Fast Cytochrome *bo* from *Escherichia coli* Binds Two Molecules of Nitric Oxide at Cu_B[†]

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ABSTRACT: The reaction of nitric oxide (NO) with *fast* cytochrome *bo* from *Escherichia coli* has been studied by electronic absorption, MCD, and EPR spectroscopy. Titration of the enzyme with NO showed the formation of two distinct species, consistent with NO binding stoichiometries of 1:1 and 2:1 with observed dissociation constants at pH 7.5 of approximately 2.3×10^{-6} and 3.3×10^{-5} M. Monitoring the titration by EPR spectroscopy revealed that the broad EPR signals at $g \approx 7.3$, 3.7, and 2.8 due to magnetic interaction between high-spin heme *o* ($S = {}^5/_2$) and Cu_B^{II} ($S = {}^1/_2$) are lost. A high-spin heme *o* signal at $g = 6.0$ appears as the 1:1 complex is formed but is lost again on formation of the 2:1 complex, which is EPR silent. The absorption spectrum shows that heme *o* remains in the high-spin Fe^{III} state throughout the titration. These results are consistent with the binding of up to two NO molecules at Cu_B^{II}. This has been confirmed by studies with the Cl^- adduct of *fast* cytochrome *bo*. MCD evidence shows that heme *o* remains ligated by histidine and water. Addition of excess NO to the Cl^- adduct leads to the appearance of a high-spin Fe^{III} heme EPR signal. Hence chloride ion binds to Cu_B, blocking the binding of a second NO molecule. These results suggest a mechanism for the reduction of NO to nitrous oxide by cytochrome *bo* and cytochrome *c* oxidase in which the binding of two *cis* NO molecules at Cu_B permits the formation of an N−N bond and the abstraction of oxygen by the heme group.

The respiratory chain of *Escherichia coli* grown aerobically is terminated by cytochrome *bo* ubiquinol oxidase (*1*). This enzyme is a member of the superfamily of protonmotive heme−copper terminal oxidases that includes mitochondrial cytochrome *c* oxidase (CCO)[1] (*2*). CCO contains a novel dinuclear copper center, called Cu_A, which accepts electrons from cytochrome *c*, whereas cytochrome *bo* is devoid of this center since quinol is the electron donor. These oxidases couple the four-electron reduction of dioxygen to water to vectorial proton translocation across the membranes in which they are situated. Cytochrome *bo* is composed of four subunits of which subunit I contains all the redox active metal centers, a low-spin heme *b*, a high-spin heme *o*, and a copper atom (Cu_B). Structural studies of the bovine mitochondrial CCO (*3*, *4*) and *Paracoccus denitrificans* CCO (*5*) revealed that the current structural models of the enzyme derived from earlier mutagenesis (*6*) and spectroscopic (*7*) studies were largely correct. These studies have demonstrated that heme *b* is coordinated by two histidine residues forming a magnetically isolated low-spin ferric species and that heme *o* is coordinated by a single histidine to form a high-spin ferric species. Cu_B is ligated by three histidine residues and together with heme *o* forms a binuclear site for dioxygen reduction.

The purification and spectroscopic characterization of wild-type *E. coli* cytochrome *bo* has been described in detail (*7*−*11*). The oxidized enzyme can exist in at least two forms which differ in their reactivity toward added ligands. One of these species, termed *fast* (*12*), can be defined by characteristic electronic absorption bands at 406.5 nm (Soret) and 624 nm (high-spin heme *o* charge transfer band) and functionally by the binding of 10 mM cyanide with an observed rate constant of 0.27 s^{-1}. In addition, the EPR spectrum shows unusual broad signals with prominent features at $g = 7.3$, 3.7, and 2.8 arising from the binuclear heme *o*-Cu_B center as a result of magnetic coupling between high-spin heme *o* Fe^{III}, $S = {}^5/_2$, and Cu_B^{II}, $S = {}^1/_2$ (*9*). In the *fast* form, heme *o* appears to be liganded by histidine and water which may itself be hydrogen bonded to a neighboring residue such as histidine (*8*).

NO is a useful spin probe for studying heme−copper oxidases as its binding characteristics somewhat resemble those of dioxygen, but also because it carries one additional unpaired electron it can transform an even-electron spin binuclear center into an odd-electron spin site. The EPR characteristics of which will be different. In the reduced state cytochrome *o* reacts with one molecule of NO to form a heme(II) nitrosyl which yields well-resolved EPR signals showing nuclear hyperfine coupling from the ^{14}N atom both of NO itself and of the proximal histidine ligand to heme *o* (*7*). It was shown several years ago that the oxidized state of bovine mitochondrial CCO will react with NO (*13*−*15*). The addition of NO to oxidized CCO in some instances results in the appearance of a high-spin heme EPR signal due to cytochrome a_3. It was proposed that NO coordinates

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[1] Abbreviations: CCO, cytochrome *c* oxidase; EPR, electron paramagnetic resonance; K_d, dissociation constant; MCD, magnetic circular dichroism; NO, nitric oxide; NOR, nitric oxide reductase; CT, charge transfer; OGP, octyl β-D-glucopyranoside.

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to Cu_B^{II}, thus breaking the magnetic coupling by forming a cytochrome $a_3^{III}-Cu_B^{II}-NO$ complex (*13*). The odd electron of NO couples with the single unpaired electron at Cu_B^{II} rendering this moiety diamagnetic ($S = 0$), and hence only the high-spin a_3^{III} heme becomes EPR detectable. However, the authors noted that the intensity of the high-spin cytochrome a_3^{III} signal depended on the method of preparation of the enzyme. For example, enzyme prepared by the Hartzell and Beinert method (*16*) gave rise to a high-spin heme a_3^{III} EPR signal, whereas little or no high-spin EPR signals were observed with CCO prepared by the method of Yu *et al*. (*17*). Recently, Moody (*12*) reported that the Hartzell and Beinert method for the preparation of bovine oxidase had significant levels of chloride present at one or more stages. Since chloride ions react with oxidized bovine CCO to produce a form of the enzyme which has different ligand-binding properties (*18*, *19*), this could explain the heterogeneity observed by Stevens *et al*. (*13*).

There is renewed interest in the reaction of NO with the heme−copper binuclear center of oxidases for several reasons. First, the discovery of NO as an important signalling molecule in the vascular system of higher organisms (for review see 20) has led to the search for examples of competitive reactions between O_2 and NO with CCO (*21*). Secondly, the realization that the bacterial enzyme, nitric oxide reductase (NOR), has a high degree of homology with CCO (*22*−*24*) has led to the proposal of a model for the active site of NOR which consists of a binuclear high-spin heme group with a non-heme iron atom liganded by three histidines. In other words, an enzyme similar to CCO and cytochrome *bo* but with Cu_B replaced by non-heme iron. The fact that the heme−Cu_B binuclear site in CCO will reduce NO to yield nitrous oxide, N_2O (*14*, *25*), mimicking the activity of NOR, suggests that mechanistic comparisons might be made. For these reasons and in order to probe further the active site of cytochrome *bo* we have undertaken a study of the reaction of *fast* cytochrome *bo* with NO.

In the present paper, the reaction of *fast* cytochrome *bo* with NO in both the presence and absence of chloride has been studied. Results are presented which indicate that when NO reacts with *fast* cytochrome *bo* two molecules of NO can bind to Cu_B generating an EPR silent binuclear center. The presence of chloride ion in the binuclear site appears to block one NO binding site, releasing a high-spin heme *o* EPR signal upon NO binding.

EXPERIMENTAL PROCEDURES

Growth Conditions. *E. coli* strain RG145 was the source of wild-type cytochrome *bo*. This strain lacks cytochrome *bd* and has been demonstrated previously to overexpress cytochrome *bo* approximately 5-fold (*26*). *E. coli* RG145 was grown in batch culture on inner membrane (IM) media, pH 7.0, at 37 °C for 12 h using a 10 L New Brunswick Bioflo IV fermenter. Cultures were agitated (50−600 rpm) to maintain dissolved oxygen at 70%. Cells were harvested as described previously (*7*).

Purification of Cytochrome bo. Cells were disrupted, membranes were washed, and cytochrome *bo* was purified as detailed by Cheesman *et al*. (*7*) with the additional manipulations described by Watmough *et al.* (*10*). Purified enzyme was stored in 1 mL aliquots at −70 °C prior to use. Estimates of protein concentration were determined optically using $\epsilon_{soret} = 183\ mM^{-1}\ cm^{-1}$ (*7*). To ensure that the purified cytochrome *bo* was *fast* (see 12), all preparations were "pulsed" according to the method of Moody and Rich (*27*). The procedure was amended by increasing each dialysis step to 2 h.

Addition of Chloride. KCl (Sigma) was added to *fast* cytochrome *bo* (62 μM) at a final concentration of 0.5 M by dialysis overnight against 50 mM hepes and 0.2% (w/v) octyl β-D-glucopyranoside (OGP), pH 7.5, containing 0.5 M KCl. The sample was concentrated to approximately 250 μM for spectroscopic studies.

Addition of NO. Gaseous NO (Aldrich) was added to both *fast* and chloride-ligated cytochrome *bo* under a pressure of 1 atm at 20 °C for 20 min to obtain NO-saturated samples. Under these conditions final NO concentration was taken to be approximately 2 mM (*28*). NO-saturated samples were generated in an anaerobic glovebox where typical oxygen levels were <1 ppm in a nitrogen atmosphere, and cytochrome *bo* samples were stirred in the glovebox for a minimum of 30 min in order to remove any traces of oxygen before exposure to NO. Following the addition of NO, samples were transferred into EPR tubes, sealed and frozen immediately in liquid nitrogen or transferred into sealed 1 mm path length cuvettes for electronic absorption studies.

For static NO titrations, 50 mM Hepes buffer pH 7.5 at 0 °C was first flushed with oxygen-free nitrogen for 20 min and then sparged with NO gas for a further 20 min. NO saturated buffer at 0 °C was assumed to be at a final concentration of 3.17 mM (*28*). For titration monitored by electronic absorption spectroscopy, small increments of NO saturated buffer were added to oxygen-free *fast* cytochrome *bo* in a sealed 1 cm path length cuvette using a gas-tight syringe. At 5 min intervals following addition of NO a difference spectrum (NO-bound cytochrome *bo* minus *fast* cytochrome *bo*) was recorded. After 15 min no further optical changes were detected. For titrations monitored by EPR spectroscopy, NO-saturated buffer was added to four separate samples of oxygen-free *fast* cytochrome *bo* in an anaerobic glovebox to give [NO]/[enzyme] ratios of 0.66, 1.34, 2.19, and 7.75. Each sample was incubated for 20 min and then transferred into EPR tubes, sealed, and frozen immediately in liquid nitrogen

Spectroscopy. Electronic absorption spectra were recorded using a Hitachi UV3000 or Aminco DW2000 spectrophotometer. EPR spectra were recorded on an X-band ER-200D spectrometer (Bruker Spectrospin) interfaced to an ESP1600 computer and fitted with a liquid helium flow cryostat (ESR-9; Oxford Instruments). MCD spectra were recorded on a circular dichrograph, JASCO J-500D using an Oxford Instruments superconducting solenoid with a 25 mm room temperature bore generating magnetic fields up to 6 T.

Sample Preparation for MCD Spectroscopy. Cytochrome *bo* samples were in D_2O buffers containing 50 mM Hepes, 0.2% (w/v) OGP and 0.5 M KCl at pH* 7.5. pH* is the apparent pH of these buffers in D_2O measured with a standard glass pH electrode. Cytochrome *bo* concentration is given in appropriate figure legends

Treatment of Binding Data. The fractional saturation (Y) of cytochrome *bo* by NO is calculated from the relationship

$$Y = \frac{\Delta A}{\Delta A_{max}}$$

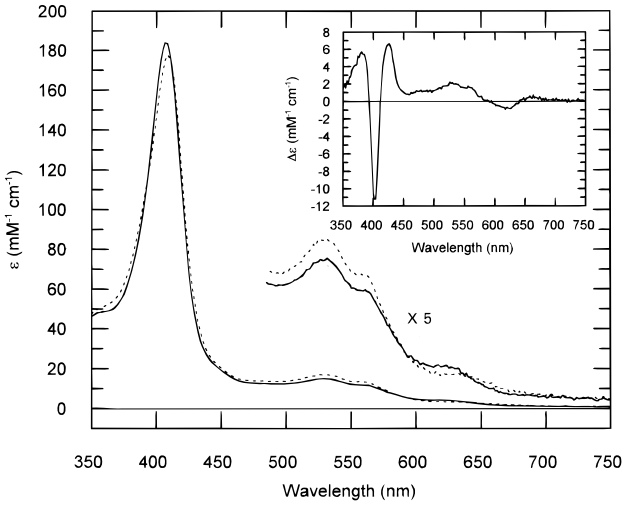

FIGURE 1: Room temperature electronic absorption spectra of *fast* cytochrome *bo* and its NO derivative. Sample concentration was 250 μM. *Fast* cytochrome *bo* (—) and cytochrome *bo* after reaction with excess NO (~2 mM) for 20 min (- - -). The inset shows the difference spectrum of *fast* cytochrome *bo* plus NO minus *fast* cytochrome *bo*.

where ΔA is the observed absorbance change at a specified wavelength in response to the addition of a known concentration of ligand and ΔA_{max} is the absorbance change observed upon saturation of the protein by that ligand. The dissociation constant K_d is defined by

$$K_d = \frac{[S](1 - Y)([L] - Y[S])}{Y[S]}$$

where [L] is the concentration of ligand added and [S] is the concentration of binding sites. Expansion of this expression gives

$$Y = \frac{[S] + [L] + K_d - \sqrt{([S] + [L] + K_d)^2 - 4[S][L]}}{[2S]}$$

The NO titration was measured at wavelengths 408 and 620 nm. At 408 nm two distinct phases could be identified. The data at 630 nm and the second binding phase at 408 nm were fitted independently. For both sets of data, the value of [S] was constrained to reflect a stoichiometry of NO binding of 1:1 and the K_d for each process was calculated using a nonlinear regression analysis done on a personal computer using Grafit v3.0 (Erithacus Software).

RESULTS

Reaction of Fast Cytochrome bo with Nitric Oxide

The changes in the electronic absorption spectrum of the *fast* cytochrome *bo* upon saturation with NO are slight (Figure 1). The difference spectrum, NO-bound cytochrome *bo* minus *fast* cytochrome *bo* (Figure 1 inset), shows a red shift in the Soret maximum from 406.5 to 408 nm and in the visible region of the spectrum there is a small increase of intensity at 530 nm and 560 nm. The shoulder at 624 nm arising from the high-spin ferric heme *o* charge transfer band is red-shifted to 634 nm. These small shifts and the retention of the charge transfer band show that heme *b* and heme *o* remain low-spin ferric and high-spin ferric, respectively.

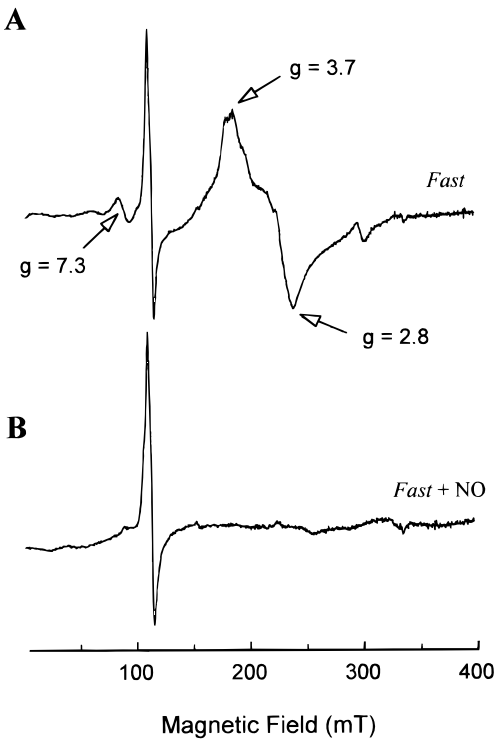

FIGURE 2: X-band EPR spectra of the *fast* and NO-bound forms of cytochrome *bo*. (A) *Fast* cytochrome *bo* and (B) NO bound-cytochrome *bo*. Sample concentration was 250 μM. NO was added to a final concentration of approximately 2 mM. Conditions of measurement were as follows: temperature, 5 K; microwave power, 103 mW; modulation amplitude, 1 mT.

The X-band EPR spectrum of *fast* cytochrome *bo*, when recorded at low temperatures, $\leq$5 K, and high powers, $\geq$100 mW, is dominated by the broad EPR features at $g = 7.3$, 3.7, and 2.8, characteristic of the heme $o-Cu_B$ pair (Figure 2A). Under these conditions the low-spin heme signals from low-spin heme *b* at $g = 2.98$, 2.26, and 1.50 are broadened and no longer detectable. The signal at $g = 6$ is probably from the high-spin heme *o* and may represent a small percentage of enzyme, either damaged or lacking Cu_B, in which the coupling at the binuclear center is broken (*7*). Figure 2B shows the spectrum recorded under the same conditions following the addition of excess NO by exposing the enzyme solution to an atmosphere of NO gas for 20 min with stirring. This clearly shows the abolition of the broad features at $g = 7.3$, 3.7, and 2.8 without the concomitant increase in the high-spin $g = 6$ signal. No EPR signals arising from Cu_B^{II} were observed. Hence the binuclear site has been rendered EPR silent by the addition of NO although heme *o* has remained high-spin Fe^{III}. This is a surprising result. If one NO molecule were to bind to Cu_B^{II}, as proposed by Chan and co-workers (*13*), high-spin heme *o* should become detectable at $g = 6.0$. Thus we suggest the possibility that more than one NO molecule might become bound to the active site.

Stoichiometry of NO Binding to Fast Cytochrome bo

Titration of *fast* cytochrome *bo* with small aliquots of NO was monitored by both electronic absorption and EPR spectroscopy. Figure 3A displays the spectral changes induced by the addition of NO in the concentration range 0–9 μM. Over this range, the NO-bound cytochrome *bo* minus *fast* cytochrome *bo* difference spectrum shows an

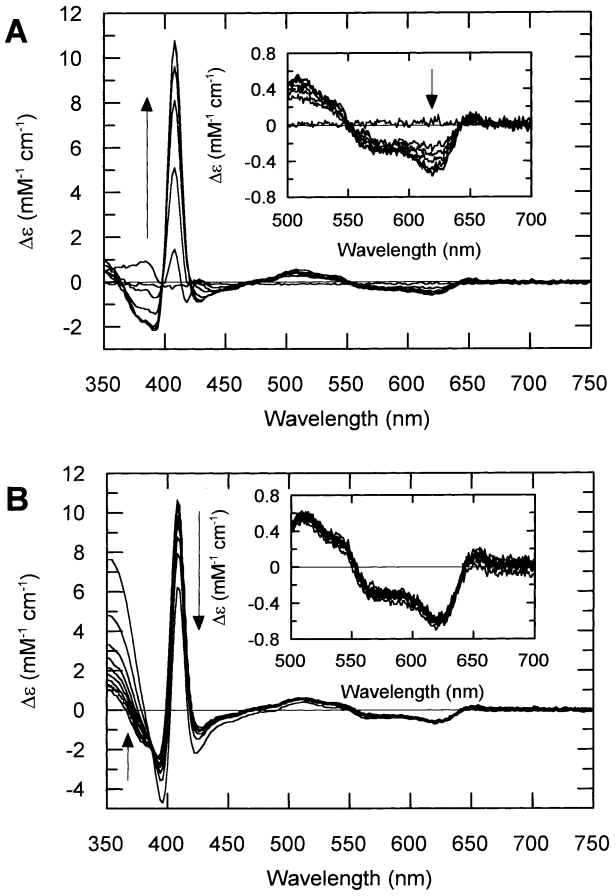

FIGURE 3: Changes in the electronic absorption spectrum of *fast* cytochrome *bo* upon titration with NO. In an anaerobic glovebox enzyme concentration was made 6.9 μM in a final volume of 10 mL in 50 mM Hepes, 0.1 mM EDTA, 0.2% (w/v) OGP, pH 7.5. Two anaerobic cuvettes, each containing 4.4 mL of enzyme solution, were used to acquire a base line. NO-saturated buffer was added incrementally to the stirred sample cuvette. After 20 min of incubation, a new difference spectrum was acquired. Longer incubation times failed to induce further optical changes. (A) Change in the spectrum upon the addition of NO in the range 0−9 μM. (B) Change in the spectrum upon the addition of NO in the range 10−70 μM. Arrows indicate the direction of absorbance changes.

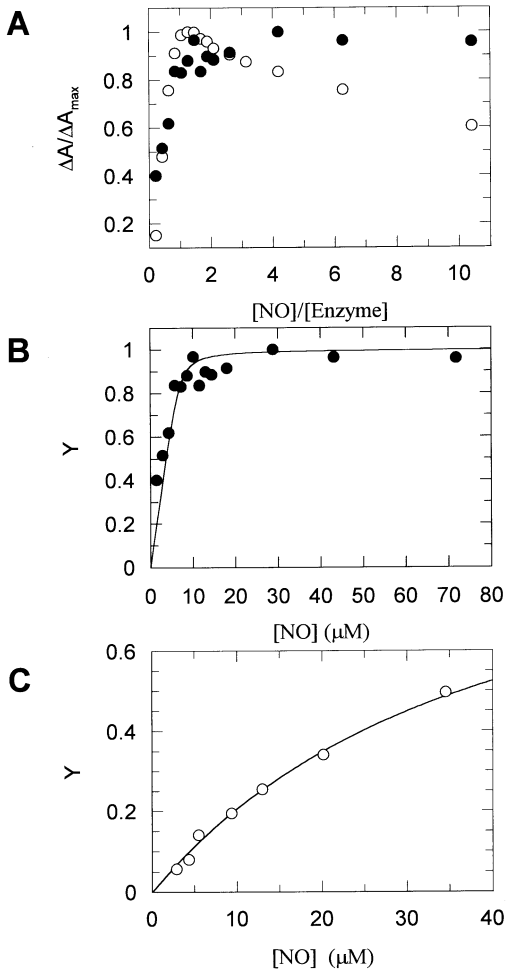

FIGURE 4: Titration of *fast* cytochrome *bo* with NO. Using the experimental data from figure 3 the change in absorption, $\Delta\epsilon_{408-400}/\Delta\epsilon_{408-400max}$ (○) and $\Delta\epsilon_{550-620}/\Delta\epsilon_{550-620max}$ (●) was plotted as a function of [NO]/[enzyme] (A). (B, C) Fractional saturation (Y) plotted as a function of the total NO concentration for the reaction at 620 nm and the second reaction at 408 nm, respectively. The solid line is the best fit as described under Experimental Procedures. The K_d values reported in the text are the means of two independent determinations from two different batches of enzyme.

increase in absorbance at 408 nm with a concomitant decrease in absorbance at 620 nm (Figure 3A inset). Figure 3B shows the spectral changes induced by the addition of NO in the concentration range 10−70 μM. Over this concentration range, the difference spectrum shows a decrease in absorbance at 408 nm, although, no further spectral changes occur at 620 nm (Figure 3B inset). Figure 4 shows the fractional saturation (Y) of cytochrome *bo* by NO ($Y = \Delta A/\Delta A_{max}$) monitored at both 620 and 408 nm. Panels B and C show the lines of best fit for the reaction at 620 nm (B) and the second reaction at 408 nm (C) as described under Experimental Procedures. These data are consistent with two binding sites for NO with observed dissociation constants at pH 7.5 of $K_d = 2.3 \pm 0.13$ and 33.06 ± 1.44 μM.

To confirm the binding of two NO molecules at the binuclear center, the NO titration was monitored by EPR spectroscopy. Figure 5 shows a series of EPR spectra of cytochrome *bo* with increasing ratios of NO to protein. The spectra are measured at 10K and 2mW microwave power in order to reveal the low-spin Fe^{III} heme *b* which can be taken as an internal standard of the protein concentration. The absorption spectra, Figure 3, show that under the conditions used no reduction of either heme group can be detected. Hence the family of spectra given in Figure 5 have been plotted to normalize the intensity of the signal at $g = 2.98$, the g_z component of the low-spin heme *b*. It can be seen that the broad signal at $g \sim 3.7$, characteristic of the coupled binuclear site, decreases when 0.66 NO to heme *o* is added and that the signal at $g = 6.0$ has increased. The intensity of the $g = 6.0$ signal was determined using computer simulation (V. S. Oganesyan, unpublished work). Assuming that the signals at $g = 2.98$, 2.26, and 1.50 account for 100% low-spin heme *b* and that the high-spin heme Fe^{III} has a D value of 6 cm^{-1}, the increased signal at $g = 6.0$ represents ~30% of total high-spin heme. The calculated occupancy of the Cu_B mononitrosyl at this NO concentration, using the K_d estimates determined from the optical titration, is 53%. This discrepancy undoubtably reflects the difficulty in obtaining an accurate K_d estimate for the low-affinity site and uncertainties in obtaining accurate spin intergrations of high-spin heme. Further additions of NO to give [NO]/[protein] ratios of 1.34, 2.19, and 7.75 cause the $g = 6.0$ signal to drop. Hence the addition of one NO to the binuclear center of *fast* cytochrome *bo* with a K_d of 2.3 μM does indeed cause the $Fe^{III}-Cu_B^{II}$ pair to become uncoupled, and the high-

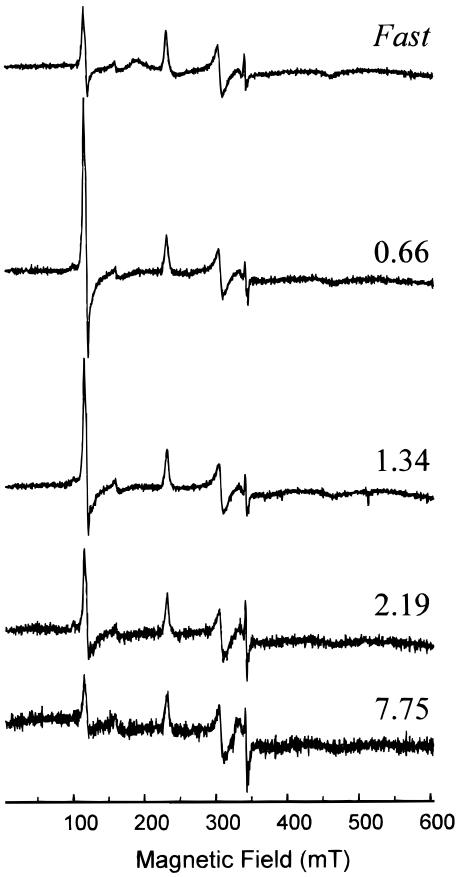

FIGURE 5: Titration of *fast* cytochrome *bo* with NO monitored by EPR spectroscopy. The inital sample concentration was 62 μM. The spectra were corrected for dilution by being normalized to the $g = 2.98$ signal from the low-spin heme of the starting enzyme. For each spectrum the [NO]/[enzyme] is given. Conditions of measurement: temperature, 10 K; microwave power, 2.03 mW; modulation amplitude, 1 mT.

spin heme *o* is observed in the EPR spectrum at $g = 6.0$. However, further addition of NO causes loss of this $g = 6.0$ signal, even though heme *o* remains high-spin Fe^{III} as judged by the absorption spectrum. Addition of a second NO molecule would supply a second unpaired electron yielding a set of spins, $S = {}^5/_2$ (Fe^{III}), $S = {}^1/_2$ (Cu_B^{II}), and two $S = {}^1/_2$ (NO), which is overall an even number of spins resulting in an EPR silent speices. This evidence strongly attests to the sequential formation of Cu_B^{II} mono- and dinitrosyls. Note that binding of NO to the Fe^{III} heme is expected to drive the heme group low-spin (*29*, *30*).

Binding of Chloride to Fast Cytochrome bo

The changes in the electronic absorption spectrum of *fast* cytochrome *bo* upon the addition of chloride are consistent with those reported by others (*31*). In the visible region, the 624 nm charge transfer band is red-shifted to 631 nm; however, its presence indicates that chloride forms a high-spin complex (data not shown). The MCD spectrum can give useful information as to the nature of the ligands bound to heme *o*. The UV−visible region spectrum for the chloride-bound cytochrome *bo* is shown in Figure 6. The Soret peak to trough intensity and the features in 400−600 nm region are consistent with only one low-spin ferric heme (*8*). However, the negative band characteristic of high-spin ferric heme *o* has moved from 635 nm (*8*) to 647 nm. This band is sensitive to changes in the ligand distal to the

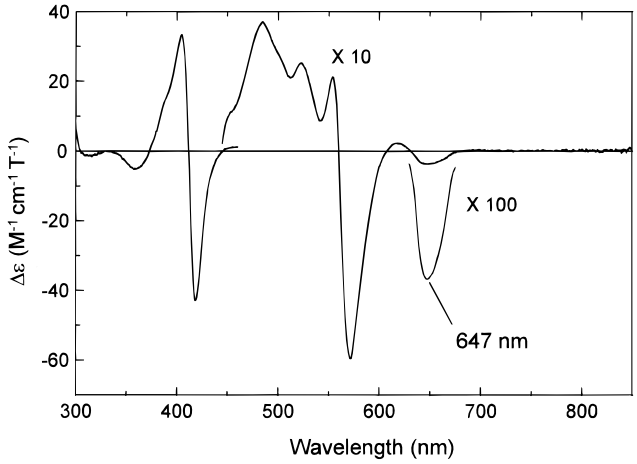

FIGURE 6: UV−visible room temperature MCD spectrum of the chloride-bound form of cytochrome *bo*. Two samples of concentration ~40 and ~400 μM were required to measure to the Soret region and the visible region, respectively.

histidine for a number of high-spin ferric hemes. A band observed in the region 635−647 nm suggests one of two possible forms of ligation, either histidine/water (*32*) or histidine/carboxylate (*33*). As no exogenous carboxylate has been added, it would seem that heme *o* remains coordinated by histidine and water.

The EPR spectra of the chloride-ligated form of the enzyme are shown in Figure 7. Signals typical of the low and high-spin hemes are observed (Figure 7A). Interestingly, the axial ($g = 6$) high-spin heme *o* signal has changed to a rhombic signal with peaks at $g = 6.27$ and $g = 6.00$. This effect may be unique to chloride as the uncoupled high-spin signal has been reported previously to be unaltered by ligand additions (*7*). Using lower sample temperatures and higher microwave powers, the broad EPR signals (Figure 7B) have been significantly altered and shifted to $g = 8.63$ with a sharp peak at $g = 3.23$. However, the general pattern is maintained of one broad derivative feature below 100 mT and a second feature in the 150−250 mT region as observed for cytochrome *bo* ligated with fluoride, formate (*9*), and azide (*11*).

Reaction of Chloride-Ligated Cytochrome bo with Nitric Oxide

Figure 7C shows the low-power (2.03 mW) EPR spectrum for chloride-ligated enzyme following exposure to an atmosphere of NO gas for 20 min giving a [NO]/[protein] ratio of approximately 8. Comparison with Figure 7A shows that the high-spin heme *o* signal ($g = 6.27$ and $g = 6.00$) has increased in intensity relative to the low-spin signal, with a concomitant decrease in the broad signals. The ratio of the intensity of the $g \sim 6.0$ signal to the signal at $g = 2.89$ is difficult to determine accurately because the signals from the binuclear center overlap with the $g = 2.89$ signal. However it is clear that there is an increase in the $g \sim 6.0$ signal by a factor of ~7. From the high-power (103 mW) spectrum (Figure 7D), the feature at $g = 3.23$ indicates that some of the high-spin heme *o* remains coupled to Cu_B. Again, no EPR signals arising from Cu_B^{II} were observed. The effect of the halides bromide and fluoride on the reaction of cytochrome *bo* with NO was also examined. Both were found to generate an axial high-spin heme *o* EPR signal of significantly increased intensity upon NO binding (data not shown). Hence we conclude that the binding of one Cl^-

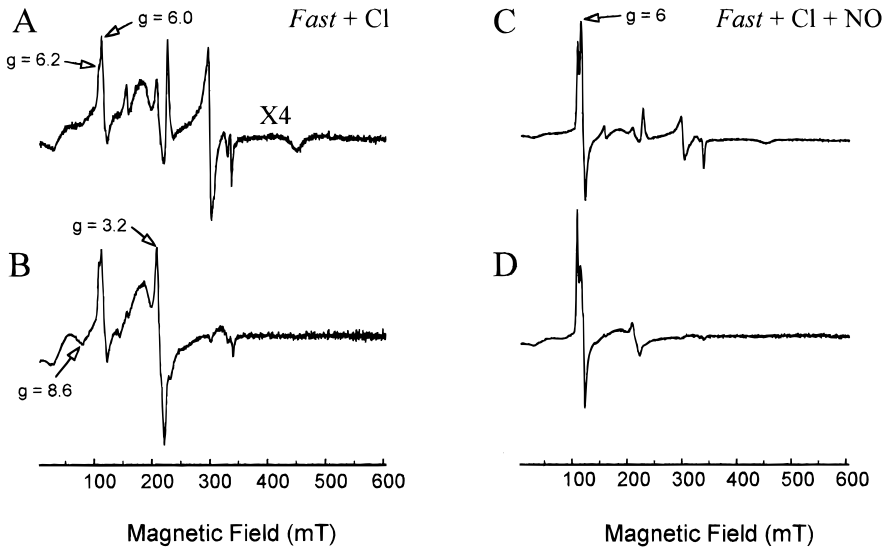

FIGURE 7: X-band EPR spectra of chloride-ligated cytochrome *bo* and its NO derivative. Chloride-bound cytochrome *bo* was NO-saturated (~2 mM) by exposure to NO gas for 20 min. Sample concentration was 250 μM. Conditions of measurement were as follows: (A, C) temperature, 10 K; microwave power, 2.03 mW; modulation amplitude, 1 mT; (B, D) temperature, 5 K; microwave power, 103 mW; modulation amplitude, 1 mT.

ion at the binuclear site does not completely inhibit NO binding, but that only one NO molecule will bind at least up to ratios of [NO]/[protein] of approximately 8. The consequence is that a mononitrosyl of Cu_B^{II} is formed and the uncoupled heme *o* high-spin Fe^{III} EPR signal is seen.

DISCUSSION

In the present study, the reaction of *fast* cytochrome *bo* with NO has been investigated. The results demonstrate that NO binds to *fast* cytochrome *bo* with an overall stoichiometry of 2:1. The observed dissociation constants for NO binding to cytochrome *bo* suggest a high-affinity site ($K_d \sim 2.3$ μM) and a low-affinity site ($K_d \sim 33$ μM). Using EPR spectroscopy, we have demonstrated that the high-affinity phase corresponds to the binding of one NO to Cu_B, breaking the coupling between the components of the binuclear center to generate a high-spin heme *o* EPR signal. The low-affinity phase corresponds to the binding of a second NO molecule which results in an EPR silent species. No redox changes have occurred at either heme *o* or heme *b* under the conditions of these experiments, hence three possible modes can be considered for the coordination of two NO molecules to the binuclear center: (i) NO binds both at Cu_B^{II} and at heme o^{III}, (ii) both NO molecules coordinate to Cu_B^{II}, or (iii) one NO molecule binds to Cu_B^{II} and the other forms a bridging ligand between Cu_B^{II} and heme o^{III}.

The dissociation constants for the binding of NO to the ferrihemoproteins metmyoglobin and oxidized cytochrome *c* are 71 and 62 μM, respectively (*34*). These K_d values are double that of the low-affinity phase (~33 μM) observed with cytochrome *bo*. Furthermore, NO acts as a strong field ligand, and so its interaction with ferric hemes yields products that have been established by EPR experiments to be low spin ($S = 0$) (*29*, *30*). The optical spectra of the Fe^{III} heme nitrosyls are also characteristic of low-spin heme and completely different from the spectra of high-spin Fe^{III} hemes (*34*). Consequently, the direct binding of NO to heme *o*, via the nitrogen to form $Fe^{III}-NO$, is considered unlikely as the retention of the ~634 nm charge transfer band demonstrates heme *o* remains high-spin.

To determine the nature of the low-affinity NO binding site, chloride-bound cytochrome *bo* was examined. The changes in the electronic absorption spectrum due to addition of chloride ion are very similar to those induced by azide binding to Cu_B, resulting in a red shift in the CT band (*11*). This is in contrast to that induced by fluoride ion which shows a blue shift in the CT band (*9*). Furthermore, the MCD spectrum of chloride-bound cytochrome *bo*, specifically the 647 nm trough, suggests that the immediate ligands of heme *o* have not changed. Again, this is in contrast to the fluoride adduct that shows a band at 625 nm, which has been interpreted as fluoride coordinating directly to heme *o* (*8*). These data provide evidence that chloride ion is a Cu_B ligand as suggested previously by Moody *et al*. (*18*) for bovine heart CCO. Because chloride-ligated cytochrome *bo* shows a strong high-spin heme EPR signal upon binding excess NO, only one NO binding site is available apparently in the chloride-bound form. Therefore we propose that chloride ion can block one of the two NO binding sites. This interpretation is consistent with the observations made by Stevens *et al*. (*13*) and would explain why the high-spin heme EPR signal was observed only with CCO prepared by the Hartzell and Beinert method (*16*).

Stevens *et al*. (*13*) also observed the high-spin heme a_3-(III) EPR signal upon the addition of fluoride to preparations of CCO isolated by the method of Yu *et al*. (*17*), that initially appeared unreactive toward NO. A similar effect was also seen with the fluoride derivative of cytochrome *bo* (data not shown). As discused above, fluoride has been demonstrated to be a heme *o* ligand (*8*). As fluoride also appears to block NO binding, we suggest that one of the NO molecules may act as a bridging ligand between heme *o* and Cu_B (Figure 8). The retention of a high-spin heme *o* complex may result from a weakening of the NO ligand strength by the oxygen moiety bridging to Fe^{III} to form the $Fe^{III}\cdots O-N-Cu_B^{II}-NO$ species.

The combined data from the studies of NO binding to the binuclear heme$-Cu_B$ site of cytochrome *bo* shows that two NO molecules can bind to this center one with an affinity about ~14 times greater than the other. The evidence is

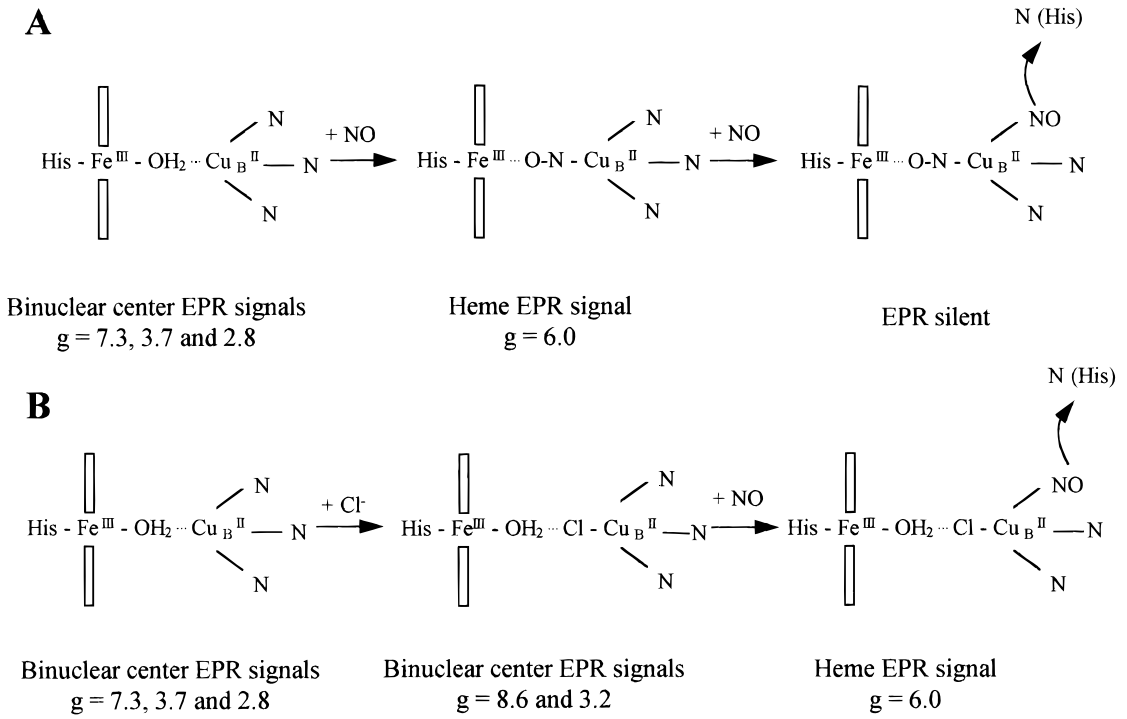

FIGURE 8: Schematic diagram showing the possible reactions of *fast* cytochrome *bo* with chloride and NO: (A) *fast* cytochrome *bo* plus NO and (B) *fast* cytochrome *bo* plus chloride and NO.

unambiguous that NO never binds to the Fe^{III} heme group to form a low-spin $Fe^{III}-NO$ species. Cu_B^{II} is the site of binding and an interesting copper(II) dinitrosyl $Cu_B^{II}(NO)_2$ is, by inference, generated. The formation of the dinitrosyl is, however, prevented if the binuclear center is pretreated with halide ion ligands. The evidence shows that F^- binds to Fe^{III} heme in preference to Cu_B^{II}, whereas Cl^-, Br^-, and I^- bind to Cu_B^{II} in preference to heme Fe^{III}. This is a manifestation of the well-known order of binding affinities of halide ions for class A (or hard) metals such as Fe^{III} which is $F^- > Cl^- > Br^- > I^-$ compared with that for class B (or soft) metals such as Cu^{II} which is $I^- > Br^- > Cl^- > F^-$ (*35*). When a halide ion is offered the choice between Fe^{III} and Cu^{II}, it seems that Fe^{III} binds F^- but Cu^{II} binds the others. Binding of more than one halide ion to the binuclear center has not been detected.

Recent studies by Wilson and co-workers (*39*) of the reaction of oxidized *fast* CCO with NO have shown partial reduction of the low-spin heme *a*, leading to the suggestion that NO reacts with Cu_B^{II} forming a nitrosonium complex ($Cu_B^{I}NO^+$) which on hydration yields HNO_2 and a proton. This leaves a single electron at the binuclear center which can be transferred to low-spin heme *a* as the proton is lost from the hydrophobic binuclear site. We consider such a mechanism unlikely for the reaction of cytochrome *bo* with NO, since we see no evidence of reduction of either the high or low-spin hemes. Furthermore the spectral changes shown in Figure 1 cannot be induced by the addition of nitrite to *fast* cytochrome *bo* (data not shown).

The evidence that Cu_B^{II} can bind up to two ligands, either two NO molecules or one halide ion plus one NO molecule, is consistent with studies of the binuclear site in CCO. For example, CN^- ion binds to the binuclear site in a bridging mode between Fe^{III} and Cu_B^{II} (*36*). Although both Fe^{III} and Cu_B^{II} sites are occupied in the cyanide form of CCO, this species can nevertheless bind one NO molecule (*37*). The NO molecule can be photolyzed from this species at low temperatures (4.2 K) and thermally recombined by warming to 100 K. Hence no significant reorganization of the site can take place. This was the first evidence that Cu_B could accommodate two exogenous ligands including one NO molecule. We have recently shown that a similar cyanide-bridge binding mode takes place in the binuclear center of cytochrome *bo* (*8*).

These findings raise the question of the maximum coordination number of Cu_B. Three histidine residues form the coordination set in the oxidized state of Cu_B in bovine mitochondrial CCO (*3*). However, in the X-ray structure of CCO from *P. denitrificans* (*5*), which was likely to be in the azide-bound form, N_3 having been added to the crystallization medium to inhibit bacterial contamination, only two histidine residues bind Cu_B. The loss of one histidine ligand of Cu_B in response to the addition of exogenous ligands has been elaborated into proposals that the movement of histidine may constitute the "handle" of the proton pump (*5*, *38*). Hence it seems unlikely that Cu_B in the dinitrosyl and in the chloronitrosyl forms detected here would increase its coordination number to 5. Therefore we propose that nitrosylation leads to the displacement of at least one histidine ligand (Figure 8). These derivatives may prove useful in probing changes at Cu_B which are proposed to be important in activating the proton pump mechanism.

Finally we return to the question of the reactivity of NO at a binuclear heme−metal site. Addition of reductant to the NO form of CCO leads to slow evolution of N_2O (*14*, *25*). To generate N_2O from two molecules of NO, the N−N bond must be formed as an oxygen atom is removed from one NO molecule. A *cis*-dinitrosyl of Cu_B, as proposed here, would certainly place two activated nitrogen atoms in close proximity. The presence of a nearby heme, to abstract an oxo atom to form [Fe^{IV}═O], may assist this process. Such a mechanism may have implications for the operation of the

enzyme NOR, in which heme $Fe^{II}-Fe^{II}$ $(His)_3$ carries out the reduction of NO to nitrous oxide.

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REFERENCES

1. Anraku, Y., and Genis, R. B. (1987) *Trends Biochem. Sci. 12*, 262−266.
2. Ferguson-Miller, S., and Babcock, G. T. (1996) *Chem. Rev. 96*, 2889−2907.
3. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh., K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) *Science 269*, 1069−1074.
4. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh., K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) *Science 272*, 1136−1144.
5. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature 376*, 660−669.
6. Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, W. J., and Gennis, R.B (1992) *J. Biol. Chem. 267*, 2105−2113.
7. Cheesman, M. R., Watmough, N. J., Pires, C. A., Turner, R., Brittain, T., Gennis, R. B., Greenwood, C., and Thomson, A. J. (1993) *Biochem. J. 289*, 709−718.
8. Cheesman, M. R., Watmough, N. J., Gennis, R. B., Greenwood, C., and Thomson, A. J. (1994) *Eur. J. Biochem. 219*, 595−602.
9. Watmough, N. J., Cheesman, M. R., Gennis, R. B., Greenwood, C., and Thomson, A. J. (1993) *FEBS Lett. 319*, 151−154.
10. Watmough, N. J., Cheeesman, M. R., Greenwood, C., and Thomson, A. J. (1994) *Biochem. J. 300*, 469−475.
11. Little, R. H., Cheesman, M. R., Thomson, A. J., Greenwood, C., and Watmough, N. J. (1996) *Biochemistry 35*, 13780−13787.
12. Moody, A. J. (1996) *Biochim. Biophys. Acta 1276*, 6−20.
13. Stevens, T, H., Brudvig, G. W., Bocian, D. F., and Chan, S. I. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3320−3324.
14. Brudvig, G. W., Stevens, T. H., and Chan, S. I. (1980) *Biochemistry 19*, 5275−5285.
15. Boelens, R., Wever, R., Van Gelder, B. F., and Rademaker, H. (1983) *Biochim. Biophys. Acta 724*, 176−183.
16. Hartzell, C. R., and Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318−338.
17. Yu, C., Yu, L. and King, T. E. (1975) *J. Biol. Chem. 250*, 1383−1392.
18. Moody, A. J., Cooper, C. E., and Rich, P. R. (1991) *Biochim. Biophys. Acta 1059*, 189−207.
19. Moody, A. J., Richardson, M., Spencer, J. P. E., Brandt, U., and Rich, P. R. (1994) *Biochem. J. 302*, 821−826.
20. Bredt, D. S., and Snyder, S. H. (1994) *Annu. Rev. Biochem. 63*, 173−195.
21. Torres, J., Darley-Usmar, V., and Wilson, M. T. (1995) *Biochem. J. 312*, 169−173.
22. Saraste, M., and Castresana, J. (1994) *FEBS Lett. 341*, 1−4.
23. van der Oost, J., deBoer, A. P. N., deGier, J.-W. L., Zumft, W. G., Stouthamer, A. H., and van Spanning, R. J. M. (1994) *FEMS Microbiol. Lett. 121*, 1−10.
24. Girsch, P., and de Vries, S. (1997) *Biochim. Biophys. Acta 1318*, 202−216.
25. Zhao, X. J., Sampath, V., and Caughey, W. S. (1995) *Biochem. Biophys. Res. Commun. 212*, 1054−1060.
26. Au, D. C.-T., and Gennis, R. B. (1987) *J. Bacteriol. 169*, 3237−3242.
27. Moody, A. J., and Rich, P. R. (1994) *Eur. J. Biochem. 226*, 731−737.
28. Kogan, V. B., Fridman, M. T., and Kafarov, V. V. (1963) in *Solubilities of Inorganic and Organic Compounds* (Stephen, H., and Stephen, T., Eds.) p 30, Pergamon, Oxford.
29. Palmer, G. (1979) in *The Porphyrins* (Dolphin, D., Ed) Vol. 4, Pt. B, Chap. 6, Academic Press, New York.
30. Yoshimura, T. (1995) in *Bioradicals Detected by ESR Spectroscopy* (Ohva-Hishiguchi, H., and Packer, L., Eds.) pp 217−235, Birkhauser Verlag, Basel, Switzerland.
31. Moody, A. J., and Rich, P. R. (1997) *Biochem. Soc. Trans. 25*, 62S.
32. Vickery, L., Nozawa, T., and Sauer, K. (1976) *J. Am. Chem. Soc. 98*, 343−350.
33. Sievers, G., Gadsby, P. M. A., Peterson, J., and Thomson, A. J. (1983) *Biochim. Biophys. Acta 742*, 637−647.
34. Hoshino, M., Ozawa, K., Seki, H., and Ford, P. C. (1993) *J. Am. Chem. Soc. 115*, 9568−9575.
35. Pearson, R. G. (1969) in *Survey of Progress in Chemistry* (Scott, A., Ed.) Chapter 1, Academic Press, New York.
36. Thomson, A. J., Eglinton, D. G., Hill, B. C., and Greenwood, C. (1982) *Biochem. J. 207*, 167−170.
37. Hill, B. C., Brittain, T., Eglinton, D. G., Gadsby, P. M. A., Greenwood, C., Nicholls, P., Peterson, J., Thomson, A. J., and Woon, T. C. (1983) *Biochem. J. 215*, 57−66.
38. Wikström, M., Bogachev, A., Finel, M., Morgan, J. E., Puustinen, A., Raitio, M., Verkhovskaya, M. L., and Verkhovsky, M. I. (1994) *Biochim. Biophys. Acta 1187*, 106−111.
39. Wilson, M. T., Torres, J., Cooper, C. E., and Sharpe, M. A. (1997) *Biochem. Soc. Trans. 25*, 905−909.

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