Oxygen Activation by Axial Ligand Mutants of Mitochondrial Cytochrome b_5: Oxidation of Heme to Verdoheme and Biliverdin

Ludivina Avila,[†] Hong-wei Huang,[‡] Juan C. Rodríguez,[†] Pierre Moënne-Loccoz,[‡] and Mario Rivera*,[†]

Department of Chemistry, Oklahoma State University Stillwater, Oklahoma 74078-3071
Department of Biochemistry and Molecular Biology Oregon Graduate Institute of Science and Technology Beaverton, Oregon 97006

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The degradation of heme in mammalian cells is catalyzed by heme oxygenase (HO).[1] Not long ago, the HO system was regarded in the context of heme homeostasis, and the reaction products, CO, Fe, and biliverdin, as toxic material. The current view has changed drastically with the discovery of important biological activity associated with these products. CO is now thought to share some of the biological properties of NO in signal transduction and communication.[2] Iron released by HO activity is known to regulate genes, including that of NO synthase. Bilirubin produced by biliverdin reductase is a potent antioxidant.[3,4]

Insight into the mechanism of heme catabolism can be gained by designing novel proteins capable of oxygenating their heme by the process known as coupled oxidation.[5] For example, we reported that replacement of one His axial ligand in outer mitochondrial membrane (OM) cyt b_5 for Met enables the H63M mutant to oxidize heme to verdoheme.[6a] More recently, it was reported that the coupled oxidation of heme in a cytochrome b_{562} mutant is also arrested at the verdoheme stage.[6b] It was postulated that the coupled oxidation of heme in the cyt b_5 and cyt b_{562} mutants is stopped at verdoheme due to the formation of a hexacoordinate verdoheme complex (H39-M63 in cyt b_5[6a] and M7-M102 in cyt b_{562}[6b]).

The H63V and H39V mutants of OM cyt b_5 were prepared for the purpose of this investigation. Replacement of either axial ligand with a non-coordinating amino acid was carried out to prevent the formation of a putative hexacoordinate verdoheme complex. We found that the coupled oxidation of the heme in H63V OM cyt b_5 is still stopped at verdoheme. In contrast the H39V mutant forms a metastable oxyheme complex and supports the oxidation of verdoheme to biliverdin.

The electronic spectra of H63V and H39V heme complexes display a Soret band maximum at 404 nm and a characteristic high-spin (HS) marker at 628 nm (Figure 1). The resonance Raman (RR) spectra of ferric H63V and H39V indicate that the heme iron is in hexacoordinate high-spin/low-spin (6cHS/6cLS) equilibrium (Figure 2).[7] This is consistent with a His−aqua axial coordination, as is observed in mammalian and bacterial HO.[8] The UV−vis and RR spectra of ferrous H63V and H39V are characteristic of HS ferrous heme, and coordination to a proximal

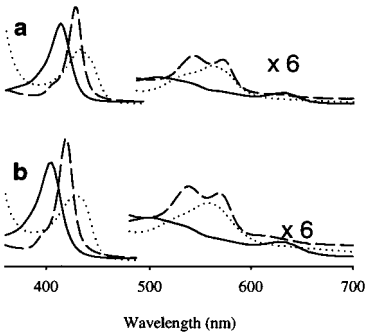

Figure 1. Electronic spectra of (a) H63V and (b) H39V OM cyt b_5. (—) Fe(III), (•••) Fe(II), (− − −) Fe(II)−CO.

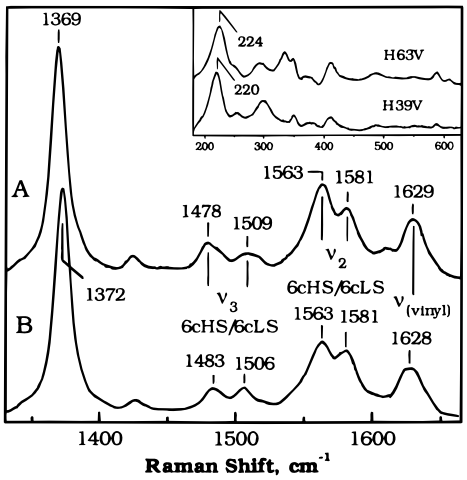

Figure 2. High-frequency RR spectra of ferric H63V (A) and H39V (B) OM cyt b_5 at room temperature acquired with 413 nm excitation (2 mW). Inset: low-frequency RR spectra of the ferrous proteins acquired with 442 nm excitation.

His ligand is confirmed by the observation of a ν(Fe−His) at ∼220 cm^{-1} (Figure 2).[9]

The coupled oxidation of the heme in the H63V mutant was carried out utilizing hydrazine as a source of reducing equivalents and monitored by absorption spectroscopy (Figure 3). The increase in the absorption at 660 nm is indicative of the formation of verdoheme. This was confirmed by the absorption spectrum obtained after the product of coupled oxidation was extracted into a chloroform−pyridine solution, which is almost identical to that of verdohemochrome.[10]

By comparison, the coupled oxidation of the heme in the H39V mutant results in bleaching of the Soret band and is not accompanied by an increase in absorption at ∼660 nm (Figure 3). These findings suggest that verdoheme does not accumulate during the coupled oxidation and is not the final product of the reaction. The nature of the final product was examined by

[†] Oklahoma State University.
[‡] Oregon Graduate Institute of Science and Technology.

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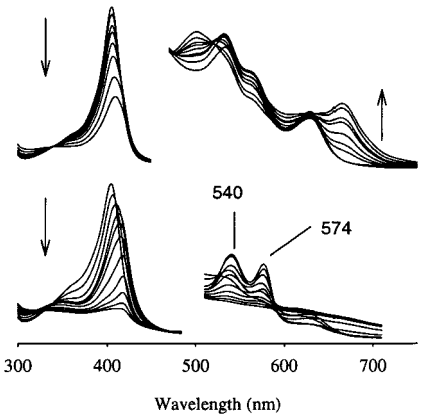

Figure 3. Electronic spectra of H63V (top) and H39V (bottom) OM cyt b_5 in the presence of hydrazine and O_2.

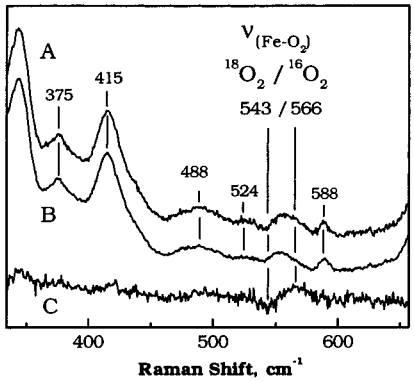

Figure 4. Low-frequency RR spectra of the oxyheme complex of H39V formed by reduction with hydrazine and exposure to $^{16}O_2$ (A), or $^{18}O_2$ (B). The difference spectrum (×2) is shown in (C). Data were accumulated for 2 min at 6 °C with 413 nm excitation.

extraction of the product into chloroform, after removal of unreacted heme with ether under acidic conditions. The UV−vis spectrum of the chloroform extract is identical to that obtained from authentic biliverdin, hence demonstrating that H39V catalyzes the coupled oxidation of heme to biliverdin.

During the coupled oxidation of the heme in the H39V mutant, a transient species with absorbances at 540 and 577 nm reaches a maxmimum concentration within the first 5 min of reaction, before it is gradually converted into product (Figure 3). The absorption spectrum of this species is reminiscent of the oxyheme complex in HO-1.[11] The formation of a metastable oxyheme complex in H39V was corroborated by the observation of a ν-(Fe−O_2) in its RR spectrum. Reduction of H39V by hydrazine in the presence of $^{18}O_2$ results in a 23 cm^{-1} downshift of a weak RR band located at 566 cm^{-1}, which was obtained in the presence of $^{16}O_2$ (Figure 4). In the oxyheme complex of rat HO-1, a ν-(Fe−O_2) was reported at the same frequency, within experimental error.[12] Similarities in ν(Fe−O_2), however, should not be overinterpreted since studies of mutant and wild-type oxy-myoglobins did not reveal clear correlations between vibration frequencies and parameters such as O_2 affinity, and on/off rates of O_2 binding.[13]

The ferrous oxyheme complex of H39V was also prepared by stoichiometric addition of sodium dithionite to an anaerobic solution of ferric H39V, followed by exposure to O_2. The UV−vis spectrum of the resultant solution also displays the peaks at 540 and 577 nm. By comparison, when ferrous H63V is exposed to air, the ferric species is formed within a few seconds without detectable accumulation of a ferrous oxyheme complex**.** An apparent autoxidation rate constant (k_{ox} = 1.4 ± 0.2 h^{-1}) was measured for the ferrous oxyheme complex of H39V by following the time-dependent decay of the peak at 577 nm.[14] The relatively low value of k_{ox} and the transient accumulation of the oxyheme complex during the coupled oxidation reaction indicate that the distal environment in H39V stabilizes the oxyheme complex.

To understand the different reactivity of H39V and H63V toward O_2, we modeled the oxyheme environment of both mutants from the X-ray coordinates of OM cyt b_5.[15] Extrapolating the heme environment in the wild-type protein to that in the mutants, the H63V model predicts the absence of a polar group susceptible to interact with the iron-bound O_2 within 6 Å of the heme iron. In contrast, the model of the H39V suggests that the amide nitrogen of Gly-41, located 4.7 Å from the iron could stabilize the oxyheme complex via the formation of a hydrogen bond. This is reminiscent of the observations made from the crystal structure of HO-1, which showed that in the absence of a distal ligand, Gly-139 (carbonyl) and Gly-143 (N−H) are the only polar groups that may interact with the iron-bound O_2.[16]

Very little is known about the conversion of verdoheme to biliverdin by HO. A ferrous verdoheme−O_2 complex has been proposed as a likely intermediate,[17] but spectroscopic and thermodynamic characterization of this complex in HO has been stymied by its high reactivity.[18] Although the hydrolytic path for the conversion of verdoheme to biliverdin by H39V cannot be ruled out at the moment, it is tempting to speculate that distal interactions promoting the stabilization of the heme−O_2 complex in H39V may play a role in the oxidation of verdoheme to biliverdin.

In summary, we have demonstrated that changes in the axial ligating environment within OM cytochrome b_5 influence the heme oxygenation process so that biliverdin is obtained when His-39 is replaced by a non-coordinating Val, but verdoheme is obtained with the H63V mutant. Further investigation aimed at elucidating the different reactivity observed with the two corresponding distal environments is currently underway.

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Supporting Information Available: UV−vis spectra of verdoheme and biliverdin extracted from the coupled oxidation reactions of H63V and H39V, respectively. UV−vis spectra for the autoxidation of the H39V−O_2 complex (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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