Structures and Oxygen Affinities of Crystalline Human Hemoglobin C (β6 Glu→Lys) in the R and R2 Quaternary Structures*

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Recent crystallographic studies suggested that fully liganded human hemoglobin can adopt multiple quaternary conformations that include the two previously solved relaxed conformations, R and R2, whereas fully unliganded deoxyhemoglobin may adopt only one T (tense) quaternary conformation. An important unanswered question is whether R, R2, and other relaxed quaternary conformations represent different physiological states with different oxygen affinities. Here, we answer this question by showing the oxygen equilibrium curves of single crystals of human hemoglobin in the R and R2 state. In this study, we have used a naturally occurring mutant hemoglobin C (β6 Glu→Lys) to stabilize the R and R2 crystals. Additionally, we have refined the x-ray crystal structure of carbonmonoxyhemoglobin C, in the R and R2 state, to 1.4 and 1.8 Å resolution, respectively, to compare precisely the structures of both types of relaxed states. Despite the large quaternary structural difference between the R and R2 state, both crystals exhibit similar noncooperative oxygen equilibrium curves with a very high affinity for oxygen, comparable with the fourth oxygen equilibrium constant ($K_{O_4}$) of human hemoglobin in solution. One small difference is that the R2 crystals have an oxygen affinity that is 2–3 times higher than that of the R crystals. These results demonstrate that the functional difference between the two typical relaxed quaternary conformations is small and physiologically less important, indicating that these relaxed conformations simply reflect a structural polymorphism of a high affinity relaxed state.

This view was largely supported by crystallographic evidence that only one quaternary conformation (classical T structure) has so far been observed in a number of different crystals forms of human wild-type, mutant, and chemically modified deoxyhemoglobins (3–6). Likewise, the early crystallographic studies demonstrate that the structures of two independent fully liganded proteins, horse methemoglobin and human oxy (or carbonmonoxy) hemoglobin, assume essentially the same quaternary conformation (classical R structure) despite different crystal-packing contacts (7–9).

However, this simple view was challenged in the early 1990s by the discovery of a second relaxed state for fully liganded hemoglobin, known as R2 or Y, the quaternary structure of which is substantially different from that of the R state (10, 11). Computational studies suggest that R2 may be the relaxed end state in a T$\rightarrow$R$\rightarrow$R2 allosteric pathway, rather than an intermediate between T and R or an off-pathway intermediate (12). Also, recent NMR studies using $^{15}$N-$^1$H residual dipolar couplings show that the quaternary conformation of human carbonmonoxyhemoglobin in solution is a dynamic ensemble of different quaternary structures that include the crystallographically identified R and R2 states (13). Consistent with this observation in solution, recent crystallographic studies identify previously unrecognized relaxed conformations that lie between R and R2 (14–16), although an additional liganded end state, R3, has also been proposed to exist (16). These recent findings strongly suggest that the fully liganded hemoglobin molecule is capable of traversing multiple conformations.

A basic question emerges as to whether R, R2, and other potential liganded conformations represent different physiological states with different oxygen affinities or whether they simply reflect a structural polymorphism of a high affinity relaxed state. To address this question directly, in this study we have determined the oxygen equilibrium curves of single crystals of human hemoglobin in the R and R2 quaternary structures by using absorption microspectrophotometry.

Previous microspectrophotometry studies on crystals of human deoxyhemoglobin in the T (tense) state demonstrated the feasibility of measuring the oxygen affinity of crystalline hemoglobin, opening up a new method for a direct comparison of the x-ray structure with its function in the same crystalline state (17, 18). However, so far this approach has not been applied to crystals of the fully liganded forms of hemoglobin, primarily because crystals of oxy (or carbonmonoxy) hemoglobin...
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bin A in the R or R2 state shatter upon deoxygenation. Moreover, because those crystals are expected to have a high affinity for oxygen, they should be equilibrated with low oxygen pressures, requiring a very long experimental time. An additional difficulty is that the R2 crystals of hemoglobin A must be preserved under low pH conditions (below pH 6) (11), where the autooxidation rate is significantly increased. Here, we now show that the use of thin flat crystals of hemoglobin C (β6 Glu→Lys) in the R and R2 state can overcome most of these problems, thus enabling determination of their oxygen dissociation curves. It is known that hemoglobin C crystallizes much more readily than hemoglobin A due to the β6 Glu to Lys surface mutation, which makes the protein less soluble but does not significantly perturb the oxygen equilibrium properties (19–21). As a result, this mutant yields both R and R2 crystals of very high quality under mild conditions at neutral pH. Note that the use of thin crystals is, on the one hand, advantageous for shortening the experimental time, but, on the other hand, disadvantageous as to the signal to noise ratio of the absorption data. Thus, unlike previous measurements using oriented crystals and linearly polarized light (17, 18), we have used unpolarized light incident on horizontally oriented, thin flat crystals, to eliminate stray light reflected from the polarizer and to keep the signal to noise ratio as high as possible. In addition to the functional study, we have refined the crystal structure of carbonmonoxyhemoglobin C, in the R and R2 state, to 1.4 and 1.8 Å resolution, respectively. These high resolution data allow us to compare precisely the structures of both types of relaxed states, enabling discussion with the observed oxygen equilibrium curves of both states.

EXPERIMENTAL PROCEDURES

Preparation of Hemoglobin C—CS human blood was kindly obtained from Dr. Kazuhiro Adachi (The Children’s Hospital of Philadelphia, Philadelphia, PA) and stripped as reported previously. The stripped hemoglobin sample was passed through a column of Sephadex G-25 equilibrated with CO-saturated 0.01 M phosphate buffer, pH 7.15, and 0.04 M phosphate buffer. The column was washed with 2 column volumes of the same CO-saturated buffer, followed by 2 column volumes of the same buffer containing 0.2% (w/v) sodium dithionite (Na2S2O4) to reduce (albeit the small amount of) methemoglobin in the original hemoglobin sample. After washing the column with 5 column volumes of the same CO-saturated buffer without dithionite, the column was eluted by a linear gradient of 0.01 M phosphate buffer, pH 7.15, and 0.04 M phosphate buffer, pH 7.60. The second major peak corresponding to hemoglobin C was collected and concentrated.

Crystallization and Structure Determination—Crystallization of carbonmonoxyhemoglobin C in the R state was carried out at 20 °C according to the batch method of Fitzgerald and Love (19), with some modifications, to give a CO-saturated 2.0% (w/v) hemoglobin solution containing 1.65 M phosphate buffer (0.528 M NaH2PO4 and 1.122 M KH2PO4, pH 7.2), 10% (v/v) glycerol, and 0.1% (w/v) D.L-homocysteine. A glass tube containing the above crystallization solution was placed into a gas-tight glass vial and quickly sealed under CO. Note that this vial also contained a small amount of Oxygen Absorbing System A-500HS (ISO, Yokohama, Japan) powder in its gas part, to maintain complete anaerobic conditions during crystallization. Similarly, crystals of carbonmonoxyhemoglobin C in the R2 state were obtained, using the same batch method at 20 °C, from CO-saturated 1.0% (w/v) hemoglobin solutions containing 11% (w/v) PEG 3350, 50 mM HEPES buffer, pH 7.6, and 10% (v/v) glycerol under complete anaerobic conditions. Both types of hemoglobin C crystals grew rapidly to their maximum dimensions within a few days. The mother liquors containing 15 and 20% (v/v) glycerol were used for the R and R2 crystals, respectively, as cryoprotectant in which the crystals were rinsed briefly before flash-freezing in liquid nitrogen.

X-ray data were collected using an ADSC Q270 CCD detector at beamlines BL17A and BL1A of the Photon Factory, Tsukuba, Japan. The wavelength of the incident x-rays was 1.0 Å. Diffraction datasets were processed with HKL2000 (22) and scaled with SCALEPACK (22). The R and R2 crystals belonged to the space group P412121 and P212121, respectively, with unit cell parameters of a = b = 53.1 Å, c = 191.53 Å, and a = 57.78 Å, b = 58.75 Å, c = 172.87 Å, respectively. Their structures were solved by molecular replacement using MOLREP (23, 24) and the previously reported structures of hemoglobin C (20, 21) (Protein Data Bank (PDB)6 IDs 1K1K and 1M9P) as a starting model. The best solution using data from 20 to 3.5 Å resolution range yielded a correlation coefficient of 0.55 and an R-factor of 0.40 for the R structure, and a correlation coefficient of 0.57 and an R-factor of 0.45 for the R2 structure, after rigid body refinement. At this stage, crystallographic refinement were pursued in PHENIX (25). After an initial round of simulated annealing refinement, several macrocycles that included bulk solvent correction, and anisotropic scaling of the data, individual coordinate refinement with minimization, and individual isotropic atomic displacement parameter refinement were carried out with maximum likelihood as the target. In the course of the refinement, water molecules were added to the models by manual inspection of their positions in both 2Fo−Fc and Fo−Fc maps, and combined TLS (translation, libration, and screw-rotation) and individual atomic displacement parameter refinement were carried out in the final stages. Map fitting and other manipulations with molecular models were performed using the graphic software COOT (26). The stereochemistry of the final models of the R and R2 state (named R-COHbC and R2-COHbC, respectively) were assessed using PROCHECK (27). Data collection and refinement statistics are summarized in Table 1.

Oxygen Equilibrium Curves of Crystals—Crystals for oxygen equilibrium measurements were grown under the above mentioned conditions except for the absence of glycerol. Among grown crystals of various shapes, thin flat crystals were chosen

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6 The abbreviations used are: PDB, Protein Data Bank; K4, fourth oxygen equilibrium constant; K0, oxygen affinity of relaxed state; Ki, oxygen affinity of tense state; R-COHbA, 1.25 Å resolution crystal structure of carbonmonoxyhemoglobin A in the R state (PDB code 2DN3); R-COHbC, final structural model for carbonmonoxyhemoglobin C in the R state; R2-COHbC, the final structural model for carbonmonoxyhemoglobin C in the R2 state; r.m.s.d., root mean square deviation; T-deoxyHbA, the 1.25 Å resolution crystal structure of deoxyhemoglobin A in the T state (PDB code 2DN2).
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| Crystal parameters, data collection, and structure refinement for the R and R2 crystals of human carbonmonoxyhemoglobin C |
|---|---|---|
| **Data collection** | **R-COHbC** | **R2-COHbC** |
| Resolution range (Å) | 50.0–1.4 | 50.0–1.8 |
| Space group | P4_1,2,2 | P2_1,2,2 |
| Unit cell dimensions (Å) | \(a = 53.1, b = 53.1, c = 191.53\) | \(a = 57.78, b = 58.75, c = 172.87\) |
| Reflections (Measured/Unique) | 471,355/54,017 | 142,988/47,832 |
| Completeness (%) | 97.9 (91.8)* | 86.4 (63.0)* |
| Mean <\(d_i/c\)\> | 18.0 | 24.6 |
| Multiplicity | 8.7 | 3.0 |
| \(R_{merge}\) (%) | 3.4 (21.3)* | 4.6 (60.1)* |
| B factor from Wilson plot (Å²) | 17 | 35 |
| **Refinement statistics** | | |
| Resolution range (Å) | 20.0–1.4 | 20.0–1.8 |
| R-factor (%)/free R-factor (%) | 20.8/23.3 | 25.0/30.5 |
| R.m.s.d. from ideals | | |
| Bond lengths (Å) | 0.007 | 0.011 |
| Bond angles (°) | 1.079 | 1.486 |
| No. of water molecules | 208 | 47 |
| Average B-factor (Hb/water, Å²) | 20/28 | 51/51 |
| Ramachandran plot | | |
| Residues in most favorable regions (%) | 93.9 | 89.2 |
| Residues in additional allowed regions (%) | 6.1 | 10.0 |
| Residues in generously allowed regions (%) | 0.0 | 0.8 |

* The numbers in parentheses refer to the outer shell with a resolution of 1.45–1.4 Å and 1.86–1.8 Å for R-COHbC and R2-COHbC, respectively.

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for measurements. Specifically, the R and R2 crystals used were hexagonal and trapezoidal in shape, respectively, each having a well developed (010) (or ac) crystal face. The crystals were washed with the mother liquors (0.8 M NaH₂PO₄ and 1.7 M K₂HPO₄ (pH 7.2) for the R crystals, and 25% (w/v) PEG 3350 and 50 mM HEPEs (pH 7.6 or 7.2) for the R2 crystals) containing 0.1 mg of catalase/ml (Sigma).

For spectral measurements a single crystal with about 20 μl of its mother liquor was placed on a cover glass and then sealed into a flow chamber. Usually the settled crystal did not move during the measurements, because its well developed (010) face made good contact with the glass surface. The flow chamber was mounted on the stage of a Zeiss UMS-80 microspectrophotometer. Before measurements, removal of CO was carried out in the flow chamber with humidified oxygen, at a gas flow of 10 ml/min, under illumination of the white light of the microspectrophotometer. The time required for this conversion was <1 h, when small thin, flat crystals were used. Humidified nitrogen-oxygen gas mixtures at defined partial oxygen pressures were prepared by a GB-4C gas blender (Kofloc, Japan) with modifications and flowed into the chamber. The oxygen pressures were detected in the outflow from the chamber using a MC-7G-L galvanic O₂ sensor (Iijima, Japan). The absorption spectra of crystals equilibrated with different oxygen pressures at 21–22 °C were recorded between 450 and 700 nm with unpolarized light incident on (010) crystal face. According to the method of Mozzarelli et al. (17), the fractional saturation of the ferrous hemes with oxygen (Y) was calculated by a least square fit of the observed absorption spectra to a linear combination of three reference spectra and a baseline offset. The reference spectra are the absorption spectra of crystals of oxy-, deoxy-, and methemoglobin C. These spectra were determined on the same crystal as used for the oxygen equilibrium measurement. Because increases in oxygen pressure above 380 torr caused no appreciable spectral changes for the R or R2 crystals, the spectrum obtained under pure oxygen, after correction for the contribution of the methemoglobin content, was taken as the reference spectrum for oxyhemoglobin C. Note that the contribution from methemoglobin was removed by subtracting increasing amounts of the methemoglobin reference spectrum (see below) until the band at 630 nm disappeared. After each oxygen equilibrium measurement, the crystal was reduced by washing with mother liquor containing 0.2% (w/v) sodium dithionite (Na₂S₂O₄) for the measurement of the deoxyhemoglobin reference spectrum. In most cases the R and R2 crystals did not crack upon deoxygenation with dithionite. The crystal was then washed with mother liquor to remove sodium dithionite, followed by washing with mother liquor containing 5 mM potassium ferricyanide to oxidize the crystal for the measurement of the methemoglobin reference spectrum.

RESULTS AND DISCUSSION

X-ray Structures—At present, in the PDB the highest resolution data for the R and R2 structures of carbonmonoxyhemoglobin C are those of Hirsh and colleagues, determined at 2.0 and 2.1 Å resolution, respectively (20, 21) (PDB codes 1K1K and 1M9P). These resolutions are sufficient to be able to assign their quaternary structures but insufficient to make a precise comparison between their heme environmental structures. To improve this situation, we have undertaken the refinement of the crystal structures of carbonmonoxyhemoglobin C, in the R and R2 state, to 1.4 and 1.8 Å resolution, respectively (Table 1). In this study, the best crystals of the R and R2 state have been obtained under high salt conditions at pH 7.2 and low salt conditions at pH 7.6, respectively.

As summarized in Table 2, in both α and β subunits the proximal His(F8) geometries relative to the heme are very similar between R-COHbC and R2-COHbC and are similar to R-COHbA. Remarkably, in both subunits distances between the proximal His(F8) side chains and the hemes are virtually
identical in these relaxed conformations and shorter than T-deoxyHbA (Table 2), suggesting that the proximal strain, which is one of the key factors determining oxygen affinity, is relieved to a similar extent in R-COHbC, R2-COHbC, and R-COHbA. Also, in both subunits the distal ligand environments are in general similar between R-COHbC and R2-COHbC (Table 2). One exception is the distal His(E7), which appears to be positioned differently between R-COHbC and R2-COHbC (Fig. 1, A–D and Table 2). However, this disagreement cannot be considered significant because the distal His(E7) side chains are not so well ordered in the α2, β1, and β2 subunits in R2-COHbC, as indicated by the lack of well defined electron density map, whereas all other heme peripheral residues in R2-COHbC and those in R-COHbC (including Hisα58 and Hisβ63) are well ordered.

The observed dynamic behavior of His(E7) is not surprising because mutagenesis and kinetics studies show that the motilities of the distal His(E7) side chains are essential for opening a major channel for ligand entry in both hemoglobin subunits (28, 29). Also, crystallographic evidence for the His(E7) gate has recently been obtained for a new relaxed state of hemoglobin A at pH 6.4 (referred to here as RR3; PDB code 3D17), which shows a rotation of the β distal His(E7) out of the distal pocket, creating a direct channel to the bulk solvent (30). By contrast, so far there has been no crystallographic evidence for an opened ligand channel in human hemoglobin α subunits. This is probably because the α distal pocket is not so flexible as the β one, as predicted by the effects of mutagenesis (28, 29) and ligand size (31). Our data on R2-COHbC now suggest that the distal His(E7) in one of the α subunits (α2) is rather disordered but appears to rotate out of the distal pocket even at pH 7.6 (Fig. 1C and Table 2). The data also indicate that the distal His(E7) side chain in the α1 subunit is well ordered in a closed conformation, and both the β distal His(E7) side chains are similarly disordered.

We also investigated how the quaternary structures of R-COHbC and R2-COHbC are different from each other. Because the structure of the α1β1 dimer itself does not vary greatly among different states, the magnitude of the quaternary structural difference between two hemoglobin tetramers can be estimated by calculating the r.m.s.d. for the main-chain atoms between their α2β2 dimers after the corresponding α1β1 dimers are superimposed. We found that this r.m.s.d. value for R-COHbC and R2-COHbC is 4.95 Å, comparable with the r.m.s.d. value of 5.54 Å found for the deoxy T and carbonmonoxy R quaternary structures of hemoglobin A (32) (PDB codes 2DN2 and 2DN3). Analysis using difference distance matrix plots produced with DMP (Center for Structural Biology, Yale University, New Haven, CT) confirmed that R-COHbC and R2-COHbC differ by the relative orientations of the α1β1 and α2β2 dimers in a way similar to that observed in the R and R2 structures of carbonmonoxyhemoglobin A (Fig. 1, E and F).

In crystals, the apparent oxygen saturation will depend on the heme orientation relative to the direction of the measuring light because the heme planes do not project equally onto the optical axis of the measuring light. Moreover, the heme orientation and crystal axes are different between the R and R2 crystals. Therefore, before oxygen equilibrium measurements, we should check the contributions made by the α and β hemes to light absorption in the R and R2 crystals. The R and R2 crystals used were hexagonal and trapezoidal in shape, respectively, each having a well developed (010) (or ac) crystal face. All of the spectra in this study were measured with light incident on this flat (010) crystal face. Based on the orientations of individual hemes in R-COHbC and R2-COHbC, the relative contributions of the individual hemes to the absorption could be calculated. A similar trend was found for both R and R2 crystals: The β hemes in the R and R2 crystals contribute 68 and 62%, respectively, of the total absorption. Thus, the measured oxygen affinities of the R and R2 crystals will be meaningfully compared with each...
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FIGURE 1. Comparison between the R and R2 crystal structures of hemoglobin C. A, stereoscopic comparison of the vicinity of the α1 heme between R-COHBc (red) and R2-COHBc (blue) by superimposing their α1 subunits. B, stereoscopic comparison of the vicinity of the β1 heme between R-COHBc (red) and R2-COHBc (blue) by superimposing their β1 subunits. C, stereoscopic comparison of the vicinity of the α2 heme between R-COHBc (red) and R2-COHBc (blue) by superimposing their α2 subunits. D, stereoscopic comparison of the vicinity of the β2 heme between R-COHBc (red) and R2-COHBc (blue) by superimposing their β2 subunits. E, difference distance matrix of carbonmonoxyhemoglobin A in the R state (PDB code 2DN3) and that in the R2 state (PDB code 1BBB). In E and F, blue regions represent residues that move closer in the R structure, whereas the opposite happens in the red regions.

other, although both affinities are somewhat biased toward the affinity of the β heme.

Oxygen Equilibrium Properties—To make a direct comparison of the x-ray crystal structure with its function, crystals for oxygen equilibrium measurements were grown under the same conditions as for x-ray studies except for the absence of glycero1. Before oxygen equilibrium measurements, these crystals were converted to the oxy form by exposure to light and oxygen. Then, they were equilibrated with different oxygen pressures, and absorption spectra were recorded at 21–22 °C. The fractional saturation with oxygen was calculated by a least square fit of the observed absorption spectra to a linear combination of the reference spectra of the oxy-, deoxy-, and met- forms of the same crystal. These three reference spectra for an R crystal and those for an R2 crystal are present in Fig. 2, A and B, respectively. Typical time courses of deoxygenation experiments at an oxygen pressure of 0.857 torr, for the R and R2 crystals, are shown in Fig. 2, C and D, respectively. As evident from the comparison between Fig. 2, C and D, the oxygen affinity of the R2 crystals is higher than that of the R crystals, and the equilibration time required by the R2 crystals (<30 min) is much shorter than that by the R crystals (about 6 h) when comparing the crystals of similar thickness. It is currently unclear why the higher affinity R2 crystals require a shorter equilibration time (in general the reverse is true), but one interesting possibility is that the more flexible distal pockets observed in R2-COHBc could facilitate the ligand binding reaction in crystals. During these deoxygenation experiments, the methemoglobin contents of the R and R2 crystals were increased to 26 and 10%, respectively (see Fig. 2, E and F). However, previous study by Rivetti et al. (18) demonstrated that the fractional saturation with oxygen in crystals is independent of the presence of ferric hemes as long as the cooperativity is very small or absent. We below show that both crystalline samples actually meet this condition. Note that in this study only the data points in the upper 50% of fractional saturation could be collected because of the slow equilibration with oxygen, resulting from the very high oxygen affinity of the crystals. For example, as expected from Fig. 2C, the overall equilibration time of this crystal at an oxygen pressure below 0.2 torr would be longer than a day, making it difficult to obtain reliable data.

Hill plots of the oxygen dissociation curves for the R and R2 crystals of hemoglobin C are shown in Fig. 3A. Both Hill plots are linear, at least in the range above half-saturation, with slopes of close to unity. Specifically, the Hill coefficient, n, for the R and R2 crystals is 0.99 ± 0.03 and 1.04 ± 0.02, respectively, demonstrating the absence of cooperativity in oxygen binding by both relaxed quaternary conformations in the crystals. The reversibility of the oxygen binding was ensured by the agreement between the deoxygenation data and the corresponding oxygenation data. The oxygen pressure at half-saturation, P50, for the R and R2 crystals is 0.29 ± 0.03 and 0.12 ± 0.01 torr, respectively, indicating that the R2 crystals have an ~2–3 times higher affinity for oxygen compared with that of the R crystals.

However, we should keep in mind that different solvent conditions used for the R and R2 crystals (i.e. high salt conditions at pH 7.2 for the R crystals, and low salt conditions at pH 7.6 for the R2 crystals) may affect differently the oxygen equilibrium properties of hemoglobin. Although both crystals are difficult to investigate under the same salt conditions, the adjustment of crystallization pH is much more feasible. Indeed, the R2 crystals grown at pH 7.2 were found to be isomorphous with those grown at pH 7.6 by determination of the unit cell parameters. Therefore, we determined the oxygen equilibrium curve of the R2 crystals grown at pH 7.2 to examine the effect of pH. As shown in Fig. 3B, the Hill plot of the oxygen equilibrium curve of the R2 crystals at pH 7.2 (n = 1.02 ± 0.03; P50 = 0.13 ± 0.01 torr) is very similar to that at pH 7.6, demonstrating that the difference in oxygen affinity between the R and R2 crystals is not due to the difference in pH. Note that although phosphate is known as an allosteric effector, available oxygen equilibrium data on hemoglobin A consistently show that the fourth oxygen equilibrium constant of human hemoglobin in solution (K0) is almost insensitive to the phosphate concentration in the range
Thus, the evidence collected to date indicates that phosphate is an allosteric effector, which lowers the oxygen affinity of hemoglobin by shifting the allosteric equilibrium toward the T state ($K_T$), but without changing the oxygen affinity of the R state ($K_R$) (34).

Our results do not provide information about functional heterogeneity between the $\alpha$ and $\beta$ subunits. However, this does not affect our conclusions because, as already mentioned, the contributions made by the $\alpha$ and $\beta$ hemes to apparent oxygen saturation are not so much different between the R and R2 crystals. Moreover, previous studies have shown that in solution the oxygen affinities of the $\alpha$ and $\beta$ subunits within high affinity state hemoglobin are nearly identical (35, 36).

Interestingly, as shown in Fig. 3, the dotted straight line corresponding to the fourth oxygen equilibrium constant of human hemoglobin in solution ($K_4 = 0.19$ torr at 20 °C) (37) falls right in the middle between the Hill plots of the R and R2 crystals, suggesting that the oxyhemoglobin solution may contain nearly equal amounts of the R and R2 state. This is consis-

![FIGURE 2. Optical absorption spectra of crystalline hemoglobin C in the R and R2 quaternary structures. Reference spectra of crystalline hemoglobin C, in the R and R2 state, are shown in A and B, respectively. In each panel, spectra of the oxy-, deoxy-, and methemoglobin are shown as solid, dashed, and dotted line, respectively. Typical time courses of deoxygenation experiments at an oxygen pressure of 0.857 torr for the R and R2 crystals, are shown in C and D, respectively. The fractional saturation of the ferrous hemes with oxygen ($Y$) was calculated by a least square fit of the observed absorption spectra to a linear combination of three reference spectra and a baseline offset. In E, the absorption spectrum of the R crystal equilibrated with oxygen at a pressure of 0.857 torr for 6 h is fitted with the reference spectra (dotted line). The fractional amounts of oxy-, deoxy-, and met-forms are calculated to be 54.6%, 19.7%, and 25.7%, respectively. In F, the absorption spectrum of the R2 crystal equilibrated with oxygen at a pressure of 0.857 torr for 2 h is fitted with the reference spectra (dotted line). The fractional amounts of oxy-, deoxy-, and met-forms are calculated to be 81.0%, 9.4%, and 9.6%, respectively.]}
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A

FIGURE 3. Hill plots of the oxygen equilibrium curves of crystalline hemoglobin C in the R and R2 quaternary structures. A, crystals of hemoglobin C in the R (circles) and R2 state (squares), which were suspended in 0.8 M NaH2PO4 and 1.7 M K2HPO4 (pH 7.2) and 25% (w/v) PEG3350 and 50 mM HEPES (pH 7.6), respectively, were equilibrated with either decreasing (open symbols) or increasing (closed symbols) oxygen pressures at 21–22 °C. B, crystals of hemoglobin C in the R2 state (triangles), which were suspended in 25% (w/v) PEG3350 and 50 mM HEPES (pH 7.2), were equilibrated with either decreasing (open symbols) or increasing (closed symbols) oxygen pressures at 21–22 °C. In each panel, the solid lines through data points represent the least squares fit of the deoxygenation data to the Hill equation. For comparison, the dotted straight line corresponding to the fourth oxygen equilibrium constant of human hemoglobin in solution (K4 = 0.19 torr at 20 °C) (37) and the dashed straight line corresponding to the lowest affinity of solution deoxyhemoglobin (K = 175 torr at 20 °C) (37) are drawn.

B

tent with the previous NMR finding that the quaternary structure of carbonmonoxyhemoglobin A in solution is a dynamic intermediate between the energetically equivalent R and R2 crystal structures (13).

Previous x-ray crystallographic studies have identified a number of relaxed states of carbonmonoxyhemoglobin A, namely, R, R2, RR2, R3, and RR3, each of which has a distinct quaternary structure (8, 9, 11, 16, 30). It has been suggested that the R2 state may be the relaxed end state in a T→R→R2 allostERIC pathway (12), and the RR2 state is an intermediate during the R→R2 transition (16). The R3 state, characterized by the smallest central water cavity among known conformations, has also been proposed as another relaxed end state in a T→R→R3 pathway (16). A recently discovered new relaxed state (referred to here as RR3) appears to lie in the pathway of the R→R3 transition (30, 38). Based on the different structural features at the central cavity and the β distal His (E7), Safo et al. have proposed that R, R2, R3 and other potential relaxed conformations represent different physiological states with different oxygen affinities, with R and R3 exhibiting the lowest and highest oxygen affinity, respectively (16, 30, 38). Until now, however, there is no direct functional evidence for, or against, this proposal.

Our study demonstrates that within single crystals both of the R and R2 states exhibit similar noncooperative oxygen equilibrium curves with a very high affinity for oxygen, comparable with the fourth oxygen equilibrium constant (K4) of human hemoglobin in solution (Fig. 3). Accordingly, little change in oxygen affinity is expected to occur during the R→RR2→R2 transition. Although our data do not provide direct information on the R and RR3 states, we would like to point out that all of the oxygen affinities of other relaxed states, R, R2, and RR2, are very similar to the oxygen affinity of solution relaxed state (K4) (34), which is an average over the ensemble of relaxed states existing in solution (such as R, R2, RR2, R3, and RR3). Therefore, it is unlikely that only the oxygen affinities of the R3 and RR3 states are substantially different from K4. If these states are significantly populated under physiological conditions, overall, we can conclude that the multiple relaxed conformations of fully liganded hemoglobin do not have a significant physiological role, but rather reflect interconvertible quaternary states all of which show a very high affinity for oxygen.

It is important to note here that the current success of determining the oxygen equilibrium curves of the R or R2 crystals is largely due to the use of hemoglobin C instead of hemoglobin A. Indeed, we found that deoxygenation of the R or R2 crystals of hemoglobin A caused irreversible behavior, presumably due to the occurrence of R-to-T transition. Increasing the concentration of the precipitant (phosphate or PEG) did not improve the reversibility, in contrast to the case of the T state crystals (18).

We speculate that the precipitant displaces the hydration water from the hemoglobin molecule, which stabilizes the less hydrated T state but destabilizes the more hydrated R (or R2) state (39).

In summary, we have shown that, in crystals, both of the R and R2 structures exhibit similar noncooperative oxygen equilibrium curves with a very high affinity for oxygen, comparable with the K4 of human hemoglobin in solution. Although the oxygen affinity of the R2 structure is 2–3 times higher than that of the R structure, this difference is energetically less important considering the entire affinity change of human hemoglobin (a 500–1000-fold change in oxygen affinity) (34, 37). More importantly, because both the affinities of the R and R2 structures are high enough to saturate hemoglobin with oxygen in the lungs, the oxygen transport activity of hemoglobin is not influenced by which conformation fully liganded hemoglobin adopts. Our high resolution x-ray data show that the heme environmental structure of the R state is not so much different from that of the R2 state despite the large quaternary difference between them. Altogether, these results indicate that multiple relaxed conformations of fully liganded hemoglobin do not play a physiological role in oxygen transport, but simply reflect a structural polymorphism of a high affinity relaxed state.
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