Genetically crosslinked hemoglobin: a structural study

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The crystal structures of three recombinant human hemoglobins, rHb1.0, rHb1.1 and rHb1.2, have been determined in the deoxy state at 1.8 Å resolution. Two of the three proteins, rHb1.1 and rHb1.2, contain a genetic fusion of the α subunits, a one- or two-glycine link, respectively, whereas rHb1.0 does not. The glycine crosslinks, localized between one N- and C-termini pair of the α subunits in the deoxy crystalline state, do not perturb the overall tertiary or quaternary or even the local structure of hemoglobin. Therefore, genetic fusion to prevent the dissociation of the hemoglobin tetramer, thereby inhibiting renal clearance based upon molecular size, is a structurally conservative method to stabilize hemoglobin for use as an oxygen-delivery therapeutic.

1. Introduction

Several forms of modified hemoglobin are currently being tested as oxygen-delivery therapeutics or 'red blood cell substitutes' in clinical applications (Chang, 1998). Solutions of unmodified hemoglobin are not a potential medical product because the $\alpha_2\beta_2$ tetrameric protein can swiftly dissociate into $\alpha\beta$ dimers (Antonini *et al.*, 1968; Ackers *et al.*, 1974) which are cleared rapidly by renal filtration (Bunn *et al.*, 1969; Bunn & Jandl, 1969) and may be toxic to the kidney over a longer term (Viele *et al.*, 1997). Methods to intramolecularly crosslink the hemoglobin tetramer include chemical alteration (Walder *et al.*, 1994) and genetic fusion (Looker *et al.*, 1992). In addition to delivering oxygen, genetically fused recombinant hemoglobin administered in animal tests maintained kidney function, *i.e.* no hemoglobin was detected in the urine, papillary damage and glomerular function indicator levels in the urine were normal and histology was also normal, resulting in an increased intravascular half-life (Looker *et al.*, 1992).

To study the effect of genetic fusion on the structure of hemoglobin, the crystal structures of three recombinant proteins were determined by X-ray analysis. The control is a true $\alpha_2\beta_2$ tetramer; the second two are pseudotetrameric as they contain a 'di-α' subunit in which both original α subunits and the linking residue(s) between them are combined into a single polypeptide chain encoded by one gene. In an attempt to image the introduced crosslinking residues themselves as well as the overall structure, these three hemoglobins were crystallized in their deoxy form under classical 'T-state' conditions (Perutz, 1968), which produces crystals with the entire tetramer in the asymmetric unit.

A naming convention is used for this series of proteins, taking the form rHb#.#. The three letters indicate that the protein is a recombinant hemoglobin, in this series the human

Table 1
Deoxyhemoglobin crystallization conditions.

Tube	Final molarity (M)	Iron citrate† (µl)	H_2O (µl)	Deoxy Hb‡ (µl)	Solution C§ (µl)
1	2.26	2	23	20	75
2	2.32	2	21	20	77
3	2.41	2	18	20	80
4	2.50	2	15	20	83
5	2.62	2	11	20	87
6	2.71	2	8	20	90
7	2.80	2	5	20	93

† 0.20 g ferrous sulfate + 0.15 g sodium citrate in 2.5 ml H_2O.   ‡ 60 g l^{-1} in 10 mM ammonium phosphate pH 7.   § 0.5 ml 2 M monobasic ammonium phosphate + 1.5 ml 2 M dibasic ammonium phosphate + 8.0 ml 4 M ammonium sulfate. Crystal mounting solution = 8.5 ml solution C + 1.5 ml H_2O + 5.2 mg sodium dithionite.

Table 2
Data-collection statistics.

	Deoxy rHb1.0	Deoxy rHb1.1	Deoxy rHb1.2
Frames	135 (1°, 30 min)	180 (1°, 30 min)	135 (1°, 27 min)
Crystal-to-detector distance (mm)	80	70	80
Limiting resolution (Å)	1.80	1.80	1.80
Space group	$P2_1$	$P2_1$	$P2_1$
Unit-cell parameters			
a (Å)	63.132	63.204	63.144
b (Å)	83.579	83.357	83.282
c (Å)	53.991	54.003	53.928
β (°)	99.246	99.065	99.067
Overall (30.0–1.80 Å, no σ cutoff)			
Measured reflections	119214	143808	133741
Unique reflections	47219	45597	45959
Redundancy	2.5	3.2	2.9
Mean signal-to-noise (I/σ)	22.1	33.3	11.3
Completeness (%)	92.0	89.0	90.2
R_{merge} (%)	6.1	4.9	6.5
Highest resolution shell (0.1 Å slice)			
Redundancy	2.2	3.2	2.9
Mean signal-to-noise (I/σ)	6.2	9.3	4.4
Completeness (%)	93.7	94.1	87.4
R_{merge} (%)	23.7	24.7	24.1

sequence, with an initiator methionine replacing the first residue of each subunit chain. The first numeric variable is 1 if the Presbyterian mutation (βN108K) is present or 0 if it is absent. This naturally occurring mutation lowers the oxygen affinity of human hemoglobin by favoring the T allosteric state (Moo-Penn *et al.*, 1978). The second variable specifies the number of glycine residues linking the α subunits of the hemoglobin tetramer, with a 0 indicating no fusion. The three proteins in this study are labelled rHb1.0, rHb1.1 and rHb1.2, for recombinant hemoglobins containing the Presbyterian mutation and having no, one or two glycines crosslinking the α subunits, respectively.

2. Materials and methods

2.1. Crystallization

The recombinant hemoglobins rHb1.0, rHb1.1 and rHb1.2 were crystallized in their deoxy form following a method adapted from Perutz (1968). Each pre-purified oxyhemoglobin was buffer-exchanged using a Sephadex G25 (Pharmacia PD-10) column equilibrated with 10 mM ammonium phosphate pH 7 and then concentrated to 60 g l^{-1} (ε = 0.8372 l g^{-1} cm^{-1} at 540 nm). Batch crystallizations of each hemoglobin were set up in a nitrogen-filled anaerobic chamber according to Table 1 after deoxygenating all solutions. The 6 × 50 mm batch tubes were sealed with Parafilm and allowed to stand in the chamber for several weeks. The resulting hemoglobin crystals were mounted in sealed quartz capillary tubes before removal from the oxygen-free atmosphere.

2.2. Data collection and reduction

A complete X-ray diffraction data set was collected for each of the three deoxyhemoglobins from one single crystal apiece at room temperature using Cu $K\alpha$ radiation from a Siemens rotating-anode generator operated at 50 kV, 90 mA and a Rigaku R-AXIS IIc imaging-plate system. The reflections were reduced (see Table 2) using the *XDS* and *XSCALE* software programs (Kabsch, 1993). The deoxyhemoglobin crystals were isomorphous to those originally grown by Perutz (1968).

2.3. Structure determination and refinement

Each of the three deoxy recombinant human hemoglobin structures was determined using coordinates based on those for deoxy native human hemoglobin (Protein Data Bank entry 2hhb; Fermi *et al.*, 1984) to calculate initial phases. Cycles of conventional positional refinement were carried out using *CNS* (Brunger *et al.*, 1998) alternated with manual fitting by means of the graphics package *O* (Jones *et al.*, 1991). Co-ordinates for the genetic crosslinker, if present, were built into difference electron density obtained from omit maps after simulated annealing (Adams *et al.*, 1997). Solvent atoms were introduced into density peaks over 4σ in a $F_{obs} - F_{calc}$ (and over 2σ in a $2F_{obs} - F_{calc}$) electron-density map. A final least-squares refinement, conjugate gradient then full matrix, was performed using *SHELX* (Sheldrick, 1997). The Fe atoms of the heme prosthetic groups in the deoxy rHb1.2 structure were fitted anisotropically with *SHELX* to account for their local difference density. Engh & Huber (1991) topology and para-meters were used throughout the refinement process. All three crystallographic refinements converged to a final residual under 19% (see Table 3).

3. Results and discussion

Differences between native human hemoglobin and the three recombinant proteins rHb1.0, rHb1.1 and rHb1.2 include (i) the N-terminal valine-to-methionine substitutions for expression in *Escherichia coli*, (ii) the Presbyterian mutation, a lysine substitution for the native asparagine in the β subunits at position 108, and, if present, (iii) the introduced genetic fusion of the two α chains in the physiological tetramer, a one- or two-glycine residue crosslink. Despite these differences, each

Table 3
Refinement statistics.

	Deoxy rHb1.0	Deoxy rHb1.1	Deoxy rHb1.2
Resolution range (Å)	8.0–1.80	8.0–1.80	8.0–1.80
Signal-to-noise (I/σ) cutoff	0.0	0.0	0.0
Unique reflections	46732	45081	45496
Free R reflections	2240 (4.8%)	2143 (4.8%)	2156 (4.7%)
Completeness (%)	92.2	89.1	90.2
$R_{working}$ (%)	18.8	17.5	18.8
R_{free} (%)	23.4	22.7	24.0
R_{final} (%)	18.9	17.6	18.9
Asymmetric unit	$\alpha_2\beta_2$ tetramer	α-Gly-$\alpha\beta_2$	α-GlyGly-$\alpha\beta_2$
R.m.s. deviations			
Bond lengths (Å)	0.005	0.005	0.011
Angle distances (Å)	0.015	0.016	0.015
Zero chiral volumes (Å^3)	0.038	0.041	0.034
Non-zero chiral volumes (Å^3)	0.042	0.041	0.038
Mean temperature factors (Å^2)			
Main chain	15.6	21.8	23.8
Side chain	23.3	29.0	31.0
Heme	15.5	22.1	24.3
Solvent	35.8	39.4	39.4

of the three deoxy recombinant hemoglobin coordinate sets overlays the deoxy native human hemoglobin structure, all in the classic T state. The root-mean-square deviations between the 574 C$^\alpha$ atoms of the four subunits in native deoxy-hemoglobin (Protein Data Bank entry 2hhb; Fermi *et al.*, 1984) and those of the three recombinant hemoglobins in this study are 0.194, 0.228 and 0.206 Å for rHb1.0, rHb1.1 and rHb1.2, respectively. The four largest r.m.s. deviations are found in the first two residues of the β chains. The genetic fusions that hold the hemoglobin tetramer together clearly do not perturb the overall secondary, tertiary or quaternary structure.

The structural role of mutated amino-termini in recombinant hemoglobin has been investigated by Kavanaugh *et al.* (1992). Also, the structure of the methionine substitution for N-terminal valine in the genetically crosslinked hemoglobin rHb1.1 (Protein Data Bank entry 1abw) has been examined by Kroeger & Kundrot (1997). The rHb1.1 structure was re-determined to a higher resolution for the present rHb1.# series to achieve similarity in crystal-growth parameters, instrumentation and refinement scenarios for comparison purposes. The previous discussions concluded that the valine-to-methionine substitutions do not cause much disruption to the structure of the N-termini regions and that the S atoms of these mutations are involved in stabilizing van der Waals interactions. These mutations in the structures of the current rHb1.# series are not well defined by electron density and refined to high temperature factors.

The $\beta108$ lysine side chain of the Presbyterian mutation present in this rHb1.# series forms a hydrogen bond with that of the native tyrosine at $\beta35$ in the deoxy state. This introduced hydrogen bond appears to be stronger in rHb1.0 than in the genetically crosslinked recombinant hemoglobins; the average distance between the N$^\xi$ of $\beta108$ lysine and the OH of $\beta35$ tyrosine is 2.6 Å for rHb1.0, 3.4 Å for rHb1.1 and 3.6 Å for rHb1.2. The Presbyterian mutation contributes to the lowered oxygen affinity of these recombinant hemoglobins

compared with the native protein either by stabilizing the T state with this hydrogen bond (Kroeger & Kundrot, 1997) or by removing the destabilizing native asparagine. A $\beta108$ alanine mutation, with no introduced hydrogen bond, also has a lowered oxygen affinity compared with the native protein (Baxter Hemoglobin Therapeutics, unpublished data).

As the asymmetric unit in this T-state hemoglobin crystal form contains one full $\alpha_2\beta_2$ tetramer or one α-Gly$_n$-$\alpha\beta_2$
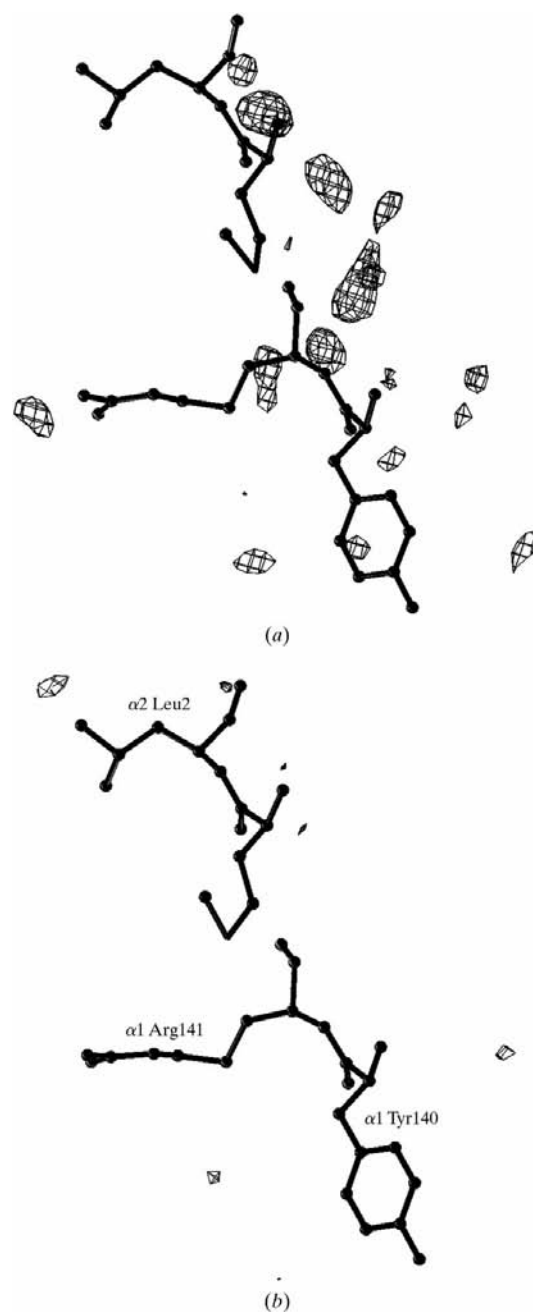

Figure 1
Deoxy rHb1.2 − deoxy rHb1.0 difference map. The refined structure of rHb1.0 is shown in black; the electron-density difference map (positive density > 4σ) determined from 30 to 1.8 Å with phases from the rHb1.0 structure is drawn as wirecage. (*a*) View of one set of α N- and C-termini: α_1C–Nα_2. (*b*) View of the opposite set of non-crystallographic symmetry-related α N- and C-termini: α_2C–Nα_1.
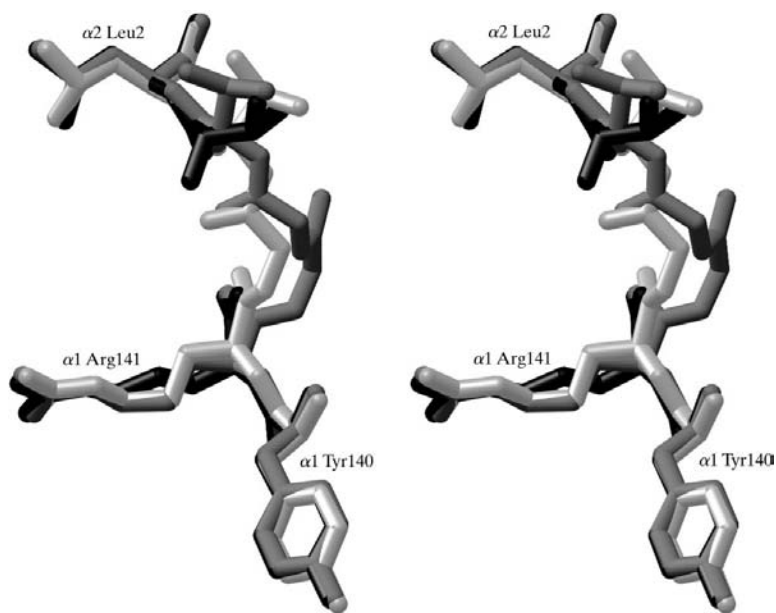
Figure 2
Overlay of HbA$_0$, rHb1.1 and rHb1.2. Stereoview includes the α subunit N- and C-termini (glycine crosslink) region; HbA$_0$ (Protein Data Bank entry 2hhb; Fermi *et al.*, 1984) with associated text is drawn in black, rHb1.1 in light gray and rHb1.2 in dark gray. Coordinates were superposed by overlaying the C$^\alpha$ atoms of the hemoglobins.

pseudotetramer, the occupancy of the one- or two-glycine link between α subunits might be averaged over both sets of non-crystallographic symmetry-related N- and C-termini, *i.e.* α_1C–Nα_2 and α_2C–Nα_1. This hypothesis was proposed by Looker *et al.* (1992) based upon their symmetry-averaged deoxy rHb1.1 − deoxy native hemoglobin difference electron-density map at 2.5 Å. Kroeger & Kundrot (1997) claim a unique position for the glycine crosslinker in their 2.0 Å structure of rHb1.1 (Protein Data Bank entry 1aby) after $2F_{obs} - F_{calc}$ and difference Fourier maps, but their coordinate file states that the alternate conformation may be present in low (20%) occupancy. The glycine crosslinkers in deoxy rHb1.1 and deoxy rHb1.2 are not occupancy averaged, but localized between one set of α N- and C-termini, based upon omit maps after simulated annealing and, more significantly, the rHb1.2 − rHb1.0 and rHb1.1 − rHb1.0 difference maps. The positive difference density in Fig. 1(*a*) clearly details the main-chain path of the two-glycine crosslink in deoxy rHb1.2; conversely, no difference density exists near the opposite set of rHb1.2 α termini (Fig. 1*b*). Remarkably, the di-α fusion appears between the same set of termini in both deoxy rHb1.1 and rHb1.2. Crystallographic analysis of a three-glycine fusion between α subunits in several other deoxy recombinant hemoglobins proved this slightly longer genetic crosslinker to be completely disordered and/or delocalized and the three glycine residues could not be modeled (data not shown).

The introduced genetic fusion of the α subunits in rHb1.1 and rHb1.2 does not appreciably disturb local structure (see Fig. 2). The orientation of the side chain in Tyr140, the second-to-last residue of the original α_1 subunit, is not affected by the glycine crosslink. The same holds true for the second and following residues of the original α_2 subunit. The side chain for Arg141, the residue immediately prior to the glycine crosslink, is slightly shifted compared with that for native hemoglobin. The position of the valine immediately following the glycine crosslink in rHb1.1 and rHb1.2, corresponding to the initial residue of the native hemoglobin α_2 subunit, is somewhat twisted to accommodate the length of the respective glycine link. Note: the initial residue of the second α chain in rHb1.0 (not shown in Fig. 2) is a methionine, not valine, as a consequence of expression in *E. coli*.

4. Conclusions

The one- and two-glycine additions employed to genetically fuse the α subunits in recombinant human hemoglobin do not disrupt the overall tertiary or quaternary structure. Furthermore, these glycine crosslinks, localized between one N- and C-termini pair in the deoxy crystalline state, have little impact on their surrounding structure. As a result of genetic fusion, the goal of crosslinking the hemo-globin tetramer to prevent dissociation into $\alpha\beta$ dimers subject to swift physiological clearance has been achieved with minimal structural repercus-sions. This structurally conservative modification allows hemoglobin to be used as an oxygen-delivery therapeutic.

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