Efficient Coupled Oxidation of Heme Performed by the H63M Variant of Outer Mitochondrial Membrane Cytochrome b_5

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Replacement of the heme axial ligand His 63 for Met in the electron transfer protein cytochrome b_5 renders a variant capable of performing the efficient coupled oxidation of heme. The experimental evidence shows that the coupled oxidation of heme catalyzed by the H63M variant is arrested after the formation of verdoheme. This may result from the formation of a hexacoordinated (His, Met) veredoheme complex.

Heme oxygenase (HO) catalyzes the regiospecific oxidative cleavage of Fe-protoporphyrin IX (heme) to form biliverdin and carbon monoxide (CO), with concomitant consumption of dioxygen and electrons provided by cytochrome P-450 reductase.[1] Using recombinant HO, many of its structural features and their corresponding functional relevance have been recently elucidated. Nevertheless, several key issues have not yet been completely resolved.[2] Several hemoproteins, perhaps the most studied of which is myoglobin, have been studied as models of HO. These proteins, are known to perform the coupled oxidation of heme by utilizing O_2 and ascorbate or hydrazine as electron donors, to yield biliverdin and CO as products,[3] albeit, in a relatively inefficient manner. Recently, however, a variant of myoglobin (V67A/V68S) was found to carry out the efficient coupled oxidation of heme with the concomitant formation of mainly Fe(III)-biliverdin.[4]

In this report we document that when His 63, one of the axial ligands in outer mitochondrial membrane cytochrome b_5 (OM cyt. b_5; an electron transfer protein), is replaced by Met, the resultant variant is capable of carrying out the efficient coupled oxidation of heme. Furthermore, this reaction as catalyzed by the H63M variant, is arrested at the verdoheme stage.

The H63M variant of OM cyt. b_5 was synthesized with the aid of the transformer™ site directed mutagenesis kit and the recombinant plasmid MRL1.[5] The mutated gene was subcloned into the pET 11a vector[5] and expressed in *E. coli* B834(DE3), a Met auxotroph, grown in M9 minimal medium supplemented with 40 mg/L of Met, as described previously.[6] After cell-lysis and ultracentrifugation, the supernatant was made 3.1 mM in $K_3Fe(CN)_6$ and 1 mM in imidazole, dialyzed against 10 mM EDTA, 50 mM Tris, 1mM imidazole (pH 7.8 at 4°C) and purified by ion exchange chromatography.[6] Fractions (A_{280}/A_{412} < 1.5) were pooled and dialyzed against 100 mM NaCl, 20 mM Tris, 1 mM EDTA (pH 7.4 at 4°C) in order to eliminate the imidazole. The protein was then purified by size exclusion chromatography, as described previously.[6] The molar absorptivity coefficient of the variant (ϵ_{410}=108 mM^{-1} cm^{-1}) was measured by the pyridine hemochrome method.

In the ferric state, the variant displays an electronic spectrum with a well defined band at 630 nm (Figure 1a), attributed to an oxygen-iron charge transfer transition, and an EPR spectrum with a peak at g = 6.45 (Figure 1d), suggesting that the H63M mutant and metaquo myoglobin are spectroscopically similar. Moreover, the variant was found to readily bind CO in the ferrous form (Figure 1c) as well as imidazole and CN^- in the ferric state, thus suggesting that the ferric heme is coordinated by His 39. The UV-vis spectrum of the variant in the ferrous state (Figure 1b), however, shows well resolved α and β bands at 558 and 532 nm, respectively, which are indicative of a low spin Fe^{2+} species resulting from axial coordination by H and M.

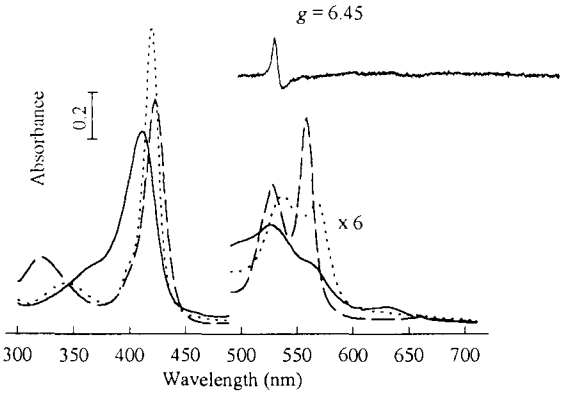

Figure 1. Electronic spectrum of the H63M variant of OM cytochrome b_5. a) Ferric (solid), b) Ferrous (dashed), c) ferrous-CO bound (dotted). d) Inset: EPR spectrum of the ferric H63M variant (77K).

The coupled oxidation of heme catalyzed by the H63M variant of OM cyt. b_5 was carried out at 35°C on a jacketed cuvette containing 1 mL of 50 mM phosphate buffer (pH 7.0), 3-4 μM of protein, and 2 mM of L-ascorbic acid (SIGMA), and monitored by UV-vis spectrophotometry. A decrease in the absorbance of the Soret band is accompanied by a decrease of the α and β bands, and the appearance of a band at 664 nm (Figure 2). Unexpectedly, the

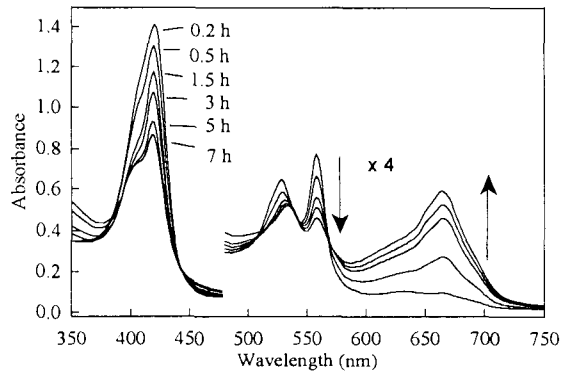

Figure 2. Changes in the UV-vis spectra of the H63M variant during the aerobic coupled oxidation of heme in the presence of L-ascorbate.

electronic spectrum obtained after 7 h of reaction closely resembles that reported for the verdoheme-HO complex.[2a,2c] Additional evidence corroborating the identity of verdoheme as the reaction product follows: a) The UV-vis spectrum of the reaction product

extracted with $CHCl_3$ and pyridine[7] (Figure 3) is almost identical to that of pure verdohemochrome.[8] b) The identity of verdoheme was also conclusively demonstrated by Electrospray Ionization Mass Spectrometric (ESI-MS) analysis of the reaction product obtained from reaction mixtures containing the aforementioned concentrations of protein and ascorbic acid.[9] The mass spectrum displays a peak at m/z = 619 (Figure 4), consistent with the mass of verdoheme, and a minor signal assigned to unmodified heme (m/z = 616). Samples of unmodified protein (no ascorbate added) analyzed by ESI-MS showed only the peak at m/z = 616. Control experiments with WT cyt. b_5 displayed no reaction.

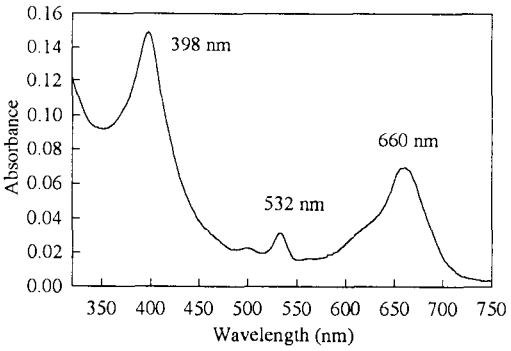

Figure 3. UV-vis spectrum obtained upon extraction of the reaction product with pyridine in chloroform.

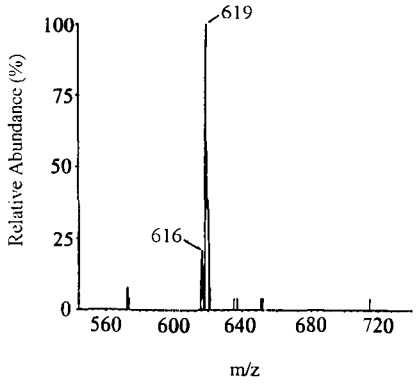

Figure 4. Mass spectrum obtained by ESI-MS analysis of the reaction mixture containing the H63M mutant and L-ascorbate under aerobic conditions.

The regiospecificity of the reaction was assessed by complexing the reaction product with 2-picoline, extracting the complex into $CHCl_3$ and converting it into biliverdin.[10] Biliverdin was subsequently esterified in 5% methanolic H_2SO_4 and analyzed by HPLC.[11] The corresponding chromatogram showed only one peak, which displayed a retention time identical to that of authentic biliverdin (SIGMA), thus indicating that heme oxygenation as catalyzed by the H63M variant occurs exclusively at the α-meso position.

The first step in the coupled oxidation process requires the formation of a transient O_2-Fe^{2+} complex at the heme binding site. Evidently, for this to occur, one of the heme-iron axial positions must be available. This particular characteristic likely determines to a large extent that only pentacoordinate proteins are capable of performing coupled oxidation reactions. It is therefore interesting, that despite the fact that the H63M variant is His-Met coordinated in the ferrous state, as indicated by its electronic spectrum, it is still capable of carrying out the coupled oxidation of heme. A plausible explanation may be that under aerobic conditions, a competition is established between one of the axial ligands (probably M) and O_2 for the 6th coordination site on Fe^{2+}. Once the O_2-Fe^{2+} complex is formed, dioxygen may be activated by a second reducing equivalent. Further steps in the coupled oxidation process may follow the mechanism proposed for the heme degradation reaction performed by HO. Furthermore, it is also unusual that the coupled oxidation carried out by the H63M variant does not result in cleavage of the porphyrin ring, but is arrested upon formation of the verdoheme-protein complex. This unique property may arise from the formation of a hexacoordinated (His-Met) verdoheme complex, which prevents subsequent binding of oxygen to the ferrous heme. Further studies to determine the oxidation and coordination states of the verdoheme-H63M complex are currently underway.

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References

1 R. Tenhunen, H. S. Marver, and R. Schmid, *J. Biol. Chem.*, **244**, 6388 (1969).
2 a) S. Takahashi, K. Mansfield Matera, H. Fujii, H. Zhou, K. Ishikawa, T. Yoshida, M. Ikeda-Saito, and D. L. Russeau, *Biochemistry*, **36**, 1402 (1997). b) K. Mansfield Matera, H. Zhou, C. T. Migita, S. E. Hobert, K. Ishikawa, K. Katakura, H. Maeshima, T. Yoshida, and M. Ikeda-Saito, *Biochemistry*, **36**, 4909 (1997). c) Y. Liu, P. Moënne-Loccoz, T. M. Loehr, and P. R. Ortiz de Montellano, *J. Biol. Chem.*, **272**, 6909 (1997).
3 J. E. Kench, *Biochem. J.*, **56**, 669 (1954).
4 D. P. Hildebrand, H. Tang, Y. Luo, C. L. Hunter, M. Smith, G. D. Brayer, and A. G. Mauk, *J. Am. Chem. Soc.*, **118**, 12909 (1996).
5 M. Rivera, C. Barillas-Mury, K. A. Christensen, J. W. Little, M. A. Wells, and F. A. Walker, *Biochemistry*, **31**, 1233 (1992).
6 a) M. Rivera and F. A. Walker, *Anal. Biochem.*, **230**, 295 (1995). b) M. J. Rodríguez, F. Qiu, R. E. Stark, S. P. White, X. Zhang, S. I. Foundling, V. Rodríguez, C. L. Schilling, R. A. Bunce, and M. Rivera, *Biochemistry*, **35**, 16378 (1996).
7 The reaction product (800 μL) was extracted with 100 μL of pyridine and 1 mL of $CHCl_3$. The $CHCl_3$ phase was transferred to a test tube, and dried with anhydrous Na_2SO_4. The electronic spectrum of the resultant solution is shown in Figure 3.
8 a) E. Y. Levin, *Biochemistry*, **9**, 2845 (1966). b) T. Yoshida and M. Noguchi, *J. Biochem.*, **96**, 563 (1984).
9 The reaction (10 mL) was stopped after 10 h by cooling at 4 °C, dialyzed against 40 mM NH_4HCO_3 at 4 °C, and concentrated to ~0.6 mL. A 100 μL aliquot was mixed with 100 μL of 10 mM ammonium acetate and analyzed by ESI-MS. The sample was introduced at 5 μL/min into a Sciex API III triple-quadrupole mass spectrometer equipped with an atmospheric pressure ion source. Sampling of the positive ions was achieved in the first quadrupole using a voltage difference of 125 V. Increments of 0.1 amu were collected in the range of 500 to 750 amu.
10 S. Saito and H. A. Itano, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 1393 (1982).
11 M. Noguchi, T. Yoshida, and G. Kikuchi, *J. Biochem.*, **91**, 1479 (1982).