Reaction of Cytochrome bo_3 with Oxygen: Extra Redox Center(s) are Present in the Protein†

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ABSTRACT: The reaction of oxygen with cytochrome bo_3, a quinol oxidase from *Escherichia coli*, has been studied by resonance Raman scattering after initiation of the reaction by CO photolysis in a continuous flow apparatus and by directly mixing the enzyme with oxygen. The high-frequency region of the spectrum was monitored to determine the time evolution of the spin, oxidation, and coordination states of heme O and the oxidation state of heme B by using newly established marker lines for each heme. Three phases of the reaction were detected. In phase I, complete in 75 μs, O_2 reacted with heme O and formed a low-spin ferric or ferryl adduct without significant oxidation of heme B. In phase II, between 75 and 120 μs, a small fraction of heme B was oxidized. In phase III, at ~1 s, the majority of heme B was oxidized and heme O reverted to a high-spin ferric state. The high rate of oxygen reduction at heme O to the three- or four-electron reduced level, despite a very low rate of heme B oxidation, indicates that there are electron donors active in the enzyme other than the metal centers. Assays of our enzyme preparations rule out a quinol in the tight binding (Q_H) site as a possible donor but instead suggest electron donation from the protein matrix, such as from tryptophans or tyrosines. Three Tryptophans (W280, W282, and W331) and one tyrosine (Y288) are postulated as candidates for such a role, and their location near the binuclear center suggests that the donor electrons follow a pathway directly to the heme O−Cu_B binuclear center without passing through heme B. The donors that participate in the catalytic mechanism *in vitro* may also play a functional role under physiological conditions.

Cytochrome bo_3, a ubiquinol oxidase from aerobically grown *Escherichia coli* (Anraku & Gennis, 1987), catalyzes the reduction of molecular oxygen to water by the oxidation of ubiquinol-8 and contributes to the proton gradient across the cytoplasmic membrane (Puustinen et al., 1989). Unlike the other proteins in the terminal oxidase superfamily, such as cytochrome *c* oxidases which possess four metal centers, cytochrome bo_3 contains only three redox metals: two heme irons and one copper atom (Minghetti *et al.*, 1992). The low-spin heme (heme B) is coordinated by two axial histidines and is analogous to cytochrome *a* in mammalian cytochrome *c* oxidases. The high-spin heme (heme O) has a proximal histidine and thus, along with the nearby Cu_B, forms a binuclear binding site for O_2 (or CO) that is analogous to the cytochrome a_3−Cu_B binuclear center in aa_3-type oxidases (Hosler *et al.*, 1993; Chepuri et al., 1990; Hill et al., 1992). The properties of the CO-bound adduct of cytochrome bo_3 are similar to those of other terminal oxidases (Wang et al., 1993).

Since cytochrome bo_3 contains only three redox metals, in experiments designed to study the reaction of the fully-reduced enzyme with O_2 there is an insufficient number of electrons present to bring about the full reduction of molecular oxygen to water. The study of the catalytic reaction is further complicated by the recent finding that cytochrome bo_3 contains a novel internal (high-affinity) quinol binding site, referred as Q_H (Sato-Watanabe et al., 1994), in addition to the substrate (low-affinity) binding site (designated as Q_L) (Welter et al., 1994). While the internal quinol (Q_H) has been proposed to play an important part in mediating the electron flow from substrate (ubiquinol) (Q_L) to the cytochrome o_3−Cu_B binuclear center as does Cu_A in mammalian cytochrome *c* oxidase, its involvement in the catalytic mechanism of the enzyme is not known as yet. An additional complication results from the recent report that the heme pocket may contain a chloride ion depending on the preparative protocol (Orii et al., 1995).

The CO-flash (flow−flash−probe) method for initiating the reaction of terminal oxidases with O_2, originally pioneered by Gibson and Greenwood (1963), has been very powerful for studying the catalytic mechanism in cytochrome *c* oxidase. The reaction between cytochrome bo_3 and molecular oxygen has been probed with the CO-flash method by following the changes in the optical absorption spectrum (Svensson & Nilsson, 1993; Orii et al., 1994, 1995). Despite complexities apparently caused by differences in sample preparation, some common properties were found. Two fast phases in the decay of the oxygen-bound enzyme were detected, followed by a very slow phase (lifetime of ~1 s). Although molecular changes were proposed for each of the phases, it was recognized that it is difficult to extract detailed molecular structural properties from the optical spectrum. Recently, Kitagawa and co-workers (Hirota et al., 1994) used resonance Raman scattering to study the reaction between

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fully-reduced cytochrome bo_3 and dioxygen in the low-frequency region and were able to identify two of the reaction intermediates at very early time: an oxy species and a ferryl−oxo species. They noted that the intensities of the iron−oxygen modes of these intermediates were low and that their lifetimes were short in comparison to those of cytochrome aa_3 from mammalian cytochrome *c* oxidase. However, they did not report the high-frequency resonance Raman spectra in which modes are present that allow the oxidation state of each heme to be monitored.

To determine the molecular basis of the reaction between cytochrome bo_3 and oxygen, we followed the variations in redox state of each heme and the intramolecular electron transfer by using resonance Raman scattering combined with the CO-flash and the direct mixing techniques. Our data confirm the presence of complex multiple phases in the reaction. The coordination of oxygen to the heme O and the oxidation of heme B are clearly distinguished in our Raman spectra. Despite the absence of quinol in our preparation, the reaction progresses without complete oxidation of heme B, suggesting contributions from as yet unidentified redox-active center(s).

MATERIALS AND METHODS

Cytochrome bo_3 from aerobically grown *Escherichia coli* was isolated in our laboratories (in the presence of chloride) by the procedure published previously (Hill *et al.*, 1992; Minghetti *et al.*, 1992). Quantification of the ubiquinol content of the isolated enzyme was done since two quinol binding sites and/or tightly associated ubiquinol in pure cytochrome bo_3 have been reported (Sato-Watanabe et al., 1994). Approximately 1 nmol of protein (60−200 μL) was diluted into 340−200 μL of absolute ethanol and mixed vigorously, as described in Sato-Watanabe et al. (1994). The hemes were extracted from the mixture by the addition of an equal volume (400 μL) of n-hexane followed by vigorous mixing. The mixture was equilibrated for 5 min at room temperature, and mixing was repeated. Phases were resolved by centrifugation at 10 000 rpm for 3 min in an Eppendorf microcentrifuge. The upper layer (organic phase) was removed and dried under a stream of nitrogen. A small oily yellow pellet was recovered and resuspended in 100 μL of absolute ethanol. Extracted ubiquinol was quantitated by monitoring peak fractions eluted from a Rainin Microsorb-MV C18 HPLC column (25 cm) with a mobile phase of ethanol, methanol, and acetonitrile (4:3:3). The flow rate was adjusted to 0.8 mL/min using a Milton-Roy Spectro-Monitor 3100 variable wavelength detector at 278 nm. Peaks were quantitated with the Milton-Roy CI4000 computing integrator. Full scale was adjusted to 0.02−0.05 absorbance units, and the response time was set to 1 s. Under these conditions, ubiquinol-8 eluted at 13.7−14.0 min, menaquinone-8 at 23.0−23.4 min, and ubiquinone-10 at 28.1−29.5 min. All ubiquinone-8 quantification was determined in relation to a ubiquinone-10 internal standard. This assay showed that our preparation has very low quinol (~10%). In addition, the cytochrome bo_3 composition was found to be very high (>85% cytochrome bo_3; <15% cytochrome oo_3).

The samples for direct mixing or CO-flash experiments were prepared as follows: The reduced samples were prepared by solubilizing the enzyme (final concentration, ~100 μM) in 100 mM Tris-HCl buffer (Sigma), pH 7.5, containing 0.05% *N*-laurylsarcosyl (Sigma), 10 μM 2,3,5,6-tetramethylphenylenediamine (TMPD) (Aldrich), and 10 mM L-ascorbic acid (Sigma). The enzyme solution was incubated, anaerobically, at 4 °C, for several hours until the protein was completely reduced. For CO-flash experiments, the CO adducts of the enzyme were prepared by flushing gaseous CO (Matheson) over the fully-reduced cytochrome bo_3. The oxygen-saturated buffer was obtained by shaking (~30 min) a syringe containing 20 mL of the buffer and 5 mL of oxygen gas under pressure. The design of our CO-flash apparatus has been described in detail elsewhere (Han *et al.*, 1990a). Typically, samples of CO adducts of fully reduced cytochrome bo_3 ($B^{2+}O^{2+}$-CO) (100 μM) were placed in one syringe of the continuous flow rapid mixing device and dioxygen-saturated (1.4 mM O_2) buffer was placed in the other syringe; the solutions were mixed and flowed continuously into a small Raman sampling cell (cross section, 0.25 mm × 0.25 mm). The laser output was split into two beams such that the first beam at high power (~700 mW) initiated the reaction by photodissociating the CO from the heme O and the second beam at low power (~12 mW), so as not to photodissociate the products, probed the subsequent reaction between O_2 and photogenerated ligand-free cytochrome o_3. For given flow rates, the separation between the pump and probe beams resulted in a reaction time evolution from tens of μs to hundreds of ms.

In the direct mixing experiments, the reduced enzyme was directly mixed with O_2-saturated buffer without the presence of any CO and only one laser beam (413.1 nm) was used to probe the resonance Raman spectrum. The flow rate could be varied so that the time delay between the mixing point and the probe beam ranged from 65 ms to 8 s. The laser power was carefully attenuated to obtain a constant laser flux despite differences in the residence time of the enzyme in the laser beam due to changes of the flow rate. At flow speeds corresponding to the CO-flash measurements the power was 7 mW. As the flow speed was lowered to obtain longer delay times, the power was reduced accordingly.

To obtain the resonance Raman spectra the scattered light was projected from the probe region onto the entrance slit of a single monochromator (Spex) for dispersion (1.25 m) and subsequent detection by a charge-coupled device camera. Typically, the spectra reported here are averages of those accumulated for 30 s. The frequencies of the Raman lines were calibrated against an indene standard or laser fluorescence lines. To characterize the instrumentation for the rapid mixing and CO-flash experiments, reference samples were run through the flow apparatus at the start of each experiment. For the static experiments samples were sealed in rotating cells as described previously (Wang et al., 1995). Optical absorption spectra were recorded both prior to and after the Raman measurements to ensure the formation and stability of each species. All of the data were obtained at room temperature.

RESULTS

Static Resonance Raman Studies. Several ligand-bound complexes of the enzyme in various oxidation states were prepared to establish marker lines characteristic of coordination and redox states for each heme as was done for mammalian cytochrome *c* oxidases (Ching et al., 1985). The resonance Raman spectra in the high-frequency region of

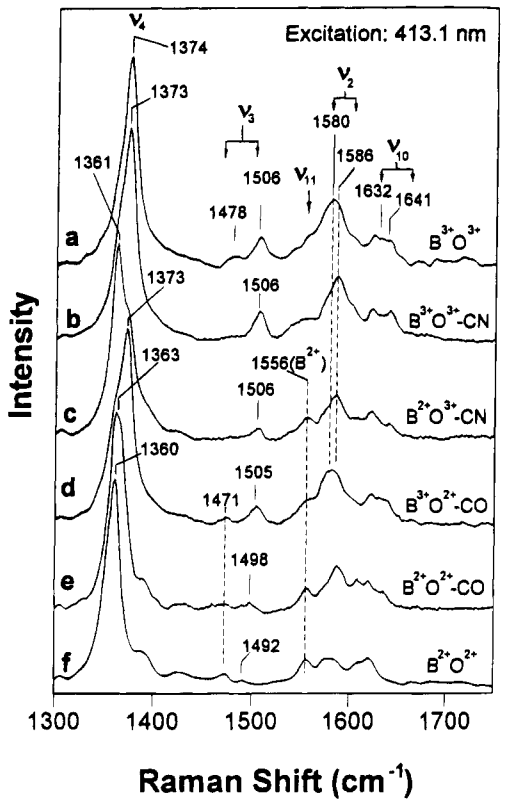

FIGURE 1: Resonance Raman spectra (in the high-frequency region) of cytochrome bo_3 in different oxidation and coordination states. Approximately 200 μL of a 100 μM enzyme solution was placed in a rotating Raman cell, and the spectra were recorded at 413.1 nm. Spectrum a is the fully-oxidized (resting) enzyme ($B^{3+}O^{3+}$) obtained at low incident laser power (1 mW). The cyanide-bound full-oxidized form (spectrum b) was prepared by adding a 10-fold excess of KCN solution to the resting enzyme. The cyanide-bound mixed-valence form ($B^{2+}O^{3+}$-CN) (spectrum c) was generated by the addition of ascorbate (~2 mM) and TMPD to the cyanide-bound fully-oxidized enzyme. The CO-bound mixed-valence form ($B^{3+}O^{2+}$-CO) (spectrum d) was made by flushing CO gas over the resting enzyme and incubating for several hours. The fully-reduced preparation (spectrum f) was formed by the anaerobic addition of a 10-fold excess of dithionite solution to the resting enzyme. The CO adduct of the fully-reduced enzyme ($B^{2+}O^{2+}$-CO) (spectrum e) was obtained by anaerobically exposing the exogenous ligand-free reduced sample to CO gas.

Table 1: Frequencies of Several Resonance Raman Marker Lines (cm^{-1}) in Cytochrome bo_3

	5C/HS	6C/LS		6C/HS	6C/LS	
mode	O^{2+} [a]	B^{2+}	O^{2+}–X[b]	O^{3+}	B^{3+}	O^{3+}–Y[b]
ν_2	1575	1585	~1589	~1580	~1586	~1586
ν_3	1471	1492	1493–1498	1478	1506	1506
ν_4[c]	1354	1361	1360	~1374	~1374	~1374
ν_{10}	~1620	~1620	1630–1635	1630	1641	1641
ν_{11}		1556				

[a] Contributions from hemes of cytochromes b and o_3 are designated by letter B and O, and the superscripts 2+ and 3+ stand for the oxidation state of each heme (ferrous and ferric, respectively). [b] X, CO and CN^-; Y, CN^-. [c] The frequencies of ν_4 are those measured in the ferric minus ferrous difference spectra.

these complexes are depicted in Figure 1, and the resulting assignments are summarized in Table 1. Resonance Raman spectra of hemeproteins in this frequency region contain several well-known porphyrin lines termed as the oxidation state (ν_4) or the ligation state (ν_3, ν_2, and ν_{10}) marker lines. We adopt the mode designations of Abe et al. (1978). With our excitation wavelength (413.1 nm), both hemes (B and O) contribute to the intensity of the Raman scattering.

The spectrum from the resting enzyme ($B^{3+}O^{3+}$) has a narrow line for ν_4 at 1374 cm^{-1} (spectrum a in Figure 1), indicating that both hemes are in the ferric state (fully-oxidized) as observed previously (Uno et al., 1985). The lines at 1506 and 1478 cm^{-1}, originating from ν_3, indicate that both the low-spin heme (B) and the high-spin heme (O) are six-coordinated, as reported in other proteins (a ferric five-coordinate high-spin species would be expected to have ν_3 at ~1495 cm^{-1}) (Dasgupta et al., 1989). Identification of ν_3 at 1478 cm^{-1} demonstrates that heme O is six-coordinate in its resting state, consistent with the proposals that in this form there is a bridging ligand between the heme iron and Cu_B (Tsubaki et al., 1993). The broad ν_2 Raman line centered at 1580 cm^{-1} is a combination of lines from both hemes. Coordination of CN^- to heme O of the resting enzyme (trace b: $B^{3+}O^{3+}$-CN) shifts the ν_3 line from heme O to higher frequency such that it coincides with that from the six-coordinate low-spin (6C/LS) heme B at 1506 cm^{-1}. In addition, the concomitant shifts of ν_2 (from 1580 to 1586 cm^{-1}) and ν_{10} (from 1630 to 1641 cm^{-1}) are consistent with the ligation of CN^- to the ferric heme O and its conversion to 6C/LS.

In one mixed-valence preparation ($B^{2+}O^{3+}$-CN) the ferrous low-spin heme B displayed a strong ν_4 line at 1361 cm^{-1} and a ν_{11} line at 1556 cm^{-1} (trace c in Figure 1). The reduction of the heme B is also apparent from the decrease in the intensities of ν_3 at 1506 cm^{-1} and ν_{10} at 1641 cm^{-1} as observed in alkaline cytochrome c peroxidase (Wang et al., 1992). The formation of the other mixed-valence complex ($B^{3+}O^{2+}$-CO) was confirmed by the detection of a strong ν_4 line at 1373 cm^{-1} (B^{3+}) and the concomitant identification of the Fe^{2+}-CO stretching mode at 521 cm^{-1} (data not shown), a similar frequency as that observed from the fully-reduced CO-adduct of cytochrome bo_3 (Wang et al., 1993). The CO-bound heme O should also have a contribution to the intensity of ν_4 at ~1373 cm^{-1}, but it is not expected to be very large based on its low intensity observed in the CO-bound fully-reduced enzyme (trace e). The spectral perturbations caused by the reduction of heme O are very limited except for the disappearance of ν_3 signal at 1478 cm^{-1} from the ferric six-coordinate high-spin (6C/HS) heme O spectrum and the concomitant appearance of the mode at 1471 cm^{-1}, a five-coordinate high-spin (5C/HS) Fe^{2+} line originating from a small fraction of photolyzed ferrous cytochrome o_3–CO complex. The spectrum of the fully-reduced enzyme (trace f: $B^{2+}O^{2+}$) is very distinct from that of the oxidized form as it shows a single ν_4 line at 1360 cm^{-1} originating from both reduced hemes and a doublet of ν_3 at 1471 and 1492 cm^{-1} from the ferrous 5C/HS heme O and the ferrous 6C/LS heme B, respectively. The spectra of the mixed-valence preparations are quite distinct from those of aa_3-type cytochrome c oxidase from bovine, especially in the ν_4 region (Ching et al., 1985; Argade et al., 1986). We attribute the differences to the different Soret maxima of the heme B and heme O derivatives as compared to heme A.

A line at 1556 cm^{-1} appears in spectra from $B^{2+}O^{3+}$-CN (trace c), $B^{2+}O^{2+}$ (trace f), and the CO adduct (trace e: $B^{2+}O^{2+}$-CO), all of which have heme B in its ferrous state. We assign this line as originating from ν_{11} of ferrous heme B and thus utilize it as marker line for reduced heme B since spectral overlap with other sharp contributions is not large.

The absence of this line in spectra a, b, and d (B^{3+}) confirms the assignment. Despite the significant difference in the intensities of ν_4 at 1361 cm^{-1}, ν_3 at 1506 cm^{-1}, and ν_{11} at 1556 cm^{-1}, between spectra b ($B^{3+}O^{3+}$-CN) and c ($B^{2+}O^{3+}$-CN), ν_2 from O^{3+}-CN remains almost unshifted (1586 cm^{-1}) and thus serves as a marker line for the low-spin complex of ferric heme O and as such is important for identification of low-spin O_2-bound intermediates in the catalytic cycle. In addition, while ν_3 from both ferric low-spin heme B (traces a and b) and CN^--bound heme O (traces b and c) coincide at 1506 cm^{-1}, ν_3 at 1478 cm^{-1} (trace a) and 1471 cm^{-1} (traces d–f), albeit weak, might be the indicators for the 6C/HS ferric form and 5C/HS ferrous form of heme O, respectively. The change in the intensity of ν_4 caused by changes in the oxidation state (and coordination with a low-spin ligand) of heme O is quite small, as evident by the comparison of the spectrum of $B^{2+}O^{3+}$-CN (trace c) to that of $B^{2+}O^{2+}$ (trace f). In contrast, a change in the redox state of heme B results in large perturbations of the ν_4 lines (traces b and c). It is thus anticipated that the variations in the relative intensity of ν_4 observed during the reaction of fully-reduced cytochrome bo_3 with oxygen primarily reflect the perturbations of oxidation state of heme B although as discussed below the contribution of each heme to ν_4 can be distinguished.

Reaction of Fully-Reduced Cytochrome bo_3 with O_2. In experiments using the CO-flash method the ligand-free fully-reduced form of cytochrome bo_3 ($B^{2+}O^{2+}$) was transiently generated (trace a in Figure 2) by photolyzing CO from the fully-reduced CO-bound enzyme. In the presence of O_2 this species reacts rapidly yielding spectrum b in Figure 2 where the time delay is 75 μs. This initial spectrum at 75 μs is largely unchanged over the full 15 ms time period we studied. The major changes all took place on a time scale shorter than 75 μs. The additional small evolution of the reaction products from the 75 μs to 15 ms time delay was followed by probing the marker lines subsequent to initiation of the reaction (traces b–h).

The presence in all of the spectra in Figure 2 of a marker line for reduced heme B (at 1556 cm^{-1}) and the small rise of ν_4 at 1373 cm^{-1} indicate that the heme B remains largely in its reduced form throughout the 15 ms series. Thus the occurrence of the ν_3 at 1506 cm^{-1} and ν_2 at 1586 cm^{-1} in the earliest spectrum (75 μs) cannot originate from the oxidation of heme B but instead demonstrates the rapid coordination of O_2 to the heme O and its subsequent oxidation. The ν_3 line at 1506 cm^{-1} further indicates that the heme O is already oxidized in our earliest spectrum (75 μs). If the heme O was coordinated by O_2 in this time domain without further reaction, a spectrum similar to the CO-bound species would be expected in which ν_3 is located at 1498 cm^{-1} or lower (Table 1).

The continuous change of ν_4 over the entire time course of our CO-flash experiment (75 μs to 15 ms) indicates a slow process occurring subsequent to the initial reaction of oxygen with reduced heme O and its rapid oxidation. In order to follow the evolution of this reaction, we have examined the ν_4 region in greater detail. The difference spectra for sets of two adjacent time delays (traces c–g in Figure 3) are compared with the spectral change in ν_4 resulting from the oxidation of heme B (trace a in Figure 3: the difference of $B^{3+}O^{3+}$-CN minus $B^{2+}O^{3+}$-CN from Figure 1) and from the ligation and oxidation of heme O (trace b in

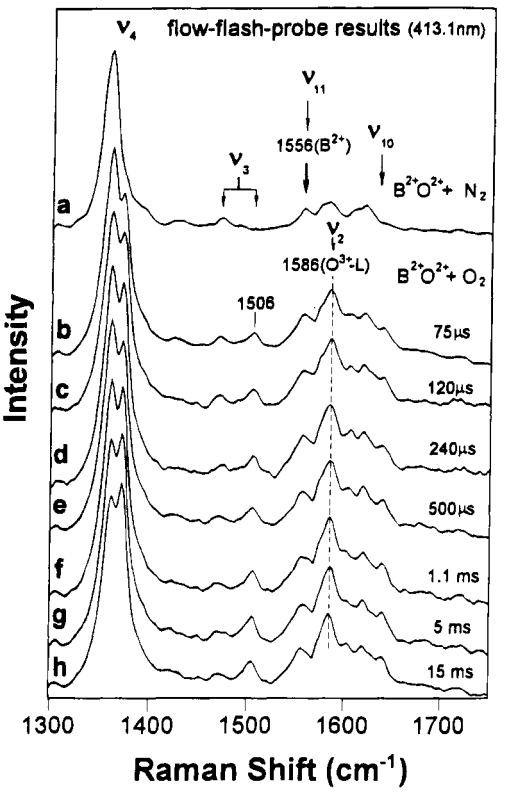

FIGURE 2: Time dependence of the reaction of fully-reduced cytochrome bo_3 with oxygen by the CO-flash method. Spectrum a is a control of the ligand-free (heme O) fully-reduced form obtained by anaerobically photodissociating the CO-bound fully-reduced enzyme in the presence of N_2-saturated buffer. Spectra b–h were observed for the enzyme photolyzed in oxygen-saturated buffer and probed with delay times, subsequent to the photolysis of bound CO, of 75 μs (b), 120 μs (c), 240 μs (d), 500 μs (e), 1.1 ms (f), 5 ms (g), and 15 ms (h). The wavelength for both photolysis and probe beams was 413.1 nm.

Figure 3: the difference of $B^{2+}O^{3+}$-CN minus $B^{2+}O^{2+}$ from Figure 1). Despite the coincidence of the ν_4 frequency (1374 cm^{-1}) from both hemes in their oxidized state, the frequencies of ν_4 in the reduced states differ by 6 cm^{-1}, presumably owing to the difference of the coordination states (O^{2+}, 5C/HS; B^{2+}, 6C/LS), and thus is able to serve as a probe of each heme. The rapid coordination of oxygen to the reduced ligand-free heme O is evident by the loss of the O^{2+} signal at 1354 cm^{-1} and the concomitant gain of ν_4 at 1374 cm^{-1} seen in the difference spectrum of 75 μs - deoxy (trace c in Figure 3). In contrast, the slight variation in the intensity of ν_4 from 75 to 120 μs originates from the oxidation of heme B as indicated by the trough at 1360 cm^{-1}. After 240 μs, very little oxidation of heme B was seen over the remaining time course of the experiments (up to 15 ms). As shown by trace g in Figure 3, however, there appears a slow ligation process of heme O in the millisecond time range. Quantitatively, this 1 ms process has a magnitude of ~10% of that of the early (<75 μs) process, and thus we consider it to represent only a minor fraction of the enzyme.

To determine when the complete conversion of cytochrome bo_3 to its resting state occurred, we directly mixed the fully-reduced enzyme with oxygen and followed the reaction up to several seconds (Figure 4). Consistent with CO-flash experiments, even at 65 ms the conversion of the hemes to the resting form was incomplete. Furthermore, by examination of ν_4 in the direct mixing experiments, it is

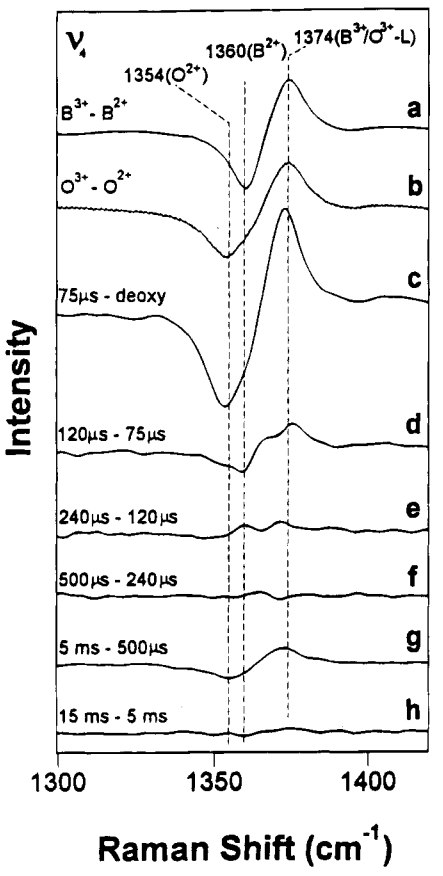

FIGURE 3: The difference spectra, in the oxidation state marker line region (ν_4), for the reaction of fully-reduced cytochrome bo_3 with oxygen. Spectra a and b are the differences between the ferric and ferrous forms of heme B (spectrum a) and heme O (spectrum b), which serve as references for the reaction products in the flow experiments. The former is the difference between the spectra of the CN^- adducts of the fully-oxidized enzyme ($B^{3+}O^{3+}$-CN) and the mixed-valence form ($B^{2+}O^{3+}$-CN); the latter is the result of the subtraction of the spectrum of the fully-reduced enzyme ($B^{2+}O^{2+}$) from that of the CN^--bound mixed-valence species ($B^{2+}O^{3+}$-CN) shown in Figure 1. Traces c–h are the differences (from the CO-flash experiments) between the spectrum detected 75 μs subsequent to the reaction of photogenerated $B^{2+}O^{2+}$ with O_2 and that of the fully-reduced form (c), between 120 and 75 μs (d), between 240 and 120 μs (e); between 500 and 240 μs (f); between 5 ms and 500 μs (g); and between 15 and 5 ms (h).

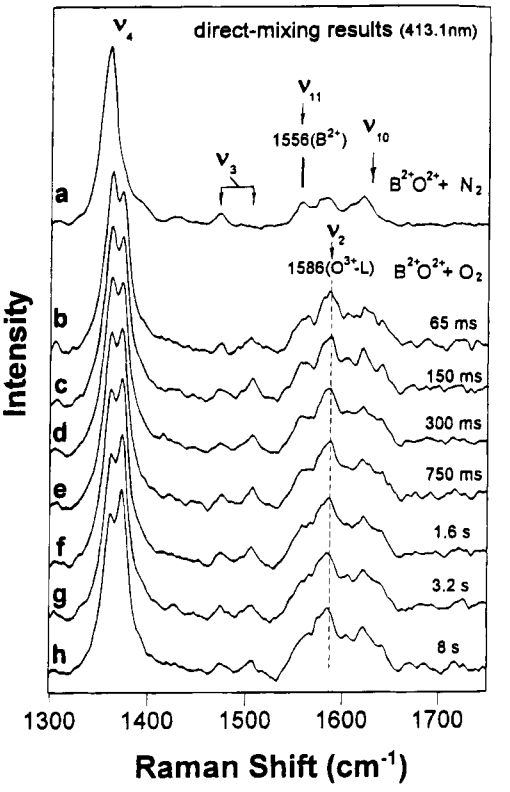

FIGURE 4: Time dependence of the reaction of fully-reduced cytochrome bo_3 with dioxygen by the direct mixing method. Spectrum a is a control of the ligand-free (heme O) fully-reduced form observed when mixed anaerobically with N_2-saturated buffer. Spectra b–h were probed at 65 ms (b), 150 ms (c), 300 ms (d), 750 ms (e), 1.6 s (f), 3.2 s (g), and 8 s (h), subsequent to the mixing of fully-reduced enzyme with O_2-saturated buffer. The laser power (at 413.1 nm) was kept low to prevent both laser-induced photoreduction of the oxy intermediates and possible photoinitiated intramolecular electron transfer.

apparent that the reaction at our first time point (65 ms) is less complete than at 15 ms in the CO-flash experiments. The origin of this result is unclear at present, although notably similar observations were made in mammalian oxidase (Takahashi et al., 1995). Furthermore, whereas in mammalian cytochrome *c* oxidase the Cu_B–CO off-rate is $\sim 10^6$ s^{-1}, in cytochrome bo_3 it is $\sim 10^3$ s^{-1} (Lemon et al., 1993). Thus, the CO-bound Cu_B could influence the catalytic reaction when initiated by the flow–flash–probe protocol. The possibility is currently being investigated. The remainder of the spectra in the 1400–1700 cm^{-1} region shows most of heme B is in its reduced state and that heme O has a 6C/LS iron. By using the line at 1556 cm^{-1} as an indicator of the heme B reduction, we found that significant oxidation took place on a time scale on the order of 1 s but the residual shoulder of ν_{11} at 1556 cm^{-1} (compared to the spectrum of the fully-oxidized enzyme shown in Figure 1a) and the contribution of ν_4 at $\sim$1360 cm^{-1} indicate even at 8 s the oxidation of heme B was not complete. The slight shift to lower frequency of ν_2 (1586 cm^{-1}) at long times is consistent with relaxation of heme O to a ferric high-spin form as reported by others on this time scale (Svensson & Nilsson, 1993; Orii et al., 1994, 1995).

DISCUSSION

The reaction of cytochrome bo_3 with oxygen detected by monitoring the optical absorption spectrum (Svensson & Nilsson, 1993; Orii et al., 1994, 1995) and the resonance Raman spectrum (Hirota et al., 1994) has been reported in the past. The results from these studies are summarized in Table 2. Although the interpretation of the data is different, there are several common features in the observations. In each case a very rapid process and a very slow process are detected ($k_{app} = 2-7 \times 10^4$ and ~ 1 s^{-1}, respectively). An intermediate phase was also detected in some experiments, although the apparent rate constants differed (700 and 5000 s^{-1}). Aspects of the reaction pathway are clearly complicated by differences in preparative procedures. Orii et al. (1995) concluded that the rate constant for the oxidation of heme B could vary by 2 orders of magnitude, depending on whether the enzyme was isolated in the presence or absence of chloride.

In the optical absorption study of the reaction of cytochrome bo_3 with O_2 by Orii et al. (1994, 1995), it was observed that in samples prepared in the absence of chloride, the product at 10 ms had a difference spectrum (10 ms minus photodissociated reactant) with a maximum at $\sim$403 nm, the

Table 2: Reported Apparent Rate Constants for the Reaction of Cytochrome bo_3 with Oxygen[a]

phase	ref *b*	ref *c* (chloride)	ref *d* (sulfate)	ref *e*	this work
I	4.5×10^4 s^{-1} O → peroxide $B^{2+} \rightarrow B^{3+}$	5×10^4 s^{-1} O → pulsed $B^{2+} \rightarrow B^{3+}$	5×10^4 s^{-1} O → pulsed	7×10^4 s^{-1} O → primary 2.3×10^4 s^{-1} O → ferryl	2×10^4 s^{-1} O → ferryl or hydroxy
II	5×10^3 s^{-1} O → ferryl $B^{2+} \rightarrow B^{3+}$?		700 s^{-1} $B^{2+} \rightarrow B^{3+}$		7×10^3 s^{-1} $B^{2+} \rightarrow B^{3+}$ (partial)
III	1 s^{-1} O → resting	0.7 s^{-1} O → resting	0.7 s^{-1} O → resting		1 s^{-1} $B^{2+} \rightarrow B^{3+}$ O → high spin

[a] The assignments made for each of the processes are also listed. [b] Svensson & Nilsson, 1993. [c] Orii et al., 1994. [d] Orii et al., 1995. [e] Hirota et al., 1994.

same as that observed by Svensson and Nilsson (1993) after their second phase was complete ($k = 5 \times 10^3$ s^{-1}). Therefore, in both of these experiments the end product appears the same. They both observe a rapid process ($k \approx 5 \times 10^4$ s^{-1}) and a slower process which Orii et al. (1995) assign as the oxidation of heme B ($k \approx 700$ s^{-1}) and which Svensson and Nilsson (1993) assign as conversion from a peroxy to a ferryl intermediate ($k \approx 5000$ s^{-1}). However, in the presence of chloride described in the experiments of Orii et al. (1995), a different product was generated since the absorption maximum in the difference spectrum appeared at ~410 nm rather than at 403 nm. In the presence of chloride, Orii et al. (1995) interpreted the data as a single kinetic process, but it is noteworthy that their spectral changes in the fast phase do not have an isosbestic point, suggesting that more than one process is occurring.

The resonance Raman spectra we report here allow for identification of the intermediates and determination of the electron transfer processes that form during each phase (Table 2). The first phase, which occurs within 75 μs of the initiation of the reaction, consists of the rapid coordination of O_2 to heme O and reaction of it to a low spin species with the iron in a high oxidation state. The spectra can be assigned as a ferryl−oxo intermediate or a ferric hydroxide intermediate. The resonance Raman results reported by Hirota et al. (1994), who detected the ferrous−oxy and the ferryl−oxo intermediates within a 40 μs time period, are in agreement with this interpretation. The changes reported in the optical absorption spectra by Orii et al., 1994, 1995 were also taken as evidence for conversion of heme O to a high oxidation state (Table 2). On the basis of the Raman results of Hirota et al. (1994), the optical results of Orii et al. (1994, 1995), and the apparent absence of a stable hydroperoxide species in terminal oxidases (Weng & Baker, 1991; Proshlyakov et al., 1994; Cheesman et al., 1994), we interpret our results as further evidence that at a very early time (<75 μs) the O_2 bond has been cleaved and heme O is converted to at least the ferryl species. Additional work is needed to determine if the heme O intermediate is a ferryl−oxo species or a ferric−hydroxy species, both of which have been detected in the reaction of mammalian cytochrome *c* oxidase with oxygen (Han et al., 1990b; Ogura et al., 1993; Varotsis et al., 1993). Some small degree of heme B oxidation also appears to have taken place during this phase, but clearly the bulk of heme B remains reduced.

The rate constant for the second phase is not well defined, and the specific process that is taking place is less clear than it is in the early phase. While Svensson and Nilsson (1993) attributed this phase to the conversion of a peroxy to a ferryl−oxo intermediate, they could not exclude some oxidation of heme B. The data reported by Orii et al. (1995) were interpreted as oxidation of heme B ($k \approx 700$ s^{-1}). Our data show that the major change during this phase is the partial oxidation of heme B. The comparison of our time-resolved spectra to the reference spectra clearly illustrates that in both these early phases of the reaction, species are produced with spectra very similar to spectrum c in Figure 1, namely, a mixed-valence species in which heme B is primarily in its reduced state (i.e., see line at 1556 cm^{-1}) and heme O is primarily in an oxidized low-spin state (i.e., lines at 1506 and 1586 cm^{-1}).

The third phase of the reaction which occurs on a time scale of ≈1 s involves the oxidation of heme B and the conversion of heme O from low to high spin. Others, using optical absorption to monitor the reaction, also concluded that heme O converts from low to high spin on this time scale. The transition in those studies was interpreted as either the pulsed (Orii et al., 1994, 1995) (although a pulsed state has not been defined in cytochrome bo_3) or the ferryl (Svensson & Nilsson, 1993) species converting to a resting form. The oxidation of heme B has been observed by others to take place at a much earlier time than we observe. However, Orii et al. (1994, 1995) found that the rate constant for heme B oxidation was very dependent on the enzyme preparation, varying from 5×10^4 to 700 s^{-1}. In our samples the bulk of the heme B oxidation was even slower, only occurring with a rate constant of ~1 s^{-1} even though chloride was used in our preparation.

The slow oxidation the heme B is a very surprising result since it is clear from our data as well as those of others that the reaction on heme O progresses rapidly to the ferryl intermediate or possibly a hydroxy intermediate in the early stages of the reaction. Cytochrome bo_3 contains only three redox metals (heme B, heme O, and Cu_B). As a result, the cytochrome bo_3-catalyzed reduction of molecular oxygen to water is unable to be completed with the electrons available on the metal centers of the fully-reduced enzyme (only three) and a "ferryl" intermediate has been anticipated to be the final product of the reaction. If, as found here, heme B does not participate in the early phases of the reaction, then only two electrons are available from the metal centers, making the rapid reaction of heme O even more surprising.

The rapid reaction of heme O associated with the slow oxidation of heme B suggests that additional sources of electrons must be available in the cytochrome bo_3. We can rule out quinol as a source of electrons since an assay of the

quinol in our samples shows that very little quinol (less than 10% per molecule) is present in our preparation. A potential source of additional electrons is suggested by considering reports on the properties of the "peroxy" intermediate in mammalian cytochrome *c* oxidase and in cytochrome bo_3. Weng and Baker (1991) and Proshlyakov et al. (1994) presented evidence indicating that when hydrogen peroxide is added to resting mammalian oxidase, instead of forming a ferric−peroxy intermediate, a ferryl−oxo intermediate was formed, implicating donation of an additional electron to the binding site. Both groups proposed that the intermediate is similar to compound I of horse radish peroxidase, a porphyrin π-cation radical, or compound ES of cytochrome *c* peroxidase in which the heme iron is in a ferryl state and an additional oxidizing equivalent is stored in the protein moiety. Similarly, Cheesman et al. (1994) observed a ferryl−oxo intermediate when hydrogen peroxide was added to cytochrome bo_3.

These observations (Weng & Baker, 1991; Proshlyakov et al., 1994; Cheesman et al., 1994) suggest that extra reducing equivalents are available from sources other than the metal centers in terminal oxidases. In the experiments reported here, we propose a similar origin for the extra electrons in cytochrome bo_3, namely, electron donation from amino acid residues. The spectra of heme O, characteristic of a ferric or ferryl species, preclude the formation of electron donation from the porphyrin with the formation of a porphyrin π-cation radical. To date, only tryptophan in compound ES of cytochrome *c* peroxidase (Sivaraja et al., 1989) and tyrosine in ribonucleotide reductase (Sahlin et al., 1982), prostaglandin H synthase (Tsai et al., 1994), and signal II of photosystem II (Debus et al., 1988) have been identified as protein cation radical carriers. In cytochrome bo_3 three tryptophans and one tyrosine are present on helices VI (W280, W282, and Y288) and VII (W331), the helices which contain the three ligands (H284, H333, and H334) shown to coordinate to Cu_B (Thomas et al., 1994) as illustrated in Figure 5. Thus, we postulate that one or more of the Trp residues or the tyrosine play a role in supplying electrons to the binuclear site during the oxygen reduction in our measurements. Since these tryptophans and the tyrosine are in the helices to which Cu_B is coordinated, they have direct access to the binuclear center. Significantly, W280 lies directly over Cu_B, and in a recent crystallographic study Y288 was shown to form a hydrogen bond to the hydroxy group of the hydroxyethylfarnesyl side chain of cytochrome a_3 (Tuskihara et al., 1995). Thus, direct electron transfer to the binuclear center from these residues should be facile, eliminating the need for an electron pathway involving heme B. There are no tryptophans or tyrosines in the helices (II and X) to which heme B is coordinated. W331 is totally conserved in terminal oxidases, and W280 and Y288 are highly conserved in all but a few oxidases so these residues may also serve as electron donors in mammalian cytochrome *c* oxidases.

In summary, the data reported here demonstrate that the reaction of oxygen with fully-reduced cytochrome bo_3 is very different from that of the aa_3-type enzyme from cytochrome *c* oxidase. Although it was originally suspected that the difference between these enzymes would lie in the inability for cytochrome bo_3 to fully reduce the O_2 to H_2O, owing to the lack of a sufficient number of electrons; instead, all of the available electrons on the metal centers do not appear to be used. Differences in the kinetic results obtained in different laboratories may result from preparative differences of the enzyme. In any case the data presented here demonstrate that the enzyme is capable of supplying electrons from donors other than the identified metal centers. Other donors, such as tryptophans or tyrosines on helices VI and VII, are postulated to be supplying electrons to the binuclear center, leaving heme B in its reduced state. The electron donors, whether they are the tryptophans, tyrosine, or some as yet unidentified groups, clearly have a strong influence on the *in vitro* measurements. What role they play, if any, in the catalytic process under physiological conditions remains to be explored.

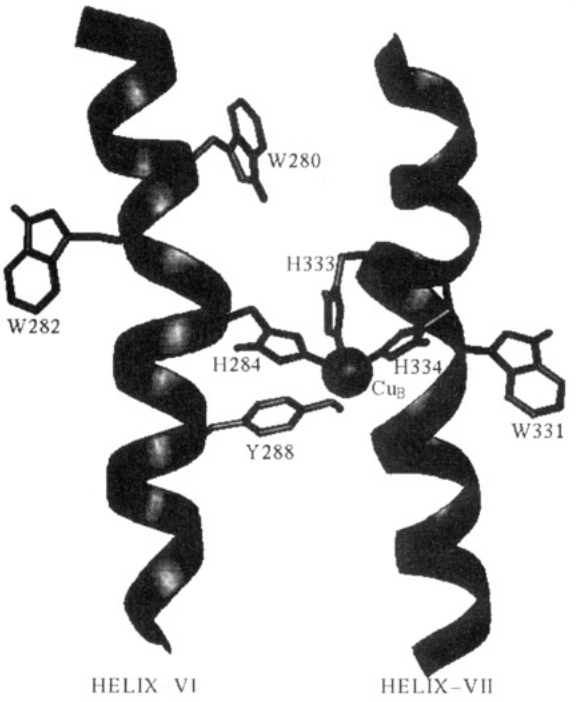

FIGURE 5: Computer-generated model of the coordination site of Cu_B in cytochrome bo_3. Showing the positions of the three tryptophans and the tyrosine that are proposed to be possible electron donors to the binuclear site. The three histidine residues identified as axial ligands for Cu_B are also labeled.

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REFERENCES

Abe, M., Kitagawa, T., & Kyogoku, Y. (1978) *J. Chem. Phys. 69*, 4526−4534.
Anraku, Y., & Gennis, R. B. (1987) *Trends Biochem. Sci. 12*, 262−266.
Argade, P. V., Ching, Y.-c., & Rousseau, D. L. (1986) *Biophys. J. 50*, 613−620.
Chepuri, V., Lemieux, L., Hill, J. J., Alben, J. O., & Gennis, R. B. (1990) *Biochim. Biophys. Acta 1018*, 124−127.
Cheesman, M. R., Watmough, N. J., Gennis, R. B., Greenwood, C., & Thomson, A. J. (1994) *Eur. J. Biochem. 219*, 595−602.
Ching, Y.-c., Argade, P. V., & Rousseau, D. L. (1985) *Biochemistry 24*, 4938−4946.
Dasgupta, S., Rousseau, D. L., Anni, H., & Yonetani, T. (1989) *J. Biol. Chem. 264*, 654−662.
Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988) *Proc. Nat. Acad. Sci. U.S.A. 85*, 427−430.

Gibson, Q. H., & Greenwood, C. (1963) *Biochem. J. 86*, 541−554.
Han, S., Ching, Y.-c., & Rousseau, D. L. (1990a) *J. Am. Chem. Soc. 112*, 9445−9451.
Han, S., Ching, Y.-c., & Rousseau, D. L. (1990b) *Nature (London), 348*, 89−90.
Hill, J. J., Goswitz, V. C., Calhoun, M., Alben, J. O., & Gennis, R. B. (1992) *Biochemistry 31*, 11435−11440.
Hirota, S., Mogi, T., Ogura, T., Hirano, T., Anraku, Y., & Kitagawa, T. (1994) *J. Biol. Chem. 269*, 28908−28912.
Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., Thomas, J. W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M. M. J., Babcock, J. T., & Gennis, R. B. (1993) *J. Bioenerg. Biomembr. 25*, 121−136.
Lemon, D. D., Calhoun, M. W., Gennis, R. B., & Woodruff, W. H. (1993) *Biochemistry 32*, 11953−11956.
Minghetti, K. C., Goswitz, V. C., Gabriel, N. E., Hill, J. J., Barassi, C., Georgiou, C. D., Chan, S. I., & Gennis, R. B. (1992) *Biochemistry 31*, 6917−6924.
Ogura, T., Takahashi, S., Hirota, S., Shinzawa-Itoh, K., Yoshikawa, S., Appelman, E. H., & Kitagawa, T. (1993) *J. Am. Chem. Soc. 115*, 8527−8536.
Orii, Y., Mogi, T., Kawasaki, M., & Anraku, Y. (1994) *FEBS Lett. 352*, 151−154.
Orii, Y., Mogi, T., Sato-Watanabe, M., Hirano, T., & Anraku, Y. (1995) *Biochemistry 34*, 1127−1132.
Proshlyakov, D. A., Ogura, T., Shinzawa-Itoh, K., Yoshikawa, S., Appelman, E. H., & Kitagawa, T. (1994) *J. Biol. Chem. 47*, 29385−29388.
Puustinen, A., Finel, M., Virkki, M., & Wikstrom, M. (1989) *FEBS Lett. 249*, 163−167.
Sahlin, M., Graslund, A., Ehrenberg, A., & Sjoberg, B.-M. (1982) *J. Biol. Chem. 257*, 366−369.
Sato-Watanabe, M., Mogi, T., Ogura, T., Kitagawa, T., Miyoshi, H., Iwamura, H., & Anraku, Y. (1994) *J. Biol. Chem. 269*, 28908−28912.
Sivaraja, M., Goodin, D. B., Smith, M., & Hoffman, B. M. (1989) *Science 245*, 738−740.
Svensson, M., & Nilsson, T. (1993) *Biochemistry 32*, 5442−5447.
Takahashi, S., Ching, Y.-c., Wang, J., & Rousseau, D. L. (1995) *J. Biol. Chem. 270*, 8405−8407.
Thomas, J. W., Calhoun, M. W., Lemieux, L. J., Puustinen, A., Wikstrom, M., & Gennis, R. B. (1994) *Biochemistry 33*, 13013−13021.
Tsai, A.-l., Hsi, L. C., Kulmarcz, R. J., Palmer, G., & Smith, W. L. (1994) *J. Biol. Chem. 269*, 5085−5091.
Tsubaki, M., Mogi, T., Anraku, Y., & Hori, H. (1993) *Biochemistry 32*, 6065−6072.
Tsukihara, T., Aoyame, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., & Yoshikawa, S. (1995) *Science 269*, 1069−1074.
Uno, T., Nishimura, Y., Tsuboi, M., Kita, K., & Anraku, Y. (1985) *J. Biol. Chem. 260*, 6755−6760.
Varotsis, C., Zhang, Y., Appelman, E. H., & Babcock, J. T. (1993) *Proc. Nat. Acad. Sci. U.S.A. 90*, 237−241.
Wang, J., Zhu, H., & Ondrias, M. R. (1992) *Biochemistry 31*, 12847−12854.
Wang, J., Ching, Y.-c., Rousseau, D. L., Hill, J. J., Rumbley, J., & Gennis, R. B. (1993) *J. Am. Chem. Soc. 115*, 3390−3391.
Wang, J., Caughey, W. S., & Rousseau, D. L. (1995) in *Methods in Nitric Oxide Research* (Feelisch, M., & Stamler, J., Eds.) Wiley, New York (in press).
Welter, R., Gu, L.-Q., Yu, L., Yu, C.-A., Rumbley, J., & Gennis, R. B. (1994) *J. Biol. Chem. 269*, 28834−28838.
Weng, L., & Baker, G. M. (1991) *Biochemistry 30*, 5727−5733.

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