Allosteric intermediates indicate R2 is the liganded hemoglobin end state

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Communicated by Quentin H. Gibson, Rice University, Houston, TX, May 16, 1997 (received for review April 10, 1997)

ABSTRACT Hemoglobin has been a long-standing paradigm for understanding protein allostery. Here, the x-ray structures of two chemically crosslinked, fully liganded hemoglobins, αβ2CA82β and αβ2ND82β, are described at 2.3 Å and 2.6 Å resolution, respectively. Strikingly, these crosslinked hemoglobins assume intermediate conformations that lie between those of R and the controversial liganded hemoglobin state R2 rather than between R and T. Thus, these structures support only a T ↔ R ↔ R2 allosteric pathway and underscore the physiological importance of the R2 conformation.

The quaternary end-state structures of human hemoglobin have long been accepted as the unliganded T and liganded R conformations (1–7). However, the appearance of a second fully liganded conformation, R2 (8–10), has stirred debate as to whether this conformation is an intermediate that lies between T and R (8, 11), an off-pathway structure (12), or the physiologically relevant end state (11, 13). On the basis of the dislocation of the imidazole side chain of residue β2His-97 from the αC-helix, the R2 conformation was first proposed as an intermediate between the R and T states because it suggested a mechanism by which this residue switches from its T to R state position (8). However, the results of the calculated trajectory of the atomic coordinates in transiting from the T to R2 structure casted doubt on the validity of this proposal (13). Specifically, that trajectory was shown to pass close to the R conformation and thereby suggested R2 might be the physiologically relevant liganded end-state conformation. Clearly, the relevance of the R2 conformation and its position along hemoglobin’s allosteric pathway is critical to our complete understanding of the function of hemoglobin. Here, we present the structures of two chemically crosslinked, fully liganded hemoglobins that capture R ↔ R2 conformational intermediates and thus clarify the relevance of the R2 state.

MATERIALS AND METHODS

The crosslinked hemoglobins, which display only slightly lower oxygen affinities than uncrosslinked hemoglobin and normal cooperativities (14, 15), were prepared by reacting human deoxyhemoglobin with the bis(methylphosphate) derivatives (15, 16) of either 4-carboxycinnamic acid (CA) or 2,6-napthalene dicarboxylic acid (ND), respectively, and saturated with carbonmonoxide (CO) before their crystallization. Both αβ2CA82β and αβ2ND82β are crosslinked between the Nζ atoms of β1Lys-82 and β1Lys-82. Although crystallized under the high phosphate conditions that result in the tetragonal crystals of unmodified carbonmonoxide hemoglobin (COHbA) (17), αβ2CA82β and αβ2ND82β assume the orthorhombic space group P212121 (Table 1). The structure of αβ2CA82β was solved by molecular replacement (18) and found to contain a tetramer in the asymmetric unit. Refinement converged to a final R factor of 18.4% at 2.3 Å resolution (20) (Table 1). This structure served as the starting model, minus the crosslinker and water molecules, for refinement of the αβ2ND82β structure, which converged to a final R factor of 15.4% at 2.6 Å resolution. The contour level is 4.0 α.

Fig. 1. (A) An Fobs-Fcalc omit map of αβ2CA82β in which the cinnamyl crosslinker has been omitted from the model refinement. The contour level is 3.5 α.

Abbreviation: COHbA, unmodified carbonmonoxide hemoglobin.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (references 1hab (αβ2CA82β) and 1hae (αβ2ND82β)).

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RESULTS AND DISCUSSION

Table 1. Summary of selected crystallographic data

<table>
<thead>
<tr>
<th>Crosslinked hemoglobin</th>
<th>$\alpha_2\beta_2^{CA}$</th>
<th>$\alpha_2\beta_2^{ND}$</th>
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<tbody>
<tr>
<td>Space group</td>
<td>$P2_1$</td>
<td>$P2_1$</td>
</tr>
<tr>
<td>Cell dimensions, Å</td>
<td>$a = 86.8$, $b = 87.1$, $c = 97.6$</td>
<td>$a = 86.8$, $b = 87.1$, $c = 97.6$</td>
</tr>
<tr>
<td>$\alpha_2$ dimers per ASU</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Data collection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution, Å</td>
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<td>2.6</td>
</tr>
<tr>
<td>Number of observations/reflections</td>
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<td>65,290/19,979</td>
</tr>
<tr>
<td>Rsym, †</td>
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<td>7.0</td>
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<tr>
<td>Refinement, Å</td>
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<td>10.0–2.6</td>
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<tr>
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<td>78</td>
</tr>
<tr>
<td>$R$ factor, †</td>
<td>18.4</td>
<td>15.4</td>
</tr>
<tr>
<td>Number of atoms</td>
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<td>4,578</td>
</tr>
<tr>
<td>Number of solvent molecules</td>
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<td>51</td>
</tr>
<tr>
<td>Root-mean-squared deviations</td>
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<td></td>
</tr>
<tr>
<td>Bond distances, Å</td>
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<td>0.018</td>
</tr>
<tr>
<td>Bond angles, degrees</td>
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<td>2.58</td>
</tr>
<tr>
<td>R factors and $\text{symmetry-related reflections}$</td>
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<td></td>
</tr>
<tr>
<td>$R_{\text{m}} = \sum</td>
<td>I_o - \langle I\rangle</td>
<td>/\langle I\rangle$, where $I_o$ is the observed intensity, and $\langle I\rangle$ is the average intensity from multiple observations of</td>
</tr>
<tr>
<td>$R_{\text{sym}} = \Sigma</td>
<td>F_{\text{obs}}</td>
<td>-</td>
</tr>
</tbody>
</table>

$\alpha_2\beta_2^{CA}$ and $\alpha_2\beta_2^{ND}$ were crosslinked in their deoxy forms by reacting human deoxyhemoglobin with the bis(methylphosphonate) derivatives of either 4-carboxyaminic acid (CA) or 2,6-naphthalene dicarboxylic acid (ND), respectively (15, 16). The $\alpha_2\beta_2^{CA}$ and $\alpha_2\beta_2^{ND}$ crosslinkers are confined to a small region near the site of crosslinker attachment, near the CD structural anomalies that are observed are confined to a small region near the site of crosslinker attachment, near the CD

To compare the quaternary structures of $\alpha_2\beta_2^{CA}$ and $\alpha_2\beta_2^{ND}$ with those of T, R, and R2 hemoglobin, superimpositions were carried out using the method of Baldwin and Chothia (1). This method involves the superimposition of the appropriate Cα residues of the $\alpha_2\beta_2$ dimers followed by the determinations of the rigid body rotations necessary to align the appropriate Cα residues of the corresponding $\alpha_2\beta_2$ dimers (1). To superimpose the $\alpha_2\beta_2$ dimers of $\alpha_2\beta_2^{CA}$ and $\alpha_2\beta_2^{ND}$ onto the $\alpha_2\beta_2$ dimer of the R2 structure requires rotations of 9.0° and 8.4°, respectively (Fig. 2). These are to be compared with rotations of 13.3° for COHbA (R ↔ R2) and 23° for deoxyhemoglobin (T ↔ R2). The tertiary and quaternary differences between the crosslinked hemoglobins and deoxyHbA, COHbA, and R2 hemoglobin were analyzed in more detail by a series of carbon-coordination difference plots (CDPs) (Fig. 3). The CDPs confirm the nearly identical tertiary and quaternary structures of $\alpha_2\beta_2^{CA}$ and $\alpha_2\beta_2^{ND}$. Furthermore, they demonstrate that there is little difference in the tertiary structures of the fully liganded forms, i.e., R2, R, and $\alpha_2\beta_2^{CA}$ and $\alpha_2\beta_2^{ND}$, and the slight structural anomalies that are observed are confined to a small region near the site of crosslinker attachment, near the CD turn of the $\alpha$ subunit and the first few residues of the A helix of the $\alpha$ subunit (Fig. 3, labeled $\beta$EF, $\alpha$CD, and $\alpha$A, respectively). The structural identity also applies to the $\alpha$ and $\beta$ hemes that are planar in these crosslinked hemoglobins. Finally, although closer to the R structure than to the R2 structure, the CDPs demonstrate unequivocally that the structures of $\alpha_2\beta_2^{CA}$ and $\alpha_2\beta_2^{ND}$ lie directly on the path between the R and R2 conformations.
than that of R hemoglobin (8, 13). Moreover, that these waters are hallmarks of the R2 structure (8). One of these water molecules (Wat1) maintains the identical hydrogen-bonding network observed in the R2 structure that links the OD2 of molecules (Wat1) to the OD2 of the R and R2 liganded structures.

 Perhaps the most striking feature that demonstrates the R ↔ R2 intermediate nature of $\alpha\beta^2\text{CA}^2\beta$ and $\alpha\beta^2\text{ND}^2\beta$ is the location of the $\beta_2\text{His-97}$ side chain (Fig. 4). In the R structure the imidazole side chain of $\beta_2\text{His-97}$ is positioned between $\alpha_1\text{Thr}38$ and $\alpha_1\text{Thr}41$ (7), whereas in the R2 conformation it is disengaged from this pocket (8). For both $\alpha\beta^2\text{CA}^2\beta$ and $\alpha\beta^2\text{ND}^2\beta$ the $\beta_2\text{His-97}$ side chain is found between its R and R2 locations such that the distances between the corresponding $\text{C}^\beta$ atoms of the imidazole moieties are 1.1 Å for $\alpha\beta^2\text{CA}^2\beta$ to R, and 2.1 Å for $\alpha\beta^2\text{ND}^2\beta$ to R2 (Fig. 4). Thus, the structures of $\alpha\beta^2\text{CA}^2\beta$ and $\alpha\beta^2\text{ND}^2\beta$ provide the first experimental support of the T ↔ R ↔ R2 transitional pathway.

Although clearly more R-like, both $\alpha\beta^2\text{CA}^2\beta$ and $\alpha\beta^2\text{ND}^2\beta$ contain two water molecules in the $\alpha_2\beta_2$ interface that are hallmarks of the R2 structure (8). One of these water molecules (Wat1) maintains the identical hydrogen-bonding network observed in the R2 structure that links the OD2 of $\alpha_1\text{Asp-94}$ to the amide nitrogen of $\beta_2\text{Asp-99}$ (Fig. 5), thereby directly supporting the supposition that the $\alpha_2\beta_2$ interface of R2 and its R ↔ R2 intermediates are more solvent accessible than that of R hemoglobin (8, 13). Moreover, that these waters are found in the transitional but more R-like structures of $\alpha\beta^2\text{CA}^2\beta$ and $\alpha\beta^2\text{ND}^2\beta$ (Figs. 2 and 3) suggests that their removal from or addition to the $\alpha_2\beta_2$ interface constitutes a major determinant in the R ↔ R2 pathway.

Central to the cooperative quaternary transition of hemoglobin are COOH-terminal residues $\alpha140$-$\alpha141$ and $\beta145$-$\beta146$ (1–7). In the unliganded state, these residues participate in interactions that are essential for the stabilization of the T conformation. However, upon ligand binding these T-state interactions are lost, and in the high-phosphate environment (greater than 2 M) of the crystallized R state (17), the COOH-terminal residues are very mobile. The continued transition to the R2 state, crystallized under physiologically relevant anion concentrations (8, 10), results in the repositioning of the $\beta_1$ and $\beta_2\text{His-146}$ imidazole side chains, which then stack against one another, and the establishment of a salt bridge between the $\alpha$-carboxylate group of $\beta_1\text{His-146}$ and the $\beta_1\text{Lys-82}$ side chain (8). Thus, the $\beta_1\text{Lys-82}$-$\beta_2\text{His-146}$ salt bridge appears to be an important determinant in the choice between the R and R2 conformations and highlights the importance of the environment on the liganded conformations of hemoglobin. Specifically, we propose that the high-
phosphate concentration used to crystallize COHbA (17), which is 20-fold greater than the $K_d$ of orthophosphate for oxyHbA (22, 23), disrupts the Lys-82–His-146 interaction and thereby destabilizes the R2 conformation. In the absence of high-phosphate concentrations, or in the presence of lower ionic strength solutions that are physiologically more relevant, the R2 conformation would be favored. The intermediate nature of the crosslinked hemoglobins is dictated by the opposing forces of a high-phosphate environment, which stabilizes many structural aspects of the R conformation, and the long bridging distances of the crosslinking reagents, which indirectly favor the R2-like $\alpha_1\beta_2$ interface.

**CONCLUSION**

The structures of liganded $\alpha_1\beta_2\text{CA}^{82}\beta$ and $\alpha_1\beta_2\text{ND}^{82}\beta$, together with those of crosslinked hemoglobins $\alpha_1\beta_1\text{Tm}^{82}\beta$ and $\alpha_1\beta_1\text{Tm}^{82}\beta$, which display quaternary structures intermediate to those of T and R (24), support only a T $\leftrightarrow$ R $\leftrightarrow$ R2 transitional pathway (Fig. 6, red arrows). That is, for the R2 structure to represent a T $\leftrightarrow$ R intermediate, an unlikely backward trajectory of the quaternary structure is required that would also have to link the structures of $\alpha_1\beta_2\text{Tm}^{82}\beta$ and $\alpha_1\beta_1\text{Tm}^{82}\beta$ to R2 (Fig. 6, blue arrows). In light of this proposed conformational pathway, there now is a need to reassess the liganded state of hemoglobin; specifically, its increased plasticity (3, 8–10, 25), the effects of environment on the quaternary structure, and the functional importance of these different conformers in a given physiological context.

We thank Ms. X. Hong for preparing the crosslinked hemoglobins and determining their oxygen affinities and Hill coefficients. This work was supported in part by the National Institutes of Health (HL-20142), the Department of Defense, the Oregon Health Sciences Foundation, and the Natural Sciences and Engineering Research Council of Canada.