Cooperativity in Scapharca Dimeric Hemoglobin: Simulation of Binding Intermediates and Elucidation of the Role of Interfacial Water

Yaoqi Zhou1,2, Hongyi Zhou2 and Martin Karplus1,3*

1Department of Chemistry and Chemical Biology
Harvard University
12 Oxford Street
Cambridge, MA 02138 USA
2Department of Physiology and Biophysics
State University of New York at Buffalo, Buffalo, NY 14214 USA
3Laboratoire de Chimie Biophysique, ISIS Université Louis Pasteur, 67000 Strasbourg, France

Cooperative binding of ligands to proteins can serve to increase their efficiency and to regulate their activity. Thus, understanding of the mechanism of cooperativity is one of the central concerns of molecular biology. For the tetrameric human hemoglobin (HbA), the cooperative mechanism involves a reasonably well understood combination of tertiary and quaternary changes that occur during the binding process. The dimeric hemoglobin of Scapharca (HbI), which is composed of subunits with the same fold as in HbA, is also highly cooperative but the structural changes on ligand binding are small. A re-orientation of Phe97 in the binding pocket and changes in the number of interfacial water molecules have been implicated in the cooperative mechanism. To explore the role of these factors, we have investigated models of partially liganded intermediate states of HbI with molecular dynamics simulation methods. Since, unlike HbA, no structures for intermediates are available, they were constructed by combining subunits from the unliganded and liganded dimers. Two structurally distinct intermediates were examined, and it was shown that the transition between the two intermediates is directly coupled to the number of interfacial water molecules. Further, it was found that there is a well-defined water channel that connects the interface between the subunits to bulk water. The bottleneck (gate) of the channel, which can be open or closed, is made of hydrophilic residues. The implication of the present results for the cooperative mechanism of HbI is discussed.

Keywords: Scapharca; dimeric hemoglobin; molecular dynamics; cooperativity; oxygen binding

Introduction

Cooperativity in multimeric proteins provides one of the essential control mechanisms of living systems.1 The homodimeric hemoglobin from the blood clam Scapharca (HbI) binds oxygen cooperatively to its two equivalent binding sites with a Hill coefficient of 1.5.2,3 This is similar to the value (1.36–1.72) for human hemoglobin (HbA) when two Fe2+ are replaced by non-reactive Ni2+.4 Although the individual subunits of HbI have the myoglobin fold, the assembly of the subunits is very different from that in HbA; i.e. the interface in HbI is formed by the E and F helices, which face the solvent in HbA. As a result, the two heme groups are nearly in direct contact (Figure 1). Also unlike HbA, no major quaternary changes occur upon binding of oxygen to HbI (the heavy-atom rmsd between the liganded and unliganded structure of HbI is 1.3 Å). Thus, the mechanism of cooperativity is not evident, though it must be very different from that of vertebrate hemoglobins.5–10

As experimental data (crystallographic, thermodynamic, kinetic) have become available, several suggestions have been made concerning the origin of cooperativity in HbI. From high-resolution crystal structures,11,12 the most significant tertiary change between deoxy and oxy HbI is the orientation of the side-chain of Phe97, whose χ1 angle changes from ~50° to ~160° on binding of oxygen (Figure 1). As a result, the phenyl group is displaced from the heme pocket into the interface...
between the two subunits and disrupts the well-ordered water molecules in that region. A total of 17 water molecules are observed in deoxy HbI and only 11 in oxy HbI. Mutation of Phe97 to Leu results in a reduction of the Hill coefficient to 1.2,13 thus supporting a role for Phe97 in cooperativity. It has also been proposed that the ordered water molecules act as key allosteric “mediators” for intersubunit communication,14 although no specific mechanism has been proposed. Other small tertiary structure changes on ligation have been noted,12 but no role in cooperativity has been suggested for them.

The X-ray data show only the differences between the liganded and unliganded structures and do not demonstrate the origin of the structural changes nor their energetic consequences. Consequently, molecular dynamics simulations can aid in obtaining a more detailed understanding of the cooperative mechanism. Here, we simulate HbI intermediates with only one liganded subunit. Such partially liganded intermediates have played an important role in the analysis of HbA,5,7,9,15 although they have had to be generated experimentally by modifying the protein (e.g. by partial oxidation or by introducing alternative metals); i.e. they occur only in small concentrations under normal conditions as a consequence of the high cooperativity.7 Since structures for monoligated intermediates are not available for HbI, we generate these species by modeling and use simulations to obtain insights concerning the communication between the liganded and unliganded subunit. Our results indicate that the number of interfacial water molecules serves as the switch that controls the tertiary structural change in the interface and that a specific channel exists for the entrance and exit of the water molecules.

Results
General behavior

The root mean-square deviation (rmsd) from the corresponding crystal structure as a function of time is shown in Figure 2. The rmsd values for both deoxy and oxy HbI are stable around 1.5 Å for the main-chain and 2.0 Å for all atoms, respectively. This confirms that CHARMM force field with explicit solvent and the PME treatment of the long-range electrostatics provides an accurate description of the structure and dynamics of HbI in its various forms.

The rmsd of the individual subunits is somewhat smaller; it is 1.2 Å for main-chain atoms and ~1.8 Å for all atoms. Thus, there is a small overall relative translation (1.4 Å) and relative rotation (8°) of the subunits. As pointed out in Methods, there is also a small inequivalence between the two subunits in the crystal (rmsd of 0.90 Å (0.81 Å) for the main-chain and 1.44 Å (1.34 Å) for all atoms in the deoxy (oxy) subunits). Interestingly, this inequivalence is essentially preserved during the simulation, suggesting that the relaxation of the system to a symmetric structure is
longer than the simulation time, even though the differences are small, or that the solution structure is not symmetric; there is no requirement for the structure to be symmetric. In a study of the resonance Raman spectra in the low frequency of the deoxy HbI, only one set of lines was observed, suggesting that the dimeric structure is, in fact, symmetric.\textsuperscript{16}

Comparison of the details of the simulated average structures with the corresponding crystal structures demonstrated that there are only small differences. They involve certain turn/loop regions; e.g. the exposed loop connecting helices C and E has a main-chain deviation of \( \sim 3.6 \text{ Å} \). The helices and the position of the heme are well preserved. The side-chains of Phe97 in both subunits maintain the respective interface (oxy) or heme-pocket (deoxy) positions (Figure 3). The root-mean-square fluctuations of the deoxy and oxy simulations are in good agreement with the X-ray temperature factors (Figure 4). The numbers of water molecules within 10.4 Å from the centers of geometry fluctuate around 20\textsuperscript{4} and 12\textsuperscript{5} for deoxy and oxy HbI, respectively, slightly larger than X-ray values of 17 and 11. The geometry within the heme pocket and between the two subunits is reproduced with great accuracy (see Table 1), except that in the simulations the O2 (oxygen)–N\textsubscript{e} (distal His69) distance is 0.5 Å longer and the iron–iron distance is 0.6 Å longer, though the difference of about 2.5 Å in the iron–iron distance between the deoxy and oxy structures is preserved; the monomer–monomer center of mass distances are about 1.4 Å (1.3 Å) larger. The increase of the center-to-center distance mostly comes from the surface loop regions that move more into the surrounding water. These small changes reflect the fact that the protein in solution is packed less tightly than in the crystal.

The structures for the intermediates were stable during the simulations. The all-atom rmsd values of the end structures are 2.4 Å, 2.2 Å, 2.0 Å from the simulated average structure of oxy and 1.9 Å, 2.3 Å, 2.4 Å from the simulated average structures of deoxy, respectively. Each subunit structure remains closer to its initial structure.

**Figure 2.** The root-mean-square deviation (rmsd) (in Å) from the corresponding crystal structure as a function of simulation time (in ns) for (a) the deoxy and (b) the oxy hemoglobins, respectively. The vertical continuous line indicates the start of unconstrained simulation at 0.65 ns. The results for main chains and all atoms are shown in broken and continuous lines, respectively.

**Figure 3.** The population distribution of the dihedral angle \( \chi_1 \) (involving atoms C, C\textsubscript{a}, C\textsubscript{b}, and C\textsubscript{g}) of Phe97 for deoxy and oxy hemoglobin simulations, as labeled. The distributions are based on the final nanosecond results.
Tertiary structural changes in intermediate simulations

Of primary interest in the simulations of the intermediates are the behavior of Phe97 and the interfacial water molecules. Figure 5 shows the time dependence of $\chi_1$ for the two Phe97 residues in the three simulations defined in Methods; 1o2d (1d2o) denotes that the structure of the first (second) subunit is from oxy HbI and that of the second (first) is from deoxy HbI. Transitions of $\chi_1$ from the heme pocket position ($\chi_1 \sim 170^\circ$) to the interface position are observed in the deoxy subunit in both simulations of the 1o2d structure; the final position of Phe97 is very close to that in the oxy structure ($\chi_1 \sim 170^\circ$). It is interesting to note that neither value is that of highest probability in tabulations of protein structures; i.e. the highest probability for $\chi_1$ is $\sim 300^\circ$ (52%), and the rotamers with $\chi_1$ equal to 60° and 170° correspond to most of the remaining observed orientations, suggesting that only little strain is present. In the first 1o2d simulation, the transition takes place at 90 ps after the structural constraints are removed and is completed within 1 ps. The resulting structure is stable with small fluctuations for the remainder of the simulation (3 ns). In the duplicate simulation of 1o2d, the conformational transition begins with large fluctuations in $\chi_1$ at around 2 ns and requires more than 40 ps to complete. Unlike the first simulation, a few attempts to transit back to the heme pocket position are observed. By contrast, no transition of deoxy Phe97 side-chain (except for some unsuccessful attempts) is observed during the entire 6 ns simulation of the 1d2o structure. In all three simulations, the side-chain of the residue Phe97 in the oxy subunit maintains its interfacial position ($\chi_1 \sim 170^\circ$), demonstrating that it is harder, if not impossible, for it to return to the pocket position in the presence of the liganded heme. Having the Phe side-chain with $\chi_1 \sim 60^\circ$ would lead to a stronger van der Waals repulsion between the heme and Phe97 in oxy than in deoxy HbI. The shortest atom–atom distance between heme and Phe97 would be 0.53 Å between HB (heme) and H$_1$2 (Phe97) in the former and 1.42 Å between carbon C4A (heme) and H$_1$2 (Phe97) in the latter; HB is the hydrogen atom on CHB (Figure 1). After minimization, the interaction energies are positive and similar in magnitude in the two cases.

To further analyze the differences among the three simulations of the binding intermediates, the time evolution of the interfacial water molecules and iron–iron separation are shown in Figure 6. In the first 1o2d simulation, the Phe97 conformational change coincides with a drop in the number of interfacial water molecules from ~14 to ~12.5. The number of interfacial water molecules continues to decrease and stabilizes at 10.5 (the oxy value) for the rest of the simulation. In the duplicate 1o2d simulation, the presence of slightly more water molecules (~15) in the interface apparently inhibits early attempts of the Phe97 displacement into the interface. In fact, the number of water molecules increases up to 20 before it drops to 15 at about 2 ns. After that point the iron–iron distance (i.e. the spacing between the subunits) increases and Phe97 undergoes fluctuations corresponding to partial transitions. The actual transition occurs at ~2.65 ns. Once the Phe97 transition has taken place, the iron–iron distance decreases to a value close to (but larger than) the deoxy value and the number of interfacial water molecules decreases to the normal oxy value.

Thus, the two simulations illustrate two different possible mechanisms leading to the side-chain
### Table 1. Comparison of geometry between X-ray structure and simulated average structures

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<td>11.7</td>
<td>10.6</td>
<td>11.7</td>
<td>12.9</td>
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ᵃ All are the averages of the last nanosecond simulated.
ᵇ Nₚ denotes the four pyrrole nitrogen atoms of the heme group.
ᶜ O denotes the nearest oxygen atom of the ligand; it is water for the deoxy subunit and an oxygen molecule for the oxy subunit.
ᵈ The distance between the second oxygen atom in oxygen and Nₚ atom in distal histidine.
ᵉ The dihedral angle involves C, Cₐ, C₇, and C₈.
ᶠ The dihedral angle involves C, Cₐ, C₇, and C₈.
ᵍ The dihedral angle involves C, Cₐ, C₇, and C₈. The fact that χ₂ value is close to the χ₁ value indicates the aromatic ring of Phe 97 is flipped during the conformational change in χ₂.
ʰ The distance between the geometric centers of two subunits.
ⁱ The number of water molecules within 10.4 Å from the center of geometry.

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**Figure 5.** Time dependence of the dihedral angle χ₁ of Phe97 for three independent simulations of the partially liganded intermediates. The plot starts from 650 ps after the constraints on the system have been removed. The top two simulations are for the 1o2d system and the bottom one is for the 1d2o system (see the text).
conformational change of Phe97 to the oxy position in the deoxy subunit of the intermediate structure. The transition in the first 1o2d simulation is coupled to water moving out of the interface and that in the second transition follows the transient increase in the subunit separation that provides the necessary room for Phe97 in the interface. For both 1o2d simulations, the averaged numbers of water molecules in the interface decrease to the level (~11) of the native fully liganded oxy state after the transition (Table 1). The presence of the hydrophobic side-chain of Phe97 in the interface, coupled with the overall reduction of the Fe–Fe distance, appears to expel water molecules into the bulk over the timescale of 1 ns (Figure 6). We refer to this intermediate as the dry intermediate in what follows.

In the simulation of 1d2o, attempts at a transition of Phe97 side-chain occur (e.g. at 1.9 ns (Figure 5)) but they are not successful during the 6 ns. This appears to be related to the fact that the number of interfacial water molecules constantly increases from its initial value of 15 until it reaches the value ~20 corresponding to the fully unliganded deoxy state (Figure 6). The influx of water into the interface is accompanied by an increase in the iron–iron distance to the value for the fully oxygenated system (Table 1). The large number of interfacial water molecules prevents the movement of the Phe97 side-chain into the interface (Figure 7). This intermediate is referred to as the wet intermediate. Table 1 compares certain geometric parameters of the deoxy and oxy X-ray structures and the average structures of the intermediates during the last nanosecond of the simulations. The 1o2d and its duplicate have essentially the same structure, although the latter takes about 1 ns longer to reach the final state. The side-chain of Phe97 in the deoxy subunit of 1o2d and its duplicate has the oxy-like conformation with essentially the same χ1 angle (χ1 ~ 165°). During the simulations there is a 180° flip of the ring; it occurs at 2.9 ns and 1.1 ns after the main χ1 transition in the first and second 1o2d simulations, respectively.

The distance between Nε (His101) and the main-chain carboxyl O of Phe97 is shorter than that in the deoxy structure and similar to that in the oxy structure, as the result of the side-chain conformational change of Phe97. The flip of Phe97 ring into the interface moves its main-chain oxygen atom deeper into the protein, so that it is closer to Nε of His101. The O2–Nε distance between the distal histidine residue (His69) and the oxygen molecule of both 1o2d simulations is shorter by 0.25 Å than the corresponding value in the oxy simulation. The number of interfacial water molecules is essentially the same as that of the fully liganded oxy interface. The iron–iron distance is equal to 18 Å, which is intermediate between 17.2 Å and 19.7 Å, the corresponding distances from the fully unliganded deoxy and fully liganded oxy simulations, respectively (Table 1). The heme–heme distance based on the distance from center of mass of four Nε atoms is essentially the same as the iron–iron distance.

Despite the structural difference, the potential energies and their fluctuations averaged over the last 1 ns of the simulations are very similar. They are ~75,479 (118) kcal/mol, ~75,458 (134) kcal/mol, and ~75,464 (187) kcal/mol for 1o2d, 1o2d duplicate, and 1d2o, respectively.

The role of the interfacial water

To study the role of the interfacial water molecules in more detail, we used the number of interfacial water molecules as the reaction coordinate in several simulations (see Methods). This is required because, as we have seen, both wet and dry intermediates are stable during the simulations, suggesting that the transition between them takes place on a longer time-scale. It is of interest in this regard that Rousseau et al.18 have shown that the half-life for the structural transition between the R and T states is around 1 μs, three orders of magnitude longer than the simulation.

We first examined the hypothesis that the large number of interior water molecules prevented the ring flip of Phe97 in the simulation of the 1d2o system. This was done by using the umbrella potential to gradually reduce the amount of water in the interface from \( n_{\text{w0}} = 20 \) to \( n_{\text{w}} = 10 \) with \( k_\omega = 5.0 \) kcal/mol and \( n_{\text{w0}} \) decreased by one for every...
100 ps (see Methods). The initial structure was obtained from the end of the 1d2o simulation and the ring flip was observed at $\sim 900$ ps, where the number of interfacial water molecules has decreased to 11, and corresponds to that of oxy HbI (Figure 8). The iron–iron distance decreases slowly throughout the simulation from $\sim 20$ Å to $\sim 19$ Å while the stable structure found in the 1o2d simulations has an iron–iron distance of 18 Å. Moreover, other properties of the 1d2o simulation at this stage are very similar to those of the 1o2d simulation. This demonstrates that the number of water molecules in the interface controls the switch from the deoxy conformation to an oxy conformation for the side-chain of Phe97 of the unliganded subunit in the intermediate.

We then tried the inverse case by introducing water into the interface to see whether this would force the side-chain of Phe97 in the deoxy subunit back to the heme pocket position. The initial structure was that of the dry intermediate with both Phe97 in the oxy position. It was obtained from

![Figure 7](image-url)
the final structure of the first 1o2d simulation. We found that increasing the number of water molecules over a 2 ns simulation time did not induce a stable conformational transition of Phe97. The iron–iron distance responded too slowly to the increase of interfacial water molecules; it increases by only 0.6 Å after 2 ns, rather than the value of ~2 Å required. We then applied an umbrella potential to both the number of water molecules and iron–iron distance (see Methods). During the simulation, \( n_{w0} \) was gradually increased from 9.5 to 20.5 and \( R_{\text{Fe–Fe,0}} \) from 18 Å to 20.5 Å. The ring flip occurs at ~3 ns for the system with \( n_{w0} = 20.5 \) and \( R_{\text{Fe–Fe,0}} = 20.5 \) Å (Figure 9). This result is in accord with the relation between the number of interfacial water molecules and the stability of the intermediate with the side-chain of Phe97 in the deoxy subunit in or out of the heme pocket. However, the increase in the number of water molecules also has to result in an increase in the interfacial Fe–Fe distance, which requires motions of entire subunits and is a slow process on the simulation time-scale.

Because of the important role of the interfacial water, it is of interest to determine the channel that connects the interface and the bulk water. The exit pathway shown in Figure 10(a) was determined from the “dehydration” simulation in which the number of interfacial water molecules was constrained to decrease gradually from 20 to 10 in 1 ns (see Methods). The background structure in the Figure is the initial structure of the simulation. It can be seen that there is a bottleneck (gate) where the channel narrows before it widens again. In the initial wet structure, the opening is large and made up of Lys96, Asn100, and propionate groups of the heme in both subunits (Figure 11(a)). In the final structure with \( n_{w} = 10 \), the opening for water is narrower and is made up of Lys96 (deoxy subunit), Asn100 (deoxy subunit), and the heme propionate group (oxy subunit) (Figure 11(b)). Thus, it appears that the gate partially closes after the water has drained out of the interface. It is interesting to note that the two gate residues (Lys96 and Asn100) are on the F helix, as is Phe97, and there

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**Figure 9.** As in Figure 8 but with harmonic constraints on both the number of water molecules and the iron–iron distance. The simulation was started from a dry intermediate.
appears to be some coupling between their motions. The gate to the interface shown in Figure 10(a) was found also in the hydration simulation in which the number of interfacial water molecules was constrained to increase slowly from 9.5 to 20.5 in 3 ns (Figure 10(b)). The background structure in Figure 10 is the final wet structure from the constrained simulation; i.e. it corresponds to the initial structure of the previous simulation that has a similar water content at the interface. The entrance is also made up of Lys96, Asn100, and propionate of the heme groups. But the hole (Figure 12(b)) is somewhat smaller than that in the initial structure.

Figure 10. Pathway for entrance and exit of water in the wet to dry (the dry to wet) transition. (a) Pathway found during water extrusion simulation; (b) pathway found during water entrance simulation. (The results are essentially the same.) The bottleneck is made up of Lys96, the heme, and Asn100 in both subunits (shown as sticks). The water molecules that appear at least once in the interface are shown as dotted spheres.

Figure 11. The gate made by Lys96, the heme and Asn100. (a) The initial structure in the dehydration simulation. (b) The final structure.
used in the dehydration simulation (Figure 11(a)). Moreover, no channel is visible in the initial dry intermediate structure (Figure 12(a)). Apparently, an opening of the gate between the interface and the bulk is required to permit water to flow in and out.

The various simulations indicate that there is a unique channel with the gate for the interfacial water made of three hydrophilic groups (Lys96, Asn100, and the two propionate groups of the heme). The gate can be wide open (formed by all six residues in two subunits, Figure 11(a)), narrow
(formed by three or four residues, Figures 11(b) and 12(b)), or completely closed (Figure 12(a)). It is of interest to compare the structures of these intermediates with those of the oxy and deoxy HbI. Figure 13 shows that while there are openings involving the same six residues in the deoxy structure (Figure 13(a)), no such opening exists in the oxy structure (Figure 13(b)). The comparison between the two structures further shows that during the open to close transition, the salt-bridge between Lys96 in one subunit and a propionate group of the heme of the other subunit is replaced by the salt-bridge between Lys96 and a different propionate group of the same heme. The opening and closing behavior is analogous to that observed by Henchman et al. for water and ligands entering the active-site region of acetylcholinesterase.\textsuperscript{19}

**Discussion**

To investigate the nature of the structural changes and the role of water in the cooperative transition of HbI, intermediates with one liganded and one unliganded subunit were constructed and simulated by molecular dynamics. These intermediates are analogous to the doubly liganded intermediates in HbA, which have played an important role in understanding cooperativity in that molecule; e.g. it is the concentration of double liganded intermediates that distinguishes between the MWC\textsuperscript{1} and KNF models.\textsuperscript{20} It would be very interesting to investigate whether such intermediates can be obtained experimentally using valence hybrids or mixed metal forms of HbI, in analogy with the work on HbA.

Starting out with slightly different models, two different intermediates, referred to as wet and dry according to the number of interfacial water molecules present at the end of the simulation (20 and 11, respectively), were obtained. In the wet intermediate, the structures of deoxy and oxy subunits are unchanged from corresponding starting structures; e.g. the distance between $N_e$ (His101) and main-chain atom O (Phe97) in both subunits of the intermediate are essentially the same as those of corresponding fully unliganded deoxy or fully liganded oxy state. Moreover, there is no re-orientation of either Phe97. In the dry intermediate, by contrast, a flip of the Phe97 side-chain of the deoxy subunit into the oxy position is observed and leads to a short distance between $N_e$ (proximal His101) and main-chain atom O (Phe97). In this case, the number of interfacial water molecules (11) is closer to the fully liganded oxy state, while the iron–iron distance is between that for the unliganded deoxy state and that for the fully liganded oxy state (Table 1). The O2–$N_e$ (distal histidine 69) distance decreases slightly from 3.6 Å to 3.3 Å; this is in the opposite direction from that in the wet intermediate.

The two dry and one wet simulations of singly liganded intermediates suggest that the interfacial water is important in determining the structural changes that contribute to the cooperative mechanism. Because the interchange time (wet to dry) is long relative to the simulation time, the number of interfacial water molecules was used as a reaction coordinate to further investigate the factors involved in stabilizing the different structures. It was shown that using the wet intermediate and expelling the excess water led to the same conformational transition as in the dry intermediate. Causing water to enter the dry intermediate led to a conformational transition only if the Fe–Fe distance was increased; this is probably due to the fact that the motion apart of the two subunits occurs on a slower time-scale.

Study of the intermediates has made possible a clear demonstration of the role of the interfacial water molecules in the major tertiary conformational change between deoxy and oxy HbI. By use of the number of interfacial water molecules as an umbrella potential, it has been shown that reduction in their number (to a dry, oxy-like interface) promotes the conformational change of Phe97 from its deoxy to oxy position. Moreover, the latter appears to lead to a higher binding affinity as a result of stronger H-bonding to the proximal histidine residue. A wetter, deoxy-like, interface on the other hand, inhibits the conformational change of Phe97 and allows the distal histidine to move slightly toward the interface, resulting in weaker H-bonding between oxygen and the distal histidine residue.

It was found that there is a channel with a bottleneck that connects the interface with the bulk. From the X-ray deoxy/oxy structures, the salt-bridge between Lys96 and the propionate group of heme seems to play an important role in opening and closing of the gate. The salt-bridge is switched between the two propionate groups of the heme during the open/close transition. We hypothesize that as water approaches the hydrophilic bottleneck, the solvation of Lys96, Asn100, and the propionate group of heme weakens the charged and polar interactions and leads to the dilation of the bottleneck. When water is absent, the reduced screening of the charged and polar interactions forces the gate to close. The discovery of a water channel in HbI is of interest in relation to other water channels in soluble proteins (e.g. acetylcholinesterase\textsuperscript{19}).

Although the simulations cannot prove that the dry and wet species exist during the HbI allosteric transition, it is possible that the cooperativity in HbI, in fact, is mediated by the two partially liganded intermediates found in the present simulations. This is consistent with two different mechanisms (one dominant and one minor) proposed to explain the experimental result that the Phe97Leu mutation\textsuperscript{13} has a residual cooperativity (Hill coefficient $\sim 1.2$), despite the fact that the Leu residue, unlike the Phe residue, remains in the heme pocket upon binding. The residual cooperativity was related to the larger heme–heme
separation found in the X-ray oxy structure of the Phε97Leu mutant, in accord with the large heme–heme separation found in the wet intermediate. Further simulations and experiments are needed to determine the possible role of the two intermediates.

Conclusions
A direct coupling between the number of interfacial water molecules and the tertiary structural changes in HbI has been demonstrated. The major tertiary structural changes (ring flip of Phε97 and the change of iron–iron distance) found by comparing the two end X-ray structures are reproduced in molecular dynamics simulations of half-ligated intermediates by using the number of interfacial water molecules as a reaction coordinate. It will be of interest to have direct measurements on such intermediates to complement the simulations.

Methods
The initial protein structures were obtained from the Protein Data Bank (deoxy 4SDH; oxy 1HBI); the missing first residue (proline) and the positions of hydrogen atoms were built using the CHARMM program; 101 and 111 crystallographic water molecules were included in the deoxy and oxy structure, respectively. The dimers were minimized for 200 steepest descent steps in vacuum, in the deoxy and oxy structure, respectively. The dimers and crystallographic water molecules were included in the simulation system. The first structure, 1o2d, consisted of subunit 1 from oxy HbI and subunit 2 from deoxy HbI and the second structure, 1d2o, was the reverse. There were 15 and 14 interfacial water molecules in 1o2d and 1d2o, respectively; in 1o2d (1d2o), seven (five) and eight (nine) water molecules were contributed by the oxy and deoxy subunits, respectively. The model structures were minimized for 100 steps to remove bad contacts.

Since the X-ray structure of the partially liganded intermediate state is not available, models were generated by taking one subunit from deoxy HbI and the other from oxy HbI. Starting with the crystal structures of dimeric deoxy HbI, one of the two subunits and its interfacial water molecules (defined as the water molecules that are closer to its center of geometry) was replaced by the corresponding subunit from the oxy HbI structure with its interfacial water molecules. The mixing of subunits from the two different X-ray structures is feasible because there is only a negligible quaternary structural change upon binding (see below) and the tertiary structural changes are small (see above). To provide a check on the simulation results, two initial structures were generated based on the slight inequivalence of the subunits in the deoxy (oxy) crystal structure. The first structure, 1o2d, consisted of subunit 1 from oxy HbI and subunit 2 from deoxy HbI and the second structure, 1d2o, was the reverse. There were 15 and 14 interfacial water molecules in 1o2d and 1d2o, respectively; in 1o2d (1d2o), seven (five) and eight (nine) water molecules were contributed by the oxy and deoxy subunits, respectively. The model structures were minimized for 100 steps to remove bad contacts. Starting with the minimized structures, the same simulation protocols were used as for the deoxy and oxy dimers (see above). Three simulations were done for the intermediates to compare their behavior with that of the stable deoxy and oxy HbI dimers. The first two were a 4 ns and a 5 ns simulation of the 1o2d structure with
different initial velocities and the third was a 6 ns simulation of the 1d20 structure.

The role of the interfacial water in controlling the structural transitions observed in the intermediates during the dynamics was investigated directly by using their number as a reaction coordinate. This was accomplished by applying an umbrella potential, \( E = k_w(n_w - n_{w0})^2 \) to the hemoglobin system. Here, the number of interfacial water molecules, \( n_w \), is given by the equation \( n_w = \sum_i \theta(r_i - r_{cut}) \) where the summation is over all the water molecules and \( r_i \) is the distance of water molecule \( i \) from the geometric center of the hemoglobin. To calculate the force for the dynamics simulations, a smoothed step function was used. \( \theta(x) = 1 \) for \( x < 0 \) and \( \theta(x) = (1 - x^2/\sigma^2)^2 \) for \( 0 < x < \sigma \) and \( 0 \) for \( x > \sigma \), where \( \sigma \) is set equal to 3 Å, the approximate thickness of a water layer. Since \( x = r_i - r_{cut} \), the force is non-zero only for those water molecules located between \( r_{cut} < r_i < r_{cut} + 3 \) Å. Thus, the effect of the external potential is limited to the edges of the interfacial region and its computational overhead is minimal. The value of \( r_{cut} \) is taken to be 8.4 Å, rather than 10.4 Å, since this provides a smooth cutoff between 8.4 Å and 11.4 Å.

The water reaction coordinate was also used in conjunction with a reaction coordinate for the iron–iron distance. In this case:

\[
E = k_w(n_w - n_{w0})^2 + k_{fe}(R_{Fe-Fe} - R_{Fe-Fe})^2
\]

Acknowledgements

We particularly thank Professor W. E. Royer for sending structural data prior to their release and for many useful discussions. Helpful discussion on the simulations with Drs Bernard Brooks, Mike Crowley, Qiang Cui, and Stefan Boresch are gratefully acknowledged. The work at Harvard was supported, in part, by a grant from the National Institutes of Health (M.K.), a grant from the Pittsburgh Supercomputing Center, and a grant from the National Energy Research Scientific Computing Center (NERSC). The work at Buffalo was supported by a grant from HHMI to SUNY Buffalo, by the Center for Computational Research at SUNY Buffalo and Keck Center for Computational Biology. The calculations at Harvard were conducted on HP 9000/735, SUN UltraSparc, SGI Origin 2000, and DEC Alpha workstations. All three-dimensional structures were drawn with VMD.\(^{33}\)

References


(Received 20 August 2002; received in revised form 11 November 2002; accepted 15 November 2002)