltia, T., Gennis, R. B. & Wikström, M. (1991) *Biochemistry* **30**, 3936–3942.
2. Anraku, Y. (1988) *Annu. Rev. Biochem.* **57**, 101–132.
3. Minghetti, K. C. & Gennis, R. B. (1988) *Biochem. Biophys. Res. Commun.* **155**, 243–248.
4. Rice, C. W. & Hemphling, W. P. (1978) *J. Bacteriol.* **134**, 115–124.
5. Cotter, P. A., Chepur, V., Gennis, R. B. & Gunsalus, R. P. (1990) *J. Bacteriol.* **172**, 6333–6338.
6. Kita, K., Konishi, K. & Anraku, Y. (1984) *J. Biol. Chem.* **259**, 3368–3374.
7. Iuchi, S., Chepur, V., Fu, H.-A., Gennis, R. B. & Lin, E. C. C. (1990) *J. Bacteriol.* **172**, 6020–6025.
8. Sotiriou, C. & Chang, C. K. (1988) *J. Am. Chem. Soc.* **110**, 2264–2270.
9. Timkovich, R., Cork, M. S., Gennis, R. B. & Johnson, P. Y. (1985) *J. Am. Chem. Soc.* **107**, 6069–6075.
10. Meinhardt, S. W., Gennis, R. B. & Ohnishi, T. (1989) *Biochim. Biophys. Acta* **975**, 175–184.
11. Rothery, R. & Ingledew, W. J. (1989) *Biochem. J.* **262**, 437–443.
12. Lorence, R. M., Koland, J. G. & Gennis, R. B. (1986) *Biochemistry* **25**, 2314–2321.
13. Lorence, R. M. & Gennis, R. B. (1989) *J. Biol. Chem.* **264**, 7135–7140.
14. Ingledew, W. J., Rothery, R. A., Gennis, R. B. & Salerno, J. C. (1991) *Biochem. J.* **282**, 255–259.
15. Miller, M. J., Hermodson, M. & Gennis, R. B. (1988) *J. Biol. Chem.* **263**, 5235–5240.
16. Green, G. N., Kranz, R. G., Lorence, R. M. & Gennis, R. B. (1984) *J. Biol. Chem.* **259**, 7994–7997.
17. Green, G. N. (1986) *Biochemistry* **25**, 2309–2314.
18. Lorence, R. M., Carter, K., Green, G. N. & Gennis, R. B. (1987) *J. Biol. Chem.* **262**, 10532–10536.
19. Poole, R. K., Scott, R. I. & Chance, B. R. (1981) *J. Gen. Microbiol.* **125**, 431–438.
20. Rothery, R. A., Houston, A. M. & Ingledew, W. J. (1987) *J. Gen. Microbiol.* **133**, 3247–3255.
21. Poole, R. K. & Williams, H. D. (1987) *FEBS Lett.* **217**, 49–52.
22. Hata-Tanaka, A., Matsuura, K., Itoh, S. & Anraku, Y. (1987) *Biochim. Biophys. Acta* **893**, 289–295.
23. Springer, B. A., Egeberg, K. D., Sligar, S. G., Rohlf, R. J., Mathews, A. J. & Olson, J. S. (1989) *J. Biol. Chem.* **264**, 3057–3060.
24. Wallace, W. J., Houtchens, R. A., Maxwell, J. C. & Caughey, W. S. (1982) *J. Biol. Chem.* **257**, 4966–4977.
25. Kahlow, M. A., Zuber, T. M., Gennis, R. B. & Loehr, T. M. (1991) *Biochemistry* **30**, 11485–11489.
26. Pudek, M. R. & Bragg, P. D. (1976) *Arch. Biochem. Biophys.* **174**, 546–552.
27. Green, G. N. & Gennis, R. B. (1983) *J. Bacteriol.* **154**, 1269–1275.
28. Alben, J. O. & Fiamingo, F. G. (1984) in *Fourier Transform Infrared Spectroscopy*, ed. Rousseau, D. L. (Academic, New York), pp. 133–179.
29. Alben, J. O. (1987) in *Polarization in Heme Proteins and Cytochrome c Oxidase*, eds. Papa, S., Chance, B. & Ernster, L. (Plenum, New York), pp. 361–369.
30. Alben, J. O. & Caughey, W. S. (1968) *Biochemistry* **7**, 175–183.
31. Shapleigh, J. P., Hill, J. J., Alben, J. O. & Gennis, R. B. (1992) *J. Bacteriol.* **174**, 2338–2343.
32. Fish, K. M., Massey, V., Sands, R. H. & Dunham, W. R. (1990) *J. Biol. Chem.* **265**, 19665–19671.
33. Alben, J. O., Hemann, C. F. & Hill, J. J. (1989) *SPIE J.* **1145**, 618–621.
34. Jiang, G. J., Person, W. B. & Brown, K. G. (1975) *J. Chem. Phys.* **62**, 1201.
35. Hill, J. J., Gennis, R. B. & Alben, J. O. (1991) *SPIE J.* **1575**, 453–456.
36. Frauenfelder, H., Sligar, S. G. & Wolynes, P. G. (1991) *Science* **254**, 1598–1607.
37. Steinbach, P. J., Ansari, A., Berendzen, J., Braundstein, D., Chu, K., Cowen, B. R., Ehrenstein, D., Frauenfelder, H., Johnson, J. B., Lamb, D. C., Luck, S., Mourant, J. R., Nienhaus, G. U., Ormos, P., Philipp, R., Xie, A. & Young, R. D. (1991) *Biochemistry* **30**, 3988–4001.
38. Srajer, V. & Champion, P. M. (1991) *Biochemistry* **30**, 7390–7402.
39. Oden, K. L. & Gennis, R. B. (1991) *J. Bacteriol.* **173**, 6174–6183.
40. Fang, H., Lin, R.-J. & Gennis, R. B. (1989) *J. Biol. Chem.* **264**, 8026–8032.
41. Saraste, M. (1990) *Q. Rev. Biophys.* **23**, 331–366.
42. Babcock, G. T. & Wikström, M. (1992) *Nature (London)* **301**–309.
43. Fiamingo, F. G., Altschuld, R. A., Moh, P. P. & Alben, J. O. (1982) *J. Biol. Chem.* **257**, 1639–1650.
44. Larsen, R. W., Pan, L.-P., Musser, S. M., Li, Z. & Chan, S. I. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 723–727.
45. Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A. & Finazzi-Agró, A. (1992) *J. Mol. Biol.* **224**, 179–205.
46. Green, N. G., Fang, H., Lin, R.-J., Newton, G., Mather, M., Georgiou, C. & Gennis, R. B. (1988) *J. Biol. Chem.* **263**, 13138–13143.
47. Georgiou, C. D., Dueweke, T. J. & Gennis, R. B. (1988) *J. Biol. Chem.* **263**, 13130–13137.
48. Newton, G., Yun, C.-H. & Gennis, R. B. (1991) *Mol. Microbiol.* **5**, 2511–2518.
49. Traylor, T. G., Magde, D., Taube, D. J., Jongeward, K. A., Bandhopadhyay, D., Luo, J. & Walda, K. N. (1992) *J. Am. Chem. Soc.* **114**, 417–429.
50. Alben, J. O., Moh, P. P., Fiamingo, F. G. & Altschuld, R. A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 234–237.