II-D Structure and Function of Metalloproteins and Its Molecular Design

Metal ion is a common cofactor that is crucial for active centers of proteins involved in many biologically important processes in cells, and a relatively small number of metal-based prosthetic groups are utilized to serve numerous and diverse chemical functions. A typical metal-based prosthetic group, which represents a fascinating example in this respect, is heme. Heme promotes a variety of functions, such as dioxygen storage, activation of small molecules, electron transfer reactions, and sensing gaseous molecule. In the field of protein design and engineering, hemoproteins also make particularly attractive targets. There are many reasons for this, including the exciting possibility of engineering protein-based molecules with useful catalytic, electronic or optoelectronic properties. Based on various kinds of spectroscopies, we have functionally and structurally characterized some hemoproteins including newly identified heme-regulated proteins, and designed hemoproteins showing improved activities and new functions.

II-D-1 L358P Mutation on P450cam Simulates Structural Changes upon Putidaredoxin Binding. The Structural Changes Trigger Electron Transfer to Oxy-P450cam from Electron Donors

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To investigate the functional and structural characterization of a crucial cytochrome P450cam (P450cam)-putidaredoxin (Pdx) complex, we utilized a mutant whose spectroscopic property corresponds to the properties of the wild type P450cam in the presence of Pdx. The ^{1}H NMR spectrum of the carbonmonoxy adduct of the mutant, the Leu-358 3 Pro mutant (L358P), in the absence of Pdx showed that the ring current-shifted signals arising from D-camphor were upfield-shifted and observed as resolved signals, which are typical for the wild type enzyme in the presence of Pdx. Signals from the β-proton of the axial cysteine and the γ-methyl group of Thr-252 were also shifted upfield and downfield, respectively, in the L358P mutant as observed for Pdx-bound wild type P450cam. The close similarity in the NMR spectra suggests that the heme environment of the L358P mutant mimics that of the Pdx-bound enzyme. The functional analysis of the L358P mutant has revealed that the oxygen adduct of the L358P mutant can promote the oxygenation reaction for D-camphor with nonphysiological electron donors such as dithionite and ascorbic acid, showing that oxygenated L358P is "activated" to receive electron from the donor. Based on the structural and functional characterization of the L358P mutant, we conclude that the Pdx-induced structural changes in P450cam would facilitate the electron transfer from the electron donor, and the Pdx binding to P450cam would be a trigger for the electron transfer to oxygenated P450cam.

II-D-2 Structural Diversities of Active Site in Clinical Azole Bound Forms between Sterol 14α-demethylases (CYP51) from Human and *Mycobacterium tuberculosis*

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To gain insights into the molecular basis of the design for the selective azole anti-fungals, we compared the binding properties of azole-based inhibitors for cytochrome P450 sterol 14α-demethylase (CYP51) from human (HuCYP51) and *Mycobacterium tuberculosis* (MtCYP51). Spectroscopic titration of azoles to the CYP51s revealed that HuCYP51 has higher affinity for ketoconazole (KET), an azole derivative that has long lipophilic groups, than MtCYP51, but the affinity for fluconazole (FLU), which is a member of the antifungal armamentarium, was lower in HuCYP51. The affinity for 4-phenylimidazole (4-PhIm) to MtCYP51 was quite low compared with that to HuCYP51. In the resonance Raman spectra for HuCYP51, the FLU binding induced only minor spectral changes, whereas the prominent high frequency shift of the bending mode of the heme vinyl group was detected in the KET- or 4-PhIm-bound forms. On the other hand, the bending mode of the heme propionate group for the FLU-bound form of MtCYP51 was shifted to high frequency as found for the KET-bound form, but that for 4-PhIm was shifted to low frequency. The EPR spectra for 4-PhIm-bound MtCYP51 and FLUbound HuCYP51 gave multiple g values, showing heterogeneous binding of the azoles, whereas the single g_x and g_z values were observed for other azole-bound forms. Together with the alignment of the amino acid sequence, these spectroscopic differences suggest that the region between the B' and C helices, particularly the hydrophobicity of the C helix, in CYP51s plays primary roles in determining strength of interactions with azoles; this differentiates the binding specificity of azoles to CYP51s.

II-D-3 Two Heme Binding Sites Are Involved in the Regulated Degradation of the Bacterial Iron Response Regulator (Irr) Protein

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The iron response regulator (Irr) protein from *Bradyrhizobium japonicum* is a conditionally stable protein that degrades in response to cellular iron availability. This turnover is heme-dependent, and rapid degradation involves heme binding to a heme regulatory motif (HRM) of Irr. Here, we show that Irr confers iron-dependent instability on glutathione *S*-transferase (GST) when fused to it. Analysis of Irr-GST derivatives with C-terminal truncations of Irr implicated a second region necessary for degradation, other than the HRM, and showed that the HRM was not sufficient to confer instability on GST. The HRM-defective mutant IrrC29A degraded in the presence of iron but much more slowly than the wild-type protein. This slow turnover was heme-dependent, as discerned by the stability of Irr in a heme-defective mutant strain. Whereas the HRM of purified recombinant Irr binds ferric (oxidized) heme, a second site that binds ferrous (reduced) heme was identified based on spectral analysis of truncation and substitution mutants. A mutant in which histidines 117–119 were changed to alanines severely diminished ferrous, but not ferric, heme binding. Introduction of these substitutions in an Irr-GST fusion stabilized the protein *in vivo* in the presence of iron. We conclude that normal iron-dependent Irr degradation involves two heme binding sites and that both redox states of heme are required for rapid turnover.

II-D-4 Involvement of Heme Regulatory Motif in Heme-Mediated Ubiquitination and Degradation of IRP2

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Iron regulatory protein 2 (IRP2), a regulator of iron metabolism, is modulated by ubiquitination and degradation. We have shown that IRP2 degradation is triggered by heme-mediated oxidation. We report here that not only Cys201, an invariant residue in the heme regulatory motif (HRM), but also His204 is critical for IRP2 degradation. Spectroscopic studies revealed that Cys201 binds ferric heme, whereas His204 is a ferrous heme binding site, indicating the involvement of these residues in sensing the redox state of the heme iron and in generating the oxidative modification. Moreover, the HRM in IRP2 has been suggested to play a critical role in its recognition by the HOIL-1 ubiquitin ligase. Although HRMs are known to sense heme concentration by simply binding to heme, the HRM in IRP2 specifically contributes to its oxidative modification, its recognition by the ligase, and its sensing of iron concentration after iron is integrated into heme.