Crystal Structure of Deoxy-Human Hemoglobin β6 Glu → Trp

IMPLICATIONS FOR THE STRUCTURE AND FORMATION OF THE SICKLE CELL FIBER*

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An atomic-level understanding of the interactions between hemoglobin molecules that contribute to the formation of pathological fibers in sickle cell disease remains elusive. By exploring crystal structures of mutant hemoglobins with altered polymerization properties, insight can be gained into sickle cell hemoglobin (HbS) polymerization. We present here the 2.0-Å resolution deoxy crystal structure of human hemoglobin mutated to tryptophan at the β6 position, the site of the glutamate → valine mutation in HbS. Unlike leucine and isoleucine, which promote polymerization relative to HbS, tryptophan inhibits polymerization. Our results provide explanations for the altered polymerization properties and reveal a fundamentally different double strand that may provide a model for interactions within a fiber and/or interactions leading to heterogeneous nucleation.

Sickle cell disease is caused by the mutation of human hemoglobin (HbA) at the sixth position of the β chain from glutamate to valine (1). The consequence of this single amino acid substitution is a drastically reduced solubility of deoxygenated sickle cell hemoglobin (HbS) leading to the formation of long polymers that distort and rigidify the normally pliable erythrocytes. Such sickled cells can occlude the capillaries of the microcirculation and decrease the oxygen supply to the surrounding tissue, which is believed to be what is responsible for the clinical manifestations of sickle-cell disease.

Structural analysis of HbS fibers using single crystal x-ray diffraction, fiber x-ray diffraction, and electron microscopy, as well as complementary information from gelation experiments, has established the basic fiber architecture (2). Crystals of deoxy-HbS grown in low salt at low pH (5.0 to 6.0) display a monoclinic space group in which hemoglobin molecules are assembled in double strands (3). Axial contacts within each strand and lateral contacts between strands that involve the mutated β6 Val stabilize these “Wishner-Love” double strands (3, 4). Structural details of the double strand interactions have been elucidated from the refined structure of HbS at 2.05-Å resolution (5). X-ray fiber diffraction data, from both extracellular gels and sickle erythrocytes, are consistent with a similar arrangement of molecules in the crystal and fiber (6). Electron microscopy with image reconstruction has revealed that the basic HbS fiber is 210-Å thick and formed from 14 filament strands that associate as half-staggered pairs (7). Although some disagreements exist as to the arrangement of the double strands within the HbS fiber (8, 9), there is broad agreement that the basic building block of the fiber is a Wishner-Love double strand with a slight helical twist. Additional convincing evidence that the crystalline double strand is physiologically relevant comes from copolymerization studies of HbS with naturally occurring variants (10, 11). These experiments show significant changes in polymerization properties as a result of altering residues that participate in the crystal double strand contacts. In contrast, most mutations of residues not involved in double strand contacts do not lead to alterations of the polymerization properties (2).

To probe the polymerization reaction, site-directed mutants of the β chains of HbA have been made using HbA overexpression systems developed in bacteria (12) and yeast (13). A number of mutations at the β6 position have been characterized (14–17). Polymerization is promoted with mutations of β6 Glu → Leu and β6 Glu → Ile, whereas mutation of β6 Glu → Trp (Hb6W) and β6 Glu → Phe results in lower polymerization relative to HbS, albeit without the delay period characteristic of HbS polymerization (14). To explore the structural basis for the striking inhibition of polymerization with a significantly larger residue at β6 (Trp, Phe), we have determined the crystal structure of Hb6W. This structure helps explain the polymerization behavior and provides insight into HbS polymerization.

**EXPERIMENTAL PROCEDURES**

**Preparation and Purification of Hb6W—**Recombinant human hemoglobin containing tryptophan at the β6 position was expressed in yeast and purified as described previously (14).

**Crystalization—**Deoxy-Hb6W was crystallized in a similar manner to that of deoxy-HbS (5), with several modifications. Twinned crystals initially formed in batch solutions containing 4–7 μl of 33% PEG 8000, 5 μl of 200 mM sodium citrate buffer (pH 4.8), and 10 μl of hemoglobin solution containing 160 mg/ml Hb6W suspended in 30 mM phosphate buffer (pH 7.0). Several crystals from this setup were stabilized in a 2-ml solution containing 11.6% PEG 8000 and 50 mM sodium citrate, then crushed in preparation for microseeding. The crushed crystal solution was serially diluted 3125-fold, and 1 μl was added to a batch crystallization setup containing 10 μl Hb6W (160 mg/ml), 5 μl of 33% PEG 8000, 5 μl of 200 mM sodium citrate buffer (pH 4.8). Single crystals
grew directly from solution after several hours, instead of from gelled hemoglobin, as in the case of HbS.

**Data Collection**—Crystals of Hbβ6W were very radiation sensitive, and had to be frozen under deoxygenated conditions before mounting. Prior to freezing, crystals were allowed to equilibrate with a stabilizing solution containing 11.6% (w/w) PEG 8000 (Fluka), 10 mM 2,3-diphosphoglyceric acid (Sigma), 75 mM bis-Tris (pH 7.0) (Sigma), and several grains of sodium dithionite (Hoechst-Celanese, Charlotte, NC) for 1 day, then placed in a freezing solution containing 45% PEG 20,000 (Fluka), 10 mM 2,3-diphosphoglyceric acid, 50 mM bis-Tris (pH 7.0), 10% glycerol, and several grains of sodium dithionite for another day. Crystals equilibrated in this solution were scooped up in nylon loops mounted on pins (Yale Medical Inst. Facility, New Haven, CT) and frozen in liquid propane placed in 2 ml Corning Cryovials (Fisher Scientific, Pittsburgh, PA). The cryovials were then plunged into liquid nitrogen, which solidifies the propane, permitting mounting of the frozen crystals in a cooled nitrogen cold stream (Molecular Structure Corp., The Woodlands, TX). All these manipulations, as well as the crystallization, were performed in an anaerobic chamber (Anaerobe Systems, Santa Clara, CA), and placement of the frozen crystal in the cooled nitrogen (approximately 100 K) stream kept the crystal deoxygenated. Data were collected on an RAXIS-IIc image plate system mounted on a Rigaku RU-200 rotating anode generator (Molecular Structure Corp.) operating at 50 kV, 100 mA. The crystals diffracted to better than 2.0-Å resolution, and a data set was collected from a single

| Table I | Data collection and refinement statistics on Hbβ6W |
|---------|--------------------------------------------------|
| Resolution limits (Å) | 20–2.0 |
| Total observations | 112,861 |
| Unique reflections | 35,597 |
| \( R_{\text{sym}} \) (%) | 7.6 |
| Completeness (%) | 89.2 |
| Number of reflections used in refinement \( (I > 2\sigma I) \) | 35,002 |
| R-factor | 0.198 |
| Number of reflections used for \( R_{\text{free}} \) \( (I > 2\sigma I) \) | 2,112 |
| \( R_{\text{free}} \) | 0.264 |
| Number of non-hydrogen atoms: | |
| Hemoglobin | 4,566 |
| Solvent | 368 |
| r.m.s. deviation from ideality: | |
| Bond lengths (Å) | 0.011 |
| Bond angles (°) | 1.829 |
| Improper (°) | 1.586 |
| Average temperature factors: | |
| Protein (Å²) | 14.0 |
| Water (Å²) | 22.7 |

**FIG. 1.** Ribbon diagram comparing the double strand from HbS and Hbβ6W. Parts of five tetramers of HbS are shown on the left depicting the association of molecules within the crystal to form a double strand. On the right, the fundamentally different strand found in crystals of Hbβ6W is depicted. In both strands the axial contacts are located between molecules within a single strand in the vertical direction. Lateral contacts involving the mutant residues act to associate the two single strands into the double strands depicted here. Ribbons are shown in gray, while heme groups (four in each tetramer) are depicted as dark bonds (figure generated using MOLESCRIPT (31)).
Crystal Structure of a β6 Hemoglobin Mutant

The crystal structure of deoxy-HbS was solved by molecular replacement using the program AMORE (18). The deoxygenated HbA structure (19) was stripped of solvent and the B-factors set to 35,597 were unique. These data were 89.2% complete to 2.0 Å, comprised of 70 oscillations yielded 112,861 observed reflections, of which 35,597 were unique. These data were 89.2% complete to 2.0 Å, comprised of 70 oscillations yielded 112,861 observed reflections, of which 35,597 were unique. These data were 89.2% complete to 2.0 Å, comprised of 70 oscillations yielded 112,861 observed reflections, of which 35,597 were unique.

Molecular Replacement—The structure of Hb6W was solved by molecular replacement using the program AMORE (18). The deoxygenated HbA structure (19) was stripped of solvent and the B-factors set to 8.0 Å² in preparation for its use as the initial search model. Data between 8.0 and 4.0 Å were used in the search. A single, unambiguous solution was obtained, with a correlation coefficient of 70.4 and an initial R-factor of 33.5% following rigid body refinement with AMORE.

Refinement—Deoxy-human hemoglobin (19) was mutated to contain β6 Trp, and the solution matrix from AMORE was applied to its coordinates before using it as the initial model for refinement. Powell minimization and overall B-factor (single B-factor for all atoms in structure) refinement was performed against 20.0–3.0 Å data using the program XPLOR (20). 10% of the data was set aside for use as a free R test set (21). The resolution was increased in 0.25-Å increments, with data from 20.0 to 2.0 Å included in the refinement. After these refinement runs, the free R-factor dropped from 38.4 to 35.1%, and the R-factor fell from 38.6 to 31.1%. At this time, a simulated annealing protocol was performed that further dropped the free R to 34.8% and the conventional R to 29.0%. To this structure, a shell of water was added using density greater than 4.0 Å in E₁ – Fo maps. After each shell placement, positional and B-factor refinement was performed, followed by model building with O (22). Next a bulk solvent correction was applied, and a final shell of water was added to corrected maps, followed by positional refinement.

RESULTS

Crystals of deoxy-Hb6W grew under similar conditions to HbS, but formed directly from solution rather than forming a gel first as in deoxy-HbS crystallization. These crystals have 1 hemoglobin tetramer per asymmetric unit and resemble crystal forms of HbC (23) and HbM Iwate (24). Data collection required flash freezing of crystals, under anaerobic conditions, to prevent the significant radiation damage that occurred when these crystals are exposed to the x-ray beam.

Fig. 2. The Hb6W axial and lateral contacts. a, axial contact made between symmetry-related molecules of the crystal translated along the crystallographic A axis. The space filling models depict atoms of the α and β subunits with carbons shown as yellow, oxygens as red, nitrogens as dark blue, and waters as light blue. The bonds decorating this surface model β1 and α1 loops. b, lateral contact showing the mutant tryptophan and β1 Pro-5 positioned above the binding pocket on another tetramer. The bonds of these two residues are shown in magenta. The atoms of the acceptor pocket are depicted as van der Waals radii, with color scheme as in panel a (figure created using MIDAS (32)).

Table II

| Axial contacts |
|----------------|
| Residues | Hb6W | Strand 1 | Strand 2 |
| α, Pro-114 (O)–α, Lys-16 (N) | 3.36 | 5.85 | 3.10 |
| α, Ala-115 (Cα)–α, Lys-16 (Ce) | 4.81* | 3.74 | 4.79* |
| α, Pro-114 (Cβ)–α, Glu-116 (Oε1) | 3.71 | 3.92 | 3.38 |
| β, Gly-16 (O)–β, Lys-120 (Nz) | 3.08 | 4.69* | 6.99* |
| β, Gly-16 (O)–β, Lys-120 (Ce) | 4.20* | 3.46 | 6.84* |
| β, Lys-17 (Cγ)–β, Phe-118 (O) | 3.76 | 3.91 | 3.72 |
| β, Lys-17 (Nζ)–β, His-117 (O) | 2.96 | 3.42 | 2.93 |
| β, Lys-17 (Nζ)–β, His-116 (O) | 6.12* | 3.42 | 2.89 |
| β, Val-18 (O)–β, Lys-120 (Nz) | 3.21 | 2.93 | 6.01* |
| β, Glu-22 (Oε2)–α, His-20 (Nε2) | 3.55 | 4.85 | 6.38 |
| β, His-117 (O)–α, Pro-114 (Cγ) | 3.36 | 3.47 | 3.74* |
| β, His-117 (O)–α, Ala-115 (Cβ) | 3.37 | 3.63 | 3.63* |
| β, Phe-118 (Cα)–α, Ala-115 (Cβ) | 3.82 | 3.69 | 3.88 |
| β, Phe-118 (Cζ1)–α, Pro-114 (Cγ) | 3.92 | 3.55 | 3.94* |
| β, Gly-119 (N)–α, Ala-115 (Cβ) | 3.78 | 3.63 | 3.84 |
| β, Glu-121 (Oε2)–α, Pro-114 (Cβ) | 3.71 | 3.79 | 4.58 |

Table III

| Hb6W lateral contact |
|----------------------|
| Residues | A |
| β, His-2 (Nζ)–β, Asp-73 (Oε2) | 3.35 |
| β, His-4 (Cγ)–β, Asp-73 (Cγ) | 3.59 |
| β, Pro-5 (Cβ)–β, Asp-73 (Oε2) | 3.74 |
| β, Pro-5 (Cβ)–β, Leu-88 (Cζ1) | 3.67 |
| β, Pro-5 (Cγ)–β, Ala-70 (Cβ) | 3.60 |
| β, Trp-6 (Cβ)–β, Thr-84 (O) | 3.89 |
| β, Trp-6 (Ne1)–β, His-77 (Ce1) | 3.19 |
| β, Trp-6 (C2)–β, Asp-73 (O) | 3.23 |
| β, Trp-6 (C3)–β, Phe-85 (Ce1) | 3.85 |
| β, Trp-6 (Cγ1)–β, Ala-70 (O) | 3.50 |
| β, Trp-6 (Cγ2)–β, Gly-74 (O) | 3.38 |
| β, Ser-9 (Oγ)–β, Thr-87 (Cγ2) | 2.91 |
crystals were exposed to x-rays at room temperature. The structure was solved by molecular replacement and has been refined to a final $R$-factor of 19.8%, with a free $R$ of 26.4%, and excellent stereochemistry (Table I). A Luzzati plot (25) suggests an upper limit for the overall positional error of the model to be approximately 0.23 Å (data not shown).

In the crystal lattice of Hbβ6W, tetramers are arranged in double strands that run along the crystallographic $a$ axis, as in crystals of HbS. However, the nature of these double strands is fundamentally different from those of HbS. The double strands of Hbβ6W and HbS are shown in Fig. 1. The differences become apparent upon strand alignment. When double strands of Hbβ6W are aligned with double strands of HbS, only very poor agreement is obtained as indicated by an r.m.s. deviation of 6.38 Å for $\alpha$-carbon atoms and 6.29 Å for all atoms. However, if tetramers are aligned as single strands, quite good agreement is found with an r.m.s. deviation of 0.64 Å for $\alpha$-carbon atoms and 1.09 Å for all atoms. Thus, the arrangement of molecules in single strands is very similar in the two crystal structures, but the association of pairs of strands is different for Hbβ6W and HbS.

The similarity of single stands of Hbβ6W and HbS is reflected in an analysis of the axial contacts between molecules within a single strand. A view of the axial contact is shown in Fig. 2s, which is quite similar to that of HbS (see Fig. 7 of Harrington et al. (5)). The Hbβ6W contact contains more water molecules than the HbS axial contacts, possibly because of the presence of more ordered water molecules in the frozen crystal. The distances between atoms across this interface are comparable with the distances in HbS, particularly around the core hydrophobic interactions involving $\alpha_2$ Pro-114 and $\alpha_2$ Ala-115 (Table II).

In contrast to the axial contacts, lateral contacts between strands are quite distinct in Hbβ6W and HbS. Lateral contacts

**Fig. 3. Stereo diagram of the lateral contacts.** The lateral contacts are depicted for HbS (a) and Hbβ6W (b). Within each stereo diagram, the backbone worms and residues to the right depict two $\beta_2$ subunits for HbS, or the $\beta_1$ subunit for Hbβ6W, both of which contain their respective mutated residue. These are shown interacting with the one $\beta_2$ acceptor pocket for HbS, or the $\beta_1$ pocket for Hbβ6W shown on the left. Side chains that closely associate with the mutant residues or are involved in hydrogen bonds are shown in these figures. Hydrogen bonds were included if the donor and acceptor atoms were within 3.2 Å of one another, and these bonds are represented as dashed lines. Residues are labeled according to the standard one-letter amino acid code, and the helices of the globin fold are also labeled by capital letters (figure generated using MOLSCRIPT (31)).
TABLE IV

| Residues | HbS | Hb6W |
|----------|-----|------|
| \( \beta I_{,} L y s-66 (O) \rightarrow \beta I_{,} P r o-5 (C) \) | 3.61 | 3.65* |
| \( \beta I_{,} G l y-69 (C) \rightarrow \beta I_{,} P r o-5 (C) \) | 3.89 | 3.96 |
| \( \beta I_{,} A l a-70 (N) \rightarrow \beta I_{,} P r o-5 (C) \) | 3.83 | 3.88 |
| \( \beta I_{,} A l a-70 (C) \rightarrow \beta I_{,} V a l-6 (C2) \) | 3.83 | 3.82* |
| \( \beta I_{,} A s p-73 (O) \rightarrow \beta I_{,} V a l-6 (C) \) | 3.11 | 3.01 |
| \( \beta I_{,} A s p-79 (O) \rightarrow \beta I_{,} S e r-49 (C) \) | 3.46 | 3.48 |
| \( \beta I_{,} A s p-79 (O) \rightarrow \beta I_{,} H i s-50 (N) \) | 3.92* | 3.84* |
| \( \beta I_{,} A s p-79 (O) \rightarrow \beta I_{,} H i s-50 (C2) \) | 3.66 | 3.65 |
| \( \beta I_{,} A s p-79 (O) \rightarrow \beta I_{,} H i s-125 (C) \) | 3.99 | 4.10 |
| \( \beta I_{,} T h r-84 (O) \rightarrow \beta I_{,} A l a-6 (C2) \) | 3.62 | 3.71 |
| \( \beta I_{,} A l a-10 (C) \rightarrow \beta I_{,} A l a-12 (C2) \) | 3.76 | 4.14 |
| \( \beta I_{,} V a l-6 (C) \rightarrow \beta I_{,} A l a-12 (C2) \) | 3.65 | 4.10 |
| \( \beta I_{,} S e r-5 (C) \rightarrow \beta I_{,} A l a-12 (C2) \) | 3.94 | 3.75 |
| \( \beta I_{,} A l a-10 (C) \rightarrow \beta I_{,} A l a-12 (C2) \) | 3.43 | 3.75 |
| \( \beta I_{,} A l a-12 (C2) \rightarrow \beta I_{,} A l a-13 (C) \) | 3.82 | 3.93 |
| \( \beta I_{,} T h r-87 (O) \rightarrow \beta I_{,} A l a-13 (C) \) | 3.83 | 12.13 |
| \( \beta I_{,} V a l-6 (C2) \rightarrow \beta I_{,} A l a-16 (C) \) | 3.99 | 4.10 |
| \( \beta I_{,} A l a-8 (C) \rightarrow \beta I_{,} A l a-16 (C) \) | 3.50 | 3.76 |
| \( \beta I_{,} G l u-90 (O) \rightarrow \beta I_{,} L y s-17 (N) \) | 5.03 | 3.49 |
| \( \beta I_{,} L y s-95 (N) \rightarrow \beta I_{,} L y s-17 (N) \) | 3.93 | 5.78 |

TABLE V

| Root mean squared differences (Å) between tetramers |
|----------|
| Hb6W tetramer 1 | 0.59 (1.01) |
| Hb6W tetramer 2 | 0.57 (0.96) |
| Hb6W | 0.36 (0.90) | 0.54 (1.02) | 0.53 (0.97) |

TABLE VI

| Surface area of crystal contacts (Å²) |
|----------|
| Contact |
| Hb6W |
| Lateral 1 | 1277 |
| Lateral 2 | 1341 |
| Axial 1 | 1272 |
| Axial 2 | 1033 |

DISCUSSION

We undertook the structural analysis of deoxy-Hb6W to investigate the basis for its altered polymerization properties relative to HbS and to gain insight into the polymerization of HbS. The complicated polymerization kinetics of deoxy-HbS (26) reflects the fact that fiber formation requires the interplay of multiple interactions between tetramers. Our results reveal details of a new mode of assembly available to human hemoglobin when the βε position is occupied by a residue with an aromatic side chain. The interactions leading to this assembly may play a role in HbS polymerization.

A prominent feature of the crystals of deoxy-Hb6W is a double strand of hemoglobin tetramers that shares many structural characteristics with the HbS double strand. The individual strands of HbS and Hb6W molecules can be superimposed nearly identically. In addition, both double strands are stabilized by lateral contacts between the A helix of a β subunit on one tetramer with the EF pocket of a β subunit on another tetramer. However, the size of tryptophan βε prevents it from packing into the EF pocket in the same manner as does βε Val. As a result, an alternate lateral contact is formed in which βε Trp participates peripherally whereas βε Pro packs into the EF pocket at nearly the same position as βε Val. The alternate lateral contact in Hb6W is characterized by significantly less surface area buried than the HbS lateral contact (Table VI). Interestingly, the water molecules located between the F and F helices are conserved in the lateral contacts, which may be useful for design of anti-sickling agents that bind in the acceptor pocket.

The inability of the bulky tryptophan residue to pack tightly into the acceptor pocket results in altered polymerization properties and formation of an alternate double strand assembly. Unlike HbS, crystals of Hb6W grew directly from solution, suggesting that the mutation precludes gel formation. Such an alternate polymerization reaction is consistent with the lack of a delay time prior to polymerization as observed in high phosphate experiments (14). The double strand observed in crystals of Hb6W, although similar to that of HbS, is fundamentally different. In both double strands, one β subunit on each tetramer acts as a donor, and the other β subunit provides an acceptor pocket leaving another donor/acceptor pair within...
using one common strand (one strand from the crystal structures of HbS and Hbβ6W were least-squared aligned). In panel a, three tetramers of a single strand are shown as surface representations. Acceptor pockets are red in this figure, while yellow surfaces represent the position of the β6 residue. In panel b, another strand represented as violet backbone worms is shown interacting with the surfaced strand, in a manner analogous to that found in HbS crystals. Panel c depicts the association of double strands found in crystals of Hbβ6W. Finally, in panel d, both strands are shown interacting with the common, least-square aligned strand, forming a triple strand (model representation done with GRASP (33)).

The different roles of β subunits in the two double strands suggests the possibility that both modes of association could occur simultaneously and contribute to formation of the HbS fiber. Fig. 4 shows the formation of the two double strands as they would associate with a single strand. As can be seen, all three strands can coexist, albeit with slight interpenetration. Additionally, a four-stranded assemblage can be modeled by aligning one strand of HbS with the blue strand of Hbβ6W shown in Fig. 4. Although there is slight interpenetration between the two parallel double strands assembled in this way, the clashes are minimal and are reduced by application of the right-handed twist (27) found in the HbS fiber. It is interesting to note that parallel pairs of double strands have been observed as part of the 14-stranded HbS fiber (8, 9). Alternatively, the interactions seen in the Hbβ6W double strand could contribute to formation of multiple fibers as described by the heterogeneous nucleation model (26). This would provide another possible mode of nucleation, related to that proposed by Mirchev and Ferrone (28). Such interactions result from one strand (the single strand represented as a molecular surface) utilizing both acceptor pockets and both donor regions, unlike the crystal structures.

The crystal structure of Hbβ6W provides evidence for the stereospecific importance of side-chains at the β6 position of human hemoglobin in promoting the formation of certain double strands. Additionally, this structure may represent a model for physiologically relevant contacts within the fibers, or model interactions responsible for promotion of heterogeneous nucleation. Conserved features in the structures of HbS and Hbβ6W, particularly with regard to solvent structure, could be exploited in the design of sickling inhibitors. Together, these models provide important building blocks for future studies aimed at elucidating the structure of HbS fibers at atomic resolution, which may ultimately contribute to the design of specific inhibitors for HbS polymerization.

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Crystal Structure of a β6 Hemoglobin Mutant

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