Accelerated Publications

The Gateway to the Active Site of Heme–Copper Oxidases†

Douglas D. Lemon,‡ Melissa W. Calhoun,§ Robert B. Gennis,§ and William H. Woodruff*,‡

Los Alamos National Laboratory, INC-14, Mail Stop C345, Los Alamos, New Mexico 87545, and School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

Received July 20, 1993; Revised Manuscript Received September 20, 1993•

ABSTRACT: The spectroscopy and dynamics of CO binding were measured for wild-type and mutant cytochromes *bo*, members of the superfamily of heme–copper oxidases. The results suggest that access of ligands, including substrate O_2, to the binuclear Fe–Cu active site is controlled at two levels. CO recombination to the wild-type ubiquinol oxidase exhibited saturation kinetics (k_{max} = 190 s^{-1}, K_m = 2.4 mM), indicative of the existence of an intermediate in the ligand-binding pathway. FTIR spectroscopy and TRIR spectroscopy were used to demonstrate conclusively that this intermediate was a Cu_B–CO complex. Two mutant oxidases (His333Leu, His334Leu) which lack Cu_B showed no evidence of saturation of CO rebinding, even up to 21 mM CO. Also, the absolute rates of CO binding to the mutant oxidases were much greater than for wild type, even at CO concentrations well below the apparent K_m for wild-type enzyme. These results clearly indicate that the copper ion at the binuclear site acts as an obligatory way station, or gate, severely limiting the approach of ligands to the heme active site. Further, an analysis of the rate constants for CO binding to Cu_B suggests that the protein structure external to the binuclear site regulates ligand entry into this site. We propose that these control mechanisms for substrate binding are operative throughout this general class of enzymes.

The superfamily of heme–copper oxidases is responsible for some 90% of the biological O_2 reduction on earth and for nearly half of the redox energy of cellular respiration (Chan & Li, 1990; Wikström *et al.*, 1981). All members of the superfamily are integral membrane proteins which share in common the function of reducing dioxygen (O_2) to water, the electrons for reduction coming ultimately from the oxidation of substrates derived from foodstuff. The energy of these redox reactions is conserved as electrochemical potential in the form of a transmembrane hydrogen ion gradient. It is a major objective in bioenergetics to understand how the heme–copper oxidases perform these functions, including how they admit and bind substrate O_2 as the essential first step in their energy conversion and storage. In this paper we report the ligand binding mechanisms of cytochrome *bo*, a heme–copper oxidase from *Escherichia coli* which functions as a ubiquinol oxidase (Chepuri *et al.*, 1990; Hill *et al.*, 1992).

All heme–copper oxidases appear to be structurally and functionally similar with regard to their O_2 binding and activation apparatus. A five-coordinate, high-spin heme (a_3 or *o*) is associated with a single copper, Cu_B, in a "binuclear center" which is the site of O_2 ligation and reduction. All members of the superfamily also have a second heme (*a* or *b*) which is six-coordinate, low spin. This low-spin heme mediates the flow of electrons from donor substrate to the binuclear center. The aa_3-type oxidases utilize cytochrome *c* as reductant and contain a fourth metal center, known as Cu_A, located in subunit II. Cytochrome *bo*, being a ubiquinol oxidase, has no Cu_A. However, the homology among members of the superfamily, particularly for subunit I which contains the binuclear site, is high enough that mechanistic features of the reduction of O_2 for this enzyme are likely to apply to all heme–copper oxidases (Holm *et al.*, 1987; Saraste, 1990). Six histidines are totally conserved among all known sequences for subunit I (Saraste, 1990). These histidines are thought to be ligands for the metals in the binuclear site and for the low-spin heme found in all subunits I. Recently, assignments of the metals to which these histidines are bound have been made using site-directed mutagenesis on cytochrome *bo* and an aa_3-type oxidase from *Rhodobacter sphaeroides* (Hosler *et al.*, 1993; Lemieux *et al.*, 1992; Minagawa *et al.*, 1992; Shapleigh *et al.*, 1992b). Because cytochrome *bo* can be manipulated genetically and overproduced (Au & Gennis, 1987; Chepuri *et al.*, 1990), this enzyme is particularly valuable in providing insight into the function of the heme–copper oxidases.

The sixth coordination position of the heme at the binuclear site is available to exogenous ligands, such as O_2, CO, and CN^- (Blackmore *et al.*, 1991; Moody *et al.*, 1993; Yoshikawa *et al.*, 1985). Binding of O_2 to the high-spin heme is the obligatory first step in the mechanism of reduction to water (Babcock & Wikström, 1992). Recent evidence from a variety of measurements of spectroscopy and dynamics (Alben *et al.*, 1981; Blackmore *et al.*, 1991; Dyer *et al.*, 1989; Einarsdóttir *et al.*, 1993) suggests that exogenous ligands bind first to Cu_B and then are transferred to the high spin heme. Here we utilize wild-type and two mutant (His333Leu, His334Leu) cytochromes *bo* to confirm the existence of a Cu_B–CO complex prior to ligation at the high-spin heme. We also show that the formation of the Cu_B–CO complex occurs at a rate 100-fold slower than expected for a diffusion-controlled reaction, which indicates that the structure of the protein can limit access to the active site of heme–copper oxidases.

† This work was supported by National Institutes of Health Grant DK 36263 (W.H.W.) and Department of Energy Grant DE-FG02-87ER13716 (R.B.G.). Work at Los Alamos National Laboratory was performed under the auspices of the U.S. Department of Energy.

* To whom correspondence should be addressed.

‡ Los Alamos National Laboratory.

§ University of Illinois.

• Abstract published in *Advance ACS Abstracts,* October 15, 1993.

0006-2960/93/0432-11953$04.00/0

EXPERIMENTAL PROCEDURES

Construction of mutants and preparation of membrane samples were performed as in Lemieux *et al.* (1992).

Low-temperature FTIR[1] difference spectra of native and-mutant cytochrome *bo* were collected in the same manner as in Hill *et al.* (1992).

A vacuum line equipped with a MKS Barytron pressure transducer and gauge was used for preparation of anaerobic and carbon monoxide samples. Reduced samples were prepared by repeated cycles of vacuum and nitrogen, followed by the addition of a small excess of sodium dithionite which had been prepared anaerobically. The CO form was then made by replacing the nitrogen atmosphere with carbon monoxide, at a pressure of 760 Torr. A special cell was used for higher pressures of CO. This cell consisted of a 25-mm length of thick-walled pyrex tube, 2-mm inner diameter, sealed on one end and attached at the other to a short length of $^1/_4$ in. stainless steel tubing via a glass-to-metal seal. Swagelok fittings and connectors were used to attach the cell to a pressure gauge and valve and for connection of the assembly to a carbon monoxide cylinder. Pressures of CO above atmospheric were established by direct addition of the gas to reduced samples containing 760 Torr of CO, following thorough removal of air from the lines which connected the cell to the CO tank and vacuum pump.

Room temperature electronic absorbance kinetics were performed at the Center for Fast Kinetics Research (CFKR), University of Texas at Austin. The second harmonic (532 nm, ca. 12 ns) pulse from a Quantel YG481 Nd:Yag laser was used as an excitation source. A 12-V tungsten lamp was used as a monitoring source. Band-pass or long-pass filters were placed between the lamp and the sample, and a monochromator was used between the sample and a Hammamatsu R928 photomultiplier tube. Kinetic measurements were made at 585 nm. Signals were fed to a Biomation 8100 transient digitizer and then analyzed by a microcomputer.

Room temperature TRIR measurements were made as in Dyer *et al.* (1989).

RESULTS AND DISCUSSION

Low-Temperature FTIR Spectroscopy. The light-minus-dark spectrum of wild-type membranes showed a sharp trough at 1959 cm^{-1} due to absorbance of Fe–CO and peaks at 2063 and 2054 cm^{-1} attributed to Cu–CO (Hill *et al.*, 1992). This result clearly indicates that CO molecules which have been photolyzed from the heme *o* iron bind to Cu_B. Raising the temperature of the sample accelerated the return of CO to Fe_o. This behavior has been observed in other heme–copper oxidases, including aa_3 from beef heart muscle (Fiamingo *et al.*, 1982) and from *R. sphaeroides* (Shapleigh *et al.*, 1992a) and ba_3 from *Thermus thermophilus* (Einarsdóttir *et al.*, 1989).

The corresponding difference spectra were taken for the His333Leu and His334Leu mutant *bo* proteins (Calhoun *et al.*, 1993). While bleaches of the Fe–CO absorbances were observed, there was no appearance of absorbance attributable to the Cu_B–CO species in either mutant. We conclude that there is no binding of CO to Cu_B in the His333Leu and His334Leu proteins, because Cu_B was not incorporated during assembly of the mutant enzymes. This view is supported by the results of Anraku and co-workers, who showed by atomic absorption analysis that His333Ala and His334Ala mutants

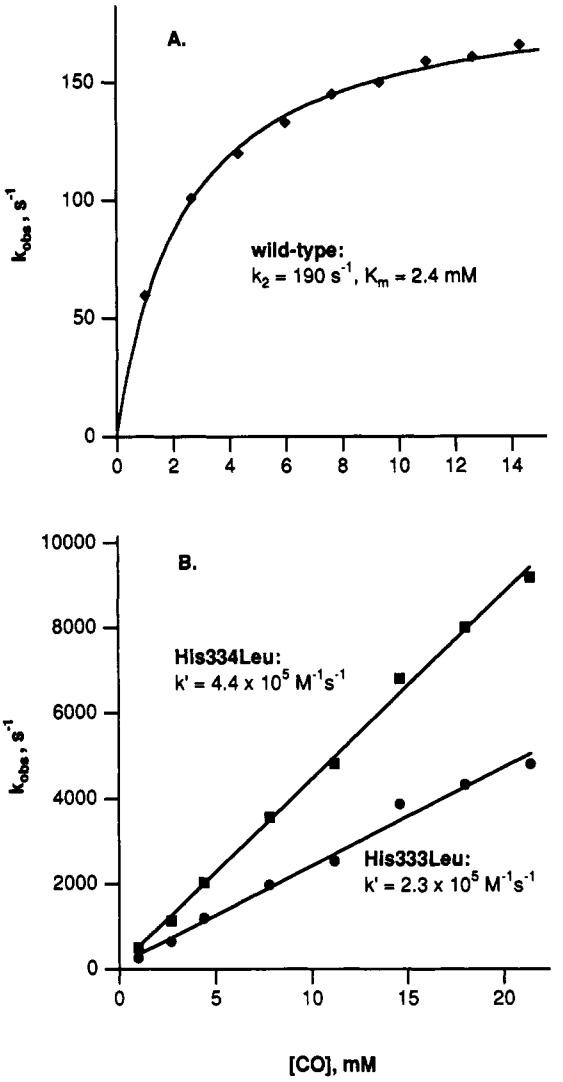

FIGURE 1: Room temperature CO rebinding to (A) wild-type and (B) mutant cytochromes *bo*. The symbols represent observed data collected after photolysis of the oxidase–CO complexes. The solid lines were calculated from linear fits to the mutant data and a hyperbolic fit to the wild-type data. Note the dramatic difference in the ordinate scales.

of cytochrome *bo* contained significantly less copper than wild-type enzyme (Minagawa *et al.*, 1992). These data strongly support the theory that the two completely conserved histidine residues His-333 and His-334 serve as ligands for Cu_B in the native oxidase (Lemieux *et al.*, 1992; Minagawa *et al.*, 1992).

Room Temperature Electronic Absorbance Kinetics. E. coli membranes containing the overproduced ubiquinol oxidases were used for all kinetic measurements. The room temperature kinetic behavior of these three ubiquinol oxidases is shown in Figure 1, plotted as the dependence of the observed rate of CO recombination to heme *o* as a function of CO concentration. Figure 1A shows that the rate of rebinding of CO to the native enzyme does not depend linearly on CO concentration but instead approaches a maximum value. This type of behavior has been observed in all six species of heme–copper oxidases tested thus far.[2] Saturable kinetics is indicative of an intermediate in the ligand binding pathway. This fact and the FTIR results indicated above demonstrate that the following scheme applies to the binding of CO to heme–copper

[1] Abbreviations: FTIR, Fourier transform infrared; TRIR, time-resolved infrared.

[2] D. D. Lemon, Ó. Einarsdóttir, R. B. Dyer, and W. H. Woodruff, manuscript in preparation.

oxidases:

$$Fe_{a_3}^{2+}, Cu_B^{+} + CO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe_{a_3}^{2+}, Cu_B^{+}\text{–}CO \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Fe_{a_3}^{2+}\text{–}CO, Cu_B^{+} \quad (1)$$

where k_1 and k_{-1} represent the reversible binding of CO to Cu_B and k_2 is the first-order transfer of CO from Cu_B to the heme iron. Because the thermal dissociation rate of the heme–CO complex in cytochrome oxidase is very slow (Gibson & Greenwood, 1963), k_{-2} can be neglected. This scheme is then analogous to the Michaelis–Menten analysis of enzyme kinetics (Lehninger, 1982). By fitting the data to the Michaelis–Menten equation, we determined that the K_m for CO binding to wild-type *bo* was 2.4 mM and the limiting rate of recombination (k_2) was 190 s^{-1}. Both of these constants are about 5 times larger in the case of mammalian aa_3-type oxidase. Because these two constants are compensatory (*i.e.*, well below saturation, $[CO] < K_m$ and $k_{obs} \approx k_2[CO]/K_m$), the observed rate at 1 atm of CO (1 mM in solution) for *bo* was not very different from that for aa_3, 60 s^{-1} vs 90 s^{-1}. These observations demonstrate the importance of determining the concentration dependence of ligand-binding kinetics.

Figure 1B shows the CO concentration dependence of the rate of CO recombination to the mutant cytochromes *bo*. Clearly, there is no evidence for saturation, even up to 21 mM CO. In addition to the lack of saturation kinetics, the magnitude of the rates is much greater for the mutant proteins, with values at 1 mM CO of about 250 and about 500 s^{-1}, already well above the asymptotic limit of the wild-type enzyme. This greatly increased accessibility of heme *o* to ligands suggests that Cu_B is not present in the mutants, consistent with the results from low-temperature FTIR (Calhoun *et al.*, 1993). Because these rate dependences are linear in CO concentration, and there was no binding to Cu_B detectable by FTIR, it is evident that a simple, psuedo-first-order reversible binding process exists for the mutants. Accordingly, linear fits were applied to the data, with the slopes representing the second-order association rate constants. The results were 2.3×10^5 M^{-1} s^{-1} for His333Leu and 4.4×10^5 M^{-1} s^{-1} for His334Leu. Interestingly, this type of kinetic behavior and even the values of the rate constants compare remarkably well with those for sperm whale myoglobin, 5.1×10^5 M^{-1} s^{-1} (Gibson *et al.*, 1986).

Room Temperature TRIR Spectroscopy. The decay of the absorbance of Cu_B–CO in wild-type enzyme was measured by time-resolved infrared spectroscopy. A clear transient exists at 2063 cm^{-1}, the frequency attributed to the stretching of CO when bound to Cu_B (Hill *et al.*, 1992), while in a control measurement at 2046 cm^{-1} no transient was observed. The rate of decay of this transient was 2100 s^{-1}. Assuming reversible kinetics for the association of CO with Cu_B, $k_{obs} = k_1[CO] + k_{-1}$. The free CO concentration was 1 mM. Given this equation along with the relationship $K_m = (k_2 + k_{-1})/k_1$, we have a system of equations which allows the determination of both k_1 and k_{-1}. Using the previously determined values of 190 s^{-1} for k_2 and 2.4 mM for K_m, we calculate a value of 1400 s^{-1} for k_{-1}, the dissociation rate constant for Cu_B–CO. We also calculate that k_1, the association rate constant for the Cu_B–CO complex, is about 7×10^5 M^{-1} s^{-1}. Three points are to be made about this value: (1) k_1 for wild-type enzyme is similar to the values of the association rate constants for CO binding to heme *o* in the mutant oxidases, (2) k_1 is also nearly equal to the association rate constant of CO binding to sperm whale myoglobin, which must undergo rearrangement of amino acid side chains to allow ligands access to heme iron (Carver *et al.*, 1990), and (3) k_1 is at least 2 orders of magnitude slower than the rate expected for a diffusion-limited process. While the first point serves to validate the calculated value of k_1, the second and third points are conclusive evidence that, in the heme–copper oxidases, protein structures external to the binuclear site provide kinetic control over the ligand-binding steps, which are fundamental to the mechanism of O_2 reduction. This is further supported by the factor of 2 difference in the association rate constants of the mutants, which must have slightly different structures in the vicinity of the heme *o* site. A second level of control is exerted by the ligand-binding equilibrium involving Cu_B. Our species-dependent studies[2] have shown that K_m varies by more than a factor of 100 among the six heme–copper oxidases tested, demonstrating that Cu_B ligation can provide thermodynamic control over substrate binding in close proximity to the heme.

CONCLUSIONS

These results dramatically illustrate the importance of both protein structural features and the Cu_B site in the ligation reactions of heme–copper oxidases. Mutations where Cu_B or at least the ability to bind to Cu_B was lost resulted in completely different kinetic behavior. In addition to a loss of saturation kinetics, the mutant proteins had high spin hemes which were much more accessible to exogenous ligands, as shown by the greatly increased magnitude of the rates of rebinding. Because of the similarity in the ligation chemistry of CO and O_2, we conclude that Cu_B is the site of the initial ligation step in the physiological function of heme–copper oxidases. This ligation step may be involved in the crucial link between the redox and proton-pumping functions of heme–copper oxidases, as we have previously suggested (Woodruff *et al.*, 1991). Finally, we suggest that the effect exerted by the cytochrome *bo* protein structure on access to Cu_B may correspond to previously undiscovered gating features inherent in the O_2 reduction mechanisms of all heme–copper oxidases.

ACKNOWLEDGMENT

The authors gratefully acknowledge Steven J. Atherton and Stephan M. Hubig for assistance in the measurement of electronic absorbance kinetics and R. Brian Dyer and Tim P. Causgrove for their help with TRIR measurements.

REFERENCES

Alben, J. O., Moh, P. P., Fiamingo, F. G., & Altschuld, R. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 234–237.

Au, D. C.-T., & Gennis, R. B. (1987) *J. Bacteriol. 169*, 3237–3242.

Babcock, G. T., & Wikström, M. (1992) *Nature 356*, 301–308.

Blackmore, R. S., Greenwood, C., & Gibson, Q. H. (1991) *J. Biol. Chem. 266*, 19245–19249.

Calhoun, M. W., Hill, J. J., Lemieux, L. J., Ingledew, W. J., Alben, J. O., & Gennis, R. B. (1993) *Biochemistry* (in press).

Carver, T. E., Rohlfs, R. J., Olson, J. S., Gibson, Q. H., Blackmore, R. S., Springer, B. A., & Sligar, S. G. (1990) *J. Biol. Chem. 265*, 20007–20020.

Chan, S. I., & Li, P. M. (1990) *Biochemistry 29*, 1–12.

Chepuri, V., Lemieux, L. J., Au, D. C.-T., & Gennis, R. B. (1990) *J. Biol. Chem. 265*, 11185–11192.

Dyer, R. B., Einarsdóttir, Ó., Killough, P. M., Lopez-Garriga, J. J., & Woodruff, W. H. (1989) *J. Am. Chem. Soc. 111*, 7657–7659.

Einarsdóttir, Ó., Killough, P. M., Fee, J. A., & Woodruff, W. H. (1989) *J. Biol. Chem. 264*, 2405–2408.

Einarsdóttir, Ó., Dyer, R. B., Lemon, D. D., Killough, P. M., Hubig, S. M., Atherton, S. J., López-Garriga, J. J., Palmer, G., & Woodruff, W. H. (1993) *Biochemistry* (in press).

Fiamingo, F. G., Altschuld, R. A., Moh, P. P., & Alben, J. O. (1982) *J. Biol. Chem. 257*, 1639–1650.

Gibson, Q. H., & Greenwood, C. (1963) *Biochem. J. 86*, 541–554.

Gibson, Q. H., Olson, J. S., McKinnie, R. E., & Rohlfs, R. J. (1986) *J. Biol. Chem. 261*, 10228–10239.

Hill, J., Goswitz, V. C., Calhoun, M., Garcia-Horsman, J. A., Lemieux, L. J., Alben, J. O., & Gennis, R. B. (1992) *Biochemistry 31*, 11435–11440.

Holm, L., Saraste, M., & Wikström, M. (1987) *EMBO J. 6*, 2819–2823.

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, G. T., & Gennis, R. B. (1993) *J. Bioenerg. Biomembr. 25*, 121–136.

Lehninger, A. L. (1982) *Principles of Biochemistry*, pp 213–217, Worth Publishers, Inc., New York.

Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, W. J., & Gennis, R. B. (1992) *J. Biol. Chem. 267*, 2105–2113.

Minagawa, J., Mogi, T., Gennis, R. B., & Anraku, Y. (1992) *J. Biol. Chem. 267*, 2096–2104.

Moody, A. J., Rumbley, J. N., Gennis, R. B., Ingledew, W. J., & Rich, P. R. (1993) *Biochim. Biophys. Acta 1141*, 321–329.

Saraste, M. (1990) *Q. Rev. Biophys. 23*, 331–366.

Shapleigh, J. P., Hill, J. J., Alben, J. O., & Gennis, R. B. (1992a) *J. Bacteriol. 174*, 2338–2343.

Shapleigh, J. P., Hosler, J. P., Tecklenburg, M. M. J., Kim, Y., Babcock, G. T., Gennis, R. B., & Ferguson-Miller, S. (1992b) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4786–4790.

Wikström, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, New York.

Woodruff, W. H., Einarsdóttir, Ó., Dyer, R. B., Bagley, K. A., Palmer, G., Atherton, S. J., Goldbeck, R. A., Dawes, T. D., & Kliger, D. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2588–2592.

Yoshikawa, S., O'Keefe, D. H., & Caughey, W. S. (1985) *J. Biol. Chem. 260*, 3518–3528.