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Human Carboxyhemoglobin at 2.2 Å Resolution: Structure and Solvent Comparisons of R-State, R2-State and T-State Hemoglobins

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Abstract

The three-dimensional structure and associated solvent of human carboxyhemoglobin at 2.2 Å resolution are compared with other R-state and T-state human hemoglobin structures. The crystal form is isomorphous with that of the 2.7 Å structure of carboxyhemoglobin reported earlier [Baldwin (1980). *J. Mol. Biol.* **136**, 103–128], whose coordinates were used as a starting model, and with the 2.2 Å structure described in an earlier report [Derewenda *et al.* (1990). *J. Mol. Biol.* **211**, 515–519]. During the course of the refinement, a natural mutation of the α-subunit, A53S, was discovered that forms a new crystal contact through a bridging water molecule. The protein structure shows a significant difference between the α and β heme geometries, with Fe—C—O angles of 125 and 162°, respectively. The carboxyhemoglobin is compared with other fully ligated R-state human hemoglobins [Baldwin (1980). *J. Mol. Biol.* **136**, 103–128; Shaanan (1983). *J. Mol. Biol.* **195**, 419–422] with the R2-state hemoglobin [Silva *et al.* (1992). *J. Biol. Chem.* **267**, 17248–17256] and with T-state deoxyhemoglobin [Fronticelli *et al.* (1994). *J. Biol. Chem.* **269**, 23965–23969]. The structure is similar to the earlier reported R-state structures, but there are differences in many side-chain conformations, the associated water structure and the presence and the position of a phosphate ion. The quaternary changes between the R-state carboxyhemoglobin and the R2-state and T-state structures are in general consistent with those reported in the earlier structures. The location of 238 water molecules and a phosphate ion in the carboxyhemoglobin structure allows the first comparison of the solvent structures of the R-state and T-state structures. Distinctive hydration patterns for each of the quaternary structures are observed, but a number of conserved water molecule binding sites are found that are independent of the conformational state of the protein.

1. Introduction

Human hemoglobin functions as an oxygen carrier, transporting oxygen from the lungs to the other tissues of the body. The tetrameric molecule found in the erythrocytes has two α- and two β-subunits, each composed of 141 and 146 amino-acid residues, respectively. A heme group that is capable of binding oxygen or other ligands is associated with each of the polypeptide chains. Binding of ligands is cooperative, such that when oxygen binds to one of the subunits, it facilitates the binding of oxygen to the other subunits. This cooperativity of hemoglobin is associated with a conformational change of the protein, or allosteric transition, from what is commonly referred to as the T-state to the R-state (T for tense and R for relaxed). The T-state is stabilized by the heterotropic ligands, H^+, Cl^-, CO_2 and 2,3-diphosphoglycerate, that lower the oxygen affinity upon binding. H^+, the most important of these ligands, is involved in what is known as the Bohr effect. In this process the uptake of protons by hemoglobin reduces its oxygen affinity and the release of protons increases its oxygen affinity.

Our understanding of the function and regulation of human hemoglobin has been enhanced by high-resolution crystallographic studies of the T-state and R-state (for reviews see Dickerson & Geis, 1983; Perutz, 1990). For human deoxyhemoglobin the T-state structure was reported at 2.5 Å resolution (Fermi, 1975) and later at 1.74 Å resolution (Fermi *et al.*, 1984). The low-resolution structure was solved by the multiple isomorphous replacement method and refined by the real-space refinement method (Diamond, 1971, 1974). These coordinates were used as the starting model in the high-resolution structure determination that employed the combined least-squares and energy-refinement method of Jack & Levitt (1978). The high-resolution structure provided the first detailed look at the heme geometry and provided the basis for comparison with ligated hemoglobins. Recently, another deoxyhemoglobin structure that has been refined using a conventional least-squares optimization of coordinates and temperature factors (Hendrickson & Konnert, 1980)

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has been reported at 2.2 Å resolution (Fronticelli *et al.*, 1994; Pechik *et al.*, 1996). Overall this structure is very similar to the earlier reported deoxyhemoglobin structure of Fermi and co-workers (Fermi *et al.*, 1984), but the two differ in their representations of the sulfate ions, the number of water molecules, and the conformations of a number of side chains, in particular those that are positively and negatively charged.

The first R-state structure of human carboxyhemoglobin at 2.7 Å resolution, 2HCO,† was solved by molecular replacement in a novel way using the 5.5 Å electron-density map of horse methemoglobin (Cullis *et al.*, 1961, 1962) as the probe, not the coordinates. Once the structure was oriented in the unit cell, map fitting and real-space refinement (Diamond, 1971, 1974) augmented by energy refinement (Levitt, 1974) were used to obtain the final coordinates. Baldwin & Chothia (1979) compared the R-state carboxyhemoglobin structure with that of the T-state deoxyhemoglobin determined at 2.5 Å resolution (Fermi, 1975) and another R-state structure, horse methemoglobin at 2.0 Å resolution (Ladner *et al.*, 1977). Their extensive analysis established that the residues at the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimer interfaces maintain their conformations, and thus the subunits in the dimers maintain their relative positions to one another. In contrast, the interface between the dimers (involving the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces) changes dramatically with a variety of specific interactions between residues being interrupted and formed during the quaternary conformational change. The comparison study also identified many subtle changes in the structure that result from the T-state to R-state transition.

Another R-state structure, human oxyhemoglobin at 2.1 Å resolution, 1HHO, was reported (Shaanan, 1983) that was virtually isomorphous with the earlier reported carboxyhemoglobin structure. In fact, the 2HCO structure with O_2 substituted for CO was the starting model in the refinement. The protein was refined using the combined X-ray least-squares positional and energy-minimization method of Jack & Levitt (1978). The structural analysis provided the first details of how oxygen interacts with the heme in the α- and β-subunits, and it identified a number of new salt bridges that stabilize the R-state. New details of the R-state conformations of the α- and β-subunit penultimate tyrosines, αY140 and βY145 and of βC93 were also observed that led to proposing a new role for these residues in the allosteric mechanism. The similarities found in the comparison of this structure with the earlier human carboxyhemoglobin (Baldwin & Chothia, 1979; Baldwin, 1980) and horse methemoglobin (Ladner *et al.*, 1977) supported proposals for the allosteric mechanism that were based on models for oxyhemoglobin constructed from these early R-state structures (Perutz, 1970; Baldwin & Chothia, 1979).

More recently, the R-state carboxyhemoglobin crystal structure has been reported at 2.2 Å resolution (Derewenda *et al.*, 1990). The carboxyhemoglobin A structure was compared with the 2.3 Å structure of carboxyhemoglobin Cowtown, a variant with His146β is replaced by leucine that is characterized by a reduced Bohr effect. The starting coordinates for the refinement of both structures were those of 2HCO (Baldwin, 1980). The structures were refined using the restrained least-squares procedure of Hendrickson & Konnert (1980). The two structures were compared along with the structure of oxyhemoglobin, 1HHO (Shaanan, 1983). The comparison of the carboxyhemoglobin A and Cowtown showed that, although the carboxyhemoglobin structures are similar to the oxyhemoglobin structure, there are subtle differences between them, especially in the ligand binding pocket.

Finally, a carboxyhemoglobin structure, 1BBB, in a unique quaternary conformation has been reported (human R2-state oxyhemoglobin, Silva *et al.*, 1992). The molecular replacement method (Rossmann, 1990) using the program package *MERLOT* (Fitzgerald, 1988) was used to solve the structure. The structure was refined using the restrained least-squares procedure of Hendrickson & Konnert (1980). The final structure revealed a novel quaternary structure designated as the R2-state. It was proposed, based on an extensive structural analysis, that the R2-state represents a crystallographically trapped intermediate in the transition between the T-state and R-states. Later modeling studies argued that the R2-state was actually the endpoint of the transition from the T-state (Srinivasan & Rose, 1994), and these studies suggested that the earlier reported R state was the 'geometric intermediate' between the T-state and R2-state. This implies that the R-state represents a trapped intermediate in the transition between the T-state and R2-state.

Reported here is the structure, including bound water molecules, of human carboxyhemoglobin (HbCO) at 2.2 Å resolution along with its comparison with other refined T-state, R-state and R2-state hemoglobins. The studies were undertaken to provide an R-state structure for comparison with the T-state and R-state structures of human hemoglobin that are chemically modified (*e.g.* Bucci *et al.*, 1996) or are variants with site-directed mutations (*e.g.* Fronticelli *et al.*, 1994; Pechik *et al.*, 1996). The redetermination of carboxyhemoglobin at 2.2 Å was carried out to obtain coordinates that were refined using a similar protocol, providing a basis for detailed comparisons with the new

† Abbreviations: HbCO, human αA53S carboxyhemoglobin; 2HCO, crystal structure of human R-state carboxyhemoglobin the determined by Baldwin (1980); 1HHO, crystal structure of human R-state oxyhemoglobin determined by Shaanan (1983); 1BBB, crystal structure of human R2-state oxyhemoglobin determined by Silva *et al.* (1992); 2HHD, crystal structure of human T-state deoxyhemoglobin determined by Fronticelli *et al.* (1994); r.m.s.d., root-mean-square deviation.

Table 1. *Comparative statistics of HbCO and deoxyhemoglobin, 2HHD R-state, carboxyhemoglobin, 2HCO; R2-state, carboxyhemoglobin, 1BBB; fully oxygenated hemoglobin, 1HHO*

	HbCO	2HCO†	1HHO‡	1BBB§	2HHD¶
Allosteric state	R	R	R	R2	T
Space group	$P4_12_12$	$P4_12_12$	$P4_12_12$	$P2_12_12_1$	$P2_1$
Cell constants					
a (Å)	54.1	53.7	53.7	97.5	62.5
b (Å)	54.1	53.7	53.7	101.5	82.1
c (Å)	195.1	193.0	193.8	61.1	53.8
β (°)	90	90	90	90	98.9
R factor (%)	15.5	31.6	22.3	18.4	13.7
R.m.s.d.					
Bond distance (Å)	0.018	–	0.02	0.012	0.016
Bond angle distance (Å)/ bond angle (°)††	0.039	–	–	2.5	0.038
Subunits/asymmetric unit	$\alpha\beta$	$\alpha\beta$	$\alpha\beta$	$\alpha_1\alpha_2\beta_1\beta_2$	$\alpha_1\alpha_2\beta_1\beta_2$
Resolution (Å)	2.2	2.7	2.1	1.7	2.2
Protein atoms	2193	2192	2192	4384	4384
Heme atoms	90	90	90	180	172
Water molecules	238	0	109	302	474
Sulfate/phosphate atoms	5 (PO_4^{-2})	0	5 (PO_4^{-2})	0	10 ($2SO_4^{-2}$)
Average B factor (Å^2)	24.1	NA	40.0	25.0	16.1
Average normalized B factor (Å^2)	24.1	NA	27.5	26.1	27.6

† Baldwin (1980). ‡ Shaanan (1983). § Silva *et al.* (1992). ¶ Fronticelli *et al.* (1994). †† The bond angle distances are not reported for 1BBB, but bond angles (°) are reported.

structures, and because the 2.7 Å coordinates of human carboxyhemoglobin (Baldwin, 1980) are currently the only ones available from the Protein Data Bank (Bernstein *et al.*, 1977). Only the comparison of the overall features and the solvent of HbCO with refined natural human hemoglobin structures in the T-state, R-state and R2-state whose coordinates (2HCO, 1HHO and 1BBB) have been deposited in the Protein Data Bank (Bernstein *et al.*, 1977) are provided here (only those hemoglobins that are fully deoxy or fully ligated). A complete analysis of the ligand binding and its comparison with other ligated hemoglobins and myoglobins are reported elsewhere (Vásquez, Ji, Fronticelli & Gilliland, in preparation).

2. Materials and methods

2.1. *Protein source, purification and ligation procedures*

Human hemoglobin was prepared from outdated blood samples obtained from the University of Maryland Blood Bank. The red cells were washed several times with 0.09% NaCl, and hemolyzed in 0.005 *M* phosphate buffer at pH 7.0, and the stroma eliminated by treatment of the solution with 5 g dl^{-1} chloroform. The oxyhemoglobin was purified by high-pressure liquid chromatography (HPLC) (DELTAPREP, Millipore)† using an MCI-DEAE (Mitsubishi) resin. The elution gradient was obtained using 0.015 *M* Tris–acetate at pH 8.4 and 0.01 *M* Tris–acetate with 0.2 *M* sodium acetate at pH 7.2. The purity of the sample was confirmed by Paragon electrophoresis (Beckman) and by reverse-phase HPLC (P2000, Thermo Separation Products) with a Vydak C4 column using a gradient made by mixing 0.1% trifluoroacetic acid in 20% acetonitrile and 0.1% trifluoroacetic acid in 60% acetonitrile. The purified oxyhemoglobin was concentrated using Centripep 20 (Amicon) and dialyzed against several changes of deionized water. 2,3-Diphosphoglycerate was removed by recycling the hemoglobin on a column of Dowex MR-3 (Sigma). Hemoglobin was equilibrated with CO at atmospheric pressure. The amount of methemoglobin present, as determined by deconvolution of absorption spectra, was less than 1%.

2.2. *Protein crystallization and X-ray data collection and processing*

The crystallization of carboxyhemoglobin used an adaptation of the protocol described by Perutz (1968). Crystals were grown using the batch method from solutions containing ~2.5 *M* sodium/potassium phosphate, pH 6.7 and 1%(*w*/*v*) carboxyhemoglobin. Crystals were mounted and sealed in thin-walled glass capillaries in a glove box under nitrogen to minimize exposure to oxygen. The crystals are tetragonal with space group $P4_12_12$ containing four tetramers in the unit cell. The procedures for X-ray data collection and processing are similar to those described by Fronticelli *et al.* (1994). Diffraction data from four orientations of a single crystal of dimensions 0.25 × 0.25 × 0.25 mm were

† Certain commercial equipment, instruments, and materials are identified in this paper in order to specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified are necessarily the best available for the purpose.

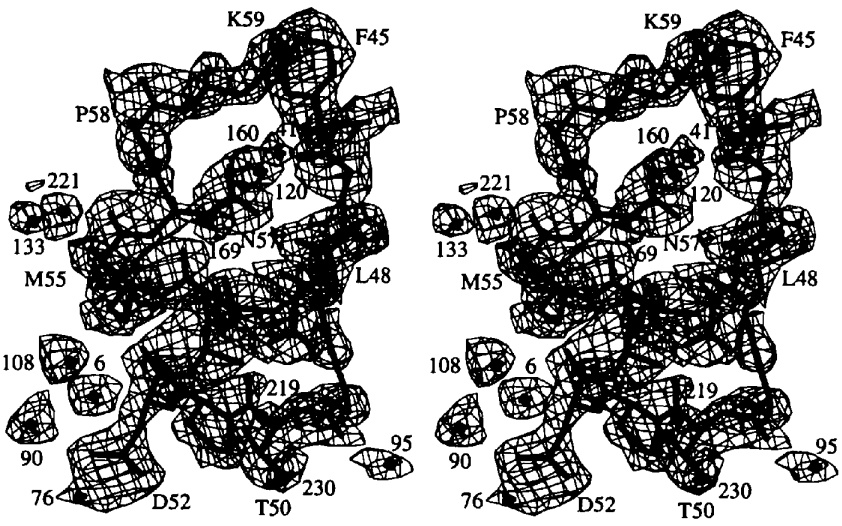


Fig. 1. The EF corner of the α-subunit of HbCO showing the $2F_o - F_c$ electron-density map contoured at 1.0σ.

collected. The X-ray source was a Rigaku RU-200B rotating anode operated at 40 kV and 90 mA with a graphite crystal monochromator and 0.3 mm collimator. The data were collected at a 2θ angle of 25°, with an ω scan of 0.25° and a 450 s exposure per frame using a Siemens electronic area detector. The *XENGEN* program system (Howard *et al.*, 1987) was used to determine the unit-cell parameters and for data processing. The crystals are isomorphous with those reported by Baldwin (1980). The crystal unit-cell parameters are given in Table 1. A total of 59 019 observations of 12 412 reflections were collected. The R_{sym}† is 0.11 with 10 496 reflections having $I > 1.0\ \sigma(I)$.

2.3. *Crystal structure and refinement of HbCO*

The map fitting and refinement protocol of carboxyhemoglobin, HbCO, were similar to that used for human deoxyhemoglobin, 2HHD (Fronticelli *et al.*, 1994). The starting coordinates for the HbCO structure were those of carboxyhemoglobin, 1HCO (Baldwin, 1980). The initial calculations were carried out using the *X-PLOR* 3.1 program package (Brünger, 1992). First, because of the slight differences between the unit-cell parameters of the HbCO and 1HCO, the position of the $\alpha\beta$ dimer was adjusted by a rigid-body refinement followed by rigid-body refinement of the individual α- and β-subunits. Next, simulated annealing was performed with a slow-cooling protocol (Brünger *et al.*, 1990). The force fields used were based on the stereochemistry dictionary compiled by Engh & Huber (1991). Empirical energy parameters for the water molecules were taken from TIP3p model of the program *CHARMM* (Brooks *et al.*, 1983). The full charges of Asp, Glu, Arg and Lys were turned off during both dynamics and conventional minimization. Prior to dynamics, the structure was minimized with 100 cycles of conjugate-gradient minimization to relieve bad contacts. The minimized system was then heated to 4000 K by 5 ps dynamics using velocity scaling. It was then cooled to 300 K in 25 K temperature decrements every 50 steps. The time step for molecular dynamics integration was set to 0.5 fs. Following dynamics, 150 cycles of conjugate-gradient minimization were performed to optimize the geometry and stereochemistry of the coordinates.

Further refinement for HbCO was carried out on a CRAY Y-MP computer with *GPRLSA* (Furey *et al.*, 1982), a restrained least-squares refinement procedure (Hendrickson & Konnert, 1980), with periodic adjustments of the coordinates using *FRODO* (Jones, 1978) or *O* (Jones *et al.*, 1991). 15 rounds of refinement followed by manual coordinate adjustment were performed to eliminate all difference peaks in the $F_o - F_c$ map above 3.0σ and below -3.0σ and to account for all of the $2F_o - F_c$ electron density. All water molecules, the phosphate ion, the mutated residue (α53) and CO ligand positions were verified by omit maps (Bhat, 1988). The omit maps were calculated after six to ten cycles of least-squares refinement without the omitted atoms. The geometry of the final coordinates was examined using the *PROCHECK* suite of programs (Laskowski *et al.*, 1993).

2.4. *Protein structure comparisons*

HbCO was compared with the structures of R-state, carboxyhemoglobin, 2HCO (Baldwin, 1980), and oxyhemoglobin, 1HHO (Shaanan, 1983), of R2-state, carboxyhemoglobin, 1BBB (Silva *et al.*, 1992), and of T-state, deoxyhemoglobin, 2HHD (Fronticelli *et al.*, 1994). The comparisons used the program *ALIGN* (Satow *et*

† $R_{sym} = \sum(I_{ij} - G_{ij}\langle I\rangle_j) / \sum|I_{ij}|$, where $G_{ij} = g_i + A_i s_j + B_i s_j^2$; $s = \sin\theta/\lambda$; g, A and B are scaling parameters.

al., 1986) to superpose the structures. The structures were visualized, and distances and angles measured using the *O* (Jones *et al.*, 1991) or the *TURBO-FRODO* (Roussel & Cambillau, 1992) program packages. Using this software, crystal symmetry operators were applied to generate coordinates of complete tetramers for those structures with dimers in their asymmetric units, HbCO 2HCO and 1HHO.

2.5. *Water structure comparisons*

The comparison of solvent structures of the R-state, R2-state and T-state hemoglobins was restricted to the HbCO, 1BBB and 2HHD structures, respectively, because all are refined using the same restrained least-squares method (Hendrickson & Konnert). The water structures of HbCO, 1BBB and 2HHD were compared in the following way. First, for each structure symmetry molecules (including water molecules) were generated, and a coordinate file was created containing the original structure and the symmetry related ones. These calculations were carried out using *TURBO-FRODO* (Roussel & Cambillau, 1992). Distance calculations were then used to exclude residues and water molecules from symmetry-related structures that were greater than 5.0 Å from the reference structure. The expanded 1BBB and 2HHD coordinate sets were then transformed into the HbCO reference frame for the solvent comparison. (The expansion of the 1BBB coordinates results in a number of close contacts that indicate symmetry constraints were not included in the refinement and that make it difficult to assign conserved water molecules in a few cases.) Water molecules were considered to be conserved if they met two criteria. First, corresponding solvent positions are required to be within 1.05 Å of one another, and second, similar hydrogen-bonding patterns must be observed for the corresponding solvent positions. In the comparison, if a water molecule in one of the structure corresponds to the position of a polar atom (N, O or S) of a symmetry-related molecule and has a similar hydrogen-bonding pattern, then it was also considered to be a conserved site.

3. Results

3.1. *Structure of HbCO*

The HbCO structure is composed of all 141 and 146 residues of the α- and β-subunits, respectively, their associated porphyrins, Fe and CO ligand atoms. The protein dimer is associated with 238 water molecules and one phosphate ion. During the structure refinement, a natural α-chain mutation, αA53S, was discovered, and a serine residue replaces alanine in the final structure. The HbCO coordinate set agrees well with the diffraction data having a crystallographic R factor for data between 6.0 and 2.2 Å resolution of 0.155. The geometry and stereochemistry of the coordinates is similar to that of other well determined structures as indicated by the root-mean-square deviations (r.m.s.d.) in bond and angle distances of 0.018 and 0.039 Å, respectively. The average temperature-factor value for all atoms of HbCO is 24 Å^2, with an average temperature factor of all protein atoms of 23 and 30 Å^2 for the 238 water molecules.

The overall quality of the final $2F_o - F_c$ electron-density map is illustrated in Fig. 1 for the EF corner of the α-subunit. In general the electron-density map is quite good, but a few regions, where the electron density is weak, are observed. These regions include the N-terminal αVal1, the final two C-terminal residues of the α-chain and the N-terminal residue of the β-chain. The interpretation of the electron-density map has resulted in a final model that has a number of differences in side-chain conformations from the starting coordinates (1HCO). These differences are found primarily for surface residues. Several of the changes are described below in the comparison of HbCO and 2HCO. One particularly interesting difference is the interaction between βH146 and βH143. In HbCO an on-face hydrogen bond is found between βH146 N_ε and

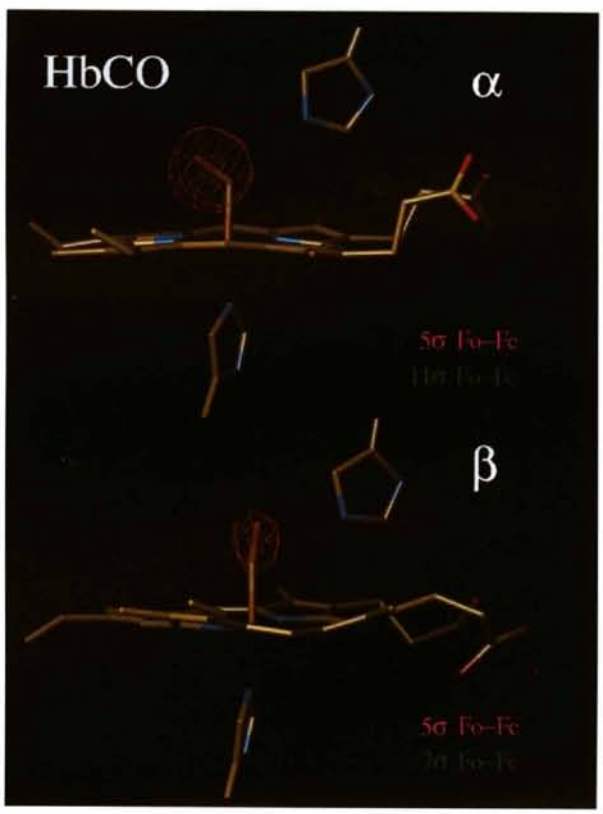


Fig. 2. The $F_o - F_c$ electron-density omit maps of the HbCO carbonmonoxide ligands. The $F_o - F_c$ omit map (*a*) contoured at 5σ (magenta) and 11σ (green) for the α-heme region, and (*b*) contoured at 5σ (magenta) and 7σ (green) for the β-heme region.

Table 2. *Comparison of HbCO with 2HCO, 1BBB, 1HHO and 2HHD*

The r.m.s.d. (Å) is calculated for corresponding C_α pairs used in each comparison. The number of C_α pairs used in each calculation is shown in parentheses.

Alignment unit	2HCO	1HHO	1BBB	2HHD
α_1	0.33 (139)	0.33 (139)	0.41 (136)	0.55 (138)
β_1	0.44 (142)	0.39 (144)	0.37 (145)	0.83 (145)
α_2	—	—	0.45 (136)	0.47 (139)
β_2	—	—	0.36 (142)	0.78 (145)
$\alpha_1 b_1$	0.39 (280)	0.37 (283)	0.44 (279)	0.85 (283)
$\alpha_2\beta_2$	—	—	0.50 (283)	0.71 (277)
$\alpha_1\alpha_2\beta_1\beta_2$	0.41 (556)	0.43 (566)	1.58 (560)	2.04 (531)

References: deoxyhemoglobin, 2HHD (Fronticelli *et al.*, 1994); R-state, carboxyhemoglobin, 2HCO (Baldwin, 1980); R2-state, carboxyhemoglobin, 1BBB (Silva *et al.*, 1992); fully oxygenated hemoglobin, 1HHO (Shaanan, 1983).

the π cloud of the βH143 imidazole ring. This interaction is not observed in the previously reported carboxyhemoglobin structures.

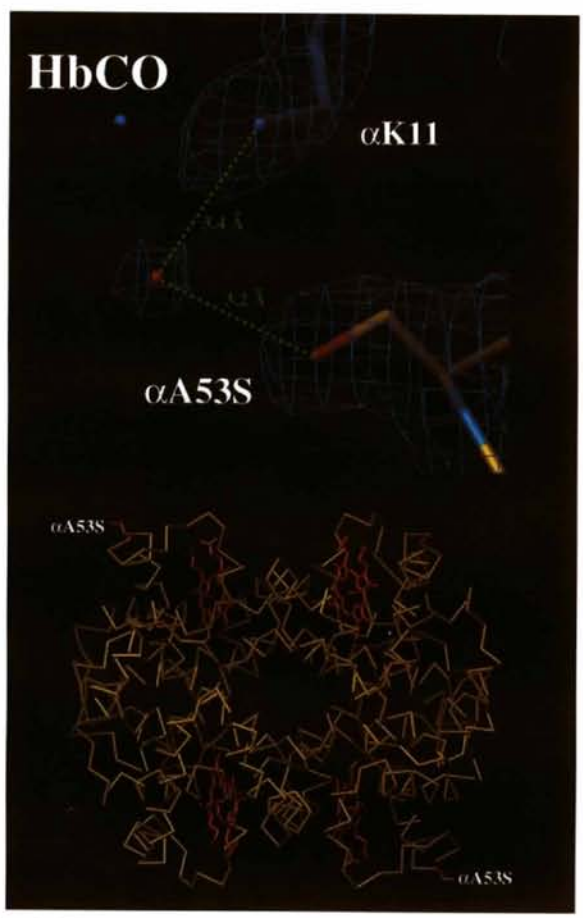


Fig. 3. The natural mutation αA53S of HbCO is shown (top), displaying the water bridging between αSer53 and αLys11 of the symmetry-related tetramer. The $2F_o - F_c$ electron-density map is contoured at 1.0σ. The locations of the mutation sites in the C_α trace of tetramer are shown in magenta (bottom).

Both the α- and β-chain hemes and associated ligands are well defined by the electron-density map, thus the geometry of the ligand–heme interactions can be determined. The geometry of the ligands and a comparison with other ligated hemoglobins and myoglobins are presented elsewhere (Vásquez, Ji, Fronticelli & Gilliland, in preparation) thus, only a brief description is reported here. The C—O bond distance is 1.2 Å, and the Fe—C—O angle for the α-subunit, heme is 125°. The β-subunit C—O bond distance is 1.2 Å, and the Fe—C—O angle is 162°. The orientation of the ligands with respect to the α- and β-hemes is shown in Fig. 2. The temperature factors of the ligands are relatively low (18.0 $Å^2$ for carbon and 17.4 $Å^2$ for oxygen in the α-subunit, and 25.5 and 26.6 $Å^2$ in the β-subunit, respectively) and consistent with the temperature factors of the adjacent atoms in their respective hemes.

The tetragonal crystals of HbCO have a solvent content of 45.0% corresponding to a V_m of 2.23 $Å^3\ Da^{-1}$. In this R-state structure, 476 water molecules are found associated with the tetramer, since 238 water molecules are associated with the dimer in the asymmetric unit. (The numbers below indicate the waters associated with the tetramer or two asymmetric units.) Most of the water molecules are located on the outer surface of the tetramer, but a significant number line the central cavity. At the $\alpha_1\beta_1/\alpha_2\beta_2$ interface 22 water molecules bridge the two dimers. There are 12 water molecules involved in contacts with other crystallographically related tetramers. More than half of the observed water molecules form hydrogen bonds with the protein, and 200 outer-shell water molecules form hydrogen bonds with other waters that in turn form hydrogen bonds with protein atoms or other water molecules.

3.2. *The alpha A53S mutation*

From the beginning of the structure determination of HbCO, the $2F_o - F_c$ electron-density maps revealed unaccounted-for electron density associated with the αA53 C_β atom, and the $F_o - F_c$ electron-density maps also had a positive peak greater than 3σ centered at the same position. The alanine was replaced with a serine that behaved well in the subsequent refinement. This residue is located on the solvent-exposed N-terminal end of the *E* helix (Fig. 3). Nearby, at 3.2 Å, is a water molecule that bridges the serine O_γ atom and the N_ζ atom of αK11 in a symmetry-related tetramer 3.4 Å away. The temperature factors of the serine C_α, C_β and O_γ atoms are 26.7, 28.0 and 31.5 $Å^2$, respectively, values that are similar to those observed for other surface-residue atoms. The nearby water molecule has a fractional occupancy of 0.35, and the temperature factor, 43.8 $Å^2$, is within the range of values observed for other water molecules, but it is higher than that of the average value reported above.

Table 3. *Comparison of the diagnostic hydrogen bonds and salt bridges*

R-state carbonmonoxy- and oxyhemoglobins, HbCO (this work), 2HCO (Baldwin, 1980), and oxyhemoglobin, 1HHO (Shaanan *et al.*, 1983), 1BBB (Silva *et al.*, 1992), and deoxyhemoglobin, 2HHD (Fronticelli *et al.*, 1994).

	Distances (Å)					
Hydrogen bond/salt bridge	HbCO	2HCO	1HHO	1BBB	2HHD	Allosteric
Allosteric state	R	R	R	R2	T	state
β_1K144–β_1H146(COO$^-$)	7.4	4.2	3.1	7.0	12.4	R
β_2K144–β_2H146(COO$^-$)	7.4	4.2	3.1	6.5	12.0	R
α_1D94–β_2N102	3.2	2.9†	2.8†	2.8	5.9	R/R2
α_2D94–β_1N102	3.2	2.9†	2.9†	2.8	5.5	R/R2
α_1S84–α_1S138	7.3	6.2	4.2	2.6	4.3	R2
α_2S84–α_2S138	7.3	6.2	4.2	3.8	4.8	R2
α_1P77–α_1Y140	15.6	15.4	15.4	2.7	13.9	R2
α_2P77–α_2Y140	15.6	15.4	15.4	2.9	13.9	R2
α_1S84–α_1Y140	10.6	8.8	10.3	4.0	9.0	R2
α_2S84–α_2Y140	10.6	8.8	10.3	2.9	9.4	R2
α_1N78–α_1R141	19.0	18.9	19.2	2.8	22.8	R2
α_2N78–α_2R141	19.0	18.9	19.2	2.9	23.4	R2
β_1K82–β_2H146(COO$^-$)	6.2	7.8	7.9	3.1	22.2	R2
β_2K82–β_1H146(COO$^-$)	6.2	7.8	7.9	7.8	21.4	Absent
α_1K127–α_2R141(COO$^-$)	4.9	3.4	2.4	7.1	2.9	R/T
α_2K127–α_1R141(COO$^-$)	4.9	3.4	2.4	7.8	3.2	R/T
α_1D126–α_2R141	11.3	8.4	7.8	20.3	2.8	T
α_2D126–α_1R141	11.3	8.4	7.8	20.6	2.7	T
α_1Y42–β_2D99	8.0	8.5	8.5	9.4	2.7	T
α_2Y42–β_1D99	8.0	8.5	8.5	9.4	2.7	T
α_1K40–β_2H146(COO$^-$)	16.1	14.4	13.2	20.3	2.7	T
α_2K40–β_1H146(COO$^-$)	16.1	14.4	13.2	20.7	2.5	T
β_1D94–β_1H146	11.2	10.0	11.0	12.1	2.8	T
β_2D94–β_2H146	11.2	10.0	11.0	11.7	2.7	T

† Amide of N102 is oriented improperly, resulting in the hydrogen bond being between the carboxylate O atom and the amide O atom.

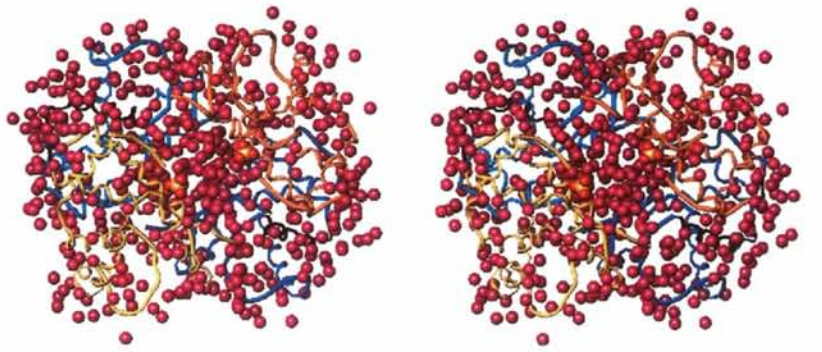
3.3. *Comparison of HbCO with the R-state 2HCO and 1HHO*

In the comparisons of the isomorphous R-state structures of HbCO and 2HCO (Baldwin, 1980), and of HbCO and 1HHO (Shaanan, 1983), the r.m.s.d. of equivalent C_α's in both cases is less than 0.4 Å for the intact dimer, and for their α- and β-subunits (Table 3). Slightly larger r.m.s.d. values are observed for the complete tetramers (Table 2) perhaps resulting from the slight differences in the unit-cell parameters (Table 1). The largest differences in C_α positions (~1.8 Å) in the comparisons of HbCO with 2HCO and 1HHO are for the N- and C-terminal residues of both α- and β-subunits. Another significant difference between HbCO and 1HHO is the movement of the βHis77 main-chain atoms by ~1.4 Å due to a conformational difference in a turn of the EF loop region.

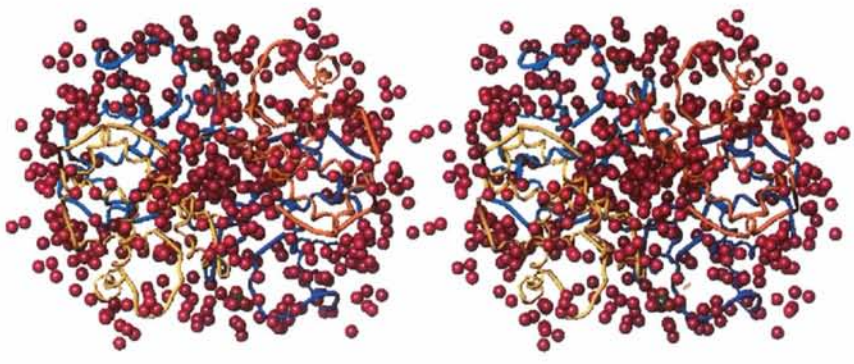
The T-state, R-state and R2-state are each characterized by the diagnostic hydrogen bonds and salt bridges listed in Table 3 (Baldwin & Chothia, 1979; Shanaan, 1983; Silva *et al.*, 1992). From the table it is evident that HbCO, 2HCO and 1HHO do have similarities, but there are also a number of differences. All three structures have the hydrogen bond between the side chains of βN102 and symmetry-related αD94, but only 1HHO has the salt bridge between the side chain of βK144 and the C-terminal carboxylate of βH146, raising the question as to whether it should be considered diagnostic of the R-state (Perutz, 1990). Another difference in the electrostatic interactions of the R-state structures is salt bridge between the side chain of αK127 and the symmetry-related C-terminus of αR141 found in 2HCO and 1HHO, but which is not found (or at least not as strong an interaction) in HbCO, since the residues are 4.9 Å apart (Table 3). This is because in HbCO, αK127 is making a salt bridge with αD6 and has rotated away from the carboxylate group of αR141, and the carboxylate group of αR141 is hydrogen bonded to the backbone carbonyl group of αY140.

3.4. *Comparison of HbCO with the R2-State 1BBB*

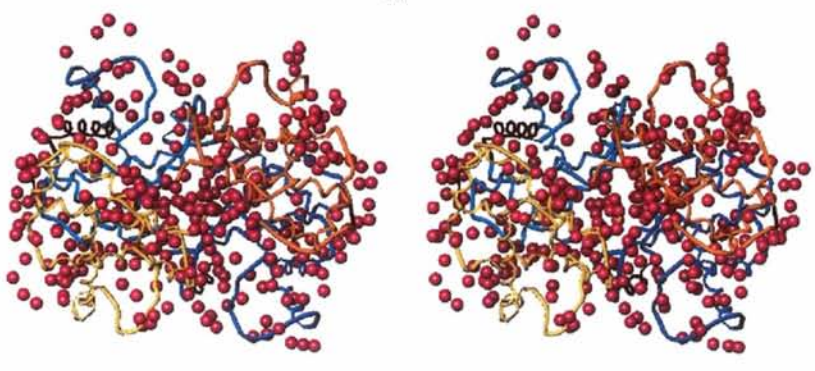
The comparison of R-state HbCO and R2-state 1BBB (Silva *et al.*, 1992) show significant differences in the structures (see Table 2). The r.m.s.d.'s of the C_α's of the individual α- and β-subunits, and the $\alpha_1\beta_1$- and $\alpha_2\beta_2$-dimers are less than 0.5 Å, but the r.m.s.d. of the C_α's of the intact tetramers increases to 1.6 Å, reflecting the change in quaternary structure of the tetramer. The individual residues of the β-subunits and most of the α-subunit residues of 1BBB have less than 2.0 Å C_α r.m.s.d.'s when compared with those of HbCO. The largest shifts of atomic positions between residues in each subunit of 1BBB and those of HbCO are at the N- and C-termini. The largest movement is observed for



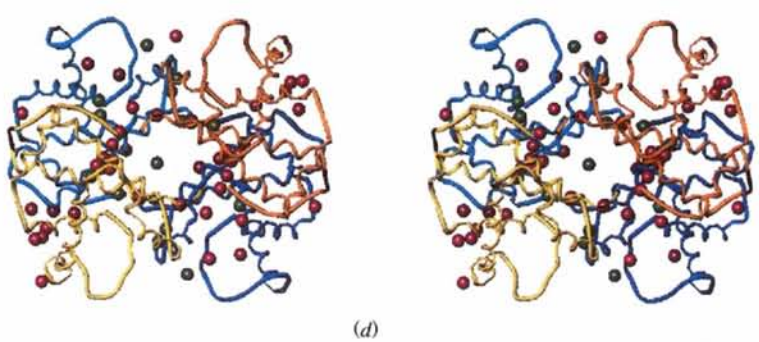
(*a*)



(*b*)



(*c*)



(*d*)

Fig. 4. A comparison of the solvent structures of T-, R- and R2-states of human hemoglobin. In each of the stereoplots the α_1 subunit is shown as cyan (upper left), the α_2 subunit is shown as blue (lower right), the β_1-subunit is yellow–green (lower left), and the β_2-subunit is shown as orange (upper right). The panels show the complete water structure associated with (*a*) the T-state deoxyhemoglobin tetramer 2HHD (Fronticelli *et al.*, 1994), (*b*) the R-state carboxyhemoglobin, 2HCO (Baldwin, 1980), and (*c*) the R2-state carboxyhemoglobin, 1BBB (Silva *et al.*, 1992). In (*d*) the conserved solvent positions of all three states are shown associated with the R-state HbCO structure. Conserved water molecules bridging two subunits are shown in green, and those in general positions are shown in magenta.

Table 4. *Conserved water molecules of the R-state and R2-state carboxyhemoglobins, HbCO and 1BBB and T-state deoxyhemoglobin, 2HHD*

	HbCO†	1BBB†	2HHD‡
R-R2-T state solvent	42	34	29
Bound to protein	38	31	26
Not bound to protein	4	3	3
Bridges subunits	8	16	11
Residues replacing water	0	1	2
R-R2-state solvent (Not R-R2-T)	16	16	–
Bound to protein	16	16	–
Not bound to protein	0	0	–
Bridges subunits	6	0	–
R-T-state solvent (Not R-R2-T)	2	–	2
Bound to protein	2	–	2
Not bound to protein	0	–	0
Bridges subunits	0	–	0

† Silva *et al.* (1992). ‡ Fronticelli *et al.* (1994).

the last two C-terminal residues of the α-subunits, the C_α r.m.s.d.'s range from 8.0 to 11.5 Å for residues, due to a repositioning of the C-termini. HbCO does not share any of the diagnostic hydrogen bonds reported for the R2-state 1BBB structure (Silva *et al.*, 1992), such as the intrachain αPro77$\cdots\alpha$Tyr140 and the αAsn78$\cdots\alpha$Arg141 hydrogen bonds, found in both 1BBB α-subunits (Table 3).

3.5. *Comparison HbCO with the T-state 2HHD*

The tertiary structures of the α- and β-subunits of the R-state HbCO and the T-state 2HHD are quite similar as indicated by C_α r.m.s.d. values of less than 0.8 Å (Table 2). The largest differences in atomic positions are at the C-termini of both α- and β-subunits, with the terminal residues having the largest change. The C_α r.m.s.d. values from the comparisons of the HbCO $\alpha\beta$ dimer with the 2HHD $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers are smaller than 0.9 Å. In contrast, the comparison of the intact tetramers results in differences that are more than twofold greater, an r.m.s.d. value of 2.04 Å for corresponding C_α positions, indicating that the relationship between the two $\alpha\beta$ dimers is the source of the greatest differences between the two structures. In addition, all of the salt-bridge and hydrogen-bond differences previously described between the R-state and T-states are evident (see Table 3).

3.6. *The HbCO phosphate-binding site*

Two phosphate ions, the one in the crystallographic asymmetric unit and a symmetry-related one, are tightly bound to the protein at the interface between α_1- and β_2-subunits and the symmetry-related α_2- and β_1-subunits (Fig. 4, top). Each of the phosphate groups has three of its O atoms involved in hydrogen bonds. At one site O2 is 2.4 Å from β_1Arg40 $N_{\eta 2}$, O3 is 2.8 Å from α_2Tyr42 O and β_1Arg40 $N_{\eta 2}$, and O4 is 2.4 Å from α_2His45 $N_{\varepsilon 2}$ and 2.6 Å from α_2Tyr42 O. At the symmetry-related site residues from the β_2- and α_1-subunits replace those from the β_1- and α_2-subunits, respectively. These distances are short implying the formation of strong hydrogen bonds. This phosphate binding site has not been reported in either of the earlier R-state structures, 2HCO and 1HHO. However, a phosphate was found bound in the central cavity of the 1HHO tetramer (Shaanan, 1983) near the T-state, diphosphoglycerate binding site (Arnone, 1972). This site lies on a crystallographic twofold axis. No phosphate is observed at this site in HbCO, but two symmetry-related waters are found at the phosphate location. Extraneous electron density does lie between the water molecules in HbCO, but it cannot accommodate a phosphate ion.

3.7. *Comparison of the HbCO, 1BBB and 2HHD water structures*

Analysis of the solvent structures of HbCO, 1BBB and 2HHD shows that many water molecules are conserved; that is, they have the same relative location with respect to the protein atoms, despite the quaternary state of the protein (Table 4). The crystallographically determined water molecules for R-state, R2-state and T-state hemoglobins and the conserved water molecules (those common to all three structures) are shown in Fig. 4. Many of the conserved water molecules are primarily bound directly to the subunits at the $\alpha_1\beta_1/\alpha_2\beta_2$ dimer interface. A number of the conserved water molecules are located within the cavity of the core of the tetramer where their location is constrained by the surrounding protein. Most of the waters within this cavity are bridging the $\alpha_1\beta_1$- and $\alpha_2\beta_2$ dimers either directly or indirectly through extensive solvent–solvent hydrogen bonds. The water molecules around the α-heme assist in anchoring the heme to the protein, either by bridging the heme carboxylates to the protein, or by hydrogen bonding to surrounding residues and providing a potential van der Waals barrier. It should be noted that the alignments of the β-subunits were not as good as those of the α-subunits for establishing a positional reference frame for solvent due to larger conformational changes in the β-subunits resulting from the allosteric transition. Therefore, fewer solvent molecules associated with the β-subunits meet the criteria for a conserved water molecule.

4. Discussion

4.1. *HbCO structure and comparison with low-resolution carboxyhemoglobin*

The HbCO structure provides new information on R-state hemoglobin including the first details of the solvent structure. The overall conformations of HbCO and 2HCO (and 1HHO) are, as expected, quite similar,

but there are differences in the side-chain conformations and the interaction of ligand with the heme. These differences can be attributed to the difference in the resolution of the diffraction data and to the difference in refinement methods, the restrained least-squares procedure of Hendrickson & Konnert (1980) for HbCO and the method of real-space refinement (Diamond, 1971, 1974) augmented by energy refinement (Levitt, 1974) for 2HCO.

The most obvious global differences between HbCO and 2HCO are in the side-chain positions. These include differences in the diagnostic hydrogen bonds and salt bridges summarized in Table 3. Two of the salt bridges found in 2HCO, one between α_2K127 and α_1R141(COO^-) and the other between βK144 and βH146, are absent in HbCO (see Table 4). Another interesting side-chain conformation difference is the orientation of βH146 and βH143. The on-face hydrogen bond of βH146 N_ε to the π cloud of the βH143 imidazole ring of HbCO is likely to be the result of crystal packing that might stabilize the highly mobile C-terminus, which might not be the case for the solution state of the molecule. Slight rotations of the χ angles of these residues allow the interconversion between orientations observed in the two structures. This arrangement is usually found between hydrogen-bond donors and the aromatic rings of tyrosine or phenylalanine (Burley & Petsko, 1986; Levitt & Perutz, 1988), and has not yet been reported for histidines. The overall conformation of HbCO is very similar (0.4 Å r.m.s.d.; Table 2) to that of 2HCO (Baldwin, 1980), and the side-chain differences appear to reflect the differences in the resolution and possibly minor differences in the crystallization conditions.

A major difference between HbCO and previous carboxyhemoglobin structures is in the heme pockets, specifically the ligand binding geometry. The Fe—C—O angle of 125° in the α-subunit and 162° in the β-subunit is a dramatic difference from the 180° Fe—C—O angle for both subunits of 2HCO. The Fe—C—O angle was restrained to be linear in the refinement of 2HCO, resulting in significant tilts (14 and 12° tilts for the α- and β-subunits, respectively) of the ligands with respect to the heme plane normals (Baldwin, 1980). The Fe—C—O angle was not restrained during the refinement of HbCO. This resulted in a significant decrease of the Fe—C—O angle in the smaller α-heme pocket, while allowing the ligand in the larger β-heme pocket to assume a more linear conformation. The differences between the close contacts of the distal histidines with the CO ligands and in histidine protonation of the α- *versus* the β-subunits are possible causes for the magnitude of the ligand angle variation observed in the HbCO. A comprehensive comparison of CO ligation of HbCO and other ligated hemoglobins and myoglobins is presented elsewhere (Vásquez, Ji, Fronticelli & Gilliland, in preparation).

4.2. *αA53S mutation*

The interpretation of the HbCO electron-density map included replacement of αA53 with a serine. The residue difference reflects a natural mutation since the source of this hemoglobin was obtained from outdated blood from the University of Maryland Blood Bank. Revision of amino-acid sequences by crystallography, in hemoglobins in particular, has been reported by Ladner *et al.* (1976) with their correction to the horse hemoglobin amino-acid sequence. The presence of this mutation on the surface of the α-subunit appears to cause no observable perturbation on the fold of the protein in either the quaternary association or in local secondary structure (Fig. 3). The presence of the αS53 O_γ atom allows the formation of a new interaction with a symmetry-related dimer through a bridging water with the N_ζ atom of αK11 N_ζ. Thus, the presence of this natural mutation that introduces a stabilizing crystal contact may have contributed to the higher resolution diffraction data.

4.3. *Comparison of HbCO with R-state, R2-state and T-state hemoglobins*

The comparison of HbCO with other fully ligated R-state human hemoglobins 2HCO and 1HHO, with the R2-state hemoglobin 1BBB and with T-state deoxyhemoglobin 2HHD, indicate that HbCO is equivalent to the earlier reported R-state structures and that the quaternary changes between the R-state carboxyhemoglobin and the R2-state and T-state structures are in general consistent with those reported in the earlier structures. These results were expected based on the isomorphism of the HbCO, 2HCO and 1HHO crystal forms. There are no gross differences between the HbCO and the two R-state structures, 2HCO and 1HHO, but significant differences in side-chain orientations and the heme-pocket geometries are found along with differences in the location of the phosphate binding sites of HbCO and 1HHO.

The general overall differences between HbCO and 1BBB result from the orientation of the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers with respect to each other (Table 2). A large difference is seen in the C-termini of the 1BBB α-chains, where two hydrogen bonds form when the ultimate and penultimate residues, αArg141 and αTyr140, loop back to interact with the residues αAsn78 and αPro77, respectively (Table 4), as previously reported by Silva *et al.* (1992).

HbCO shows both the expected tertiary and quaternary differences from 2HHD. The quaternary differences also involve the orientation of the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers with respect to each other. The 2HHD structure has two sulfates associated with the tetramer and these sulfates bind in different positions from either

of the two R-state phosphate-binding positions of HbCO and 1HHO.

4.4. *Hemoglobin water structures*

The comparison of the solvent structures of HbCO, 1BBB and 2HHD clearly indicate distinctive hydration patterns for each of the quaternary states, but the comparison also reveals a number of conserved waters sites between the different allosteric states (see Fig. 4 and Table 4). Overall, there is more solvent conservation between the R-state HbCO structure and the R2-state 1BBB structure than either of these structures to the T-state 2HHD structure. This is consistent with the C_α alignments of the structures (Table 2). The concentration of the conserved waters at the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ interfaces, particularly in the central cavity of the tetramer (Fig. 4), is consistant with the fact that the $\alpha\beta$ dimers are relatively stable, and that the allosteric changes primarily occur at the dimer–dimer interface. The waters in the central cavity are constrained by the surrounding protein, limiting the available positions and orientations for them to occupy. This would also explain the observation of conserved waters in the cavity that are not bound to the protein directly, but rather through solvent-mediated interactions. A conserved water is found that has a hydrogen bond to the protein backbone near the α heme. This water could provide a van der Waals barrier that helps maintain the heme in the heme pocket. Other waters anchor the hemes *via* their carboxylate groups to the protein subunits. The function of these waters appears to be similar; however, these are not considered to be positionally conserved since variations in the positions of the carboxylates occur in the different structures.

5. Conclusions

The HbCO R-state carboxyhemoglobin structure has many changes in side-chain conformations and in the ligand heme geometry from those of the starting model. The structure also provides the first details of the bound solvent associated with the R-state. The comparison of HbCO with two other R-state structures, 2HCO and 1HHO, indicates discrepancies in side-chain conformations and ligand orientation. In addition, the comparison of HbCO with the 1BBB and 2HHD, R2-state and T-state human hemoglobins, respectively, find variance in important details of the structures not found in earlier comparisons. Certainly many of the observed differences result from the changes in the allosteric state of the structure, but contributing factors such as the experimental conditions for crystal growth, the method of X-ray data acquisition, the presence of bound ligand, and the refinement methods cannot be ruled out. Thus, further studies that minimize experimental and computational differences are required to further establish the changes that hemoglobin undergoes during its allosteric transition.

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