Engineering a Ligand Binding Pocket into a Four-Helix Bundle Protein Cytochrome b_{562}

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Many of the reactions of molecular oxygen and its derivatives in biological systems are orchestrated by heme-containing enzymes. The capacity of these proteins to direct their cofactors to perform a variety of chemical reactions makes them attractive candidates for the engineering of novel redox catalysts. A ligand binding pocket is a prerequisite for generating functional diversity and considerable effort has been directed at the construction of a heme pocket in which exogenous ligands can be bound. Four helix bundle structures having histidine residues as heme iron ligands[1] have advantages for protein engineering because they are amenable to total synthesis and they provide a robust scaffold for protein design. However, a serious limitation of this approach has been the low affinity of the engineered polypeptides/proteins for the cofactor, resulting in facile heme loss during handling. We therefore analyzed the amino acid residues surrounding the cofactor in the structures of a four-helix bundle heme protein, cytochrome b_{562} from *Escherichia coli*, and developed a strategy for designing an exogenous ligand binding pocket into this protein. Here we report the construction and characterization of a cytochrome b_{562} variant which binds CO reversibly to form stable ferrous CO adducts, as revealed by resonance Raman spectroscopy. This represents the first step in the engineering of proteins designed to perform a variety of catalytic and electron-transfer reactions utilizing heme chemistry.

Our approach was simple and straightforward. In cytochrome b_{562} the iron is axially coordinated[2] to $N_{\epsilon 2}$ of His-102 and the sulfur of Met-7 (Figure 1). To create a pocket for an exogenous ligand, one of the axial heme ligands Met-7 was replaced by Gly or Ala using site-directed mutagenesis. The resulting single mutants M7G and M7A were, as anticipated, unstable, and the heme group escaped easily from the proteins during purification. We therefore analyzed the structure of wild-type cytochrome b_{562} in an attempt to establish a rationale for restoring heme stability to the mutants.

Three major factors contribute to cofactor retention in heme proteins:[3] (1) covalent bonding between the heme iron and axial ligand residues, (2) apolar interactions between the heme and surrounding hydrophobic residues, and (3) polar interactions between the heme propionates and surrounding hydrophilic residues. Leaving the remaining axial ligand, His-102, and the apolar pocket residues unchanged to satisfy criteria 1 and 2, we

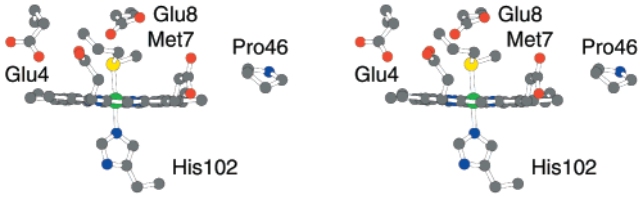

Figure 1. Residues surrounding the heme propionate oxygens of wild-type cytochrome b_{562}. The figure was drawn using the atomic coordinate set 256B.pdb[2]. The coloring is by atom type: C (gray), O (red), N (blue), S (yellow), and Fe (green).

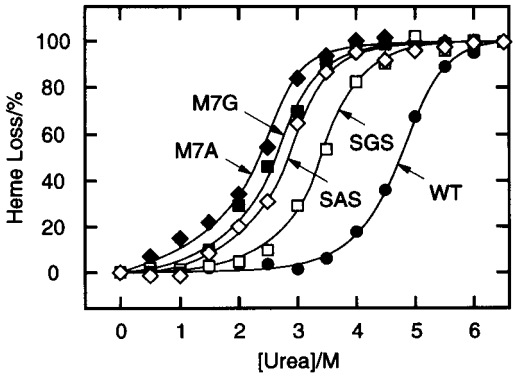

Figure 2. Urea denaturation of wild-type and mutant cytochromes b_{562}. The absorbance at the Soret maxima was monitored following addition of increasing amounts of urea and was normalized. The spectra of ~5 μM ferric protein were measured in 10 mM sodium phosphate and 0.1 M NaCl (pH 7.0). Key: solid circles, wild-type cytochrome b_{562}; open squares, SGS; open diamonds, SAS; solid squares, M7G; solid diamonds, M7A.

confined our analysis to the residues surrounding the propionate oxygens. Although there are minor differences between the two molecules in the asymmetric unit of crystals of wild-type cytochrome b_{562},[2] in both molecules two Glu side chains are in close proximity to the heme propionate oxygens (Figure 1). This is expected to result in an energetically unfavorable electrostatic repulsion between the negatively charged propionates and the glutamates. Unlike in the wild-type protein, Fe coordination by Met-7 no longer provides compensating effects for heme binding in the mutants. Thus, these two residues, Glu-4 and Glu-8, were the target for further mutation. Positively charged Arg or Lys are plausible replacements, but the aliphatic stems of these amino acids are likely to be too long to permit favorable interactions of the guanidino and amino groups respectively with the propionates. We thus replaced the two Glu residues by Ser simultaneously. Although Ser is a neutral amino acid, the hydroxyl group is expected to stabilize the heme through the formation of hydrogen bonds to the propionates, assuming that the side chain geometries remain unchanged.

Heme stability was evaluated by urea-induced denaturation of the triple mutants, E4S/M7G/E8S (SGS) and E4S/M7A/E8S (SAS) (Figure 2).[4] The midpoint urea concentration is 4.8 M for the wild-type protein corresponding to a free energy change in

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(4) The cytochrome gene was amplified by PCR from an *E. coli* B strain (NCIB11595) and cloned into the *Hin*dIII-*Eco*RI site of pBluescriptII SK(+) plasmid with XL-1 Blue MRF′ as the host cell. The amino acid sequence was the same as cytochrome b_{562} from another *E. coli* B strain.[13,16] Recombinant cytochromes were expressed from *E. coli* NM554 cells grown in (2×) TY broth, and the holoproteins were purified directly under air without the need for reconstitution with hemin. The protein was purified as reported previously.[17] The mutants were oxidized by the addition of potassium ferricyanide, followed by extensive dialysis to remove excess oxidant. Ferrous protein was prepared with the addition of sodium dithionite under nitrogen gas.

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water[5] (ΔG_{H_2O}) of 6.6 kcal/mol, as reported previously.[6] This change is thought to reflect heme loss.[6] The ΔG_{H_2O} values for the triple mutants SAS (3.3 kcal/mol) and SGS (3.9 kcal/mol) are lower but significantly higher than those for the single mutants M7A (2.7 kcal/mol) and M7G (3.2 kcal/mol), indicating that the strategy for the heme stabilization has been successful and that the stabilization energy conferred by the Ser mutations amounts to ~0.6 kcal/mol. This value corresponds to almost half of the heme stabilization conferred by a covalent thioether linkage of the heme to Cys-98 in the cytochrome b_{562} mutant R98C (1.3 kcal/mol).[7] Our results demonstrate that altering protein/cofactor propionate interactions is a viable strategy for reducing heme loss in cytochrome b_{562}. This supports the proposal based on observations with myoglobin[3] that engineering interactions with the heme propionates may be the most efficacious route to engineering proteins resistant to heme loss. Although the increases in heme affinity are modest, alterations of residues around the propionates are unlikely to influence strongly the chemistry at the iron.

The purified triple mutants exhibit absorption maxima at 423, 540, and 572 nm. These spectral profiles are distinct from those of the deoxy (433 and 559 nm) and ferric (407, 503, and 632 nm) forms of the mutants, suggesting the metal is 6-coordinate and ferrous. The presence of a CO ligand is suggested by the absorption maxima of the carbonmonoxy form (424, 541, and 571 nm). Irradiation of freshly purified samples of the triple mutants at 10 °C with a xenon lamp at 420 nm (200 W/m^2) led to progressive oxidation of the sample ($t_{1/2}$ = 34.7 min) presumably due to CO photodissociation followed by oxygen-mediated oxidation. Although recombinant heme proteins are most commonly isolated in the ferric forms, a distal histidine mutant of myoglobin (H64L) with an unusually high CO affinity was purified as the carbonmonoxy form,[8] the source of the CO being intracellular heme degradation.

Resonance Raman analysis (Figure 3) revealed CO-isotope sensitive lines at about 490 and 1970 cm^{-1} in the SGS mutant. These bands, which can be assigned to the ν(Fe−CO) and ν(C−O) stretching modes, respectively, were also observed for the SAS mutant. The ν(Fe−CO)/ν(C−O) frequency pair is similar to that of H64L myoglobin (489 and 1966 cm^{-1}),[9] suggesting an apolar environment in the heme pocket consistent with expectations based on the crystal structure and with the implied high CO affinity. The CO form shows no spectral change after storage for a month at 10 °C; moreover it is stable under the illumination of a 406.7 nm focused laser beam in a deoxygenated solution. This character of the CO form is striking in comparison with other heme proteins, and implies that the ligand is enclosed. Collectively, these observations demonstrate that we have engineered heme stability and reversible ligand binding in cytochrome b_{562} by triple mutation.

Although cytochromes c' from photosynthetic bacteria share a common four-helix bundle architecture with cytochrome b_{562}, no significant sequence similarity is observed[10] and the two classes of protein may have evolved independently.[11] The cytochromes c' contain five-coordinate high-spin heme, which is covalently

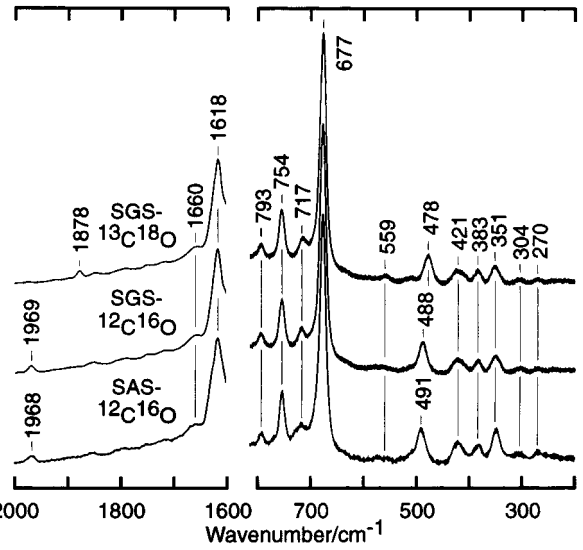

Figure 3. Resonance Raman spectra of the ferrous CO-bound triple mutants. From top: SGS-^{13}C^{18}O, SGS-^{12}C^{16}O, and SAS-^{12}C^{16}O. The spectra were recorded with a double monochromator (Jasco R-800), following excitation by a krypton ion laser (406.7 nm line, Coherent I-302) with a laser power of 50 mW at the sample. A spinning Raman cell was used. The samples contained 100 μM mutant proteins in 10 mM sodium phosphate and 0.1 M NaCl (pH 7.0).

attached to the protein through two thioether linkages to cysteine residues.[12] The physiological function (if any) of cytochrome b_{562} is unknown. It has a curious periplasmic location and is dispensable in *E. coli*, indeed the K strain lacks an initiation codon for this protein and its gene is presumably not expressed.[13] Nature has developed alternative strategies for stabilizing heme in these four-helix bundle cytochromes (1) through coordination of the Fe to two axial protein ligands or (2) through covalent linkage of the heme vinyl groups to Cys residues.[14] Here, we have exploited a quite different method of heme stabilization and, in some sense, revitalized a protein that may be regarded as an evolutionary molecular fossil. The simple four-helix bundle module is a useful platform on which to graft specific ligand binding sites, this work perhaps representing the first stage in the engineering of biocatalysts with novel functions based on heme chemistry. The next step is the introduction of suitable polar residues into the heme pocket with the aim of stabilizing a bound O_2 ligand through hydrogen bonding.[15]

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