Mutation of Residue Phe\textsuperscript{97} to Leu Disrupts the Central Allosteric Pathway in Scapharca Dimeric Hemoglobin\textsuperscript{*}

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Residue Phe\textsuperscript{97}, which is thought to play a central role in the cooperative functioning of Scapharca dimeric hemoglobin, has been mutated to leucine to test its proposed role in mediating cooperative oxygen binding. This results in an 8-fold increase in oxygen affinity and a marked decrease in cooperativity. Kinetic measurements of ligand binding to the Leu\textsuperscript{97} mutant suggest an altered unliganded (deoxy) state, which has been confirmed by high resolution crystal structures in the unliganded and carbon monoxide-liganded states. Analysis of the 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\textsuperscript{97} side chain, whose tight packing in the heme pocket of the deoxy state normally restricts the heme from assuming a high affinity conformation. The absence of the Phe\textsuperscript{97} side chain is also associated with diminished cooperativity, since Leu\textsuperscript{97} packs in the heme pocket in both states. Residual cooperativity appears to be coupled with observed structural transitions and suggests that parallel pathways for communication exist in Scapharca dimeric hemoglobin.

The homodimeric hemoglobin (Hbl)\textsuperscript{1} from the blood clam Scapharca inaequivalvis offers a simple model system for studying communication between two chemically identical subunits. Analysis of the mechanistic details of this intersubunit communication is being pursued to provide insights into the regulation of protein function.

Scapharca Hbl binds oxygen cooperatively, with a p50 of 7.8 torr and a Hill coefficient (n) of 1.5 at 20 °C, neither of which changes as pH varies from 5.5 to 9.0 (1). Although the individual subunits of Hbl have the same myoglobin fold as mammalian hemoglobins, the quaternary assemblage is radically different (2). Upon oxygen binding by Hbl, only small tertiary changes are seen at the subunit interface in contrast to the relatively large quaternary changes observed with mammalian hemoglobins (3). Analysis of structures of this hemoglobin at 1.6 Å for the deoxygenated molecule, 1.4 Å for the CO-liganded form, and 1.7 Å for the oxygenated form has provided a framework for understanding the role of individual side chains and interfacial water in mediating cooperativity (2, 4).

Phenylalanine 97 has a central role in the proposed structural mechanism of cooperativity (2, 4). The side chain of Phe\textsuperscript{97} undergoes the largest ligand-linked conformational change in Hbl. 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. The lowered oxygen affinity of the deoxy state appears to result primarily from the packing of Phe\textsuperscript{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. Upon ligation, movement of Phe\textsuperscript{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 effects within a subunit alter the nature of interactions between subunits, which presumably encourages movement of the Phe\textsuperscript{97} side chain (second subunit) into the interface, allowing the second subunit to attain a high oxygen affinity state prior to ligand binding (2). In view of its significant role in the proposed cooperative mechanism of Hbl, Phe\textsuperscript{97} is clearly indicated as a target for mutation. Phe\textsuperscript{97}, four residues from the proximal histidine at position 101, is analogous to the fourth residue of the F helix 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 paper, we present functional and high resolution crystallographic studies of the Leu\textsuperscript{97} Hbl (F97L) mutant. This mutation results in increased oxygen affinity and diminished cooperativity that appear correlated with observed ligand-linked structural transitions. Our results confirm the crucial role of residue Phe\textsuperscript{97} in modulating oxygen binding by Hbl, while demonstrating the persistence of residual cooperativity and suggesting 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 W3110lac\textsuperscript{+}L8 was the host used for overexpression of recombinant Hbl (5).

Mutagenesis—In vitro oligonucleotide-mediated, site-directed mu-
tagenesis was used to generate the F97L mutant. The initially constructed gene for HbI bearing 5 and 3' termini complementary to Kan1 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 rounds of plaque purification using TG1 cells as hosts, recombinant single-stranded DNA was isolated (6). Subsequently, a 19-mer oligonucleotide (University of Massachusetts Medical Center DNA synthesis facility) complementary to the region of the gene coding for residue Phe was used in a site-directed mutagenesis protocol (Sculptor in vitro mutagenesis system, Amersham Life Science Inc.) 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 (U. S. Biochemical Corp.). Double-stranded DNA of the replicative form was then prepared and subjected to AccI and SacI restriction cleavage to isolate a segment of the HbI gene that contains the leucine 97 mutation. This fragment was then subcloned into a restriction site in the expression vector that, as reported earlier, has allowed for significant overexpression of recombinant, wild-type HbI protein (5).

**Protein Expression and Purification—Recombinant F97L was overexpressed in E. coli and purified as described for wild type (5).**

**Spectroscopic Characterization—**Spectroscopic measurements were carried out at 20 °C in a Cary 3 spectrophotometer with a Cary 3 spectrophotometer accessory (Varian, Inc.). The buffer and solid dithionite was added (10).

Circular dichroism spectra were measured at 20 °C in 0.1 M phosphate buffer at pH 7.0 in a Jasco J710 spectropolarimeter equipped with a Jasco J700 processor in the range 380–650 nm. The deoxy- and CO-liganded derivative was obtained by dissociation of the oxygenated protein of a small volume of a 1.0 M sodium dithionite solution prepared in deoxygentuated buffer.

**Kinetic Measurements—**Kinetic measurements using flash photolysis were performed using a YAG laser giving a 9-ns flash of 25 mJ at 532 nm. The beam was telescoped to give a parallel beam 2 mm in diameter collinear with the observing beam sampling from a 57-W xenon arc passing through a blue filter and imaged at the sample within the photolysis beam. The observing beam continued through a Spex 250-mm monochromator to a photomultiplier and amplifier (Teledyne-Philbrick 1321). The output from the amplifier was digitized by a DAS-50 12-bit A/D converter (Metabyte) and transferred to an IBM personal computer. The combination had an overall response time of 2 µs.

Stopped flow measurements were performed with the apparatus described by Gibson and Milnes (9) in the slightly modified version of the Durrum Corp. (Palo Alto, CA). Data were digitized and recorded as described for flash photolysis using a tungsten lamp as light source. The dead time of the stopped flow experiment (2-cm path) was 2.4 ms. For the oxygen-pulse experiments, the sample was filled with its own buffer bubbled with pure nitrogen. For the low oxygen experiments the apparatus was filled with a dilute solution of sodium dithionite and allowed to stand overnight. It was then washed out repeatedly with buffer bubbled with pure nitrogen before introducing the working solutions. Low oxygen concentrations were obtained by mixing air-equilibrated buffer with nitrogen-bubbled buffer in a syringe. For the oxygen-pulse experiments a strong (5 mM) solution of deoxyhemoglobin was diluted into nitrogen-bubbled buffer and solid dithionite was added (10).

A kinetic equivalent of the two-state model was then developed to simulate the progress of oxygen saturation in oxygen-pulse and other kinetic experiments using a two-step Runge-Kutta process (11). To simulate the progress of oxygen saturation in oxygen-pulse and other kinetic experiments using a two-step Runge-Kutta process (11).

**Results**

Functional and Spectroscopic Characterization of F97L—Mutation of phenylalanine 97 to leucine (F97L) in Scapharca HbI resulted in increased ligand affinity and diminished cooperativity as observed by both equilibrium and kinetic measurements. The p50 value for oxygen binding, based on equilibrium measurements, decreased from 7.8 to 1.0 torr, and the Hill coefficient decreased from 1.5 to 1.16 ± 0.05. The statistical analysis of all the data sets (n = 12) indicated that the Hill coefficient was significantly higher than 1.0, such that cooper-
activity, although drastically reduced, was not abolished.

A summary of kinetic experiments on ligand binding is presented in Table I. The increased oxygen affinity observed in equilibrium measurements was primarily due to a decrease in the oxygen dissociation rate, particularly at low O₂ saturation. Oxygen-pulse and CO-replacement experiments (15) showed a greater than 10-fold decrease in the dissociation rate at low oxygen saturation compared with HbI. In contrast to HbI, there was little or no change in the oxygen-dissociation rate for F97L as a function of varying levels of oxygen saturation (Fig. 1).

Additionally, measurements of CO binding to F97L by both stopped flow and flash photolysis showed a clear increase in combination rates compared with wild type.

The absorption spectra of the oxygenated and CO-liganded derivatives of F97L were almost identical to those reported for native HbI (1). In contrast, significant differences were observed for the deoxygenated derivative. In the Soret region, the band line shape was wider and more symmetric and the absorption maximum was shifted to lower wavelengths by 4 nm. In the visible region, the unusual shoulder present at 590 nm

| Ligand binding parameters to Scapharca HbI and F97L
| Parameters are intrinsic (i.e. no statistical factors). Subscripts define the binding step based on a consecutive two-step scheme following Adair (22). A prime designates an on rate, and no prime indicates an off rate.

| Oxygen on rates | Oxygen off rates | CO on rates |
|-----------------|------------------|-------------|
|      | k₁ s⁻¹ | k₂ s⁻¹ | l₁ s⁻¹ | l₂ s⁻¹ |
| F97L | NDᵃ | 26 | 40ᵇ | 45ᵇ | 0.5 | 2.3 |
| HbI | 11¹ | 16¹ | 490ᵇ | 50ᵇ | 0.09ᵈ | 0.2ᵈ |

ᵃ ND, not determined.
ᵇ Oxygen pulse measurements (15).
ᶜ From Ref. 23.
ᵈ From Ref. 24.
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band, which gives rise to splitting of the Soret band, was diminished in the mutant in which symmetry was restored. The overall decrease in molar ellipticity in F97L supports the coupled oscillator theory of Hsu and Woody (17), since the removal of an aromatic ring from within 4 Å of the heme plane is expected to decrease the rotational strength of the coupled π-π* transitions of aromatic residues and heme, thus decreasing the intensity of the Cotton effect.

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 II). An electron density map of the heme region of one subunit is shown in Fig. 3. Both the unliganded and liganded structures of F97L were 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 Fig. 4.

As shown in Tables III and IV, the overall B-factors were slightly higher in deoxy F97L than in deoxy wild type and were nearly the same for the CO-liganded structures. In contrast to the similarity in overall B-factors, the core interface water molecules showed significantly higher B-factors in F97L than in wild type. This indicates that these water molecules, which have been implicated in cooperative function in wild-type HbI (18), 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 was very similar to that in the deoxy wild-type structure, where it is packed in the heme pocket (Figs. 4 (top) and 5). Unlike the phenylalanine side chain, which is packed tightly between the heme group and the proximal histidine (residue 101), the smaller Leu97 side chain appeared to be accommodated without strain. This was reflected in differences in heme conformation, position of the heme iron, and subtle changes in F-helix geometry compared with deoxy wild-type HbI (18), as discussed further below.

Upon ligand (CO or O2) binding to wild-type HbI, the Phe97 side chain is displaced into the subunit interface, since its tight pocket is reduced when the iron moves into the heme plane (2). In contrast, the leucine side chain remained in the heme pocket in F97L upon ligand binding (Fig. 4, bottom).

F-helix Geometry—In deoxy wild-type HbI, there is a sharp bend in the F-helix (residues 87–103) (Figs. 4 (top) and 5) that was postulated to result from wedging of the Phe97 side chain in the heme pocket (2). Unexpectedly, this bend, although attenuated, persisted in F97L, where the smaller leucine side chain was readily accommodated in the heme pocket. Subtle differences in F-helix geometry were present including a movement of the backbone toward the heme group, most markedly seen involving the C-o of residue Leu97 (0.5 Å), but also involving Lys96 (0.3 Å) and Glu95 (0.3 Å), as shown in Fig. 5. This change presumably resulted from loss of the tight packing of Phe97 between the F-helix backbone and the heme group. Additionally, the distances between the carbonyl oxygens of residues 96 and 97 and the amide nitrogens of residues 100 and 101, respectively (4.5 and 3.9 Å), were slightly less than the corresponding distances in the wild-type deoxy structure. These 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 Nδ of the proximal histidine, which is hypothesized to play a role in determining oxygen affinity of the heme iron (2, 19, 20), was optimized in F97L (Tables V and VI).

### Table II

|                   | CO       | Deoxy    |
|-------------------|----------|----------|
| Resolution limits (Å) | 10.0–1.5 | 10.0–1.7 |
| No. of reflections used in refinement (F o > 1 σ) | 37,557   | 24,848   |
| R-factor*          | 18.6%    | 18.3%    |
| No. of test reflections for Rfree (F o > 1 σ) | 3038     | 2742     |
| Rfree              | 23.1%    | 24.4%    |
| No. of nonhydrogen atoms | 2316     | 2312     |
| Hemoglobin         | 222      | 174      |
| Root mean square deviation from ideality |          |          |
| Bond lengths (Å)   | 0.010    | 0.011    |
| Bond angles (°)    | 2.2      | 2.2      |
| Dihedrals (°)      | 18.5     | 18.2     |
| Improper (°)       | 1.5      | 1.5      |
| Average B-factors (Å²) |        |          |
| Main chain         | 16.7     | 20.4     |
| Side chain         | 19.8     | 23.4     |
| Heme atoms         | 16.0     | 17.7     |
| CO ligands         | 14.4     | –        |
| Solvent atoms      | 37.2     | 36.6     |

* R = ∑|Fo| - |Fs| / ∑Fo, where Fo is the observed structure factor and Fs is that calculated from the model.

![Fig. 3. Simulated annealing omit F₀ - Fₐ 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 II, which includes the heme group, CO-ligand, Phe97, His102, and His95. The map is contoured at the 3σ level.](image)
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 Phe97 side chain from the heme pocket (2). Our structural results argue against such a determinative role for Phe97. 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 intersubunit interactions with the heme propionates likely contribute to this effect.

Interface Water Structure—There exists an elaborate net-

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**FIG. 4.** Stereo diagram showing an $\alpha$-carbon trace of HbI (solid lines) and F97L (dashed lines) for the deoxy state (top) and the CO-liganded state (bottom). In both panels, the view is approximately down the molecular dyad. In addition to the $\alpha$-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 $\alpha$-carbon positions and the heme groups. The Leu97 side chain remained 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.

**TABLE III**

| Comparison of average B-factors ($\AA^2$) between deoxy HbI and deoxy F97L structures |
|-----------------------------------------------|
| Deoxy Hb | Deoxy F97L |
|-----------|-------------|
| Main chain atoms | 18.0 | 20.4 |
| Side chain atoms | 19.4 | 23.4 |
| Heme atoms | 15.1 | 17.7 |
| Water atoms | 34.2 | 36.6 |
| Core interface water molecules (17) | 19.6 | 29.5 |

**TABLE IV**

| Comparison of average B-factors ($\AA^2$) between HbI-CO and F97L-CO structures |
|-----------------------------------------------|
| HbI-CO | F97L-CO |
|-----------|-------------|
| Main chain atoms | 16.6 | 16.7 |
| Side chain atoms | 18.2 | 19.2 |
| Heme atoms | 14.3 | 16.0 |
| CO atoms | 13.9 | 14.4 |
| Water atoms | 35.2 | 37.2 |
| Core interface water molecules | 24.7 (14) | 34.5 (18) |
work 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 (2, 18).

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 noncrystallographic 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 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 Fig. 6 (top), the interfacial water architecture for this structure was found to be virtually iden-
FIG. 6. Stereo diagram of the core ordered water molecules in the subunit interface. In all three panels, the view is approximately down the molecular dyad. Shown are the heme group, the F-helix (residues 94–102), and side chains for residues 97 and 101 for each subunit. Additionally, a van der Waals representation of the interface water molecules is shown. Top, 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. Middle, ordered water molecules in the subunit interface of HbI-CO. Note the disruption of the water network and the associated extrusion of the Phe97 side chain into the subunit interface. 14 water molecules are present in this subunit interface. Bottom, ordered water molecules in the subunit interface of F97L-CO. Note that the Leu97 side chain stays packed in the heme pocket. This preserves 4 water molecules (black shading) that are normally expelled by the extrusion of the Phe97 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. 18 water molecules are present in this subunit interface.
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tical to that of wild type, with 17 water molecules that were structurally equivalent (<0.60 Å) to the waters described above. These water molecules, however, were significantly less well ordered than in wild type, with an average B-factor of 29.5 Å². The deepest cluster of 4 water molecules maintained the lowest B-factors, whereas those closer to the bulk solvent were increasingly disordered. The stability of water molecules correlated with the extent to which they were hydrogen-bonded to protein groups as opposed to other water molecules. Water molecules whose sole hydrogen-bonding partners were other water molecules exhibited the highest B-factors. For instance, the 5 water molecules that hydrogen-bond only with other water molecules showed an average B-factor of 32.7 Å² in F97L compared with 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 resulted in a marked loosening of the interfacial water network.

In contrast to the deoxy form, striking differences in the interfacial water architecture were observed between the CO structures of F97L and wild-type HbI. Upon ligand binding to HbI, the displacement of each Phe97 side chain into the subunit interface results in the direct expulsion of 3 water molecules (Fig. 6, middle). The liganded wild-type interface thus has fewer and less well ordered water molecules than the deoxy interface (Tables III and IV). As shown in Fig. 6 (bottom), loss of the Phe97 side chain resulted 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 Phe97 side chain were now preserved, since the Leu97 side chain remained in the heme pocket. The other water molecules, including those participating in additional aspects of ligand-linked transitions, however, resembled wild-type HbI-CO water molecules. Second, the interfacial water molecules in the F97L-CO structure were significantly more disordered, with average B-factors of 34.5 Å² compared with 24.7 Å² for wild-type HbI-CO. The interfacial water architecture for F97L-CO thus appeared 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 was asymmetric, in contrast to the wild-type structures. This asymmetry was seen to persist in simulated annealing omit maps.

**DISCUSSION**

HbI represents a simple allosteric system that is useful for the investigation of intersubunit communication. The experiments described here were undertaken to test the hypothesis that Phe97 is critical for cooperativity. Based on a comparison of high resolution crystal structures of liganded and unliganded HbI, Phe97 was suggested to play a key role in both regulation of oxygen affinity and transfer of information between subunits (2). By virtue of its tight packing in the heme pocket of deoxy HbI, Phe97 was thought to lower oxygen affinity by (i) acting as a wedge, restricting movement of the heme iron into the heme plane and (ii) lengthening and weakening a hydrogen bond between the Nδ of proximal histidine and the main chain carbonyl oxygen of Phe97. Furthermore, it was suggested that its movement into the interface upon ligand binding would enhance the oxygen affinity of the second subunit through two pathways: (i) by a direct effect of its side chain in the interface and (ii) by triggering a movement of the heme group deeper into its subunit that would alter the hydrogen bonding of the propionate at the interface. As a result, one would predict that mutation of Phe97 to Leu would result in a substantial increase in oxygen affinity and a loss of cooperativity.

F97L exhibited an 8-fold increase in oxygen affinity over that of wild type. From the kinetic results, it appeared that the increased oxygen affinity results primarily from an increased affinity of the deoxy state relative to the deoxy state of wild-type HbI. This was 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 (Fig. 1, Table I). An altered deoxy state was also suggested by spectral results and was clearly shown in the crystallographic analysis. The crystal structures demonstrated that the smaller leucine side chain is accommodated without strain in the heme pocket of both deoxy and F97L-CO. This permitted the heme iron unrestricted movement into the heme plane (Fig. 5, Tables V and VI). 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, was nearly optimal in both the deoxy and CO structures. Thus, our analysis of the F97L mutant strongly supports the proposal that Phe97 plays the central role in determining oxygen affinity.

Cooperativity in F97L was largely decreased due to a loss of the ligand-linked movement of the side chain of residue 97 into the subunit interface. Kinetic measurements showed 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 Phe97 into the interface. Perhaps surprisingly, cooperativity was not fully lost in this mutant. This appears to be associated with our structural finding that the ligand-linked movement of the heme groups continued to occur in this mutant, refuting the earlier hypothesis that this movement was linked to the disposition of the Phe97 side chain. Thus, other interactions with the heme are responsible for this movement, which results in communication via an alternative pathway that presumably supplements the primary Phe97-based pathway in wild-type HbI.

Royer et al. (18) have recently compared the ligand binding parameters of wild-type HbI and the mutant T72V, which exhibits very high oxygen affinity (p50 = 0.2 torr) in combination with high cooperativity (n = 1.7). 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 (21). These model properties can never be fully expressed in the experimentally accessible ligand binding behavior of the protein but can be simulated to interpret the experimental data for the liganded and unliganded proteins. That analysis suggested 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 Kc and Kc for unliganded protein, where Kc = Tc/Tof and Kc = Ruc/Ruc). Fig. 1 shows the agreement between experimental results and calculated values based on reducing L by 25-fold and decreasing Tc 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 that of wild type.

Previous work has shown that the well ordered water mole-
cules in the subunit interface are crucial for stabilization of the deoxy state of HbI and suggested that these water molecules act as allosteric mediators in the cooperative mechanism (18). In this regard, 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.

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