CONTRIBUTION OF ORDERED WATER MOLECULES AND A CRUCIAL PHENYLALANINE TO COOPERATIVE PATHWAY(S) IN SCAPHARCA DIMERIC HEMOGLOBIN

A Dissertation Presented
by
Animesh Dev Pardanani

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CONTRIBUTION OF ORDERED WATER MOLECULES AND A CRUCIAL PHENYLALANINE TO COOPERATIVE PATHWAY(S) IN SCAPHARCA DIMERIC HEMOGLOBIN

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Abstract

The homodimeric hemoglobin (HbI) from the blood clam Scapharca inaequivalvis binds oxygen cooperatively and thus offers a simple model system for studying communication between two chemically identical sites. Although the individual subunits of HbI have the same myoglobin-fold as mammalian hemoglobins, the quaternary assemblage is radically different. Upon oxygen binding by HbI, only small tertiary changes are seen at the subunit interface in contrast to the relatively large quaternary changes observed with mammalian
hemoglobins. Analysis of structures of this hemoglobin in the liganded (O2 or CO) and unliganded states has provided a framework for understanding the role of individual amino acid side-chains in mediating cooperativity. The work presented in this dissertation has directly tested the central tenets of the proposed structural mechanism for cooperativity in HbI, illuminating the key roles played by residue Phe 97 and interface water molecules in intersubunit communication.

**Heterologous expression of Scapharca dimeric hemoglobin**: A synthetic gene has been utilized to express recombinant HbI in *Escherichia coli*. The HbI apoprotein constitutes 5-10% of the total bacterial protein in this system. Addition of the heme precursor δ-aminolevulinic acid to the expression culture results in a ~3-fold increase in the production of soluble hemoglobin. Recombinant HbI has been successfully purified to homogeneity, resulting in a final yield of 80-100 mg of pure holoprotein from a 12 L expression culture. Analysis of recombinant HbI reveals its oxygen binding properties to be indistinguishable from native HbI. It was necessary to correct a protein sequence error by mutating residue Asn 56 to aspartate in order to obtain diffraction quality crystals, that are isomorphous to native HbI crystals. These recombinant HbI crystals diffract to high resolution, permitting the functional effects of mutant HbI proteins to be correlated with detailed structural analysis.

**Role of residue Phe 97**: Residue Phe 97, which is proposed to play a central role in the cooperative functioning of *Scapharca* dimeric hemoglobin, has been mutated to leucine. In wild-type HbI, the side-chain of residue Phe 97 packs in the heme pocket in the deoxy state but is extruded into the subunit interface upon ligand binding. Mutation to leucine results in an eight-fold
increase in oxygen affinity and a marked decrease in cooperativity. Kinetic measurements of ligand binding to the Leu 97 mutant suggest an altered unliganded (deoxy) state. Analysis of high resolution crystal structures at allosteric end points reveals them to be remarkably similar to the corresponding wild-type structures with differences confined to the disposition of residue 97 side-chain, F-helix geometry and the interface water structure. Increased oxygen affinity results from the absence of the Phe 97 side-chain, whose tight packing in the heme pocket of the deoxy state normally restricts the heme from obtaining a high affinity conformation. Diminished cooperativity results from a lack of the ligand-linked extrusion of Phe 97 into the interface, as Leu 97 packs in the heme pocket in both states. These results confirm the central role of Phe 97 in determining the oxygen binding properties of HbI and clearly illustrate the role of proximal strain in regulating ligand affinity. Residual cooperativity is coupled with the observed heme movements and suggests that parallel pathways for communication exist in Scapharca dimeric hemoglobin.

Role of well-ordered interface water molecules: Ligand-induced structural transitions lead to the disruption of a network of well-ordered water molecules in the subunit interface of Scapharca dimeric hemoglobin. These water molecules have been suggested to be involved in stabilizing the two allosteric end-points. Residue Thr 72, whose side-chain is positioned to form a hydrogen bond with a water molecule at the periphery of the deoxy water cluster, has been mutated to valine to test the importance this cluster. This isosteric mutation results in a more than 40-fold increase in oxygen affinity and, surprisingly, enhancement of cooperativity. The only significant structural effects of this mutation are a loss of the water molecule that is normally hydrogen bonded to
each Thr 72 side-chain, and destabilization of the remaining water molecules. High resolution crystal structures of a related mutant, Thr 72 to Ile, which exhibits a similar increase in oxygen affinity but markedly diminished cooperativity, have also been determined at the allosteric end points. The deoxy Ile 72 structure is virtually identical to the corresponding Val 72 structure, revealing a destabilized interface water cluster, while the CO-structure comparison shows more extensive van der Waals interactions between the side-chains of Phe 97 and Ile 72. These studies confirm the crucial role of crystallographically observed interface water molecules in cooperative functioning and illustrate how the intricate balance between alternate allosteric states can be significantly altered by subtle structural perturbations.

The experiments described here have directly demonstrated the contributions of residue Phe 97 and the interface water cluster in modulating the ligand binding properties of Scapharca HbI. This has confirmed important aspects of the previously proposed mechanism for cooperativity, but has additionally revealed a crucial role of interface water molecules in intersubunit communication. As a result, an elegantly simple mechanism for cooperativity emerges in which the ligand-induced movement of Phe 97 into the interface, disrupts a cluster of well ordered water molecules, whose integrity is key for stability of the low affinity state. The results described here thus reveal how ligand-induced changes in heme conformation of one subunit are coupled to the transfer of information across the dimer interface in HbI.
ACKNOWLEDGEMENTS

I dedicate this thesis, and the effort it embodies, to my parents, Nirmala and Dev Pardanani, for their unwavering love and support in every quest, weighty or trivial, that I have undertaken, and to my sister, Samira Pardanani, who, with panache and aplomb, makes our family complete in more ways than one.

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I thank Dr. Franca Ascoli for providing me with purified T72I hemoglobin and Dr. Gianni Colotti, for oxygen binding studies with the F97L mutant.

Lastly, I thank all my friends at UMMC for their support and camaraderie and in making graduate school a truly enjoyable experience.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>ALA</td>
<td>δ-aminolevulinic acid</td>
</tr>
<tr>
<td>B-factor</td>
<td>isotropic temperature factor</td>
</tr>
<tr>
<td>c</td>
<td>ratio between intrinsic affinities of the T and R states of unliganded hemoglobin</td>
</tr>
<tr>
<td>CHES</td>
<td>(2-[N-cyclohexylamino]-ethanesulfonic acid)</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>F</td>
<td>crystallographic structure factor</td>
</tr>
<tr>
<td>F97A</td>
<td>Phe 97 to Ala mutant of <em>Scapharca</em> HbI</td>
</tr>
<tr>
<td>F97L</td>
<td>Phe 97 to Leu mutant of <em>Scapharca</em> HbI</td>
</tr>
<tr>
<td>F97Y</td>
<td>Phe 97 to Tyr mutant of <em>Scapharca</em> HbI</td>
</tr>
<tr>
<td>F_c</td>
<td>calculated structure factor</td>
</tr>
<tr>
<td>F_0</td>
<td>observed structure factor</td>
</tr>
<tr>
<td>HbA</td>
<td>human hemoglobin</td>
</tr>
<tr>
<td>HbI</td>
<td>wild-type <em>Scapharca</em> dimeric hemoglobin</td>
</tr>
<tr>
<td>HEPES</td>
<td>(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])</td>
</tr>
<tr>
<td>I</td>
<td>Intensity of measured reflections</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>KNF</td>
<td>Koshland-Nemethy-Filmer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>L</td>
<td>Allosteric constant (Ratio between R and T states of unliganded hemoglobin)</td>
</tr>
<tr>
<td>Mb</td>
<td>Myoglobin</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular Dynamics</td>
</tr>
<tr>
<td>MWC</td>
<td>Monod-Wyman-Changeux</td>
</tr>
<tr>
<td>n</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>p50</td>
<td>partial pressure of ligand at half-saturation of hemoglobin</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methane-sulfonyl fluoride</td>
</tr>
<tr>
<td>R-state</td>
<td>Relaxed or high affinity state</td>
</tr>
<tr>
<td>RMSD</td>
<td>root mean square deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>T72I</td>
<td>Thr 72 to Ile mutant of <em>Scapharca</em> HbI</td>
</tr>
<tr>
<td>T72S</td>
<td>Thr 72 to Ser mutant of <em>Scapharca</em> HbI</td>
</tr>
<tr>
<td>T72V</td>
<td>Thr 72 to Val mutant of <em>Scapharca</em> HbI</td>
</tr>
<tr>
<td>T-state</td>
<td>Tense or low affinity state</td>
</tr>
<tr>
<td>Torr</td>
<td>pressure in mm of Hg</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
CRYSTALLOGRAPHIC DEFINITIONS

B-factor - Isotropic temperature factor

F - Crystallographic structure factor

F₀ - Observed structure factor

Fₐ - Structure factor calculated from model

I - Intensity of measured reflections

R_{factor} = \sum |F₀| - |Fₐ| / \sum |F₀|, where F₀ is the observed structure factor and Fₐ is that calculated from the model

R_{merge} = \sum |I - <I>| / \sum I, where I is the measured intensity of each reflection and <I> is the mean value for the corresponding unique reflection

RMSD - Root mean square deviation

σ - Standard deviation
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Chapter I

Introduction

Many important biological phenomena, such as the regulation of gene expression and enzyme activity, are mediated by the efficient transfer of information between protein molecules. An understanding of the mechanism of this communication requires a thorough analysis of the underlying stereochemical details. The cooperative binding of oxygen by hemoglobin, which can be defined as the stepwise increase in its affinity for ligand as ligand binding proceeds, provides an excellent example of intersubunit communication in macromolecules.

The homodimeric hemoglobin (HbI), found in the blood clam *Scapharca inaequivalvis*, represents the simplest possible cooperative system in which oxygen binding is mediated by communication between two chemically identical ligand binding sites. In contrast to human hemoglobin, there are no known heterotropic effectors for HbI, hence, for instance, oxygen affinity and cooperativity remain unchanged in the pH range of 5.5 - 9.0 (Chiancone et al. 1981). High resolution crystallographic structures of HbI, in both unliganded (deoxy) and liganded forms, have revealed that although the individual subunits possess the classic "myoglobin fold", they are assembled into a quaternary complex that is entirely different from mammalian hemoglobins (Condon and
Royer 1994; Royer 1994) (Figure I-A). The inter-subunit interface in HbI is made up largely by the E and F helices, which in mammalian hemoglobins are exposed to bulk solvent. This results in the heme groups being brought close to each other, and suggests a shorter and more direct pathway of communication than observed in mammalian hemoglobins. Additionally, the crystal structures reveal that, in contrast to human hemoglobin, the structural changes underlying cooperativity in HbI involve relatively large tertiary conformational changes at the subunit interface, but only very minor quaternary changes (Figure I-A).

Analysis of the two allosteric states has suggested that cooperativity is mediated by significant conformational changes in only a few specific amino acid residues, the heme propionates and the well ordered interface water molecules. These elements are proposed to couple ligand-induced changes in heme conformation within one subunit, with rearrangements in protein structure that induce the opposite subunit to attain a high affinity conformation, prior to having bound ligand. This analysis has provided a framework for understanding the ligand binding properties of HbI in atomic detail and hence, given new insights into how proteins can function as molecular transducers to transmit information over long distances.

**Regulation of oxygen affinity:** Studies of mammalian hemoglobins have associated low oxygen affinity with a heme iron that is slightly displaced from the plane of the heme group. In human hemoglobin, this displaced position of the heme iron is proposed to be stabilized by the T quaternary structure, through its specific positioning of the F-helix and proximal histidine in relation to the heme group. Recent work, in which the covalent bond between the histidine imidazole side-chain and the protein main-chain was severed, has confirmed the
Figure I-A. Diagram of the Scapharca HbI structure.

These diagrams depict the structures of (top panel) deoxy-HbI at 1.6 Å resolution (PDB entry code 3SDH) and (bottom panel) HbI-CO at 1.4 Å resolution (PDB entry code 4SDH). Included is a ribbon diagram showing the tertiary structure of each subunit, bond representations for the heme group, and Phe 97 side-chain and spheres representing the approximate Van der Waals radii of oxygen atoms for core interface water molecules. Note the cluster of 17 ordered water molecules in the interface of deoxy-HbI for which Phe 97 is packed in the heme pocket. Upon ligation, by either CO or O2, Phe 97 is extruded into the interface and disrupts this water cluster, expelling six water molecules from the interface.
importance of this linkage in constraining the position of the iron atom within the T-state (Barrick et al. 1997). Switching to the R quaternary state, repositions the F-helix and proximal histidine, which removes the constraints that are normally exerted on the heme iron.

In sharp contrast to human hemoglobin, the affinity of *Scapharca* HbI for ligand is proposed to be primarily regulated by the position of a single amino acid (Phe 97), which undergoes a large conformational change in the course of the allosteric transition. Phe 97, which is analogous to position F4 in human hemoglobin, lies in the second half of the F-helix in HbI. This segment of the F-helix is sharply bent in deoxy HbI, which results in the disruption of several main-chain hydrogen bonds. In deoxy HbI, the iron atom is displaced out of the heme plane by the packing of the hydrophobic side-chain of residue Phe 97 in the heme pocket (Figure I-B). Here, the packing of the Phe 97 side-chain between the heme group and the proximal histidine (residue 101), is thought to lower oxygen affinity by: (i) acting as a wedge, thereby hindering the movement of the iron atom into the heme plane and (ii) lengthening and weakening a hydrogen bond between Nδ of proximal histidine and main-chain carbonyl oxygen of Phe 97. This bond has been proposed to modulate the affinity of the heme iron for ligand by stabilizing a partial positive charge that is induced in the proximal histidine upon ligand binding (Valentine et al. 1979). These effects of the side-chain conformation of Phe 97 in deoxy HbI are proposed to stabilize the heme group in a low affinity conformation. Upon ligand binding, the side-chain of Phe 97 moves out of the heme pocket, into the subunit interface, which allows the heme group to adopt a high affinity conformation.
Figure I-B. Stereo diagram showing the proximal heme region of subunit I for deoxy HbI (dashed lines) and HbI-CO (solid lines).

Shown are the heme groups, an α-carbon trace of the F-helices (residues 96 to 104), and side-chains of residues Phe 97 and His 101 (proximal histidine). Note the increased doming of the deoxy heme group as compared to HbI-CO, and the displacement of the F-helix away from the heme group in the deoxy state. These structural features are coupled to packing of the Phe 97 side-chain in the deoxy heme pocket. In this view, the two structures are centered on the FE atom of the respective heme groups.
Figure I-B
**Structural mechanism of cooperativity:** Ligand binding results in significant tertiary conformational changes at the core of the subunit interface (Figure I-C). Binding of ligand on the distal face of the heme group moves the iron atom into the heme plane, which pulls along with it the proximal histidine. These changes are associated with extrusion of the Phe 97 side-chain into the subunit interface, presumably due to its inability to fit in the diminished heme pocket of HbI-CO. This movement is the largest ligand-induced conformational change in HbI, and is proposed to constitute the central trigger for the cooperative mechanism, which eventually promotes the expulsion of residue Phe 97' from the second subunit, before the latter has bound ligand. Extrusion of Phe 97 into the subunit interface is associated with the following effects: (i) dropping of the heme groups deeper within the individual HbI subunits and (ii) partial disruption of the well-ordered water network in the deoxy HbI interface. These changes within a subunit are associated with altered contacts across the subunit interface, thus providing plausible routes for communication of allosteric information between subunits.

Coupled with ligand binding to HbI, is the movement of the heme group deeper into the subunit and away from the dimer interface (Figure I-C). This is proposed to result largely from the expulsion of the tightly packed Phe 97 side-chain from the heme pocket, although interactions of the heme propionates with other protein side-chains almost certainly contribute to this phenomenon. This movement of the heme group is proposed to be instrumental in the cooperative mechanism, by altering the nature of its contacts with several key residues from the F-helix of the symmetry-related subunit.
Figure I-C. Stereo diagram of the core region of the HbI interface.

The view is approximately down the molecular dyad. The continuous lines trace the deoxy HbI structure while the dotted lines show the HbI-CO structure. In addition to the heme groups for each subunit, an α-carbon trace for residues 96 to 101 and side-chains for residues Lys 96, Phe 97, Asn 100 and His 101 are shown. Small circles show the location of carbon atoms, slightly larger circles show position of oxygen and nitrogen atoms and the largest circles show the heme iron position. Note the large conformational changes in this region upon ligation, especially those of Lys 96, Phe 97 and the heme group.
Figure I-C
An intriguing aspect of oxygen binding in HbI is a striking rearrangement of the very well ordered water molecules in the dimer interface (Figure I-D). This results from the ligand-induced movement of Phe 97 into the subunit interface. In HbI, these interface water molecules have been proposed to stabilize the deoxygenated, low affinity conformation by: (i) stabilization of the bend in the F-helix. Here, three water molecules contribute hydrogen bonds to main chain atoms for each subunit, which compensate for backbone hydrogen bonds that are disrupted by the unfavorable F-helix conformation; (ii) stabilization of the deoxy heme conformation. Two water molecules in the core of the interface participate in stabilizing the folded conformation of each pyrrole A propionate group in the deoxy state. These combined effects of the deoxy water cluster appear to promote the packing of the Phe 97 side-chain in the heme pocket, thereby stabilizing the low affinity heme conformation. The interface water molecules are also proposed to contribute to the stabilization of the liganded state of HbI by providing new hydrogen bonds that can be substituted for the ones that are broken during the course of the allosteric transition.

Scapharca HbI provides a simple model system for cooperative oxygen binding, in which a few amino acid residues mediate communication between the two heme groups. It offers an elegant example for how the ancient myoglobin fold can be utilized, within a novel assemblage, to provide for cooperative ligand binding.
Figure I-D. Stereo diagram showing the hydrogen bond network among ordered water molecules in the central core of the dimeric interface in: (a) deoxy HbI and (b) HbI-CO.

Shown along with water molecules (circles) are the heme groups, main-chain atoms for residues 93 to 101 and 69 to 75, as well as side-chain atoms for Lys 96, Phe 97, His 101, Thr 72 and Tyr 75. Atom circles are shown for nitrogen, oxygen and iron atoms. Hydrogen bonds (< 3.25 Å) are shown by dotted lines except for those involving only main-chain atoms. Note hydrogen bonding by water molecules to main-chain atoms in the bent F-helix of deoxy HbI, and the effect of movement of Phe 97 into the interface upon ligation.
Figure 1-D
Chapter II

The heterologous expression of Scapharca HbI in Escherichia coli.

In order to examine the precise role of the structural elements involved in cooperative ligand binding, it is necessary to develop a system that allows for the expression of sufficient quantities of recombinant HbI. This approach permits a detailed analysis of the structural and functional properties of site-directed HbI mutants, that target specific amino acids implicated in inter-subunit communication and dimer assembly.

Our approach has been to use a synthetic HbI gene (Summerford et al. 1995), that was constructed in vitro on the basis of the published HbI protein sequence (Petruzzelli et al. 1985), in order to express recombinant HbI in Escherichia coli. A similar strategy has provided adequate yields of other recombinant globin chains, including those for sperm whale myoglobin (Springer and Sligar 1987) and human hemoglobin (Hoffman et al. 1990; Hernan et al. 1992). The synthetic HbI gene is expected to provide for optimal protein synthesis in this heterologous expression system due to the use of favored E. coli codons (de Boer and Kastelein 1986). Another salient advantage of a synthetic gene is the ability to incorporate unique restriction sites at convenient locations within the gene. This provides a simple approach for generating specific HbI mutants, and later combining these mutations, through the use of appropriate mutant
oligonucleotide cassettes. Initial cloning of the HbI gene into the pUHE21-2 plasmid, downstream of a bacteriophage T7 promoter, allowed for the expression of very limited quantities of recombinant protein in small-scale expression cultures, which was confirmed by Western blotting. Upon re-engineering the nucleotide sequence between the ribosomal binding site and the start codon to a more optimal sequence (Gold and Stormo 1990), much higher levels of HbI expression were obtained (Summerford et al. 1995).

In this chapter, I present results on the optimization of recombinant HbI expression, development of a protein purification protocol and crystallographic analysis of the recombinant hemoglobin.
Materials and Methods

**Bacterial Strains:** E. coli strain TG-1 (Sambrook et al. 1989) was used in the construction of the gene and strain W3110lacIq L8 (Brent and Ptashne 1981) is the host used for the overexpression of recombinant HbI.

**Protein Expression:** Expression of recombinant HbI was carried out in a Microferm Fermentor (New Brunswick Scientific Co., Edison, NJ). Twelve liters of LB broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/L NaCl, 1 ml/L, 1.0N NaOH) containing 100 μg/ml ampicillin and 0 to 60 μg/ml δ-aminolevulinic acid (ALA) were inoculated with 350 ml of an overnight culture of E. coli cells containing the appropriate expression plasmid. The culture was grown to an OD600 of 0.6-0.8 before commencing induction by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM (concentration found to be optimal). Expression times of 6 to 8 hours were found to be optimal.

Cells were harvested in a one liter volume using a Millipore Pellicon ultrafiltration system (Millipore Corporation, Bedford, MA) then centrifuged at 6,000g for 30 minutes. The cell pellets were resuspended in an equal mass of the following lysis buffer: 50 mM Tris HCl pH 8.0, 1.0 mM disodium ethylenediaminetetraacetate (EDTA), 0.5 mM dithiothreitol (DTT), 100 mM NaCl, 5% glycerol, 1 mM phenyl methane-sulfonyl fluoride (PMSF). This and all subsequent buffers were saturated with carbon monoxide (CO) to maintain the
recombinant hemoglobin in a CO bound state during the purification process. Two different procedures were used to lyse cells and release recombinant protein. In one protocol, cells were lysed in two successive steps in a French Pressure cell (SLM Instruments, Inc., Urbana, IL) using a 20K rapid fill cell at 1000 psi gauge pressure. In the second protocol, repeated freezing and thawing cycles were used as follows. The cell pellets were suspended in a three-fold excess mass of the following lysis buffer: 50mM Tris-HCl pH 8.0, 100mM NaCl, 1mM DTT, 1mM EDTA, 10% sucrose (w/v). The cell suspensions were then subjected to rapid freezing on a dry ice - ethanol bath, maximizing the surface area of the suspension in contact with the freezing bath. The frozen suspensions were then transferred to a 37°C water bath and allowed to thaw completely. The freeze-thaw cycle was repeated 3-4 times and the lysate was centrifuged at 30,000g for 60 minutes. Very efficient cell lysis was obtained with minimal DNA contamination as observed by pale pellets and clear, red supernatants. Nevertheless, following either lysis procedure, samples were treated with 40 units/ml DNase I (Sigma) and 3 units/ml RNaseA (Sigma) at 4°C for 60-90 minutes with constant stirring.

**Protein Purification:** After pelleting the cell debris at 30,000g for 30 minutes, the supernatant was saturated with carbon monoxide and subjected to ammonium sulfate fractionation at 4°C. The precipitate at 45-50% saturation in ammonium sulfate, which contained little or no hemoglobin, was removed by centrifugation at 14,000g. The supernatant was then increased to 95% saturated ammonium sulfate in which the recombinant HbI precipitated. This precipitate
(centrifuged at 16,000g) was resuspended in a minimal volume of 30mM CHES (2-\([N\text{-cyclohexylamino}]\)-ethanesulfonic acid) buffer at pH 9.0. The sample pH was then adjusted to the isoelectric point (pI) of HbI by dialyzing overnight against 30mM CHES pH 9.0 (120-fold excess volume). After centrifugation (20,000g) to remove precipitate, the supernatant was concentrated in an Amicon (Beverly, MA) 10kDa molecular mass cutoff Centriprep concentrator to a final volume of 5-10ml. This was loaded on to a 50ml Toyapearl DEAE-650S (TosoHaas, Montgomeryville, PA, USA) column that had been pre-equilibrated with 30mM CHES pH 9.0. Recombinant HbI was monitored visually and recovered with the column flow-through. The sample was then transferred into 30mM N-2-hydroxyethylpiperazine-2N\(^{-}\)-ethane sulfonic acid (HEPES) at pH 7.0 either by dialysis or by equilibration on a G-25 desalting column. This sample was loaded on a 15ml CM Biogel-A (BIORAD Laboratories, Hercules, CA) cation-exchange column that was pre-equilibrated with 30mM HEPES at pH 7.0. After washing the column with three column volumes of buffer, the protein was eluted in 30 mM NaCl.

**Oxygen binding measurements:** Oxygen binding equilibrium measurements were performed spectroscopically at 20°C using the method of (Rossi-Fanelli and Antonini 1958).

**Crystallization:** Crystals of carbonmonoxide (CO) liganded and unliganded (deoxy) recombinant HbI were grown using conditions and procedures successful with native HbI (Royer 1994). Growth of diffraction
Results

**Protein expression:** Conditions for the optimal expression of recombinant HbI protein were determined through the initial use of small-scale bacterial cultures (~ 30 mL). The use of a bacterial strain (W3110 lacIq L8) that constitutively expresses the lac repressor, provides for a tightly regulated expression system, that can be switched on by the addition of the synthetic inducer, IPTG. The initial experimental parameters that were varied included the density of bacterial culture and concentration of IPTG at induction, as well as the ambient temperature and total duration of protein expression. Preliminary experiments revealed that although significant quantities of HbI were being synthesized (5-10% of total bacterial protein) in this system, a majority of the recombinant HbI protein was insoluble (results not shown). The addition of exogenous hemin to expression cultures did not appreciably enhance the production of soluble recombinant hemoglobin. However, the addition of the heem precursor δ-aminolevulinic acid (ALA), had a dramatic impact on the level of expression of soluble hemoglobin. As shown in Figure II-A, a ~3-fold increase in soluble recombinant HbI was observed when cells were grown in the presence of 60 μg/mL of ALA as compared to cells grown in its absence. At this stage, the expression culture volumes were scaled up to 12.0 liters, while keeping all other experimental variables constant.

**Protein purification:** Purification of the soluble fraction of recombinant HbI was achieved in three steps. The initial ammonium sulfate precipitation step
Figure II-A: Effect of δ-aminolevulinic acid (ALA) on recombinant HbI expression.

a) Photograph showing crude lysates (using freeze/thaw procedure, see materials and methods) with, from left to right, 0, 25 and 60 μg/ml ALA in the expression medium. Note the significant increase in red color with increasing quantities of ALA. b) SDS-PAGE (10%) gel of the above crude lysates. Note the significant increase in expression of soluble HbI as a result of increasing ALA concentrations from 0 to 60 μg/ml.
Figure II-A
was followed by two successive column chromatography steps, using a weak anion-exchange resin, followed by a weak cation-exchange resin. Analytic isoelectric focusing experiments done with partially purified fractions of bacterial cell lysates, revealed the isoelectric point of recombinant HbI to be ~9.0. As shown in Figure II-B, the dominant purification step is the anion-exchange column, which was run at pH 9.0. As predicted from the isoelectric focusing experiments, recombinant HbI is uncharged under these conditions and is therefore not retained on this column, while a majority of the contaminating E. coli proteins are removed. Using this protocol, we obtain yields of 60-100 mg of pure recombinant HbI, from a 12.0 L starting expression culture.

Protein crystallization: Initial attempts at crystallizing recombinant HbI protein were largely disappointing. The morphology of the dominant crystal form of CO-liganded recombinant HbI was that of thin plates, which were unsuitable for X-ray diffraction experiments. Using microseeds derived from crushed native CO-HbI crystals, we inconsistently obtained small crystals that are isomorphous to native hemoglobin crystals. None of these crystals, however, exhibited diffraction extending beyond Bragg spacings of 2.1 Å in preliminary X-ray diffraction experiments, in contrast to native CO-HbI crystals, which show diffraction extending past Bragg spacings of 1.4 Å. Crystal growth showed a dramatic improvement upon mutating residue Asn 56 to aspartate. This single change not only encouraged recombinant crystals to achieve a size of up to 1.5 X 0.2 X 0.2 mm, but these crystals diffracted comparably with native hemoglobin crystals, to a resolution better than 1.5 Å. These larger recombinant HbI crystals
Figure II-B: Purification of recombinant HbI as shown on a SDS-PAGE (10%) gel with proteins visualized by staining with Coomassie brilliant blue.

Lane 1: Molecular weight standards (from top to bottom 107kDa, 76kDa, 52kDa, 36.8kDa, 27.2kDa, 19kDa); Lane 2: HbI purified from Scapharca red blood cells; Lane 3: Crude lysate (using French Press lysis procedure, see materials and methods); Lane 4: Following ammonium sulfate precipitation step; Lane 5: Following anion exchange column; Lane 6: Following elution from cation exchange column.
Figure II-B
are isomorphous to native CO-HbI crystals. Reasonably sized deoxygenated (unliganded) crystals of recombinant (Asp 56) HbI have also been obtained. These crystals are isomorphous to the corresponding native HbI crystals and show diffraction corresponding to better than 1.6 Å resolution.

**Oxygen binding measurements:** Soluble recombinant HbI, purified from *E. coli*, is fully functional and exhibits oxygen binding properties that are virtually identical to native HbI obtained from *Scapharca* erythrocytes. A series of independent oxygen binding measurements performed by Gianni Colotti, at 20°C, revealed values of 7.3 ± 0.8 Torr and 1.49 ± 0.03 for oxygen affinity and the Hill coefficient for cooperativity (n), respectively. The corresponding published values for native HbI are 7.8 Torr and 1.5, respectively (Chiancone *et al.* 1981).

**Chemical measurements:** N-terminal amino acid sequencing using the Edman degradation procedure revealed the first residue in recombinant HbI to be a proline. This confirms that the initiator methionine residue is completely cleaved from the N-terminal position. Electrospray ionization mass spectroscopy measurements on both recombinant and native hemoglobin give identical molecular masses of 15,949 Da for the apoprotein monomer.
Discussion

We have used a synthetic HbI gene (Summerford et al. 1995), which has been re-engineered to provide increased translational efficiency, in order to express soluble recombinant hemoglobin in Escherichia coli. Initial small-scale experiments, using the synthetic gene construct, revealed a majority of the expressed recombinant protein to be insoluble. One plausible explanation for this result is the relative lack of heme in comparison to the highly expressed globin chains, which retards the assembly of fully functional HbI holoprotein. In several cases for the expression of hemoglobins and myoglobins, the level of endogenous bacterial heme synthesis has been found to be sufficient for substantial holoprotein production, although rather long expression times were necessary (Springer and Sligar 1987; Guilemette et al. 1991; Hernan et al. 1992). In a previous study, exogenous hemin has been added to bacterial cultures used for the expression of recombinant human hemoglobin, in an effort to boost endogenous heme production (Hoffman et al. 1990). In our system, however, the use of hemin did not significantly enhance the production of recombinant HbI. Interestingly, addition of the key heme precursor δ-aminolevulinic acid (ALA), dramatically increases the expression of soluble HbI, which apparently results from an increase in the rate of heme synthesis to match the rate of globin synthesis. The efficacy of ALA in boosting levels of heme synthesis, as compared to hemin, may result from an increased uptake into bacterial cells during expression. A similar effect is thought to account for the increase in bacterially
expressed cytochrome P450 4A4 (Nishimoto et al. 1993). The bacterially expressed HbI is fully functional, showing oxygen binding properties that are indistinguishable from native HbI.

The successful growth of diffraction quality crystals is a key prerequisite for the utility of recombinant HbI as a model system for cooperative oxygen binding and dimer assembly (Royer 1994). Initial attempts to crystallize recombinant HbI resulted in the growth of thin plates, which were unsuitable for X-ray diffraction experiments. Using microseeds derived from crushed native CO-HbI crystals, small crystals that are isomorphous to native hemoglobin crystals were occasionally obtained. Unfortunately, in contrast to native hemoglobin, these crystals diffracted rather poorly. Cloning of the HbI gene (Gambacurta et al. 1993), revealed a sequence conflict with the synthetic gene at residue 56. While the cloned gene encoded for aspartic acid at this position, the synthetic gene sequence, which was based on HbI protein sequencing (Petruzzell et al. 1985), encoded for an asparagine. Although Asn 56 is located at the surface of the HbI molecule and does not affect HbI oxygen binding properties, the effect on crystallization of recombinant CO-HbI is drastic. As shown in Figure II-C, residue 56 appears to participate in a key lattice contact in CO-HbI crystals, with residue Lys 113 from a neighboring HbI molecule. The clear preference of aspartate to form a salt bridge with Lys 113, suggested that mutating Asn 56 to Asp might significantly improve crystal growth, especially in the dimension in which growth was stunted. With this mutation, recombinant CO-HbI crystals showed a dramatic improvement in both size and diffraction quality. These crystals are isomorphous to corresponding native HbI crystals and diffract to a comparable resolution. Deoxy crystals of recombinant HbI (Asp
The packing of four dimers in the HbI-CO crystal lattice is shown, with the crystallographic b axis (44Å) oriented in the horizontal direction. For each dimer an $\alpha$-carbon trace is shown along with side chains for Asp 56 and Lys 113 (in one subunit) and both heme groups. In the yellow dimer (top right) Asp 56 is drawn in violet and is within 3.3Å of Lys 113 from the light green dimer in the lower left. Also in the yellow dimer, Lys 113 is drawn in light blue and is interacting with Asp 56 from the light green dimer in the lower right. The importance of this contact is evident from the poor quality of crystals grown from recombinant HbI with Asn at position 56 (based on the published protein sequence) and the high quality of crystals grown from recombinant HbI with Asp at position 56 (as suggested by the cDNA sequence).
56), which are isomorphous to native deoxy HbI crystals and diffract as well, have also been grown.

In summary, a bacterial system for the expression of significant quantities of recombinant *Scapharca* dimeric hemoglobin is now available, which permits extensive structural, functional and thermodynamic characterization of HbI mutants. This system allows determination of the role of individual residues at the dimer interface in inter-subunit communication.
Chapter III

Mutation of residue Phe97 to Leu disrupts the central allosteric pathway in Scapharca dimeric hemoglobin

Introduction

Phenylalanine 97 has a central role in the proposed structural mechanism for cooperativity (Royer 1994). The side-chain of Phe 97 undergoes the largest ligand-linked conformational change in HbI. In the deoxy state, it is tightly packed in the heme pocket, whereas upon ligand binding it is displaced from the heme pocket into the subunit interface. There is evidence from both crystallographic and resonance Raman studies that ligand affinity in HbI is primarily modulated through a proximal strain mechanism (Rousseau et al. 1993; Royer 1994). The lowered oxygen affinity of the deoxy state appears to result primarily from the packing of Phe 97 in the heme pocket where it restricts movement of the iron atom into the heme plane and lengthens a hydrogen bond involving the proximal histidine. The high degree of strain in the Fe-histidine bond contributes to the low frequency (203 cm\(^{-1}\)) for the Fe-His stretching mode in resonance Raman scattering measurements with deoxy HbI (Rousseau et al. 1993). Upon ligation, movement of Phe 97 into the subunit
interface is coupled with disruption of a well-ordered interfacial water cluster and movement of the heme groups deeper within each subunit. These ligand-induced conformational changes within a subunit alter the nature of interactions between subunits, which presumably encourages movement of the Phe 97' side chain (second subunit) into the interface, allowing the second subunit to attain a high oxygen affinity state prior to ligand binding (Royer 1994). In view of its significant role in the cooperative mechanism of HbI, Phe 97 is clearly indicated as a target for mutation. Phe 97, four residues from the proximal histidine (101), is analogous to residue F4 in mammalian hemoglobins which is a leucine in all known vertebrate hemoglobins and most invertebrate hemoglobins. The smaller size of the leucine side-chain in these hemoglobins allows it to remain packed in the heme pocket in both liganded and unliganded states.

In this chapter, functional and high resolution crystallographic studies of the Leu 97 HbI (F97L) mutant are presented. This mutation results in increased oxygen affinity and diminished cooperativity. These results confirm the crucial role of residue Phe 97 in modulating oxygen binding by HbI, while demonstrating the persistence of residual cooperativity, which appears correlated with the observed heme movements. This suggests that parallel pathways exist for information transfer between subunits.
Materials and Methods

**Bacterial strains:** *Escherichia coli* strain TG1 was used as the host strain for propagating recombinant bacteriophage M13mp18 vector and for *in vitro* site-directed mutagenesis. *E. coli* strain W3110lacIq L8 was the host used for over-expression of recombinant HbI (Summerford *et al.* 1995).

**Mutagenesis:** *In vitro* oligonucleotide-mediated, site-directed mutagenesis was used to generate the F97L mutant. The initially constructed gene for HbI bearing 5' and 3' termini complementary to *KpnI* and *SacI* sites was cloned into the polylinker region of a M13mp18 bacteriophage vector (New England Biolabs, Beverly, MA) which had been cut previously with the same two restriction endonucleases. Following several rounds of plaque purification using TG1 cells as hosts, recombinant single stranded DNA was isolated. (Sambrook *et al.* 1989). Subsequently, a 19-mer oligonucleotide (University of Massachusetts Medical Center DNA synthesis facility) complementary to the region of the gene coding for residue Phe 97 was used in a site-directed mutagenesis protocol (Sculptor *in vitro* mutagenesis system, Amersham Life Science, Illinois, USA) to mutate residue 97 to leucine. Following three rounds of plaque purification, single stranded DNA was isolated and the changes in DNA sequence were confirmed by dideoxy sequencing using the Sequenase kit (US Biochemical, Cleveland, OH). Double
stranded DNA of the replicative form of the vector was then prepared and subjected to AccI and SacI restriction cleavage in order to isolate a segment of the HbI gene that contains the leucine 97 mutation. This fragment was then sub-cloned into a similarly digested wild-type HbI gene, borne on a pCS26 expression vector which, as reported earlier, has allowed for significant overexpression of recombinant, wild type HbI protein (Summerford et al. 1995).

**Protein expression and purification:** Recombinant F97L was over-expressed in *E. coli* and purified as described for wild-type (Summerford et al. 1995).

**Crystallization:** Deoxy and carbon-monoxy F97L crystals were grown as described in (Royer 1994), except that both crystal forms were grown from solutions of 40-50 mg/ml hemoglobin. Microseeds obtained by crushing corresponding wild-type HbI crystals were used to nucleate mutant HbI crystal growth. The morphologies of both crystal forms were similar to those of wild-type recombinant HbI.

**Collection of Diffraction Data:** X-ray diffraction data were collected from crystals at room temperature, on a R-AXIS IIC imaging plate system mounted on a Rigaku RU 200 rotating anode generator (Molecular Structure...
Corporation, The Woodlands, TX). Data were processed using software supplied by the manufacturer.

The F97L-CO crystals are essentially isomorphous to native crystals, showing the symmetry of monoclinic space group C2, and cell constants a=93.25 Å, b=43.98 Å, c=83.50 Å and β=122.03°. A total of 116 frames were collected from two crystals with an oscillation range of 1.5° to 1.8° for each frame. The overall Rmerge for 40,595 unique reflections with I/σ > 1 was 5.52%. This data set represents 93.5% of the predicted unique reflections to Bragg spacings greater than 1.6 Å and 88.8% to 1.5 Å.

Deoxygenated F97L HbI crystals show the symmetry of orthorhombic space group C2221, with cell constants of a=91.99 Å, b=44.27 Å and c=143.83 Å, essentially isomorphous to native deoxy crystals. The deoxy F97L HbI data set represents diffraction data from a single crystal that was collected in two different orientations. Initially the large c axis (143.8 Å) was aligned horizontally (perpendicular to the spindle axis). The close spacings between reflections along the c* axis necessitate a small oscillation range (0.9°) in this orientation for the 34 oscillation frames collected. In a second set of frames, the crystal was reoriented with its c axis vertical (parallel to the spindle axis). This allowed for the use of a larger oscillation range of 2.5°. A total of 30 oscillation frames were recorded from this crystal orientation. The overall Rmerge for 27,590 unique reflections derived from 95,839 independent observations with I/σ > 1, was 7.33%. These represent 84.1% of the predicted unique data to Bragg spacings greater than 1.7 Å.
Refinement: The starting models for both CO-liganded and deoxy Leu 97 HbI structures were the native HbI structures (Royer 1994) in which residue Phe 97 was mutated to leucine using the macromolecular modeling program 'O' (Jones et al. 1991). Also, the water molecules associated with these structures were deleted and alternate conformers of protein side chains eliminated from the pdb coordinate files (entry codes 3SDH and 4SDH).

These models were then refined using the XPLOR package (Brünger 1992). Prior to the commencement of refinement, 10% of randomly selected reflections from each data set were designated as test reflections for use with the Free-R cross-validation method (Brünger 1992). Two cycles of simulated annealing were performed, with the first cycle incorporating non-crystallographic symmetry restraints upon the model. The protocol for simulated annealing that was used called for 'heating' the molecule to 1000 K followed by 50 steps of 0.5 fs molecular dynamics. After each set of 50 steps, the temperature was lowered by 25 K and 50 new dynamics steps were performed. This procedure was continued until a temperature of 300 K was reached and followed by 60 cycles of conjugate gradient minimization combined with B-factor refinement.

Addition of water molecules to the models was initiated at this stage using the programs PEAKS and LOCATE written by Wayne Hendrickson (Columbia University). Peaks of electron density greater than 3.5 σ in Fo - Fc maps and 0.8 σ in 2Fo - Fc maps that satisfied hydrogen bonding criteria were identified as locations for water molecules. This procedure was reiterated, resulting in the addition of 5 and 4 shells of water to the CO-liganded and deoxy structures, respectively. Each model was subjected to 120 cycles of
conjugate gradient minimization combined with B-factor refinement using XPLOR, following the addition of each shell of water molecules. Water molecules with refined B-factors greater than 50 Å² were deleted.

The model was then examined in conjunction with 2Fo-Fc and Fo-Fc maps to locate poorly placed protein side-chains and missing water molecules, which were then built. This was followed by 120 cycles of conjugate gradient energy minimization combined with B-factor refinement.
Results

**Functional and spectroscopic characterization of F97L:** Mutation of phenylalanine 97 to leucine (F97L) in *Scapharca* HbI results in increased ligand affinity and diminished cooperativity, as observed by both equilibrium and kinetic experiments. The p50 value for oxygen binding, based on equilibrium measurements carried out by Gianni Colotti, decreases from 7.8 Torr to 1.0 Torr, while the Hill coefficient decreases from 1.5 to 1.16 ± 0.05. Statistical analysis of all the data sets (n=12) indicates that the Hill coefficient is significantly higher than 1.0, showing that cooperativity, although drastically reduced, is not abolished. Additionally, spectroscopic experiments performed by Gianni Colotti have provided evidence for a reduction of proximal strain in the deoxy state of F97L relative to wild-type HbI (Pardanani et al. 1997).

A summary of kinetic experiments performed by Quentin Gibson (Pardanani et al. 1997) is presented in Table III-A. The increased oxygen affinity observed in equilibrium measurements is primarily due to a decrease in the oxygen dissociation rate, particularly at low O2 saturation. Oxygen pulse and CO replacement experiments (Gibson 1973) show a greater than 10-fold decrease in the dissociation rate at low oxygen saturation as compared to HbI. In contrast to HbI, there is little or no change in the oxygen dissociation rate for F97L as a function of varying levels of oxygen saturation (Figure III-A). Additionally, measurements of CO binding to F97L by both stopped-flow and
Table III-A. Kinetic ligand binding parameters to *Scapharca* HbI and F97L

Taken from (Pardanani *et al.* 1997)

<table>
<thead>
<tr>
<th></th>
<th>Oxygen On Rates ( \mu M^{-1} s^{-1} )</th>
<th>Oxygen Off Rates ( s^{-1} )</th>
<th>CO On Rates ( \mu M^{-1} s^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k'1 )</td>
<td>( k'2 )</td>
<td>( k1 )</td>
</tr>
<tr>
<td>F97L</td>
<td>nd</td>
<td>26</td>
<td>40^a</td>
</tr>
<tr>
<td>HbI</td>
<td>11^b</td>
<td>16^b</td>
<td>490^b</td>
</tr>
</tbody>
</table>

Parameters are intrinsic (i.e. no statistical factors). Subscripts define the binding step based on a consecutive two step scheme following Adair (Adair 1925). A superscript prime designates an on rate, no prime indicates an off rate.

nd - not determined

^a oxygen pulse measurements (Gibson 1973)

^b from (Chiancone *et al.* 1993)

^c from (Antonini *et al.* 1984)
Figure III-A. Oxygen pulse experiments for HbI (a) and F97L (b).

The oxygen concentrations starting from the right are 0.59, 0.124, 0.062, 0.032, 0.016 and 0.008 mM. The lowest trace is the average of 7 individual stopped-flow runs. Panels (c) and (d) represent corresponding simulations for HbI and F97L. The first order rate constant for oxygen dissociation from HbI increases from 130 s$^{-1}$ at high and intermediate saturations, to 370 s$^{-1}$ at low saturation. The corresponding rates for F97L do not change. The experiments were done at 2.8 and 4.2 mM heme for HbI and F97L, respectively, in 0.1 M potassium phosphate buffer at pH 7.0 and 20 °C. Data were collected at a wavelength of 435 nm using a 2 cm path length.
Figure III-A

(a)

Δ Abs

Time (ms)

0 80

(b)

Δ Abs

Time (ms)

0 160

(c)

Time (ms)

0 80

(d)

Time (ms)

0 160
flash photolysis show a clear increase in combination rates as compared with wild-type.

**Overview of F97L Crystal Structures:** The models for deoxygenated and CO-liganded F97L have been refined against the X-ray data, to conventional R-factors of 18.3% and 18.6%, respectively, with excellent stereochemistry (Table III-B). An electron density map of the heme region of one subunit is shown in Figure III-B. Both the unliganded and liganded structures of F97L are found to be strikingly similar to the corresponding structures of wild-type HbI, with significant differences confined to the disposition of residue 97 side-chain, F-helix geometry and the interfacial water structure. Evidence of similarities in the overall structure is presented in Figure III-C.

As shown in Table III-C, the overall B-factors are slightly higher in deoxy F97L than deoxy wild-type and nearly the same for the CO-liganded structures. In contrast to the similarity in overall B-factors, the core interface water molecules show significantly higher B-factors in F97L than wild-type. This indicates that these water molecules, which have been implicated in cooperative function in wild-type HbI (Royer et al. 1996), are less well ordered in F97L.

**Disposition of residue Leu97 side-chain:** In the deoxy F97L structure, the disposition of the side-chain of residue 97 is very similar to that in the deoxy wild-type structure, where it packs in the heme pocket (Figure III-C a
Table III-B. Refinement statistics on *Scapharca F97L*.

<table>
<thead>
<tr>
<th></th>
<th>CO</th>
<th>DEOXY</th>
</tr>
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<tbody>
<tr>
<td>Resolution Limits (Å)</td>
<td>10.0 - 1.5</td>
<td>10.0 - 1.7</td>
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<tr>
<td>No. of reflections used in refinement (F &gt; 1 σ)</td>
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<tr>
<td>R-factor</td>
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<td>18.3%</td>
</tr>
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<td>2742</td>
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<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;</td>
<td>23.1%</td>
<td>24.4%</td>
</tr>
<tr>
<td>No. of non-hydrogen atoms</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>dihedrals (°)</td>
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<td>18.2</td>
</tr>
<tr>
<td>impropers (°)</td>
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<td>1.5</td>
</tr>
<tr>
<td>Average B-factors (Å²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>main-chain</td>
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</tr>
<tr>
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</tr>
<tr>
<td>heme atoms</td>
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</tr>
<tr>
<td>CO ligands</td>
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<td>---</td>
</tr>
<tr>
<td>solvent atoms</td>
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Table III-C a. Comparison of Average B-factors ($\text{Å}^2$) between Deoxy HbI and Deoxy F97L structures.

<table>
<thead>
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<th>DXY-F97L</th>
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<td>20.4</td>
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<tr>
<td>Side-chain atoms</td>
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</tr>
<tr>
<td>Heme atoms</td>
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<td>17.7</td>
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<td>Water atoms</td>
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<tr>
<td>Core Interface Water Molecules (17)</td>
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<td>29.5</td>
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</table>

Table III-C b. Comparison of Average B-factors ($\text{Å}^2$) between HbI-CO and F97L-CO structures.

<table>
<thead>
<tr>
<th></th>
<th>HbI-CO</th>
<th>F97L-CO</th>
</tr>
</thead>
<tbody>
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<td>16.7</td>
</tr>
<tr>
<td>Side-chain atoms</td>
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<tr>
<td>Heme atoms</td>
<td>14.3</td>
<td>16.0</td>
</tr>
<tr>
<td>CO atoms</td>
<td>13.9</td>
<td>14.4</td>
</tr>
<tr>
<td>Water atoms</td>
<td>35.2</td>
<td>37.2</td>
</tr>
<tr>
<td>Core Interface Water Molecules</td>
<td>24.7 (14)</td>
<td>34.5 (18)</td>
</tr>
</tbody>
</table>
Figure III-B. Simulated annealing omit F₀-Fc map of F97L-CO.

The F97L-CO structure was subjected to simulated annealing refinement, omitting the atoms shown. The panels show the heme region of subunit 2, which includes the heme group, CO-ligand, Phe 97, His 101 and His 69. The map is contoured at the 3σ level.
Figure III-C. Stereo diagram showing an α-carbon trace of HbI (solid lines) and F97L (dashed lines) for the deoxy state (a) and the CO-liganded state (b).

In both panels, the view is approximately down the molecular dyad. In addition to the α-carbon positions, the heme group and the side-chain for residue 97 are shown for each subunit. Note the close alignment of the two deoxy plots, with subtle differences in the region of the heme groups and the F-helices. In contrast, note the almost perfect alignment of the CO-liganded plots for the α-carbon positions and the heme groups. The Leu 97 side-chain remains packed in the heme pocket in F97L-CO. The dimer interface is largely formed by contacts between the E and F helices and the heme propionate groups.
Figure III-C
and Figure III-D). Unlike the phenylalanine side-chain which is packed tightly between the heme group and the proximal histidine (residue 101), the smaller Leu 97 side-chain appears to be accommodated without strain. This is reflected in differences of heme conformation, position of the heme iron and subtle changes in F-helix geometry compared with deoxy wild-type HbI, as discussed further below.

Upon ligand (CO or O₂) binding to wild-type HbI, the Phe 97 side-chain is displaced into the subunit interface as its tight pocket is reduced when the iron moves into the heme plane (Royer 1994). In contrast, the leucine side-chain remains in the heme pocket in F97L upon ligand binding (Figure III-C b).

**F-helix geometry:** In deoxy wild-type HbI, there is a sharp bend in the F-helix (residues 87-103) (Figure III-C a and Figure III-D), that was postulated to result from wedging of the Phe 97 side-chain in the heme pocket (Royer 1994). Unexpectedly, this bend, although attenuated, persists in F97L where the smaller leucine side-chain is readily accommodated in the heme pocket. Subtle differences in F-helix geometry are present including a movement of the backbone towards the heme group, most markedly seen involving the C-α of residue Leu 97 (0.5 Å), but also Lys 96 (0.3 Å) and Glu 95 (0.3 Å), as shown in Figure III-D. This change presumably results from loss of the tight packing of Phe 97 between the F-helix backbone and the heme group. Additionally, the distances between the carbonyl oxygens of residues 96 and 97 and amide nitrogens of residues 100 and 101 respectively (4.5 Å and 3.9 Å) are slightly less than the corresponding distances in the wild-type deoxy structure. These
Figure III-D. Stereo diagram showing the proximal heme region of subunit 1 (a) and subunit 2 (b), for deoxy-HbI (dashed lines) and deoxy-F97L (solid lines).

Shown are the heme groups, an a-carbon trace of the F-helices (residues 96-104) and side-chains of residues 97 and 101 (proximal histidine). Note the increased displacement of the F-helix backbone away from the heme group in HbI, and a correlated increase in heme group doming.
Figure III-D

(a) Helix

(b) Helix

F-Helix

F-Helix
effects reflect a subtle straightening of the sharp bend observed in the F-helix of deoxy wild-type HbI. Also, the hydrogen bond between the main-chain carbonyl oxygen of residue 97 and the N8 of the proximal histidine, that is hypothesized to play a role in determining oxygen affinity of the heme iron (Caughey et al. 1975; Valentine et al. 1979; Royer 1994), is optimized in F97L (Table III-D).

**Heme Conformation:** Coupled with ligand binding, a movement of the heme group deeper into each subunit and away from the dimer interface is observed with wild-type and F97L HbI. This movement was previously suggested to result from extrusion of the tightly packed Phe 97 side-chain from the heme pocket (Royer 1994). Our structural results argue against such a determinative role for Phe 97. Thus, other interactions between the heme group and protein must be sufficient for this heme movement. In this regard, interactions of protein side-chains with the domed deoxy-heme as well as intra- and inter-subunit interactions with the heme propionates, likely contribute to this effect.

**Interface Water Structure:** There exists an elaborate network of hydrogen-bonded water molecules in the subunit interface of wild-type HbI. These water molecules have been proposed to play an important role in the cooperative mechanism by providing stabilizing hydrogen-bond interactions to the alternate allosteric states of HbI (Royer 1994; Royer et al. 1996).
Table III-D a. Comparision of Heme region geometry between deoxy HbI and deoxy F97L.

<table>
<thead>
<tr>
<th>Subunits</th>
<th>DXY-HbI</th>
<th>DXY-F97L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe to heme plane distance (Å)</td>
<td>0.50</td>
<td>0.52</td>
</tr>
<tr>
<td>Fe to N_p^a plane distance (Å)</td>
<td>0.36</td>
<td>0.38</td>
</tr>
<tr>
<td>Mean Fe to N_p^a bond distance (Å)</td>
<td>2.04</td>
<td>2.04</td>
</tr>
<tr>
<td>Fe - Ne^2 (His^101) distance (Å)</td>
<td>2.19</td>
<td>2.18</td>
</tr>
<tr>
<td>H^101 N_d^1 - O F^97 distance (Å)</td>
<td>3.09</td>
<td>3.08</td>
</tr>
</tbody>
</table>

^a N_p denotes the 4 pyrrole nitrogen atoms of the heme group.
Table III-D b. Comparison of Heme region geometry between HbI-CO and F97L-CO HbI.

<table>
<thead>
<tr>
<th>Subunits</th>
<th>HbI-CO I</th>
<th>HbI-CO II</th>
<th>F97L-CO I</th>
<th>F97L-CO II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe to heme plane distance (Å)</td>
<td>0.04</td>
<td>0.04</td>
<td>0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>Fe to Np&lt;sup&gt;a&lt;/sup&gt; plane distance (Å)</td>
<td>0.01</td>
<td>0.00</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean Fe to Np&lt;sup&gt;a&lt;/sup&gt; bond distance (Å)</td>
<td>2.01</td>
<td>2.03</td>
<td>1.98</td>
<td>1.97</td>
</tr>
<tr>
<td>Fe - Ne&lt;sup&gt;2&lt;/sup&gt; (His&lt;sup&gt;101&lt;/sup&gt;) distance (Å)</td>
<td>2.09</td>
<td>2.14</td>
<td>2.18</td>
<td>2.23</td>
</tr>
<tr>
<td>H&lt;sup&gt;101&lt;/sup&gt; Nd1 - O F&lt;sup&gt;97&lt;/sup&gt; distance (Å)</td>
<td>2.87</td>
<td>2.87</td>
<td>2.82</td>
<td>2.86</td>
</tr>
<tr>
<td>CO ligand: Fe - C distance (Å)</td>
<td>1.83</td>
<td>1.83</td>
<td>1.87</td>
<td>1.88</td>
</tr>
<tr>
<td>Fe - C - O angle (°)</td>
<td>155.2</td>
<td>162.8</td>
<td>173.7</td>
<td>174.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Np denotes the 4 pyrrole nitrogen atoms of the heme group.
In the deoxy wild-type structure, of the 219 total water molecules, 17 are located at the central core of the dimer interface. These water molecules are symmetrically distributed about a non-crystallographic dyad (2-fold), even though this restraint was not imposed in model building or refinement, and are very well ordered, with an average B-factor of 19.6 Å². Of these, the lowest B-factors are seen for a cluster of 4 water molecules that are located at the bottom of the interfacial crevice and which are hydrogen-bonded to E-helix residues that remain largely unaltered by ligand binding.

In comparison, 174 water molecules were independently built into the deoxy F97L structure during the course of model refinement. As shown in Figure III-E a, the interfacial water architecture for this structure is found to be virtually identical to wild-type, with 17 water molecules that are structurally equivalent (< 0.60 Å) to the waters described above. These water molecules, however, are significantly less well ordered than in wild-type, with an average B-factor of 29.5 Å². The deepest cluster of 4 water molecules maintains the lowest B-factors, while those closer to the bulk solvent are increasingly disordered. The stability of water molecules correlates with the extent to which they are hydrogen-bonded to protein groups, as opposed to other water molecules. Water molecules whose sole hydrogen-bonding partners are other water molecules, exhibit the highest B-factors. For instance, the 5 water molecules that can hydrogen-bond only with other water molecules, show an average B-factor of 32.7 Å² in F97L, as compared to 19.1 Å² in HbI. The changes observed for the deoxy F97L structure with regard to water molecules therefore suggest that the subtle changes in the F-helix conformation results in a marked loosening of the interfacial water network.
Figure III-E. Stereo diagram of the core ordered water molecules in the subunit interface.

In all 3 panels, the view is approximately down the molecular dyad. Shown are the heme group, the F-helix (residues 94 to 102) and side-chains for residues 97 and 101, for each subunit. Additionally, a van der Waals representation of the interface water molecules is shown. (a) Ordered water molecules in the subunit interface of deoxy-F97L. The 17 waters shown here are structurally equivalent (< 0.60 Å) to the corresponding water molecules in deoxy-HbI. (b) Ordered water molecules in the subunit interface of HbI-CO. Note the disruption of the water network and the associated extrusion of the Phe 97 side-chain into the subunit interface. A total of 14 water molecules are present in this subunit interface. (c) Ordered water molecules in the subunit interface of F97L-CO. Note that the Leu 97 side-chain stays packed in the heme pocket. This preserves 4 water molecules (solid-black shading) that are normally expelled by the extrusion of the Phe 97 side-chain into the interface. Water molecules in medium-gray shading are structurally equivalent to the corresponding water molecules in HbI-CO (< 0.75 Å). The water molecules in light-gray shading are not; note the asymmetric distribution of these water molecules. A total of 18 water molecules are present in this subunit interface.
In contrast to the deoxy form, striking differences in the interfacial water architecture are observed between the CO structures of F97L and wild-type HbI. Upon ligand binding to HbI, the displacement of each Phe 97 side-chain into the subunit interface results in the direct expulsion of 3 water molecules (Figure III-E b). The liganded wild-type interface thus has fewer and less well ordered water molecules than the deoxy interface (Table III-C). As shown in Figure III-E c, loss of the Phe 97 side-chain results in several discrete changes in the interfacial water distribution for the F97L-CO structure. First, 4 of the 6 water molecules that are normally lost due to displacement of the Phe 97 side-chain are now preserved, as the Leu 97 side-chain remains in the heme pocket. The other water molecules, including those participating in additional aspects of ligand-linked transitions, however, resemble wild-type HbI-CO water molecules. Second, the interfacial water molecules in the F97L-CO structure are significantly more disordered, with average B-factors of 34.5 Å² as compared to 24.7 Å² for wild-type HbI-CO. The interfacial water architecture for F97L-CO thus appears to resemble a hybrid version of the deoxy and liganded HbI water distribution. Another difference is that the distribution of water molecules in the mutant CO interface is asymmetric, in contrast to the wild-type structures. This asymmetry is seen to persist in simulated annealing omit maps.
Discussion

F97L exhibits an eight-fold increase in oxygen affinity over that of wild-type. From the kinetic results, it appears that the increased oxygen affinity results primarily due to an increased affinity of the deoxy state relative to the deoxy state of wild-type HbI. This is reflected in the large decrease in oxygen dissociation rates for F97L, particularly at low oxygen saturation and a modest increase in ligand combination rates, especially for CO (Figure III-A and Table III-A). An altered deoxy state is also suggested by spectral results and clearly shown in the crystallographic analysis. The crystal structures demonstrate that the smaller leucine side-chain is accommodated without strain in the heme pocket of both deoxy and F97L-CO. This permits the heme iron unrestricted movement into the heme plane (Figure III-D and Table III-D). Additionally, the hydrogen bond between the main-chain carbonyl oxygen of residue 97 and Nδ of the proximal histidine, which has been proposed to increase oxygen affinity, is nearly optimal in both the deoxy and CO structures. Thus, analysis of the F97L mutant strongly supports the proposal that Phe 97 plays the central role in determining oxygen affinity. Recently, residue Phe 97 has also been mutated to Ala (F97A) in order to examine the relation between size of the residue 97 side-chain and the magnitude of proximal strain in deoxy HbI (A. Pardanani; unpublished results). F97A exhibits increased oxygen affinity as compared to F97L (p50=~0.3 Torr) with similar cooperativity (Hills' 'n'=~1.2). This trend in ligand affinity extends to another related mutant, Phe 97 to Tyr (F97Y), in
which the Tyr 97 side-chain is clearly observed to be displaced into the dimer interface in both the deoxy and CO-liganded F97Y crystal structures (M. Bonham, unpublished results). This hemoglobin (F97Y) exhibits the highest oxygen affinity (p50 <0.1 Torr) of all the HbI mutants studied to date, with similar residual cooperativity to F97L and F97A (Hill's 'n'=~1.2). These results suggest that the position (and size) of the side-chain of residue 97 in relation to the proximal heme pocket of HbI, plays a determinative role in modulating oxygen affinity of the unliganded "low affinity" conformation of this protein.

Cooperativity in F97L, as well as F97A and F97Y, is largely decreased due to a loss of the ligand-linked movement of the side chain of residue 97 into the subunit interface. Kinetic measurements show no distinct change in the oxygen dissociation rate for F97L as a function of varying oxygen saturation, in sharp contrast to wild-type HbI. These observations are consistent with diminished cooperativity, resulting from the loss of displacement of Phe 97 into the interface. Perhaps surprisingly, cooperativity is not fully lost in this mutant. This appears to be coupled with our structural finding that the ligand-linked movement of the heme groups continues to occur in this mutant, refuting the earlier hypothesis that this movement was linked to the disposition of the Phe 97 side-chain. Thus, other interactions with the heme are responsible for this movement which results in communication via an alternate pathway that presumably supplements the primary Phe 97-based pathway in wild-type HbI.

Royer et al. (1996) have recently compared the ligand binding parameters of wild-type HbI and the mutant Thr 72 to Val (T72V), which exhibits very high oxygen affinity (p50=0.2 Torr) in combination with high cooperativity (n=1.7), as discussed in detail in the next chapter. The functional
behavior of these proteins was analyzed by defining the properties of ideal T and R states of HbI within the context of a two state model (Monod et al. 1965). These model properties can never be fully expressed in the experimentally accessible ligand binding behavior of the protein, but can be simulated in order to interpret the experimental data for the liganded and unliganded proteins. That analysis suggests that HbI and T72V have very similar T and R states, but that large functional distinctions result from a very different balance between T and R states as reflected in the allosteric constant L, the ratio between T and R states of the unliganded protein. A similar treatment of data for F97L that assumes no fundamental change in the affinities of the T and R states and that alters the value of L alone, does not work. The results from oxygen pulse and CO-binding experiments agree in indicating more R than T state for singly liganded F97L than predicted by such a simulation as well as greatly diminished cooperativity. The experimental results for oxygen binding data with F97L, however, may be well approximated by large changes in the value of both L and c (c is defined as the ratio between KT and KR for unliganded protein, where KT=T_{on}/T_{off} and KR=R_{on}/R_{off}). Figure III-A shows the agreement between experimental results and calculated values based on reducing L by 25-fold and decreasing T_{off} by 12-fold relative to wild-type. This interpretation is in agreement with our evidence from the deoxy crystal structure, spectroscopy and kinetic data that the T-state of F97L is significantly different from wild-type.

Displacement of the Phe 97 side-chain into the subunit interface upon ligand binding is proposed to represent the central trigger in the cooperative mechanism in wild-type HbI. The expelled Phe 97 side-chain directly interacts
with the opposite subunit only via the tenuous contact between its Cζ atom and the methyl group of T72', which lies away from the ligand binding site. However, this Phe 97 movement is also coupled with disruption of the deoxy interface water cluster and dropping of the heme groups. The marked decrease in cooperativity observed in F97L, in the presence of normal ligand-induced heme movements, thus implicates the Phe 97 induced disruption of the interface water cluster in the cooperative mechanism of wild-type HbI (as discussed in much greater detail in the next chapter). Residual cooperativity in F97L may, however, be mediated by a direct heme-heme pathway that supplements the primary Fe-proximal histidine-Phe 97 based pathway. Additionally, analysis of the water network in the interface of F97L HbI reveals that quite subtle changes in the deoxy conformation result in a destabilization of the interface water cluster. This suggests that the deoxy protein conformation of wild-type HbI is finely tuned to maintain its very well ordered interface water cluster.
Chapter IV

Ordered water molecules in the core of the HbI subunit interface act as allosteric mediators.

Introduction

High resolution crystal structures of *Scapharca* HbI have revealed a network of buried water molecules at the core of the dimer subunit interface (Condon and Royer 1994; Royer 1994). This region of the molecule exhibits large tertiary conformational changes upon ligand binding, which presumably mediate cooperative oxygen binding. The interface water molecules are very well ordered and distributed symmetrically in relation to the molecular dyadic axis. Additionally, the crystal structures suggest the participation of these water molecules in an intricate lattice of hydrogen-bonding interactions that bridge the two subunits.

The deoxy interface of HbI exhibits a more extensive cluster of well-ordered water molecules as compared to the liganded dimer. The deoxy water molecules form hydrogen-bonds with protein atoms from the E and F-helices, as well as with the heme propionate groups (Figures IV-A and IV-B). The interactions contributed by bound water in the core of the deoxy interface have
Figure IV-A. Stereo diagram depicting the interface water molecules of HbI viewed down the molecular dyad.

Water molecules are shown as blue spheres representing the approximate van der Waals radii of oxygen atoms in (i) deoxy HbI (PDB code 4SDH) and (ii) oxy HbI (PDB code 3SDH). In addition to the water molecules, for both subunits an α carbon trace for residues 93-101 and 69-75 is shown in yellow, the heme groups are shown in white (with the heme iron green), side-chains for the distal (residue 69) and proximal (residue 101) histidines are shown in yellow, while the side-chains for Phe 97 are shown in red and the side-chains for Thr 72 are shown in yellow with the hydroxyl shown in red. The deoxy water molecules are more well ordered than those of the oxy structure and form a clear cluster, with five of the water molecules in position to form hydrogen bonds with other water molecules but not with protein atoms. In the oxy interface, only one water molecule has all its possible hydrogen bonds with water molecules.
Figure IV-A
Figure IV-B. Stereo diagram showing the hydrogen bond network among ordered water molecules in the central core of the dimeric interface in (a) deoxy HbI and (b) HbI-CO.

Shown along with water molecules (circles) are the heme groups, main-chain atoms for residues 93 to 101 and 69 to 75, as well as side-chain atoms for Lys 96, Phe 97, His 101, Thr 72 and Tyr 75. Atom circles are shown for nitrogen, oxygen and iron atoms. Hydrogen bonds (< 3.25 Å) are shown by dotted lines except for those involving only main-chain atoms. Note hydrogen bonding by water molecules to main-chain atoms in the bent F-helix of deoxy HbI, and the effect of movement of Phe 97 into the interface upon ligation.
Figure IV-B
been suggested to stabilize the low affinity state of HbI by promoting the following: (i) *stabilization of the bend in the F-helix*. The sharp bend in the F-helix, which is coupled to packing of the Phe 97 side-chain in the heme pocket of deoxy HbI, results in disruption of the main-chain hydrogen-bonds involving the carbonyl oxygen atoms of residues 96 and 97 with the amide nitrogen atoms of residues 100 and 101 respectively. For each subunit, three interface water molecules are hydrogen bonded to main-chain protein atoms in this region, which help in stabilizing the unfavorable F-helix conformation (Figure IV-B). The bend in the F-helix may be further stabilized by the highly hydrophilic nature of the deoxy interface which favors the packing of the hydrophobic Phe 97 side-chain in the heme pocket, (ii) *stabilization of the deoxy heme conformation*. In deoxy HbI, the two pyrrole A propionate groups are folded over to avoid the potential collision and electrostatic repulsion that would result if these groups assumed extended conformations (Figure IV-B). Two water molecules in the core of the interface help in stabilizing each propionate group in this conformation; one of these water molecules provides a bridging interaction between the carbonyl oxygen of residue 97 and a propionate oxygen atom, while the second water molecule bridges the other propionate oxygen atom with the \( N^\circ \) of Lys 96' (from the opposite subunit) (Figure IV-B).

Upon ligand binding to HbI, there is a relatively large tertiary conformational change in the core of the dimer interface which leads to a significant rearrangement of the ordered water molecules. The largest ligand-linked conformational change is the movement of the Phe 97 side-chain into the subunit interface. Each Phe 97 side-chain directly causes the displacement of three water molecules in the subunit interface due to steric reasons (Figures IV-A
and IV-B). The remaining interface water molecules adapt to the ligand-induced conformational changes and may in fact facilitate these changes by providing compensatory hydrogen bonds to replace the ones broken by the allosteric transition. An example of this role is provided by the water molecule that bridges the \( \text{N}^\varepsilon \) atom of residue Lys 96 with the oxygen atom of the bent pyrrole A' propionate group in the deoxy state (Figure IV-B). Upon ligand binding, this water molecule exchanges places with the \( \text{N}^\varepsilon \) atom of Lys 96, where it now forms a bridge between the latter and the pyrrole D' propionate and the distal histidine (residue 69). Interestingly, the average isotropic B-factor of the core water molecules in the liganded interface is higher than that of the corresponding deoxy water molecules (Table IV-C). The water network in the liganded interface is thus less extensive and more disordered as compared to the deoxy network.

The presence of ordered solvent in the interface of HbI thus appears important in stabilizing the alternate allosteric states of HbI that are required for cooperativity. Resonance Raman measurements on deoxy HbI in the presence of high concentrations of glycerol provided an early indication that perturbation of the HbI water structure could have dramatic structural consequences (Royer et al. 1996). These experiments revealed that the deoxy HbI spectrum, in the presence of glycerol, closely resembles the 10 ns photoproduct spectrum of HbI-CO, which suggests that glycerol promotes a structural transition towards the liganded conformation even in the absence of ligand. In order to further probe the effect of osmotic forces on the HbI water structure, equilibrium oxygen binding experiments have been performed in the presence of glycerol, glucose and sucrose (Royer et al. 1996). In sharp contrast to human hemoglobin
(Colombo et al. 1992; Colombo and Bonilla-Rodriguez 1996), each of these agents significantly increases the affinity of HbI for oxygen, while fully maintaining cooperativity. As shown in Figure IV-C, the measured oxygen affinity increases with osmotic pressure in an identical manner for all three solutes. This indicates that increasing osmotic pressure destabilizes the low affinity, deoxy state relative to the liganded state, probably reflecting the greater hydration of the deoxy dimer interface. It has been recently shown that the deoxy (unliganded) HbI dimer is more tightly assembled than the liganded form (Royer et al. 1997). This result unequivocally demonstrates that, as in human hemoglobin and other allosteric systems, quaternary constraint (Monod et al. 1965) underlies cooperativity in Scapharca HbI, indicating that cooperative free energy is stored in the interface of the unliganded molecule. In terms of the two-state (MWC) model (Monod et al. 1965), a specific subset of local contacts at the subunit interface constrains the molecule in a low affinity, or T-state, and binding of successive ligands weakens these contacts, which destabilizes the T-interface to the point where the quaternary switch to the high affinity R-interface is favored. These observations strongly suggest the importance of the interactions made by the interface water molecules for stabilization of the low-affinity conformation of HbI.

Coupled to the ligation state, water molecules in the dimer interface occupy any free spaces that result from protein side-chain movement. An example is the space resulting from the removal of the Phe 97 side-chain from the dimer interface upon deoxygenation, which is filled by water molecules (Figures IV-A and IV-B). These ligand-induced structural transitions of HbI would lead to the loss of hydrogen bonding interactions in the absence of water. The unique
Figure IV-C. Plot showing the dependence of oxygen affinity with osmotic pressure for HbI and human hemoglobin (HbA).

Taken from (Royer et al. 1996)

The value of p50 at π=0 was estimated from measurements in buffer alone. The line showing dependence for human hemoglobin is obtained from Colombo et al. (Colombo et al. 1992). Data points are shown for tonometer measurements with glycerol (○), glucose (♦) and sucrose (+) solutions and Hem-O-Scan measurements for glycerol (●) and glucose (♦). Note the opposite direction for HbI, in which osmotic pressure increases oxygen affinity, compared with HbA, for which osmotic pressure decreases oxygen affinity. The slope of the least squares fit of the HbI data is $-9.5 \times 10^{-4} \pm 0.5 \text{ atm}^{-1}$, which indicates that an additional $6.2 \pm 0.3$ water molecules are bound to the deoxy state relative to the oxy state.
Figure IV-C

log \left[ \frac{p50(\text{obs})}{p50(\pi=0)} \right]

\[ \pi \text{ (atm)} \]
hydrogen bonding properties of water avoids this energetic penalty by allowing new hydrogen bonds to be substituted for those that are lost. This suggests that water molecules are key for an appropriate energy balance between the alternate allosteric states of HbI. A small difference in Free energy allows for an easy transition between the two physiologic states, which permits the optimal regulation of oxygen transport. This feature is well exhibited in human hemoglobin (HbA), where the difference in free energy between the unliganded and fully liganded states is ~6.0 kcal/mol (Holt and Ackers 1995), which is approximately equivalent to the energetic contribution of two or three hydrogen bonds.

The experiments described in this chapter were performed to analyze the role of interface water molecules in the structural mechanism of cooperativity of Scapharca HbI. Residue Thr 72 was an obvious target for mutagenesis, as (i) it possesses a side-chain that directly interacts with the deoxy interface water network but not water molecules in HbI-CO, and (ii) this interaction occurs at the periphery of the water cluster, away from the heme groups. The isosteric mutation of Thr 72 to Val is predicted to result in the loss of a single hydrogen-bond between a water molecule and the side-chain of Thr 72 from each subunit. This mutation should therefore selectively perturb the deoxy interface water molecules without affecting the intrinsic heme reactivity of individual HbI subunits. The replacement of Thr 72 by valine constitutes a more conservative change than its mutation to isoleucine, which exhibits increased oxygen affinity associated with greatly diminished cooperativity (Gambacurta et al. 1995).

This chapter presents oxygen binding and high resolution crystallographic studies of the Val 72 (T72V) mutant. Crystal structures of the Ile
72 (T72I) mutant, whose functional effects were reported earlier (Gambacurta et al. 1995), are also presented. In sharp contrast to T72I, the T72V mutant exhibits enhanced cooperativity along with increased oxygen affinity. The deoxy structures of both mutants are very similar and reveal a destabilization of the interface water cluster relative to wild-type, while the CO-structure comparison reveals a more extensive van der Waals interaction between the side-chains of Phe 97 and Ile 72' than observed for the corresponding residues in T72V or wild-type HbI. These results show the critical role of the interface water cluster in modulating the intricate balance between the deoxy and liganded allosteric states.
**Bacterial strain:** *Escherichia coli* strain W3110lacIqL8 (Summerford *et al.* 1995) was used as the host strain for overexpression of recombinant T72V.

**Mutagenesis:** *In vitro* cassette mutagenesis was used to construct the T72V mutant. The unique HindIII and BclI restriction sites of the HbI expression vector, pCS-26, were used to incorporate an oligonucleotide cassette bearing the appropriate sequence changes. Recombinant T72V was expressed and purified as described for wild-type (Summerford *et al.* 1995).

**Equilibrium Oxygen Binding Measurements:** Oxygen binding measurements on T72V were carried out in 0.1 M Tris/1.0 mM EDTA buffer (pH 7.2) at 23°C, using tonometric methods (Rossi-Fanelli and Antonini 1958). These experiments were performed in the presence of an enzymatic reduction system to reduce oxidation of the heme iron (Hayashi *et al.* 1973). The measurements were performed at either a heme concentration of about 5 μM, using four wavelengths in the Soret region, or at 50 μM, using nine wavelengths in the visible region.
Crystallization: Deoxy and carbon-monox crystals of T72V and T72I were grown as described in (Royer 1994), except that the crystals were grown from solutions of 40-50 mg/ml hemoglobin. Microseeds obtained by crushing corresponding wild-type HbI crystals were used to nucleate mutant HbI crystal growth. The morphologies of both crystal forms were similar to those of wild-type recombinant HbI.

Collection of diffraction data: X-ray diffraction data were collected from crystals at room temperature, on a R-AXIS IIC imaging plate system mounted on a Rigaku RU 200 rotating anode generator (Molecular Structure Corporation, The Woodlands, TX). Data were processed using software provided by the manufacturer.

The T72V-CO and T72I-CO crystals are essentially isomorphous to wild-type crystals, showing the symmetry of monoclinic space group C2.

The cell constants for T72V-CO crystals are: a=93.34 Å, b=43.84 Å, c=83.48 Å and β=121.96°. A total of 36 frames were collected from a single T72V crystal with an oscillation range of 2.5° for each frame. The overall Rmerge for 32,461 reflections derived from 58,462 independent observations with I/σ > 1, was 5.46%. This data set represents 86.1% of the predicted unique reflections to Bragg spacings greater than 1.6 Å.

The cell constants for T72I-CO crystals are: a=93.27 Å, b=43.75 Å, c=83.35 Å and β=121.93°. A total of 54 frames were collected from a single T72I crystal with an oscillation range of 1.8° for each frame. The overall Rmerge for 33,035 reflections derived from 85,575 total observations with I/σ > 1, was 6.17%. This
data set represents 87.9% of the predicted unique reflections to Bragg spacings greater than 1.6 Å.

Deoxygenated T72V and T72I crystals show the symmetry of orthorhombic space group C222₁, essentially isomorphous to deoxy wild-type crystals.

The cell constants of deoxy T72V crystals are: \(a=91.90\) Å, \(b=44.41\) Å and \(c=144.1\) Å. A total of 57 frames were recorded from two crystals, with an oscillation range of 1.2° to 2.5° for each frame. The overall \(R_{\text{merge}}\) for 140,113 independent observations with \(I/\sigma > 1\), was 8.9%. These represent 90.2% of the predicted unique data to Bragg spacings greater than 1.8 Å.

The cell constants for deoxy T72I crystals are: \(a=91.76\) Å, \(b=44.32\) Å and \(c=143.79\) Å. A total of 30 frames were recorded from a single crystal with an oscillation range of 3.0° for each frame. The overall \(R_{\text{merge}}\) for 35,777 unique reflections from 156,949 independent observations with \(I/\sigma > 1\), was 5.64%. These represent 91.8% of the predicted unique data to Bragg spacings greater than 1.6 Å.

**Refinement:** Isomorphous molecular replacement was used to refine the T72V and T72I structures. The starting models for determination of deoxygenated and CO-liganded mutant structures were the corresponding native HbI structures (Royer 1994). Residue Thr 72 was mutated to the appropriate amino acid in these models using the macromolecular modeling program 'O' (Jones et al. 1991). The models were then refined using the X-PLOR package (Brünger 1992) using a protocol described for the F97L HbI structure
determination (Pardanani et al. 1997). Water molecules were added to these models using the programs PEAKS and LOCATE written by Wayne Hendrickson (Columbia University).
Results

Overview: The side-chain of residue Thr 72 is positioned to form a hydrogen bond with a water molecule at the periphery of the interface water cluster in deoxy *Scapharca* HbI (Figures IV-A and IV-B). Upon ligand binding, this bond is broken due to a partial disruption of the interface water cluster. We have mutated Thr 72 to the isosteric amino-acid valine (T72V) in order to probe the contribution of the interface water structure to cooperative protein function. Structures of the T72I HbI mutant, which exhibits increased oxygen affinity but greatly diminished cooperativity (Gambacurta et al. 1995), have also been determined in the deoxygenated and CO-bound form.

Oxygen binding properties of T72V: Mutation of Thr 72 to valine in *Scapharca* HbI results in increased oxygen affinity and cooperativity, as observed by equilibrium oxygen binding measurements. These measurements at 23°C show a p50 of 0.2 Torr with a Hill coefficient (n) of 1.7, as compared to the corresponding values of 9.8 Torr and 1.5 for wild-type HbI. There is no change in the observed values of p50 and 'n' in the concentration range of 5-50 μM heme for T72V.
The T72V crystal structures: The models for deoxygenated and CO-ligated T72V have been refined against the X-ray data, to conventional R-factors of 18.8% and 19.5%, respectively, with excellent stereochemistry (Table IV-A). An electron density map of the heme region from one CO-ligated subunit is shown in Figure IV-D. The deoxy and CO-ligated structures of T72V are remarkably similar to the corresponding wild-type structures, with significant differences limited to the precise orientation of the Val 72 side-chain and the interface water structure. A comparison of average atomic B-factors between CO-ligated wild-type and mutant structures reveals them to be virtually identical, while the overall values for deoxy T72V are higher than deoxy wild-type HbI (Table IV-C).

In both T72V structures, the side-chain of Val 72 rotates slightly (0.5 Å displacement of the methyl group) towards the heme group due to the loss of hydrogen bond that normally tethers each Thr 72 hydroxyl group to the backbone carbonyl oxygen of residue 68 (Figure IV-E a). Steric repulsion between the bulkier methyl group of the valine side-chain and neighboring E-helix atoms also contributes to this movement in the mutant structures. Disruption of the hydrogen bond between the Thr 72 hydroxyl group and a water molecule at the periphery of the wild-type deoxy cluster, leads to the loss of this water molecule in T72V (Figure IV-F b) and an associated destabilization of the water cluster. 15 of the total 17 water molecules in this cluster are structurally identical (< 0.8 Å) to water molecules in the wild-type interface and are symmetrically distributed about the non-crystallographic dyad (2-fold). The remaining 2 water molecules exhibit asymmetry, which persists in simulated annealing omit maps, and are located in sites that are normally unoccupied by
water molecules in wild-type HbI. These asymmetric water molecules, which exhibit relatively high B-factors (36.6 and 42.7 Å²), are hydrogen bonded to neighboring protein atoms and water molecules. As shown in Table IV-C a, the water molecules in the core of the deoxy T72V interface are significantly less well ordered than in wild-type, with an average B-factor of 30.1 Å². This is in sharp contrast to the corresponding CO-structure comparison (Figure IV-G b and Table IV-C b) and suggests a marked loosening of the interfacial water network due to the loss of just two hydrogen bonds from the periphery of the subunit interface to the deoxy water network. Interestingly, the water molecules that are normally directly linked to the ones lost from this network, exhibit the largest increase in B-factor values relative to wild-type (36.7 and 41.0 Å², as compared to 18.3 and 18.8 Å² in wild-type). This serves as a marker for instability in the interface water cluster.

The T72I crystal structures: The structures of deoxygenated and CO- liganded T72I have been refined at 1.6 Å resolution to conventional R-factors of 19.2% and 19.4%, respectively, with excellent stereochemistry (Table IV-B). While the deoxy T72I structure is nearly identical to the corresponding T72V structure, the CO-liganded structure comparison reveals significant differences that are confined to the interaction between the side-chains of residues 97 and 72' and the interface water structure. Interestingly, the side-chain of residue Phe 97 remains packed in the heme pocket in deoxy T72I, contrary to earlier predictions (Gambacurta et al. 1995).
As seen with T72V, the side-chain of residue Ile 72 rotates (0.4 Å displacement of the methyl group) towards the heme group in T72I (Figure IV-E b). Also as in T72V, the water molecule that is normally hydrogen bonded to the side-chain hydroxyl group of each Thr 72 is now lost (Figure IV-F c) and the interface water cluster simultaneously destabilized. The loss of this water molecule results from the disruption of its hydrogen bond with Thr 72, and due to steric incompatibility with the position of the Cδ methyl group of the Ile 72 side-chain. As with deoxy T72V, 15 of the total 17 water molecules are structurally identical (< 0.6 Å) to the corresponding wild-type water molecules (Figure IV-F c). The remaining 2 are hydrogen bonded solely to other interface water molecules and are located at positions that are normally unoccupied by water in the wild-type interface. Unlike deoxy T72V, all of these water molecules exhibit symmetry in their distribution about the molecular 2-fold. As shown in Table IV-C a, the interface water cluster in deoxy T72I is more disordered as compared to wild-type, but less than in T72V, with an average B-factor of 25.3 Å². Similar to the deoxy T72V structure, the greatest increase in B-factors, relative to wild-type, is seen for water molecules that are normally hydrogen bonded to the 2 lost from the periphery of this cluster (31.4 and 45.7 Å², as compared to 18.3 and 18.8 Å² in wild-type).

The interface water network in T72I-CO is more disorganized than that in CO-liganded T72V or wild-type HbI (Figure IV-G c and Table IV-C b). This is mainly due to the loss or gross disordering of the 2 water molecules that are normally hydrogen bonded to the Nε of each Lys 96 residue. These water molecules are lost due to displacement of the Lys 96 side-chain, away from the heme groups and the interface core, by the bulky Ile 72' side-chain from the
opposite subunit. The average B-factor for the interface water cluster in T72I-CO is 28.7 Å², which is greater than the corresponding values for CO-bound wild-type or T72V (Table IV-C b).

An important feature of the T72I-CO interface is the favorable van der Waals interactions between the side-chains of residues Phe 97 and Ile 72' (Figure IV-H). This interaction is maximized by a change in the conformation of the Ile 72 side-chain about its $\chi_2$ torsion angle, as indicated in the comparison between deoxy and CO-liganded T72I structures (compare Figures IV-F c and IV-G c). Surface accessibility calculations using a spherical probe of 1.4 Å radius reveals that an additional 15-16 Å² of the Phe 97 side-chain is buried through its interaction with the Ile 72' side-chain, as compared to the interaction between corresponding side-chains in T72V or wild-type HbI. This enhanced interaction appears likely to contribute to enhanced stabilization of the high affinity conformation of T72I.
Table IV-A: Refinement Statistics on *Scapharca* T72V

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<th>CO</th>
<th>DEOXY</th>
</tr>
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<td>10.0 - 1.8</td>
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<tr>
<td>CO</td>
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Table IV-B: Refinement Statistics on *Scapharca* T72I

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Table IV-C a. Comparison of average B-factors (Å²) between deoxy HbI, deoxy T72V and deoxy T72I structures

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<td>Side-chain atoms</td>
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<td>21.1</td>
<td>20.4</td>
</tr>
<tr>
<td>Heme atoms</td>
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<td>15.1</td>
<td>15.7</td>
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<tr>
<td>Water atoms</td>
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<td>37.8</td>
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<td>Core Interface water atoms (15)</td>
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<td>28.9</td>
<td>23.6</td>
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<tr>
<td>Core Interface water atoms (17)</td>
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<td>30.1</td>
<td>25.3</td>
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Table IV-C b. Comparison of average B-factors (Å²) between HbI-CO, T72V-CO and T72I-CO structures

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<th>T72I-CO</th>
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<td>16.8</td>
<td>16.2</td>
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<td>Side-chain atoms</td>
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<td>19.7</td>
<td>19.4</td>
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<tr>
<td>Heme atoms</td>
<td>14.3</td>
<td>14.1</td>
<td>16.4</td>
</tr>
<tr>
<td>Water atoms</td>
<td>35.2</td>
<td>34.4</td>
<td>35.7</td>
</tr>
<tr>
<td>Core Interface water atoms</td>
<td>23.3 (11)</td>
<td>23.7 (11)</td>
<td>28.7 (8)</td>
</tr>
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Figure IV-D. Simulated annealing omit $F_o-F_c$ map of T72V-CO.

The T72V-CO structure was subjected to simulated annealing refinement, omitting the atoms shown. The panel shows the heme region of subunit 2, which includes the heme group, CO-ligand, Phe 97, His101 and Thr 72'. The map is contoured at the 3σ level.
Figure IV-D
Figure IV-E. Stereo diagram depicting the side-chain orientation of residue 72 in T72V (solid lines) and wild-type (dashed lines), in the deoxy state (*panel a*), and T72I (solid lines) and wild-type (dashed lines), in the deoxy state (*panel b*).

In both panels, the heme group, F-helix backbone (residues 96-100), E-helix backbone (residues 69-75) and side-chains of residues 72 and 101, for subunit I, are shown. Note the rotation of the side-chain of residue 72 in both T72V and T72I structures, towards the heme group. In this representation, mutant and wild-type structures have been centered on the α-carbon of residue 72 for each panel.
Figure IV-E
Figure IV-F. Stereo diagram of the core ordered water molecules in the deoxy interface.

In all 3 panels, the view is approximately down the molecular dyad. Shown are the heme group, the F-helix (residues 94 to 102), the E helix (residues 69 to 75) and side-chains for residues 72, 97 and 101, for each subunit. Additionally, a van der Waals representation of the interface water molecules is shown. (a) Ordered water molecules in the subunit interface of deoxy wild-type HbI. (b) Ordered water molecules in the subunit interface of deoxy T72V. 15 of the 17 water molecules, shown in medium-grey shading, are structurally equivalent (< 0.8 Å) to the corresponding water molecules in deoxy wild-type HbI. However, the 2 water molecules shown in light-grey shading, are not, and are asymmetrically distributed about the molecular dyad. The water molecules in this cluster are significantly less well ordered than wild-type. (c) Ordered water molecules in the subunit interface of deoxy T72I. 15 of the 17 water molecules, shown in medium-grey shading, are structurally equivalent (< 0.6 Å) to the corresponding water molecules in deoxy wild-type HbI, while the 2 shown in light-grey shading, are not.
Figure IV-G. Stereo diagram of the core ordered water molecules in the CO-liganded interface.

In all 3 panels, the view is approximately down the molecular dyad. Shown are the heme group, the F-helix (residues 94 to 102), the E helix (residues 69 to 75) and side-chains for residues 72, 97 and 101, for each subunit. Additionally, a van der Waals representation of the interface water molecules is shown. (a) Ordered water molecules in the subunit interface of wild-type HbI-CO. (b) Ordered water molecules in the subunit interface of T72V-CO. All 11 water molecules in this cluster are structurally equivalent (< 0.9 Å) to the corresponding water molecules in wild-type HbI. (c) Ordered water molecules in the subunit interface of T72I-CO. Although 3 water molecules are lost from this interface, the remaining 8 are structurally equivalent (< 1.0 Å) to the corresponding water molecules in wild-type HbI-CO. These water molecules are significantly less well ordered than wild-type.
Figure IV-G
Figure IV-H. Diagram showing interaction between the side-chains of Phe 97 and residue 72' in HbI-CO and T72I-CO.

Apart from the side-chains of Phe 97 and residue 72', the side-chain of residue His 101 and the heme group are shown in a van der Waals surface representation for each structure. Surface area accessibility calculations reveal that \(~55 \text{ Å}^2\) of the Phe 97 side-chain is buried from its interaction with the Ile 72' side-chain in T72I-CO, as opposed to \(~39 \text{ Å}^2\) in wild-type HbI, where it interacts with Thr 72'.
Discussion

Mutation of residue Thr 72 to valine, which results in the selective removal of just two hydrogen bonds from the deoxygen interface water cluster, results in a more than 40-fold increase in oxygen affinity and enhanced cooperativity. The latter result is very surprising given that (i) the closely related mutant, T72I, exhibits sharply different oxygen binding properties (Gambacurta et al. 1995), with a similar increase in oxygen affinity relative to wild-type, but markedly diminished cooperativity (Hill coefficient=~1.2), and (ii) a panel of high affinity mutants that target residue Phe 97 exhibits greatly diminished cooperativity (Hill’s n=~1.2) (Pardanani et al. 1997) (unpublished results for mutants F97Y and F97A).

Kinetic studies carried out with T72V (Royer et al. 1996) confirm the results of equilibrium oxygen binding measurements. Stopped-flow and oxygen pulse experiments performed at varying levels of oxygen saturation reveal that the increase in oxygen affinity results largely from reductions in the ligand dissociation rates (Table IV-D). Additionally, flash photolysis studies show a significant increase in kinetic cooperativity. As shown in Table IV-D, whereas the combination rate for the first oxygen is essentially the same for wild-type HbI and T72V, there is approximately a 2.2-fold increase in the combination rate for binding of the second oxygen to T72V, relative to wild-type. This effect is even more pronounced for CO binding.
Table IV-D. Kinetic ligand binding parameters to *Scapharca* HbI and T72V

[Taken from (Royer *et al.* 1996)]

<table>
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<tr>
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<th>Oxygen On Rates $\mu$M$^{-1}$s$^{-1}$</th>
<th>Oxygen Off Rates s$^{-1}$</th>
<th>CO On Rates $\mu$M$^{-1}$s$^{-1}$</th>
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<tr>
<td></td>
<td>$k'_1$</td>
<td>$k'_2$</td>
<td>$k_1$</td>
</tr>
<tr>
<td>T72V</td>
<td>10</td>
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<tr>
<td>HbI</td>
<td>11$^a$</td>
<td>16$^a$</td>
<td>490$^a$</td>
</tr>
</tbody>
</table>

Parameters are intrinsic (i.e. no statistical factors). Subscripts define the binding step based on a consecutive two step scheme following Adair (Adair 1925) A superscript prime designates an on rate, no prime indicates an off rate.

$^a$ from ref (Chiancone *et al.* 1993)

$^b$ from ref (Antonini *et al.* 1984)
Analysis of the crystal structures of T72V and T72I reveal a significant destabilization of the water cluster in the deoxy interface of each mutant. This stems from the loss of just two water molecules, each of which is normally linked to the side-chain hydroxyl group of Thr 72 from one HbI subunit. Loss of these water molecules leads to a disordering of the remaining water molecules in this cluster. Interestingly, despite the much higher oxygen affinity of these mutants, the Phe 97 side-chain remains packed in the deoxy heme pocket in both cases. As the two water molecules directly influenced by the mutation of residue Thr 72 lie at the periphery of the interface water cluster, rather than in a position to stabilize the low-affinity conformation through direct interactions with the hemes, it is unlikely that the observed functional consequences of these mutations result from a perturbation of the intrinsic oxygen affinity of the individual subunits. Additional evidence that destabilization of the deoxy water cluster is sufficient to skew the balance away from the low affinity form, towards the high affinity conformation, is the increased oxygen affinity that results from osmotic stress (Royer et al. 1996). These results reflect the importance of an intact water cluster to the deoxy state. A novel feature of the interface of CO-bound T72I is the more extensive van der Waals interaction between the side chains of Phe 97 and Ile 72', as compared to the corresponding interaction in wild-type HbI or T72V, which presumably stabilizes the high affinity conformation of T72I.

The functional effects of the T72V mutation have been interpreted within the context of the two-state model for cooperativity (Royer et al. 1996). Although the ligand binding parameters of the canonical T and R states described by this model are not directly accessible through experiment, reasonable
approximations can be obtained by the simultaneous consideration of ligand binding parameters for HbI and T72V. If it is assumed that ligand binding properties of the T state are described by deoxy wild-type HbI and those of the R state by liganded T72V, it is possible to simulate the functional characteristics of both, wild-type HbI and T72V, with a single set of kinetic parameters. As shown in Table IV-E, a surprisingly good fit of the experimental data for these molecules is achieved by varying only the allosteric constant L, which is defined as the ratio between the T and R states of the unliganded molecule. These results suggest that, in wild-type HbI, the singly liganded species largely retains the low-affinity conformation, while in the case of T72V, the equilibrium is greatly skewed towards the high-affinity conformation (Table IV-F).

Preliminary simulations have shown that the significantly lowered cooperativity of T72I, relative to T72V, can be approximated by further decreasing the assumed value of L within the two-state model. Lowering L to 2.5, from 15 for T72V, yields values of $p50=0.13$ Torr and $n=1.3$, probably within experimental error of the reported values of $p50=0.2$ Torr and $n=1.2$ (Gambacurta et al. 1995). The structural basis for this additional change in L is the apparently increased stability of the R-state conformation that results from favorable interactions between the side-chains of Phe 97 and Ile 72'. This results in the equilibrium between T and R states being further skewed towards the R-state (Table IV-F). These simulations reveal that any further decrease in L, from that in T72V, results in a precipitous reduction in cooperativity, with only small changes in oxygen affinity (Figure IV-I).
Table IV-E. Fit of HbI ligand binding data toallostERIC model [Taken from (Royer et al. 1996)]

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<td>1.7</td>
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</table>

Constant model parameters: \( R_{on} = 36 \mu M^{-1}s^{-1} \), \( T_{on} = 11 \mu M^{-1}s^{-1} \), \( R_{off} = 5s^{-1} \), \( T_{off} = 490s^{-1} \)

a from reference (Chiancone et al. 1981)
b from reference (Chiancone et al. 1993)
c from reference (Antonini et al. 1984)
d The on rate for CO to the R-state was fixed to the experimentally determined value for T72V.
Table IV-F. Percentage of HbI molecules calculated to be in the T-state for various states of ligation; from fit of experimental ligand binding data to the MWC allosteric model

<table>
<thead>
<tr>
<th></th>
<th>Unliganded</th>
<th>Singly Liganded</th>
<th>Fully Liganded</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (L=12500)</td>
<td>99.9%</td>
<td>97.5%</td>
<td>12.1%</td>
</tr>
<tr>
<td>T72V (L=15)</td>
<td>93.8%</td>
<td>4.7%</td>
<td>0.01%</td>
</tr>
<tr>
<td>T72I (L=2.5)</td>
<td>71.4%</td>
<td>0.8%</td>
<td>0.002%</td>
</tr>
</tbody>
</table>

c (T72V and WT) = $K_aT/K_aR = \sim 1/321$ (determined from constant model parameters shown in Table X-E)
Figure IV-I. Plot showing the dependence of HbI oxygen binding properties on the allosteric constant $L$, as predicted by the MWC (two-state) model.

The calculated values of (a) p50 and (b) Hill coefficient [$n$], from simulations of oxygen pulse experiments, have been plotted as a function of $L$. Model parameters for T and R states are taken from (Royer, Pardanani et al. 1996) in these simulations. $L$ values, for wild-type HbI, T72V and T72I, that allow the best fit of individual ligand binding data to the MWC model are illustrated on the plot.
Figure IV-I

MWC simulation of HbI Oxygen Binding

\[ \log(L) \]

\[ n \]

\[ p50 \text{ (Torr)} \]
Chapter V

Summary

There are several reasons why the homodimeric hemoglobin HbI, from the mollusc *Scapharca inaequivalvis*, constitutes a unique model system for the study of cooperative protein function: (i) intersubunit communication is mechanistically simple in HbI. As compared to HbA, the lack of heterotropic allosteric effects and the shorter distance between ligand binding sites allows communication to be mediated by only a few residues; (ii) crystals of native and recombinant HbI in both unliganded and liganded states diffract to high resolution. This permits detailed correlations between protein structure and HbI ligand binding behavior to be established, and (iii) there is no large ligand-induced quaternary change in HbI. This feature allows structure-function relationships to be elucidated with a higher degree of confidence than would be otherwise possible. This is because global changes in protein conformation frequently contribute confounding structural effects, which have to be distinguished from those effects which impact directly on the protein activity being measured.

Results from this thesis project demonstrate that relatively subtle perturbations of the proximal heme pocket produce large changes in HbI ligand affinity (Chapter III). Thus, it appears that, like human hemoglobin (HbA)
(Barrick et al. 1997), HbI has utilized proximal strain very effectively to modulate the intrinsic subunit affinity for ligand. In contrast to HbA, however, most of this strain is generated through the disposition of a single protein side-chain, Phe 97, which packs in the heme pocket of deoxy HbI. Attenuation of the size of residue 97 side-chain (F97L and F97A mutants), or its removal from the heme pocket (F97Y mutant), results in a progressive, but non-uniform, increase in oxygen affinity relative to wild-type HbI. The contribution of distal heme effects towards ligand affinity has not been evaluated here. It is predicted that, like sperm whale myoglobin (Mb), where these effects have been extensively studied (Phillips 1980; Quillin et al. 1993), the precise geometry of residues in the distal heme pocket plays an important role in modulating the relative affinity of HbI for CO as compared to O2. In this regard, observed structural features of the distal heme pocket (Condon and Royer 1994) correlate well with the discrimination of CO and O2 ligands in HbI, relative to that observed in Mb and HbA.

Residue Phe 97, which plays a central role in modulating intrinsic heme affinity of the deoxy state, is also a crucial determinant in the mechanism of information transfer between subunits. Ligand-induced changes in heme conformation are coupled to large changes in the conformation of Phe 97, presumably via the proximal histidine (His 101), although the contribution of the linkage between His 101 and the heme iron has not yet been directly tested (see Future Directions, Chapter VI). The change in disposition of the Phe 97 side-chain results in communication of information regarding the ligation state of one subunit, across the interface, to the opposite subunit (Chapter III). Results described in this dissertation have established the major effect of Phe 97 in
mediating this information transfer to be disruption of a well ordered interface water cluster (Chapter IV). Disruption of this water cluster couples the large conformational change in Phe 97 to a functional state in which higher affinity binding of the second ligand is now favored, resulting in cooperative ligand binding. The interface water cluster therefore constitutes a crucial link in the pathway of information transfer between subunits.

High resolution crystallographic studies of T72V and T72I have revealed that the interface water cluster modulates ligand affinity by preferentially stabilizing the low affinity conformation of HbI. The water molecules in this cluster represent a class of molecules that are tightly bound in deep crevices or interior cavities of proteins, and are distinct from water molecules found either in bulk solvent or those interacting with the surface of macromolecules (Levitt and Park 1993). This is reflected in the low B-factors exhibited by these water molecules in the HbI crystal structures, and suggests a low vibrational amplitude of atomic motion due to optimal hydrogen bonding interactions. The residence times of these buried water molecules, typically studied by dynamic NMR measurements, are expected to be in the range of $10^{-2}$ to $10^{-8}$ seconds, as compared to surface waters, which are in rapid exchange with bulk solvent (<500ps, depending upon temperature) (Otting and Wuthrich 1989). The finding that the deoxy dimer interface, which is more highly hydrated as compared to the liganded interface, is also the more tightly assembled (Royer et al. 1997), raises an important issue regarding the role of these water molecules in the thermodynamics of ligand-induced structural transitions. Although not directly addressed in this study, it is clear that the interface water molecules must be important participants in modulating the free energy ($\Delta G$) difference between the
allosteric end points of HbI. The ordering of water molecules in the deoxy interface results in a significant entropic cost, which has been estimated to be between 0 and 2 kcal mol$^{-1}$ at 300 K (Dunitz 1994). This analysis suggests that the favorable gain in enthalpy from the optimal hydrogen bonding of these water molecules is sufficient to render their ordering in the subunit interface energetically favorable. Quite obviously, determination of the precise contribution of solvent enthalpy and entropy terms to the free energy change upon ligand binding will hinge on careful thermodynamic experiments.

Several studies using both experimental and theoretical techniques (Karplus and Faerman 1994), have implicated ordered water molecules in important structure-function relationships. Structural studies on antigen-antibody reactions have revealed, at least in specific cases, that networks of ordered water molecules can link antigen and antibody, through numerous solvent mediated hydrogen bonds (Bhat et al. 1994; Braden and Poljak 1995). Extensive thermodynamic analysis of these antigen-antibody reactions, under varying experimental conditions including a reduction in water activity, show these reactions to be enthalpically driven. This demonstrates the crucial role interface water molecules can play in stabilizing the macromolecular complex between antigen and antibody via a significant solvent-enthalpy contribution (Bhat et al. 1994; Goldbaum et al. 1996). These studies have implicated water molecules in macromolecular recognition and have provided a correlation between structural models and thermodynamics. In many examples, specific water molecules serve to increase the molecular complementarity between interacting macromolecular surfaces, which leads to interactions that are characterized by high affinity and specificity. In this regard, studies that have
addressed the specificity of protein-DNA interactions have been particularly illuminating (Schwabe 1997). These studies reveal the specific binding of trp repressor (Otwinowski et al. 1988), BamH1 restriction endonuclease (Newman et al. 1995) and the estrogen receptor DNA-binding domain (Schwabe et al. 1993), to relevant target-DNA sequences to be mediated by well ordered water molecules at the binding interface. Other examples of the essential role of water molecules in protein structure and activity include sugar binding (Quiocho et al. 1989) and enzyme catalysis (Singer et al. 1993).

A unique feature of water molecules is the ability to form alternate networks of hydrogen bonding interactions that facilitate conformational changes at interacting surfaces, and consequently, permit the binding of diverse ligands to macromolecules. An example of the role of alternate solvent networks in molecular recognition is the binding of different peptides to a single Major Histocompatibility Complex (MHC) class I allele. Here, bound water molecules in the peptide binding groove act together with polymorphisms in the MHC protein sequence to allow the binding of degenerate peptides to MHC class I molecules with high affinity, but relatively low specificity (Smith et al. 1996). The concurrent use of experimental methods such as single crystal diffraction (X-Ray or Neutron) or NMR spectroscopy, and theoretical approaches such as MD simulations provide the most detailed understanding of ordered water at the atomic level. A combination of NMR and MD (Molecular Dynamics) approaches has been utilized to provide insights into the dynamic aspects of protein-DNA recognition, and the role of water molecules therein (Billeter et al. 1996). NMR studies of the Antennapedia homeodomain-DNA complex have revealed 16 different conformers to represent the solution NMR structure of this complex.
Each of these 16 conformers exhibits a distinct pattern of intermolecular interactions, which include residues that are critical for specificity. Each conformer also exhibits a distinct hydration pattern of the interaction interface. MD simulations have indicated residence times for the interface water molecules in this complex to be approximately 1 ns, which suggests a highly mobile and constantly fluctuating network of non-bonded interactions. The authors conclude that specificity of interaction in this case must be enciphered by the ensemble of rapidly interconverting structures, which appears to reduce the entropic costs arising from a potential "locking" of the two molecules into a single complex (Billeter et al. 1996).

Water molecules can be used in allosteric proteins to stabilize one allosteric state preferentially, but also to facilitate the transitions between alternate states. The very high concentration of water in cells and its unique hydrogen bonding properties allow it to effectively mediate these properties. Although the work on HbI provides the clearest detailed mechanism to date of how water molecules might be harnessed by allosteric proteins, it by no means suggests that HbI is unique in this regard. Indeed, there is evidence that water molecules play similar important roles in other allosteric systems such as human hemoglobin (Colombo et al. 1992) and phosphofructokinase (Schirmer and Evans 1990). The unique advantages of HbI as a model system, however, stem from the small tertiary conformational changes that underlie cooperativity, which allows select structurally observed water molecules to be assigned specific roles in ligand binding and cooperativity. Ordered water molecules in the HbI subunit interface have been shown in this work to act both as "cement", that stabilizes the deoxy state, and as "lubricant", which facilitates the structural transitions that
mediate cooperativity, thus fulfilling roles of water suggested by other studies (Levitt and Park 1993).
Chapter VI

Future Directions

Distal-to proximal heme communication

According to the Perutz model for cooperativity, the bond between the heme iron and the proximal histidine constitutes the central link for distal-to-proximal heme communication in human hemoglobin (Perutz 1990). This linkage is proposed to directly couple the changes in heme conformation upon ligand binding, to the changes in protein structure that mediate the T to R quaternary switch. A recent study has directly tested this model by breaking the link between the histidine imidazole side-chain and the protein backbone (Barrick et al. 1997). Results from this work reveal that detachment of the proximal histidine significantly increases ligand affinity, while decreasing cooperativity, which is largely in accord with the Perutz model. Interestingly, residual cooperativity occurs in the absence of a T to R quaternary conformational change, which indicates that supplementary heme-heme communication pathways that are independent of the proximal histidine coupling mechanism, exist in human hemoglobin. There is evidence in Scapharca HbI of a direct heme-to-heme pathway of intersubunit communication (see
Chapter III). A similar approach, therefore, that decouples the heme-to-protein backbone linkage, will establish the precise contribution of the heme conformational changes to inter subunit communication, independently of the proximal histidine mediated pathway. This experiment will also establish whether the ligand-induced dropping of the heme groups away from the subunit interface, which has been shown to persist in the absence of the Phe 97 side-chain (Chapter III), is mediated through this linkage. The components of the supplemental pathway(s) of heme-heme communication, that mediate residual cooperativity in the Leu 97 mutant, should thus be identified. In this regard, the proximal histidine in HbI has been mutated to glycine, as part of this thesis project. The mutant protein has subsequently been overexpressed in E. coli, in the presence of exogenous imidazole, which binds the heme iron in trans, in lieu of the proximal histidine side-chain (Barrick 1994). Successful purification of this recombinant protein will allow for the analysis of its ligand binding properties through equilibrium oxygen binding studies and the determination of its structures in both allosteric states. Our hypothesis for cooperativity predicts that oxygen affinity will increase and cooperativity will decrease, once the covalent link between the heme iron and F-helix is severed.
Probing the role of the interface water structure in residual cooperativity

The crucial role of interface water molecules in stabilizing the low affinity state of *Scapharca* HbI has been demonstrated by both site-directed mutagenesis and osmotic stress techniques. These studies reveal that destabilization of the interface water cluster results in a dramatically altered balance between the two alternate allosteric states of HbI. This water network is predicted to constitute an essential element in both the proximal histidine mediated pathway of communication, and the supplemental heme based pathway. It is therefore expected that the disruption of the water cluster, by either osmotic or mutagenesis methods, on a background of HbI mutants in which the primary proximal histidine (Phe 97) based pathway has been disrupted, will establish the contribution of these water molecules in residual cooperativity.

(i) Osmotic stress experiments: Equilibrium oxygen binding experiments at elevated osmotic pressures using glycerol, glucose or sucrose, with available Phe 97 mutants (Leu 97, Ala 97 and Tyr 97) will be used as an initial approach in this effort.

(ii) Construction of double mutants: Mutations that are known to selectively destabilize the interface water cluster (T72V and T72S) will be combined with the various Phe 97 mutations to generate appropriate double mutants.
We expect that the combined perturbation of these alternative pathways of communication will reveal an opposite effect on HbI cooperativity, as compared to the isolated Phe 97 mutation. It is predicted that both oxygen affinity and and cooperativity will further increase as the water cluster is destabilized in this situation. This is because, within the context of the MWC model, we expect the value of $L$ to further decrease as compared to isolated Phe 97 mutants (see Chapters III and IV). The magnitude of the increase in residual cooperativity and oxygen affinity will reveal the precise contribution of the interface water molecules in the direct heme-to-heme pathway in HbI.

Creation of a heterodimer

Cooperativity requires that intermediate states be unstable relative to the allosteric endpoints. The low stability, and thus small populations, of intermediates complicates efforts to understand cooperativity. The absence of a large quaternary change in *Scapharca* HbI make it an attractive system for creating a heterodimeric molecule. A heterodimer, in which one subunit resembles the liganded form and the other the unliganded, is analogous to the singly liganded intermediate state of HbI. The MWC (two-state) model for cooperativity (Monod *et al.* 1965), which has been utilized to interpret the functional behavior of the T72V and F97L mutants described in this report, predicts a structural symmetry between the two subunits at all stages of ligation.
In contrast, the KNF (sequential) model allows significant structural differences between the two subunits for the singly liganded state. Crystal structures of HbI heterodimers will thus provide invaluable information regarding both, the molecular details of the cooperative mechanism, and the applicability of the two contrasting allosteric model systems to HbI.

A key prerequisite for structural studies on a HbI heterodimer is the preservation of wild-type ligand binding properties. Initial attempts at creating such a heterodimer involved using the only cysteine (Cys 92) to form a disulfide bond with another cysteine engineered at position 64 (unpublished results, M. Bonham). It was predicted that mixing of the double mutant (C92S/D64C) with wild-type HbI (C92, D64) would provide substantial quantities of the heterodimer, by driving the equilibrium towards the disulfide-stabilized molecule. However, under these conditions, the Hill coefficient drops to ~1.2, indicating a substantial change in the oxygen binding properties of the covalently linked species. This is presumably due to the disruption of intersubunit communication by the disulfide bridge, which lies in close proximity to some of the residues involved. This adverse result compels the pursuit of alternative approaches to generate HbI heterodimers. One such approach involves the use of cross-linking agents to link lysine residues from different subunits. These lysine residues can be introduced at pre-selected positions by site-directed mutagenesis. Key to success is that the pair of residues to be cross-linked lie well away from the allosteric core of the interface.
REFERENCES


