Electron Flow through Cytochrome P450

Thesis by

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for my aunt Mine, who first inspired me to pursue a PhD

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ABSTRACT

The cytochromes P450 (P450s) are a remarkable class of heme enzymes that catalyze the metabolism of xenobiotics and the biosynthesis of signaling molecules. Controlled electron flow into the thiolate-ligated heme active site allows P450s to activate molecular oxygen and hydroxylate aliphatic C–H bonds via the formation of high-valent metal-oxo intermediates (compounds I and II). Due to the reactive nature and short lifetimes of these intermediates, many of the fundamental steps in catalysis have not been observed directly. The Gray group and others have developed photochemical methods, known as "flash-quench," for triggering electron transfer (ET) and generating redox intermediates in proteins in the absence of native ET partners. Photo-triggering affords a high degree of temporal precision for the gating of an ET event; the initial ET and subsequent reactions can be monitored on the nanosecond-to-second timescale using transient absorption (TA) spectroscopies. Chapter 1 catalogues critical aspects of P450 structure and mechanism, including the native pathway for formation of compound I, and outlines the development of photochemical processes that can be used to artificially trigger ET in proteins. Chapters 2 and 3 describe the development of these photochemical methods to establish electronic communication between a photosensitizer and the buried P450 heme. Chapter 2 describes the design and characterization of a Ru-P450-BM3 conjugate containing a ruthenium photosensitizer covalently tethered to the P450 surface, and nanosecond-to-second kinetics of the photo-triggered ET event are presented. By analyzing data at multiple wavelengths, we have identified the formation of multiple ET intermediates, including the catalytically relevant compound II; this intermediate is generated by oxidation of a bound water molecule in the ferric resting state enzyme. The work in Chapter 3 probes the role of a tryptophan residue situated between the

photosensitizer and heme in the aforementioned Ru-P450 BM3 conjugate. Replacement of this tryptophan with histidine does not perturb the P450 structure, yet it completely eliminates the ET reactivity described in Chapter 2. The presence of an analogous tryptophan in Ru-P450 CYP119 conjugates also is necessary for observing oxidative ET, but the yield of heme oxidation is lower. Chapter 4 offers a basic description of the theoretical underpinnings required to analyze ET. Single-step ET theory is first presented, followed by extensions to multistep ET: electron "hopping." The generation of "hopping maps" and use of a hopping map program to analyze the rate advantage of hopping over single-step ET is described, beginning with an established rhenium-tryptophan-azurin hopping system. This ET analysis is then applied to the Ru-tryptophan-P450 systems described in Chapter 2; this strongly supports the presence of hopping in Ru-P450 conjugates. Chapter 5 explores the implementation of flash-quench and other phototriggered methods to examine the native reductive ET and gas binding events that activate molecular oxygen. In particular, TA kinetics that demonstrate heme reduction on the microsecond timescale for four Ru-P450 conjugates are presented. In addition, we implement laser flash-photolysis of P450 ferrous–CO to study the rates of CO rebinding in the thermophilic P450 CYP119 at variable temperature. Chapter 6 describes the development and implementation of air-sensitive potentiometric redox titrations to determine the solution reduction potentials of a series of P450 BM3 mutants, which were designed for non-native cyclopropanation of styrene *in vivo*. An important conclusion from this work is that substitution of the axial cysteine for serine shifts the wild type reduction potential positive by 130 mV, facilitating reduction by biological redox cofactors in the presence of poorly-bound substrates. While this mutation abolishes oxygenation activity, these mutants are capable of catalyzing the cyclopropanation of styrene, even within the confines of an *E. coli* cell. Four appendices are also provided, including photochemical heme

oxidation in ruthenium-modified nitric oxide synthase (Appendix A), general protocols (Appendix B), Chapter-specific notes (Appendix C) and Matlab scripts used for data analysis (Appendix D).

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