A New Relaxed State in Horse Methemoglobin Characterized by Crystallographic Studies

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ABSTRACT **A new relaxed state has been characterized in the crystals of horse methemoglobin grown at neutral pH at low ionic concentration and their low humidity variants. The crystals provide an example for improvement in X-ray diffraction quality with reduced solvent content. Only the classical R state has been so far observed in liganded horse hemoglobin. The state characterized in the present study lies in between the R state and the R2 state characterized earlier in liganded human hemoglobin. The results presented here, along with those of earlier studies, suggest that relaxed and tense hemoglobin can access ensembles of states. Proteins 2005; 60:547–551.** © 2005 Wiley-Liss, Inc.

Key words: **hemoglobin; X-ray crystallography; quaternary association; protein hydration; intersubunit mobility; allosteric transition**

INTRODUCTION

Hemoglobin, along with myoglobin, is the first protein to be structure analyzed using X-ray crystallography. Thanks particularly to the monumental work of Perutz,[1] it is also among the most thoroughly studied proteins. It has been the best example and model for allosteric transitions in proteins. In conformity with the suggestions of Monod, Wyman, and Chengeux,[2] tetrameric hemoglobin exists in two multimeric states, namely, the relaxed liganded R state and the tense unliganded T state. The metal ion in heme group is ferrous in both the states. The nonfunctional methemoglobin with a ferric ion instead of a ferrous ion in the heme also exists in the R state. Except for differences in the immediate vicinity of the heme group, particularly the metal ion, the molecule in both the states has nearly the same tertiary structure. The difference between the two states is primarily in the quaternary association.

Although most of the original definitive results were obtained using horse hemoglobin, the protein from other sources also received considerable attention.[3] In particular, much of the work in recent years has been focused on human hemoglobin.[4–9] An interesting result to emanate from studies on the human protein was the identification of a new relaxed state, designated R2,[10] which has quaternary structure substantially different from that of the protein in the R state.[4] The R2 state was observed in crystals grown in a medium with lower ion concentration as well as pH than the medium from which the crystals

containing R-state hemoglobin were grown. Subsequently, we analyzed crystals containing three crystallographically independent tetrameric molecules, of human methemoglobin from a medium with comparatively low ionic concentration, but high pH.[11] The three molecules in these crystals have quaternary structures intermediate between those of R and R2 states. Thus it appeared that hemoglobin can access different relaxed states with varying degrees of similarity among them. A similar conclusion was arrived at in a parallel study involving bovine carbonmonoxyhemoglobin.[12] Subsequently, we demonstrated through crystallographic studies of a low humidity crystal form of human deoxyhemoglobin,[13] that the tense state can also exist in an ensemble of structures.

Although much of the fundamental results on hemoglobin were derived from the equine protein, the R2 state has not been characterized in the crystals of horse hemoglobin. The structure of a crystal of horse methemoglobin obtained at low pH (5.4) was determined with a view to characterize this state, but it was found that the molecules in the crystal exist in the R state.[14] Thus, the variability of the relaxed state of horse hemoglobin merits further exploration. In this context, here we report the structure of a horse methemoglobin crystal form grown using polyethelene glycol at comparatively high pH (7.0). The results provide insights into the structural variability of relaxed state horse hemoglobin. Incidentally, the present work also provides an example of the improvement in the quality of the diffraction data accompanying reduction in solvent content.

MATERIALS AND METHODS

Horse methemoglobin was purchased from Sigma Chemical Company. A 25 mg/ml solution of the protein in 0.01 M ammonium phosphate buffer, pH 7.0, was first dialyzed against the same buffer using a 12 KDa dialysis membrane sack to remove impurities. The dialyzed protein solution was mixed with twice the volume of a 30% (w/v) solution of PEG 3350. Crystals grew in a week. The crystal was maintained at a relative humidity (r.h) of 98% by placing a drop of a saturated solution of $CuSO_4.5H_2O$ approximately 1 cm from the crystal in the capillary before

TABLE I. Data Collection and Refinement Statistics

	Native	r.h.98%	r.h.88%
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
a(Å)	63.30	62.80	62.52
b(Å)	83.07	80.91	80.14
c(Å)	112.09	112.16	111.32
Z	4	4	4
Unit cell Volume (Å^3)	589413	569857	557718
Solvent content (%)	46.1	44.3	43.1
Data resolution (Å)	3.1	2.6	2.3
Last Shell (Å)	3.21–3.1	2.69–2.6	2.38–2.3
Number of measured reflections	30099	69356	115564
Number of Unique reflections	10748 (1028)	18160 (1778)	24502 (2373)
Completeness (%)	95.9 (93.5)	99.9 (100)	96.0 (94.9)
R_{merge}[a] (%)	18.8 (51.1)	14.3 (47.4)	11.2 (52.3)
Resolution limits used in refinement (Å)	20.0–3.1	20.0–2.6	20.0–2.3
Number of reflections with $F > 0$	10174	17215	24883
R-factor[b] (%)	20.1	16.7	18.3
R_{free}[b] (%)	27.4	22.8	22.8
Protein atoms	4406	4406	4406
Solvent atoms	136	304	386
ligand atoms	172	172	172
RMS deviation from ideal values			
Bond lengths (Å)	0.010	0.009	0.008
Bond angles (°)	1.4	1.3	1.2
Dihedral angles (°)	20.7	19.0	19.7
Improper angles (°)	1.2	1.1	1.0
Residues (%) in Ramachandran plot			
Core region	87.7	90.9	91.9
Additionally allowed region	11.9	8.9	7.9
Generous allowed region	0.4	0.2	0.2
Disallowed region	0.0	0.0	0.0

Values within parentheses refer to the last resolution shell.
[a]$R_{merge} = \Sigma|I_i - \langle I \rangle|/\Sigma\langle I \rangle$
[b]$R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$; R_{free} is calculated in the same way but for a subset of reflections that is not used in the refinement

sealing. A saturated solution of K_2CrO_4 was used instead for maintaining the crystal at r.h.88%. Data collection commenced only 24 hr after sealing the capillary to ensure equilibration. Intensity data from the native, the r.h.98% and the r.h.88% crystals were collected at room temperature (293 K) on a MAR imaging plate mounted on a Rigaku Ru-200 rotating anode X-ray generator, with a crystal-to-detector distance of 100 mm. The data sets were processed and scaled in an identical manner using DENZO and SCALEPACK.[15] The data collection statistics are summarized in Table I. The structure of native horse methemoglobin, obtained at high salt conditions,[16] was used as the search model for molecular replacement. AMoRe[17] calculations yielded best solutions with correlation coefficients of 46.6, 53.9, and 50.1% and R-factors 40.8, 37.8, and 39.2, respectively for data from the native, r.h.98% and r.h.88% crystals. The structures were refined in a similar manner using CNS incorporating the maximum likelihood target.[18] In addition to those in the protein, the structural parameters of the heme group were also restrained.[19] To start with, the structures were refined, first treating the whole tetrameric molecules as rigid bodies and then the subunits as rigid bodies. This was followed by cycles of refinement of positional parameters and temperature factors and model building using O.[20] Simulated annealing was also carried out in between the cycles. Identification of water oxygens was undertaken when R was in the 23–24% range. Initially, peaks greater than 3σ in Fo-Fc maps and 1σ in 2Fo-Fc maps, were identified as corresponding to water oxygens. The limits were subsequently reduced to 2.5σ and 0.8σ. Except in the case of the $\alpha2$ subunit in the native structure, water oxygens coordinated to the heme iron could be identified in the first round itself. During most of the refinement cycles, noncrystallographic symmetry (NCS) constraints were applied separately to the α and β subunits in each structure. The restraints were progressively removed and no NCS restraints were used in the final cycles. Bulk solvent corrections and anisotropic scaling were used throughout. The stereochemical acceptability of the structure was validated using PROCHECK.[21] The refinement parameters are listed in Table I. The final coordinates have been submitted to the Protein Data Bank (PDB references: native, 1Y8H; r.h.98%, 1Y8I; r.h.88%, 1Y8K).

TABLE II. RMS Deviations (Å) in the Main-Chain Atoms Resulting From the Superposition of α1β1 Subunits of Pairs of Molecules[†]

	1	2	3	4	5	6	7	8	9	10
1	—	0.87	0.80	2.19	2.04	2.22	2.37	7.29	2.43	2.68
2	0.30	—	0.53	1.73	1.58	1.88	1.89	6.82	2.78	2.15
3	0.40	0.21	—	2.00	1.89	2.10	2.13	7.04	2.56	2.34
4	0.42	0.27	0.31	—	0.41	1.37	0.82	5.62	4.34	0.97
5	0.47	0.33	0.35	0.13	—	1.30	0.76	5.78	4.21	1.07
6	0.52	0.33	0.33	0.16	0.19	—	1.13	5.50	4.27	1.52
7	0.60	0.45	0.46	0.29	0.28	0.40	—	5.34	4.40	1.10
8	0.90	0.84	0.89	0.78	0.79	0.85	0.82	—	9.22	5.20
9	0.62	0.51	0.46	0.51	0.54	0.69	0.61	0.89	—	4.73
10	0.73	0.58	0.55	0.49	0.46	0.67	0.50	0.86	0.44	—

[†]Those in the α1β1 subunits are given in the lower left and those in α2β2 subunits in the upper right.
1. Native; 2. r.h.98%; 3. r.h.88%; 4. Horse met (PDB code:1NS9); 5. Horse carbonmonoxy (1G0B); 6. Horse met (1NS6); 7. Horse carbonmonoxybezafibrate complex (1IWH); 8. Horse deoxy (2DHB); 9. Human R2 (1BBB); 10. Human oxy (1HHO).

RESULTS AND DISCUSSION
Solvent Content and Quality of Diffraction Pattern

Improvement in the quality of diffraction pattern associated with the reduction in solvent content was first demonstrated in this laboratory in monoclinic lysozyme.[22–24] At that time it was perceived as, and it still is, an exception to the rule. In most cases, partial dehydration results in poorer quality of diffraction data. However, there have been a few instances where reduction in solvent has been made use of to obtain better quality data.[25]

In monoclinic lysozyme, the solvent content of the crystal was reduced by reducing environmental humidity. When the humidity is reduced below a threshold value, the crystal undergoes a structural transformation with loss of water. The solvent content reduces by about 10% to 23%. The unit cell also changes with the addition of a twofold symmetry element which results in the two independent molecules in the native form becoming crystallographically equivalent. Thus in monoclinic lysozyme, the reduction in solvent content, which leads to better diffraction data, is accompanied by large structural changes.

The crystals of horse methemoglobin discussed here present an entirely different picture. There is hardly any noticeable structural change associated with loss of water. The difference in solvent content between the native and the 98% r.h. form is only 1.8%. That between 98% and 88% forms is still lower at 1.2%. However, each reduction is accompanied by significant improvement in the resolution. (Data quality decreases with further reduction of environmental humidity.) The reduction of solvent content necessary for improvement in data quality is among the lowest in the present case when compared to other protein crystals which exhibit this property.[25] The structural basis for the improved resolution that accompany partial dehydration in some crystals, including those of horse methemoglobin reported here, is as yet unclear.

The Quaternary Structure

A major objective of the work is to explore the variability in the quaternary association of liganded horse hemoglobin.

The R and R2 states observed in the human protein provide a framework for this exploration. The observation of states intermediate between the two in human methemogloin by the authors[11] and in bovine carbonmonoxy hemoglobin by Mueser et al.[12] adds a further dimension to the problem. The R state was observed in crystals grown at pH 6.7 from a high salt medium[4] while the R2 state was characterized in crystals grown at pH 5.8 from a medium with low ionic strength.[10] The crystals of human methemoglobin were grown at low ion concentration using PEG, a condition which has been suggested to favor the R2 state. On the other hand, the pH at which they were grown is similar to that used for preparing R-state human oxyhemoglobin. In the case of bovine carbonmonoxyhemoglobin,[12] the low-salt/low pH form, crystallized under conditions that yield human R2 structure, the quaternary structure is intermediate between R and R2 states; the same is the case with crystals grown from a high salt medium at pH 7.2, a condition which favors the R state in liganded human hemoglobin. However, the R2 state is exhibited by a low-salt/high pH form. These studies on human and bovine hemoglobin show that although liganded human and bovine hemoglobin exhibits the R state, the R2 state, and states intermediate between the two, the precise conditions for their occurrence are not yet clearly established.

As indicated above, the structural variability of the liganded state of two of the three most studied mammalian hemoglobin has been clearly established. The remaining one is that from horse. The structures of liganded horse hemoglobin available in the literature include those of aquomet and carbonmonoxyhemoglobin from crystals prepared in high salt conditions at pH 7.1[14] and pH 8.5,[12] respectively. Crystals of hemichrome hemoglobin (pH 5.4)[14] grown in the high salt conditions have also been analyzed. Yet another crystal analyzed is that of a complex of carbonmonoxyhemoglobin with bezafibrate, grown in low-salt condition at pH 6.8.[26] The molecules in all these crystals are essentially in the R state. It is interesting to compare their structure with that of aquomethemoglobin obtained from the present analysis of crystals grown from a low-salt medium at pH 7.0. This has been done with the
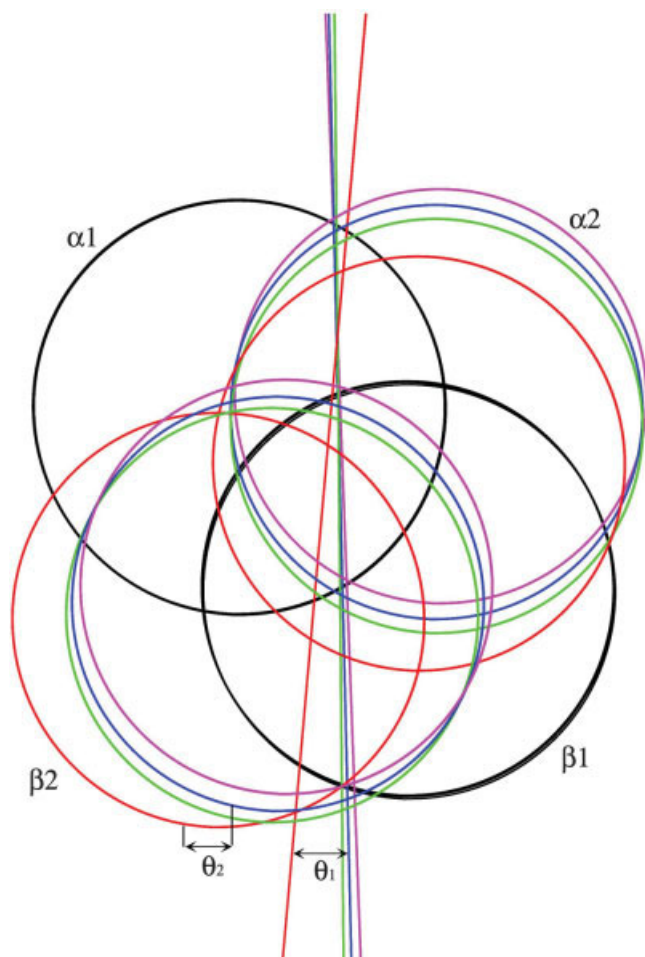
Fig. 1. Schematic diagram showing the orientation of α2β2 relative to α1β1 in the r.h.88% structure reported here and other selected relevant structures. The α1β1 dimer in all the structures are superposed (black). The α2β2 dimer and the molecular dyad are shown in different colors: r.h.88%, blue; human R2, magenta; horse deoxy, red; horse met R, green. θ_1 and θ_2 values of all the relevant structures with respect to the r.h.88% structure, are given in Table III. Please see text for details. This figure was generated using MOLSCRIPT.[29]

TABLE III. Angles (°) θ_1 (Upper Right) and θ_2 (Lower Left) That Define the Change in Quaternary Structure Between Pairs of Structures

	1	2	3	4	5	6	7	8	9	10
1	—	0.4	0.0	2.6	2.4	2.2	2.9	9.0	3.1	3.1
2	0.9	—	0.4	2.1	1.9	1.7	2.4	8.7	3.6	2.6
3	0.1	0.8	—	2.5	2.3	2.1	2.8	9.1	3.2	2.8
4	5.2	4.3	5.0	—	0.0	0.8	0.7	6.7	5.7	0.5
5	4.8	3.9	4.7	0.4	—	0.6	0.7	6.9	5.6	0.8
6	4.4	3.4	4.2	1.5	1.3	—	0.8	6.9	5.3	1.3
7	5.8	4.8	5.6	1.3	1.3	1.5	—	6.2	5.9	0.9
8	18.1	17.3	18.1	13.5	13.9	13.7	12.4	—	12.1	6.6
9	6.2	7.2	6.4	11.5	11.2	10.6	11.7	24.2	—	6.2
10	6.1	5.2	5.7	1.1	1.7	2.6	1.9	13.1	12.4	—

be described by angles θ_1 and θ_2 (Fig. 1) and a translation associated with θ_2. The angles θ_1 and θ_2 between relevant pairs of structures are listed in Table III. The screw translations are small; their magnitude is about 1 Å or less among liganded horse hemoglobin structures.

Tables II and III clearly show that the three horse methemoglobin structures with different solvent contents reported here, are in the same relaxed state. All the other relaxed horse hemoglobin structures are essentially in the R state; they and the liganded human R-state structure together form a cluster. The structures reported here are nearly as different from the R2 structure as they are from the R structure. Tables II and III and Figure 1 clearly demonstrate that, in terms of quaternary structure, the horse methemoglobin structure presented here lies nearly halfway between the R and the R2 states.

In terms of inter-subunit interactions, the "switch region(s)" in the α1β2-α2β1 interfaces serves as an indicator of the quaternary state of the hemoglobin molecule.[28] The geometry of this region in a few representative structures is illustrated in Figure 2. In the R state, His97β2 is located between Thr38α1 and Thr41α1, while in the T state the histidyl residue moves out of this region and is close only to Thr41α1. In the R2 state also the histidyl residue is situated in between two threonyl residues, but it is farther away from them than in the R state. The appropriate distances involving the α-carbon positions of the three residues in the relevant structures are listed in Table IV. These distances and Figure 2 also clearly show that the geometrical disposition of the three residues in the structures reported here are in between those in the R and the R2 states.

CONCLUSION

The present study leads to the characterization of a relaxed state other than the classical R state in horse hemoglobin. This state has geometrical features in between the R state and the R2 state described in human hemoglobin. This result, our earlier demonstration of the structural variability in the R and T states of human hemoglobin and the observation of the variability in the relaxed state of bovine hemoglobin in another study, together suggest that the relaxed as well as the tense hemoglobin molecules can access ensembles of states.

R and the R2 state structures of liganded human hemoglobin as the bench marks.

The asymmetric unit of the present structure contains a full tetrameric molecule while most other structures have only one α subunit and one β subunit in the asymmetric unit. To facilitate comparison, the coordinates of the main-chain atoms of α1β1 and α2β2 in the tetramer were averaged about the molecular dyad, thus producing a tetramer with exact twofold symmetry as in other structures. The α1β1 subunits of different pairs of structures were then superposed using main-chain atoms. The resulting RMS deviations in α1β1 subunits and α2β2 subunits are listed in Table II. These calculations were performed using the computer program ALIGN.[27] The deviations among α1β1 subunits provide a measure of the differences in the structure of the dimer; those in α2β2 largely depend upon the differences in the mutual orientation of the two dimers. Baldwin and Chothia[28] have earlier shown that the change in the mutual orientation of α1β1 and α2β2 can
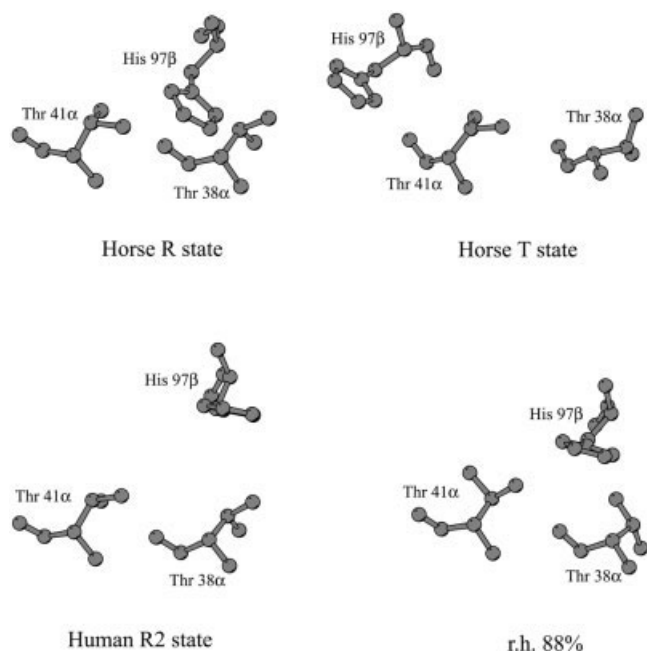
Fig. 2. Switch region in selected relevant structures. The appropriate distances in all the relevant structures are given in Table IV. This figure was generated using MOLSCRIPT.[29]

TABLE IV. Distances (Å) Between the C^{α} Atoms of the Residues Involved in Switching Action[†]

Structure		α1β2 interface	
		His97β2-Thr38α1	His97β2-Thr41α1
Native		6.11	8.42
r.h.98%		5.93	7.98
r.h.88%		5.86	7.83
Horse R	1NS9	5.34	7.50
	1G0B	5.37	7.78
	1NS6	5.29	7.38
	1IWH	5.19	7.66
Horse deoxy	2DHB	—	4.98
Human R2	1BBB	7.16	9.63
Human oxy R	1HHO	5.19	7.28

[†]The values have been averaged when two crystallographically independent sets are present.

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