Functional Cavities in Proteins: A General Method for Proximal Ligand Substitution in Myoglobin

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Received December 9, 1993

The amino acid ligands to the heme iron provide an important distinction between different classes of heme proteins. For example, the peroxidases and the oxygen carrier proteins (Mb and hemoglobin) bear a proximal His, electron-transfer proteins such as cytochrome c have His, Met, and Lys ligands, and oxidases such as cytochrome P450 and catecholase contain Cys and Tyr ligands, respectively. In contrast to chemical transfer proteins such as cytochrome P450, Mb and hemoglobin have broader implications for protein engineering in vivo, with advantages in preserving specificity, and ease of manipulation provided by the protein matrix. We report a simple method to achieve this goal for model systems, where a large range of ligands has been used to modulate heme function;1 replacement of the proximal ligand in Mb has been limited in scope to the natural amino acids Cys and Tyr.2,3 In order to understand the vast range of reactivities among heme proteins, it would be ideal to Possess the flexibility to vary the proximal ligand, while retaining the structure, recognition, specificity, and ease of manipulation provided by the protein matrix. We report a simple method to achieve this goal for myoglobin, with broader implications for protein engineering in general.4

When the proximal His residue 93 in sperm whale Mb is changed to Gly by site-directed mutagenesis and the protein is expressed in E. coli, a protein is isolated which has a molecular weight increased to about 15 kDa, a strong absorption at 410 nm, and a hyperfine-shifted 1H NMR spectra of the para-methyl group. For experimental details, see ref 8. Our strategy for substituting the proximal His was to exchange the imidazole ligand for other small organic ligands. We have discovered that a wide range of small organic ligands can replace Im in the proximal binding site simply by addition of excess of the ligand to a solution of ferric H93G(Im) and yields similar absorption spectra (Figure 2). Ligand exchange is completely reversible, as addition of Im to the other ligand-substituted proteins yields back the spectrum of the Im-ligated protein (data not shown). The hyperfine-shifted 1H NMR spectra of the paramagnetic metmyocyanin complexes of heme proteins and models are exquisitely sensitive to proximal ligand orientation.11 The pattern of the hyperfine-shifted heme methyl resonances of H93G(Im)-CN is different from that of WT MbCN (Figure 3A and B), consistent with the different orientation of the Im and His ligands observed in their respective crystal structures.12 Addition of excess deuterated Im (Im-d3) results in the loss of a peak at

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(7) Relevant differences in the structure of H93G(Im) compared to WT reported in ref 6 include (1) Fe–N bond length 0.3 Å shorter; (2) imidazole plane rotated 36° relative to the His93 plane; (3) nonliganded Im N atom 0.2 Å closer to the hydroxyl group of Ser92, adopting a favorable hydrogen-bonding geometry; (4) 0.6 Å increase in the distance between Fe and the distal His93 and Lys95; and (5) Fe 0.2 Å closer to the plane of the heme.

Figure 1. Schematic diagram of heme pocket residues in carbonmonoxy myoglobin mutant H93G(Im), illustrating the replacement of proximal His93 with free imidazole and further exchange with other small ligands L.

Figure 2. Ligand rebinding kinetics following flash photolysis of CO from H93G(Im) and H93G(L), exactly as for WT and H93G(Im), and yields similar absorption spectra (Figure 2). Ligand exchange is completely reversible, as addition of Im to the other ligand-substituted proteins yields back the spectrum of the Im-ligated protein (data not shown). The hyperfine-shifted 1H NMR spectra of the paramagnetic metmyocyanin complexes of heme proteins and models are exquisitely sensitive to proximal ligand orientation. The pattern of the hyperfine-shifted heme methyl resonances of H93G(Im)-CN is different from that of WT MbCN (Figure 3A and B), consistent with the different orientation of the Im and His ligands observed in their respective crystal structures. Addition of excess deuterated Im (Im-d3) results in the loss of a peak at
The diamagnetic and paramagnetic NMR data together indicate that ligand exchange perturbs the proximal but not the distal side. The chemical shifts of the Va168C,Ho protons of MbCO are sensitive to structural changes on the proximal side and can be used to test whether the observed shifts originate from the proximal or the distal side. For example, modification of the distal His of WT Mb (Decatur et al., unpublished observations) leads to a shift in the Val68qHs resonance ascribed to the proximal side. However, such modifications do not significantly affect the chemical shifts of the proximal imidazole, thereby indicating that the shift is a consequence of changes on the proximal side. This ligand exchange strategy proves to be remarkably general and can be used to probe more radical changes in the proximal cavity. We have initially focused on replacing Im with phenol and ethanethiol, as they are the analogs of the amino acids Tyr and Cys, which have been introduced into Mb by conventional mutagenesis of His93. Replacement of Im in H93G(Im) by these ligands leads to very large changes in the absorption spectrum. As shown in Table 1, the spectrum of H93G(phenol) is quite similar to that of H93Y and catalase, while the spectrum of H93G(ethanethiol) is very similar to that of H93C. The CO rebinding kinetics of H93G(ethanethiol) and H93G(phenol) are quite different from those of H93G(Im) (Figure 2). The CO recombination kinetics vary widely with different exogenous ligands, thus the possibility that the distal His64 is serving as the fifth ligand, with CO bound on the proximal side, is ruled out. As shown in Table 1 and Figure 2, we have also replaced Im with other ligands, such as furan and thiophene, thereby introducing entirely unnatural functionality into the cavity created by removal of His93 and producing large effects on ligand binding kinetics.

Replacement of the proximal His with exogenous ligands generates a rich variety of novel proteins. With this exchange method it is possible to systematically alter the size, chemical, and isotopic properties of the proximal ligand, using methodology which is much simpler than the chemical or biosynthetic introduction of unnatural amino acids. In combination with other distal- and proximal-side changes produced by conventional site-directed mutagenesis, it should be possible to discover the precise mechanisms by which the protein modulates heme function. Extensions of this ligand exchange strategy are in progress to examine the role of the proximal ligand in determining cooperativity in Hb and in modulating the binding of chlorophylls in photosynthetic proteins and to probe electron transfer pathways in proteins.

Acknowledgment. We thank Professor Steven G. Sligar for his gift of the sperm whale Mb expression system. G.D.P. is supported in part by an NIH Postdoctoral Fellowship; S.M.D. is the recipient of an NSF Predoctoral Fellowship. This work was supported in part by a grant from the National Institutes of Health.

(14) The chemical shift of the Val68C,H2 protons is sensitive to the geometry of the distal pocket. For example, modification of the distal His of WT SW Mb with the tetrazole moiety leads to a shift in the Val68C,H2 resonance indicative of conformational changes at His64 (Adachi, I.; Mar, F. G.; Noguchi, K.; Takahashi, K.; Kikuchi, T.; Demizu, Y.; Osbahr, A. J.; Eichhorn, G. L. J. Biol. Chem. 1962, 237, 1820-1824. High-spin ferric, camphor-bound.13) Reference 3.

(15) Im and simple thiols have been shown to bind weakly to the distal site of SW WT Mb (Sono, M.; Anderson, L. A.; Dawson, J. H. J. Biol. Chem. 1982, 257, 8308-8320) but only at a much higher concentration of ligand than employed here.

(16) The Soret band maximum in H93G(MbCO) is found at 420 nm, while the maximum of the corresponding complex in cysteine-ligated P450 is 450 nm.2 This led the authors to conclude that distal His64 replaces the proximal Cys ligand as the fifth ligand, with CO bound in the proximal pocket. However, the results are consistent with model studies of CO complexes in which both ligands, including Im, ethers, thiols, and thiocarbamates, all yield 420 nm Soret maxima. The Soret band shifts to 450 nm only when a thiol ligand is deprotonated (Collman, J. P.; Sorrell, T. N. Am. Chem. Soc. 1975, 97, 4313-4314).

(17) We assume that as for WT Mb, no geminate recombinations occur on the subnanosecond time scale for CO (see, e.g., Cornellia, P. A.; Stolle, A. W.; Chernoff, D. A.; Hochstrasser, R. M. Proc. Natl. Acad. Sci. U.S.A. 1981, 78, 7526--7529). This assumption is being tested by picosecond time-resolved measurements.

Figure 3. Comparison of 1H NMR spectra of WT MbCN and H93G-(Im)CN, taken in 90% H2O buffer, pH 9.0, 30 °C, and H93G(1-MeIm)-CN, taken in D2O buffer, pH 7.0 (500 MHz). Assignments for WT Mb are from the work of La Mar and coworkers; assignments for H93G(Im) are based on 1D NOE, 2D NOESY, and COSY spectra, and isotopic labeling of the heme and the imidazole as in ref 11. The inset above the H93G-(Im) spectrum shows the disappearance over a period of 50 min of an Im proton upon addition of an excess of Im-d5 to H93G(Im) in D2O buffer, pH 7.0. The small extra peak at 12.0 ppm in the parent spectrum taken in H2O buffer, pH 9.0, is an exchangeable amide backbone proton.

12.1 ppm (inset, Figure 3B), unambiguously demonstrating that the proximal Im ligand is reversibly exchanged with exogenous ligands.13 Substitution with other ligands yields unique and characteristic spectra (e.g., 1-methylimidazole, Figure 3C). We have also obtained the 1H NMR spectra of the diamagnetic FeII CO complexes of H93G(Im), because the chemical shifts of the Val68 protons of MbCO are sensitive to structural changes on the distal side.14 The chemical shifts of the Val68C,H2 protons of all H93G(Im) are essentially the same as WT (data not shown).

Thus, the diamagnetic and paramagnetic NMR data together indicate that ligand exchange perturbs the proximal but not the distal side of the heme pocket. Finally, addition of the same ligands to solutions of the WT protein produces none of the changes reported above for H93G.14 These findings support the contention that exogenous ligands replace Im specifically in the proximal cavity of H93G.

Table 1. Electronic Absorption Spectra of Proteins

<table>
<thead>
<tr>
<th>protein</th>
<th>soret (nm)</th>
<th>relative soret*</th>
<th>visible (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Mb</td>
<td>408 (N)</td>
<td>502, 632</td>
<td></td>
</tr>
<tr>
<td>H93G(Im)</td>
<td>409 (N)</td>
<td>1.00</td>
<td>503, 629</td>
</tr>
<tr>
<td>H93G(pyr)</td>
<td>408 (N)</td>
<td>0.92</td>
<td>497, 630</td>
</tr>
<tr>
<td>catalase</td>
<td>405 (B)</td>
<td>620</td>
<td></td>
</tr>
<tr>
<td>H93Y Mb</td>
<td>402 (B)</td>
<td>480, 598</td>
<td></td>
</tr>
<tr>
<td>H93G(phenol)</td>
<td>403 (B)</td>
<td>605</td>
<td></td>
</tr>
<tr>
<td>P450ane</td>
<td>391 (VB)</td>
<td>510, 600</td>
<td></td>
</tr>
<tr>
<td>H93C human Mb</td>
<td>391 (VB)</td>
<td>509, 629</td>
<td></td>
</tr>
<tr>
<td>H93G(ethanethiol)</td>
<td>393 (VB)</td>
<td>505, 626</td>
<td></td>
</tr>
<tr>
<td>H93G(thiophene)</td>
<td>393 (VB)</td>
<td>505, 622</td>
<td></td>
</tr>
<tr>
<td>H93G(furan)</td>
<td>408 (N)</td>
<td>497, 629</td>
<td></td>
</tr>
</tbody>
</table>

*Width at half-height: N, narrow (<30 nm); B, broad (>40 nm); VB, very broad (>50 nm). *Measured relative to H93G(Im); all H93G(L) at same concentration. **Both human* and sperm whale.13

Acknowledgment. We thank Professor Steven G. Sligar for his gift of the sperm whale Mb expression system. G.D.P. is supported in part by an NIH Postdoctoral Fellowship; S.M.D. is the recipient of an NSF Predoctoral Fellowship. This work was supported in part by a grant from the National Institutes of Health.