Oligomerization and ligand binding in a homotetrameric hemoglobin: Two high-resolution crystal structures of hemoglobin Bart's (γ_4), a marker for α -thalassemia

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Abstract

Hemoglobin (Hb) Bart's is present in the red blood cells of millions of people worldwide who suffer from α -thalassemia. α -Thalassemia is a disease in which there is a deletion of one or more of the four α -chain genes, and excess γ and β chains spontaneously form homotetramers. The γ_4 homotetrameric protein known as Hb Bart's is a stable species that exhibits neither a Bohr effect nor heme-heme cooperativity. Although Hb Bart's has a higher O₂ affinity than either adult ($\alpha_2\beta_2$) or fetal ($\alpha_2\gamma_2$) Hbs, it has a lower affinity for O₂ than HbH (β_4). To better understand the association and ligand binding properties of the γ_4 tetramer, we have solved the structure of Hb Bart's in two different oxidation and ligation states. The crystal structure of ferrous carbonmonoxy (CO) Hb Bart's was determined by molecular replacement and refined at 1.7 Å resolution ($R = 21.1\%$, $R_{\text{free}} = 24.4\%$), and that of ferric azide (N_3^-) Hb Bart's was similarly determined at 1.86 Å resolution ($R = 18.4\%$, $R_{\text{free}} = 22.0\%$). In the carbonmonoxy-Hb structure, the CO ligand is bound at an angle of 140°, and with an unusually long Fe-C bond of 2.25 Å. This geometry is attributed to repulsion from the distal His63 at the low pH of crystallization (4.5). In contrast, azide is bound to the oxidized heme iron in the methemoglobin crystals at an angle of 112°, in a perfect orientation to accept a hydrogen bond from His63. Compared to the three known quaternary structures of human Hb (T, R, and R2), both structures most closely resemble the R state. Comparisons with the structures of adult Hb and HbH explain the association and dissociation behaviour of Hb homotetramers relative to the heterotetrameric Hbs.

Keywords: Hemoglobin; hemoglobin Bart's; thalassemia; quaternary structure; subunit association

Mammalian hemoglobins (Hbs) function as heterotetramers comprising two alpha-type chains and two beta-type chains. In normal healthy humans, transcription and translation of the alpha-type and beta-type globin chains produces closely

matched quantities of each type that spontaneously aggregate to form heterotetramers (Valdes and Ackers 1977). However, in the conditions known as the thalassemias mismatched quantities of globin chains are produced. In the case of alpha (α) thalassemias, excess beta-type chains aggregate to form abnormal homotetrameric Hbs (Rigas et al. 1956; Ager and Lehmann 1958; Dance and Huehns 1962). Furthermore, as the α globin chain is expressed at all stages of human development, two main varieties of development-stage linked homotetramers are formed in α -thalassemias. During the adult stage the predominant abnormal homotetramer is HbH (β_4), while at the fetal stage Hb Bart's (γ_4) predominates. The beta (β) chains have a greater attraction than gamma (γ) chains for α chains in tetramers (Lehmann and Huntsman 1974); therefore, when both β and γ chains

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Abbreviations: Hb, hemoglobin; HbH, β_4 homotetramer; Hb Bart's, γ_4 homotetramer; CO- γ_4 , carbonmonoxy γ_4 Hb; NNN- γ_4 , met-azide γ_4 Hb; CO- β_4 , carbonmonoxy β_4 Hb; HbA, adult Hb ($\alpha_2\beta_2$); HbF, fetal Hb ($\alpha_2\gamma_2$); Hb Portland, $\zeta_2\gamma_2$; MePEG, methoxypolyethylene glycol; rms, root-mean-square.

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are present and there is an insufficient supply of α , HbA ($\alpha_2\beta_2$) forms in preference to HbF ($\alpha_2\gamma_2$). Excess γ chains then aggregate to form Hb Bart's. Only when the amount of β by itself is greater than α are significant quantities of HbH observed.

Alpha thalassemia is associated with four general clinical syndromes that correspond with the loss of increasing numbers of the four Hb α genes (Dickerson and Geis 1983; Bunn and Forget 1986): (1) *The silent-carrier state* (α -thalassemia 2). One α gene is missing. This condition is entirely symptomless with 1–2% Hb Bart's present at birth. (2) *Classical α -thalassemia trait* (α -thalassemia 1). Two α genes are missing. About 5% Hb Bart's is present at birth. The symptoms, minor red blood cell abnormalities but no anemia, are the same for either homozygous or heterozygous individuals. (3) *Hemoglobin H disease*. Three α genes are missing, resulting in the presence of Hb Bart's in infancy and HbH in adulthood. There has also been a report (Dance and Huehns 1962) of rare HbH disease individuals with a tetramer of another β -like chain, delta (δ). HbH disease is characterized by a chronic hemolytic anemia of variable severity. (4) *Hydrops fetalis*. All four α genes are missing. This condition is fatal, leading to stillbirth in the last few weeks of pregnancy. Therefore, the presence of Hb Bart's, which was first recognized as such by Ager and Lehmann (1958), is an important indicator of a major health disorder. Hb Bart's occurs in tens of millions of humans worldwide, and reaches a frequency of over 40% in the Laotian population of South-East Asia (Wasi 1986).

The β_4 homotetramer has been studied in some detail, using both naturally isolated material and protein prepared from the aggregation of β chains isolated from normal adult hemoglobin (Huehns and Beaven 1962; Benesch and Benesch 1974; Philo et al. 1988). In contrast to heterotetrameric hemoglobins, HbH has been found to have a high affinity for oxygen (Benesch et al. 1968) and almost no cooperativity in oxygen binding (Kurtz et al. 1981), and to be relatively unstable in solution (Dance et al. 1963). Crystal structures of the carbonmonoxy (Borgstahl et al. 1994a) and deoxygenated (Borgstahl et al. 1994b) forms of HbH indicate that the protein remains in the R-state quaternary structure both in the absence and presence of heme ligands.

In contrast to HbH, little is known of the structural and functional properties of Hb Bart's, partly due to the previous unavailability of large amounts of protein. It is more stable in solution than HbH (Bunn and Forget 1986), and has a lower affinity for free alpha chains than does HbH (Huehns and Beaven 1962). It has also recently been shown (Adachi et al. 2000) that the γ_4 homotetramer self-assembles by a different pathway from that of β_4 , involving stable γ_2 dimers. Hb Bart's has been crystallized previously (Czerwinski et al. 1981), but no structure is available. Indeed, although many studies on the functional properties of fetal hemoglobin ($\alpha_2\gamma_2$, HbF) have appeared (e.g., Allen et al.

1953; Weatherall et al. 1974; Huisman 1981), the 2.5 Å structure of the deoxy form of this protein (Frier and Perutz 1977) remains the only structure available for a hemoglobin incorporating the γ -chain.

Here we present the three-dimensional structure of Hb Bart's (γ_4), in two liganded forms, determined at high resolution from crystals that were unexpectedly obtained during attempts to crystallize the human embryonic hemoglobin Portland ($\zeta_2\gamma_2$) (Kidd et al. 2001). The crystal structures allow us to derive new information on the structural organization and ligand binding properties of this medically significant protein, and to obtain a second, high-resolution view of the hemoglobin γ -chain. Further, by comparing the subunit interfaces in $\alpha_2\beta_2$, β_4 , and now γ_4 , we are able to probe the factors that stabilize the γ_2 dimer in solution, and offer an explanation as to why β -type globins aggregate to form homotetramers whereas α -type globins do not.

Results

Structure determination and model quality

The crystal structure of the carbonmonoxy form of ferrous Hb Bart's (CO- γ_4) was determined by molecular replacement using a search model derived from the 1.8 Å structure of CO- β_4 (Borgstahl et al. 1994a). The structure was refined at 1.7 Å resolution to a crystallographic R -factor of 0.211 ($R_{\text{free}} = 0.244$). Refinement results are summarized in Table 1. The structure of the azide form of ferric Hb Bart's (NNN- γ_4) was also determined by molecular replacement using a search model derived from the 1.7 Å model of CO- γ_4 , and was refined at 1.86 Å resolution to an R -factor of 0.184 ($R_{\text{free}} = 0.220$). Both structures exhibit good stereochemistry with root-mean-square (rms) deviations from ideal geometry of 0.006 Å for bond lengths and 1.06° for

Table 1. Refinement and model statistics

	CO- γ_4	NNN- γ_4
Resolution limits (Å)	20.0–1.7	20.0–1.86
Number of reflections	26,914	22,315
R_{cryst} (%)	21.1	18.4
R_{free}^a (%)	24.4	22.0
Bond length r.m.s. deviation (Å)	0.006	0.005
Bond angle r.m.s. deviation (deg.)	1.06	1.06
Average B -factor (Å ²):		
Main chain	15.4	15.0
Side chain	17.0	16.8
Solvent	30.4	28.2
Number of protein atoms (2×146 residues)	2260	2260
Number of heme atoms	86	86
Number of ligand atoms	4 (2 CO)	6 (2 N ₃ [−])
Number of water molecules	287	242

^a 10% randomly omitted reflections were used for R_{free} .

bond angles for CO- γ_4 and 0.005 Å and 1.06° for NNN- γ_4 (Table 1).

The final models for both structures comprise the complete γ -chain (146 residues) for each of the two monomers in the asymmetric unit, which have been independently refined. The carbonmonoxy-dimer contains 2260 nonhydrogen protein atoms, two CO-heme groups, and 287 water molecules, and the azide-dimer 2260 nonhydrogen protein atoms, two N₃⁻-heme groups, and 242 water molecules. With the exception of several side chains on the surface of the molecule that exhibit little or no electron density and have been assigned zero occupancies (two each in molecules A and B for CO- γ_4 and one in A, four in B for NNN- γ_4) the electron density for the entire polypeptide is in each case excellent (Fig. 1). There are indications of possible minor alternate conformations for other side chains (12 in molecule A and 12 in molecule B for both structures), but these have not been modeled. Root-mean-square (rms) differences when the two crystallographically independent molecules are superimposed are 0.28 Å for CO- γ_4 and 0.30 Å for NNN- γ_4 , for all main-chain atoms. Ramachandran plots of main-chain torsion angles (ϕ, ψ) calculated with PROCHECK (Laskowski et al. 1993) show that 91.5% of residues lie in the most favored region for CO- γ_4 and 93.8% for NNN- γ_4 , with no residues in disallowed areas. The heme groups are well defined, and there is clear density visible for the ligands bound to each heme.

Humans possess two γ genes adjacent to each other on the same chromosome (Dickerson and Geis 1983). These two genes differ only by a single residue (Ala versus Gly) at position 136, and are designated $^A\gamma$ and $^G\gamma$. During the cloning of the embryonic hemoglobin genes into a yeast expression system (Mould et al. 1994; Hofmann et al. 1995) it was initially not known which γ gene had been inserted; however, DNA sequencing of the cloned γ gene indicated that $^A\gamma$ had been cloned (R. Mould and T. Brittain (unpubl.)) Based on our 1.7 Å and 1.86 Å electron density maps (Fig. 1), it is clear that the residue at position 136 is an alanine, and this result confirms the cloning of the $^A\gamma$ gene.

Tertiary structure

The overall molecular structure of Hb Bart's is shown in Figure 2. The two monomers of the asymmetric unit, labeled A and B (Fig. 2A), are analogous to subunits $\alpha 1$ and $\beta 1$ of adult HbA, respectively. The complete homotetramer (Fig. 2B) is generated by crystallographic symmetry; the two symmetry-related monomers are labeled A' and B', and are analogous to HbA $\alpha 2$ and $\beta 2$, respectively. The CO- γ_4 and NNN- γ_4 structures are very similar, with rms differences of 0.34 Å for main-chain atoms and 0.96 Å for side-chain atoms, after superposition of the entire structures.

Superposition of the γ subunits on to other β -type globin subunits shows a generally close correspondence. Taking

the A subunit of CO- γ_4 as a reference, the entire chain, with the exception of the N-terminal residue, Gly1, can be superimposed on subunit A of CO- β_4 (Borgstahl et al. 1994a) with an rms difference of 0.73 Å for 145 C α atoms. The most significant deviation involves the N-terminal A helix (residues 7–18), which is tilted slightly inwards towards helix E so that its N-terminus is displaced by about 2 Å; the movement is further propagated through the extended residues 1–6 such that the N-terminal residue of the γ -chain, Gly1, is displaced 4 Å from that of the β -chain (see Fig. 4). This displacement of the N-terminal residue seems to be a characteristic feature of the γ -chain (compared with the β -chain) as it is also seen in fetal Hb (Frier and Perutz 1977), and is implicated in the greater tetramer stability of HbF relative to HbA (Manning et al. 1999). Interestingly, superposition of the γ subunits on to either of the β subunits of heterotetrameric oxy-HbA (Shaanan 1983) shows somewhat less agreement than for the β subunits of homotetrameric β_4 , with an rms difference of 0.90 Å (145 C α atoms). This implies that quaternary structure differences related to homotetramer formation do have some slight impact on the tertiary structure.

Comparing the location and orientation of the heme group within the globin folds, if the A subunit of CO- γ_4 is superimposed on to the A subunit of CO- β_4 , using the globin C α atoms alone, the heme groups match with a mean positional difference (for all atoms except those of the vinyl and propionate groups) of 0.35 Å. Most of this difference comes from a slight translational displacement of 0.25 Å, in which the movement of the heme accompanies a similar displacement of helix F, to which it is attached.

Heme stereochemistry

The heme groups in the CO and azide complexes match very closely (rms difference 0.42 Å for 32 heme atoms when the structures are superimposed on the basis of their main chain atoms). In both complexes the hemes are remarkably planar, with little buckling or ruffling. This must be a genuine property of the heme stereochemistry for Hb Bart's, at the pH of crystallization, because the hemes in both subunits of both structures were independently refined using minimal bond length, angle, dihedral, and improper constraints, and using two different refinement protocols. In each case the iron atom lies in the plane of the porphyrin, and its bond with N $\epsilon 2$ of the proximal histidine ligand, His93, is about 2.1 Å (Table 2), as in other liganded Hbs. The only differences in heme stereochemistry are for two of the heme substituents. First, one of the vinyl groups is rotated $\sim 180^\circ$ about its C-C bond in the NNN- γ_4 structure relative to the CO- γ_4 structure, a difference that is found in both subunits. Second, one of the propionic acid groups of CO- γ_4 is in two conformations, again in both subunits, whereas only a single conformation is found in NNN- γ_4 .

Fig. 1. Electron density for CO- γ_4 and identification of the HBG1 gamma gene product. Normally, two gamma genes (HBG1 and HBG2) are expressed in humans. Their gene products differ at residue 136 (out of 146) where glycine is found in the G-gamma product (HBG2) and alanine is found in the A-gamma product (HBG1). (A) Density around residue 136 (middle of figure) modeled as glycine and refined. The $2F_o - F_c$ maps (blue) are contoured at 1.2σ . The $F_o - F_c$ maps are contoured at $\pm 3\sigma$ with positive density in green and negative density in red. (B) Density around residue 136 after modeling as alanine and refinement. The density corresponds best with an alanine, identifying the cloned gene as HBG1. Figures drawn using BOBSCRIPT (Esnouf 1997) and RASTER3D (Merritt and Murphy 1994).

Fig. 2. Structure of the carbonmonoxy form of hemoglobin Bart's (γ_4). (A) Ribbon diagram of the two subunits (labeled A and B) of γ in the asymmetric unit. The hemes are depicted as ball-and-stick models. (B) Ribbon diagram of the CO- γ_4 tetramer. The tetramer was generated through crystallographic symmetry (symmetry-related globins are labeled A' and B'). Figures were drawn using the program MOLSCRIPT (Kraulis 1991), and rendered with RASTER3D (Merritt and Murphy 1994).

Table 2. Metal–ligand geometry and interactions

	CO- γ_4			NNN- γ_4	
	A	B		A	B
A. Angles (deg.)					
Fe-C-O	140	135	Fe-N1-N2	115	115
Fe-C/heme normal	9	10	Fe-N1/heme normal	-1	1
			N1-N2-N3	5	11
B. Distances (Å)					
Fe-C	2.25	2.26	Fe-N1	2.16	2.19
Fe-Ne2(His92)	2.12	2.09	Fe-Ne2(His92)	2.14	2.10
C...Ne2(His63)	3.05	2.97	N1...Ne2(His63)	2.72	2.84
O...Ne2(His63)	3.78	3.85	N2...Ne2(His63)	3.55	3.76
C...Cδ2(His63)	3.66	3.55	N1...Cδ2(His63)	3.40	3.38
C...Cγ2(Val67)	3.35	3.34	N1...Cγ2(Val67)	3.19	3.24
O...Cγ2(Val67)	3.51	3.48	N2...Cγ2(Val67)	3.22	3.28
			N3...Cγ2(Val67)	3.66	3.79
O...Cδ2(Leu106)	3.72	3.86	N3...Cδ2(Leu106)	3.40	3.28

Ligand binding

Both the carbon monoxide and azide complexes show full occupancy of their bound ligands. The crystals of CO- γ_4 were grown in carbon monoxide-saturated conditions, in the dark, and in the presence of a reducing agent, to avoid oxidation and to promote full CO occupancy. Initial omit maps (both $2F_o - F_c$ and $F_o - F_c$) showed strong electron density for bound CO in both independent subunits. After model building and refinement, strong electron density in the $2F_o - F_c$ map (Fig. 3A) and low temperature factors for the CO atoms (average B values of 16.7 Å² and 19.2 Å² for the C and O atoms, respectively) are consistent with full occupancy. Similarly, the azide ligands in NNN- γ_4 were also represented by strong electron density in $2F_o - F_c$ and $F_o - F_c$ omit maps (Fig. 3B), and have full occupancy as judged by their low temperature factors (average B value of 15.8 Å²).

Carbon monoxide binding

The CO ligand in Hb Bart's is bound in a single well-defined conformation, with a stereochemistry that is essentially the same in both independently refined subunits of CO- γ_4 ; details are given in Table 2. Two features of the CO binding mode in Hb Bart's appear to differ significantly from that in other Hbs and from the Fe-CO geometry found in model CO-porphyrin complexes (Peng and Ibers 1976). First, the Fe-C bond length (2.25 Å) is markedly greater than in other CO-Hbs where it is consistently around 1.8 Å (Derewenda et al. 1990; Borgstahl et al. 1994a). Second, the CO ligand is bound in an angular mode, with an Fe-C-O angle of ~135–140°. This compares with Fe-C-O angles of ~170° in R-state CO-HbA (Derewenda et al. 1990), R2-

state CO-HbA (Silva et al. 1992) and CO- β_4 (Borgstahl et al. 1994a) and angles close to 180° in CO-porphyrin complexes (Peng and Ibers 1976). Accompanying this, the Fe-C bond deviates by 10° from colinearity with the heme normal through the Fe atom, markedly more than in the other CO-Hb structures where the deviation is ~6° (Derewenda et al. 1990; Borgstahl et al. 1994a), and in CO-porphyrin complexes where it is colinear (Peng and Ibers 1976). Both subunits were refined independently and without restraints on the Fe-C bond lengths or Fe-C-O bond angles, strongly suggesting that these differences are a real feature of the Hb Bart's crystal structure.

The geometry of ligand binding is likely to be influenced by the crystallization conditions, especially pH, as well as by the structural constraints of the ligand binding pocket (Ray et al. 1994). The unusually planar heme group should allow greater Fe-porphyrin back-bonding and thereby reduce Fe-CO back-bonding (Li and Spiro 1988). Considering that the extent of heme buckling varies in different CO-Hbs, however, yet the Fe-C distance is consistently around 1.8 Å, heme planarity is unlikely to be the main factor in the substantially lengthened and more angular Fe-CO bond in Hb Bart's.

The closest approach to the CO ligand is from the distal histidine, His63, whose side chain Ne2 atom is only 3 Å from the CO carbon atom. His63 is in contact with solvent (its Nδ1 atom forms a 2.8 Å hydrogen bond with a well-ordered water molecule), and at the pH of crystallization (4.5–4.9) protonation of His63 is likely. The pKa of His63 in Hb Bart's is not known but in Mb the equivalent distal histidine, His64, has a pKa of 4.4 (Yang and Phillips 1996, and references therein). Because the Ne1...C(CO) interaction cannot be a hydrogen bond, the increased displacement of the CO ligand from the heme normal is most probably due to this (repulsive) contact. In Mb, the distal histidine

Fig. 3. Heme groups with bound ligands. The maps for both structures were calculated with Fourier coefficients $2F_o - F_c$, and were contoured at 1.2σ . (A) Two views of one of the heme groups in the 1.7 Å structure of CO- γ_4 . The carbon monoxide ligand is depicted as a ball-and-stick model with carbon in yellow and oxygen in red. The Fe-C-O angle is 140° and the Fe-C distance is 2.25 Å. (B) Two views of one of the heme groups in the 1.86 Å structure of NNN- γ_4 . The azide ligand is depicted as a ball-and-stick model with nitrogen in blue. The Fe-N1-N2 angle is 115° and the Fe-N1 distance is 2.16 Å. Figures were drawn using BOBSCRIPT (Esnouf 1997) and RASTER3D (Merritt and Murphy 1994).

can relieve this repulsion at low pH by swinging out of the ligand-binding pocket at low pH (Yang and Phillips 1996), but this does not occur in the present structure. Positive charge on His63 should also change the polarization of the CO ligand (Kushkuley and Stavrov 1997), and hence, the Fe-C bond order. The CO oxygen atom is positioned between the side chains of His63 and Val67 (Fig. 3A) such that it is in van der Waals contact with His63 C ϵ 2, Val67 C γ 1 and Leu106 C δ 2. This packing is similar to that in other CO-Hb structures, however, and we suggest that the longer Fe-C bond and more angular bonding in Hb Bart's results from reduced Fe-CO back-bonding, due to the positive charge on the nearby His63.

Azide binding

In contrast to the CO binding in CO- γ_4 , the azide binding in NNN- γ_4 appears close to optimal. The average Fe-N1-N2 angle is 115° (Table 2), close to the ideal sp^2 bond angle of 120° , and very similar to that found in other azido-met globin structures; 118° in the hemoglobin-like molecule from *Vitreoscilla stercoraria* (VtHb, PDB entry 2VHB; Tarricone et al. 1997), 119° in horse myoglobin (HMb, PDB entry 1AZI; Maurus et al. 1998), and 117° in sperm whale myoglobin (SWMb, PDB entry 1SWM; Rizzi et al. 1993). This orientation allows a short, linear hydrogen bond with the distal histidine in which the lone pair on the azide N1

atom and a hydrogen on the protonated N ϵ 2 of His63 would be directed precisely towards each other. This interaction presumably also explains why the azide ligand is bound with the Fe-N1 bond colinear with the heme normal. The Fe-N1 bond length (average 2.17 Å) is also similar to that in Mb (Maurus et al. 1998). Back-bonding between Fe(III) and azide should be less significant than between Fe(II) and CO, and protonation of His63 should in this case be favorable to ligand binding. The overall orientation of the azide ligand in each subunit of NNN- γ_4 is also very similar to that in the other met-azido globin structures; it is oriented towards the hydrophobic back of the heme pocket, in contact with His63, Val67, and Leu106, with the N3 atom positioned approximately over the C1C heme atom.

Quaternary structure

Three quaternary states have been characterized crystallographically for heterotetrameric Hbs; one unliganded T state (Fermi et al. 1984; Kavanaugh et al. 1992) and two liganded states; the R (Shaanan 1983; Derewenda et al. 1990) and the R2 (Silva et al. 1992) or Y state (Smith et al. 1991; Smith and Simmons 1994). The R and R2 states are similar, and there is debate as to whether one or other is an intermediate in the transition between liganded and unliganded quaternary structures, or whether both are variants of a single R state (Tame 1999).

Multiple superpositions show that the liganded Hb Bart's structures most closely resemble the standard R state of the heterotetrameric Hbs. In the latter, the $\alpha\beta$ dimer interface ($\alpha 1\beta 1$ or $\alpha 2\beta 2$) is the most extensive in the tetramer, and changes least during the R-to-T transition, that is, the $\alpha\beta$ dimer remains largely intact. Superposition of the $\gamma\alpha$ subunit of Hb Bart's on to the individual subunits of R-state HbA (human oxy-Hb; Shaanan 1983) gives an rms difference of 1.03 Å (132 C α) for the HbA α subunit and 0.87 Å (145 C α) for the β subunit. Superposition of the entire γ_2 (AB) dimer of Hb Bart's on to the $\alpha\beta$ dimer of HbA shows that they, too, are very similar; the rms difference of 1.11 Å (277 C α) for the whole dimer is only slightly more than for the individual subunits. If one subunit pair is first superimposed (e.g., $\gamma\alpha$ on α) only a small rotation (1.8°) is required to superimpose the other ($\gamma\beta$ on β). If the R2-state HbA structure (Silva et al. 1992) is used, a larger rotation (4.0°) is needed. Considering now the tetramer; if one dimer pair is superimposed (γ_2 (AB) on $\alpha 1\beta 1$) a rotation of 8.3° is required to superimpose the other dimer pair (γ_2 (A'B') on $\alpha 2\beta 2$) if the R state is assumed, and 19.9° if the R2 state is assumed. Thus, the γ_4 homotetramer much more closely resembles the standard R state (Fig. 4).

Essentially the same quaternary structure is shared by the liganded β_4 homotetramer (Borgstahl et al. 1994a); the γ_2 dimer can be superimposed on to the β_2 dimer with an rms difference of only 0.82 Å (individual subunits 0.75 Å) and

a rotation of only 1.0° is then required to superimpose the other dimer pair; the whole γ_4 tetramer can be superimposed on to β_4 with an rms difference of only 0.90 Å. We conclude that this quaternary structure is characteristic of a liganded β -type homotetramer, and is not affected by sequence differences in the individual subunits.

To compare the subunit interfaces of CO- γ_4 with those of CO- β_4 and oxy- $\alpha_2\beta_2$ we calculated the surface area buried in each interface, using a 1.4-Å probe, with the program AREA-MOL from the CCP4 program suite (Collaborative Computational Project Number 4 1994); these results are summarized in Table 3.

The most extensive interface in HbA is the $\alpha 1\beta 1$ (or $\alpha 2\beta 2$) interface, formed primarily by residues from the BC and GH corners of each subunit; residues 30–36 and 103–126 of the α subunit and 30–35 and 111–131 of the β subunit. In CO- γ_4 and CO- β_4 , this interface is slightly reduced in size; to 1580 Å² in γ_4 and 1730 Å² in β_4 , compared with 1805 Å² in HbA. The greater reduction in γ_4 is due largely to the substitution of His116 in β by Leu116 in γ , and of Cys112 in β by Thr112 in γ , and a 2–3° rotation of helix G, to which these residues belong, in γ_4 relative to β_4 . In HbA and in β_4 , His116 N ϵ 2 is hydrogen bonded to the carbonyl oxygen of Pro114 across the interface, but this interaction cannot occur in γ_4 because of the His $\rightarrow$ Leu substitution. In β_4 , the Cys112 side chains from both subunits adopt dual conformations to avoid mutual steric conflicts; the closest approach of the two sulfur atoms is 2.99 Å. In γ_4 , however, the rotation of helix G prevents a steric clash between the two Thr112 side chains despite conformations remarkably similar to the dual conformations of the cysteine side chains of β_4 .

The greatest difference between γ_4 and HbA is in the $\alpha 1\beta 2$ interface, which is significantly enhanced in the homotetramer, to be almost as extensive as the $\alpha 1\beta 1$ interface. This enhancement is also a feature of β_4 . This interface involves primarily the 3_{10} -helix C (residues 35–42), and residues 96–101 of the FG corner, and the increased interfaces in γ_4 and β_4 arise from interactions between the Arg40 and Asp99 side chains from both subunits. In the β_4 structure (Borgstahl et al 1994a), equally populated dual conformations are found for both Arg40 and Asp99, in both subunits, allowing two populations of Arg40...Asp99 salt bridges at this interface. The equivalent γ_4 interface also has Arg40 and Asp99 in close proximity to each other and Arg40 and Asp99 of the adjacent symmetry-related subunit. However, the electron density for these residues is not as clearly defined in γ_4 , suggesting that these four residues at this interface are mobile.

Homotetramer symmetry requires that the $\alpha 1\alpha 2$ and $\beta 1\beta 2$ interfaces are equivalent in γ_4 . These interfaces involve mainly the chain termini, residues 1–2 and 143–146 and are significantly larger in γ_4 than in β_4 (Table 3) because of the displacement of the γ -chain N-terminus relative

Fig. 4. Superposition of CO- γ_4 (sky-blue) upon R-state adult oxy-hemoglobin, O₂- $\alpha_2\beta_2$ (α in yellow, β in orange). The alignment was based on superposition of the two γ -chains on to the two β -chains, and shows clearly the displacement of the A helix and N-terminus (indicated) of the γ -chain relative to the β -chain. On the other hand, the N-terminus of the α -chain in $\alpha_2\beta_2$ corresponds closely with that of the γ -chain in γ_4 . As observed for β_4 (Borgstahl et al., 1994a), γ_4 most closely resembles the R-state of liganded adult hemoglobin (Shaanan, 1983). Figures were drawn using MOLSCRIPT (Kraulis 1991) and RASTER3D (Merritt and Murphy 1994).

to that of the β -chain (Fig. 4). This brings it closer to the C-terminus of the neighboring subunit. The substitution of Ser143 in the γ -chain for His143 in the β -chain also increases the interface in γ_4 . In heterotetrameric HbA, in contrast, the $\alpha_1\alpha_2$ interface is slightly larger than that in γ_4 (and includes a salt bridge between the chain termini that is not present in either homotetramer), whereas the $\beta_1\beta_2$ interface is much smaller.

Discussion

An important feature of hemoglobin is that the individual globin chains are synthesized separately and must then as-

semble into functional heterotetrameric Hb molecules. Because the β -type (but not α -type) globin chains also have the ability to self-assemble into stable homotetramers, these homotetrameric species must dissociate prior to formation of the functional heterotetramer. Structural differences between the γ_4 , β_4 and $\alpha_2\beta_2$ Hb tetramers thus offer clues as to what factors influence their association/dissociation behavior, and ultimately correct Hb self-assembly.

Differences in association/dissociation behavior of β and γ chains

Both the β and γ globins can form stable homotetramers, and our structural comparisons show that their quaternary structures are extremely similar. Why, then, does the self-assembly mechanism for the γ chain go through a stable γ_2 dimer intermediate (Adachi et al. 2000; Kidd et al. 2001), whereas the β chain shows very little dimer formation, and the β_4 homotetramer assembles directly from monomers (Bunn and Forget 1986)? The nature of the γ_2 dimer is unknown, but the largest interface in the γ_4 homotetramer is

Table 3. Comparison of subunit interfaces^a

	HbA ($\alpha_2\beta_2$)	Bart's (γ_4)	HbH (β_4)
$\alpha_1\beta_1$ interface	1805	1580	1735
$\alpha_1\alpha_2$ interface	800	700	500
$\alpha_1\beta_2$ interface	1010	1200	1325
$\beta_1\beta_2$ interface	350	700	500

^a Buried surface areas ($\text{\AA}^2$) given for each interface.

that equivalent to the $\alpha 1\beta 1$ (or $\alpha 2\beta 2$) interface in HbA, and it is reasonable to assume that this might be the γ_2 dimer interface. Differences in this interface may therefore explain the differences in homodimer formation. Moreover, we note that the other major interface in the homotetramers, equivalent to $\alpha 1\beta 2$, is essentially the same in γ_4 and β_4 , dominated by the Arg40/Asp99 pairs contributed by both subunits.

Four residues at the $\alpha 1\beta 1$ interface in β_4 differ from those in γ_4 . These are $\beta 51\text{Pro}$, $\beta 112\text{Cys}$, $\beta 116\text{His}$, and $\beta 125\text{Pro}$ versus $\gamma 51\text{Ala}$, $\gamma 112\text{Thr}$, $\gamma 116\text{Ile}$, and $\gamma 125\text{Glu}$. Mutagenesis studies suggest that only the residue at position 116 contributes to differences in heterotetramer formation with α chains (Adachi et al. 2000). This residue also accounts for the ability of γ chains to form stable homodimers. When $\gamma 116\text{Ile}$ is mutated to His, γ chains then form monomers in addition to dimers just like β . Size-exclusion chromatography results suggest that the substitution of His for Ile at 116 promotes the dissociation of γ_4 and causes it to follow an assembly pathway analogous to the $4\beta \leftrightarrow \beta_4$ pathway instead of the wild-type $4\gamma \leftrightarrow 2\gamma_2 \leftrightarrow \gamma_4$ pathway (analogous to HbA). Curiously, the interface equivalent to $\alpha 1\beta 1$ is actually larger in β_4 than in γ_4 (Table 3), yet it is less stable. The His64Ile mutation suggests that it is the burial of the hydrophobic Ile116 in the γ oligomer, compared with the protic His116 in the β oligomer, that is critical in giving enhanced stability, both because of the hydrophobic effect and because His116 is expected to be protonated in the pH range in which oligomer dissociation occurs. In addition, repulsive interactions between the two sulfurs of $\beta\text{Cys}112$ in this interface, even if in two conformations, should be stronger in β_4 compared to the two oxygens of $\gamma\text{Thr}112$ in γ_4 , thus weakening this interface even further in β_4 .

Why are homotetramers formed by β -type chains but not by α -type chains?

Comparison of the subunit interfaces in the γ_4 and β_4 heterotetramers with those in the $\alpha_2\beta_2$ heterotetramer of HbA highlights several notable features. First, in both γ_4 and β_4 homotetramers the interface equivalent to $\alpha 1\beta 2$ is more extensive (Table 3) and more tightly packed (Borgstahl et al. 1994a) than the same interface in HbA, and includes the cluster of Arg40/Asp99 pairs. Much of this enhancement comes from the presence of β -chain residues Trp37 and Arg40 (both Thr in the α -chain). Second, the $\beta 1\beta 2$ interface in heterotetramers is very small and contains no specific interactions, whereas the equivalent interface in the homotetramers is significantly larger (Table 3) and contains specific interactions between the N- and C-terminal residues. These latter interactions are more extensive in γ_4 than in β_4 , which may perhaps imply greater homotetramer stability for γ_4 . In combination, however, these two effects, which stabilize homotetramer formation by the β - and γ -globin

chains, arise from features specific to these chains, explaining why α -chains do not form stable homotetramers.

Materials and methods

Crystallization and data collection

The Hb Bart's crystals used in this work were obtained unexpectedly during attempts to crystallize the human embryonic hemoglobin Portland $\zeta_2\gamma_2$; crystals grew over a wide range of pH (4.5–8.5) but the poorly diffracting needle-shaped crystals obtained at higher pH proved to be Hb Portland, whereas the block-shaped crystals obtained at lower pH were of Hb Bart's. Evidently, dissociation of the ζ and γ subunits occurred at pH below 5.0, leading to the reassociation of the γ subunits as the homotetramer γ_4 (Kidd et al. 2001).

The recombinant hemoglobin Portland used was expressed in *Saccharomyces cerevisiae* and purified as previously described (Mould et al. 1994; Hofmann et al. 1995). The preparation of the NNN- γ_4 crystals is described in Kidd et al. (2001). For preparation of the carbonmonoxy form of the protein, carbon monoxide gas was gently bubbled through solutions of hemoglobin Portland. To prevent oxidation, 2 mM sodium dithionite was added to the protein prior to crystallization. All crystallization trials were set up in a glove box filled with carbon monoxide gas, and all solutions were degassed and purged with carbon monoxide. Crystallization conditions were found using a search procedure based on orthogonal arrays (Kingston et al. 1994). Crystals were grown in the dark, by hanging-drop vapor diffusion at 18°C. Equal volumes of protein solution (40 mg/mL in 0.1 M HEPES buffer (pH 7.0) with 2 mM sodium dithionite) and reservoir solution were mixed, with the best-diffracting crystals ($0.2 \times 0.2 \times 0.2$ mm) obtained with a reservoir solution of 0.2 M acetic acid/KOH (pH 4.5–4.9) and 21% MePEG 5000. The best met-azido crystals were similarly obtained at low pH (pH 4.9).

Both the carbonmonoxy and met-azido crystals were orthorhombic, $P2_12_12$, with nearly identical unit-cell parameters $a = 61$, $b = 82$, and $c = 53$ Å (Table 4), and differed from those originally obtained for the met form of Hb Bart's (Czerwinski et al. 1981). Assuming two globin chains in the asymmetric unit, the crystal density (V_m) is calculated to be $2.1 \text{ Å}^3 \text{ Da}^{-1}$ with a solvent

Table 4. Crystal and data collection statistics

	CO- γ_4	NNN- γ_4
Space group	$P2_12_12$	$P2_12_12$
Cell dimensions:		
a (Å)	60.62	60.54
b (Å)	82.84	81.63
c (Å)	53.58	53.04
Reflections measured	105,161	156,828
Unique reflections	27,848	22,691
Maximum resolution (Å)	1.7	1.86
Completeness ^a (%)	91.5 (98.5)	99.7 (98.3)
Multiplicity ^a	3.8 (3.7)	6.9 (5.9)
$R_{\text{merge}}^{\text{a,b}}$ (%)	7.4 (28.3)	6.4 (26.1)
Mean ^a $I/\sigma I$	8.7 (3.0)	14.1 (6.9)

^a Figures in parentheses are for the outermost resolution shell: 1.76–1.70 Å for CO- γ_4 , and 1.93–1.86 Å for NNN- γ_4 .

^b $R_{\text{merge}} = \sum_i \sum_j |I_{h,i} - \langle I_h \rangle| / \sum_i \sum_j I_{h,i}$.

content of 41% (v/v) for both structures (Matthews 1968). A data set to 1.86 Å resolution was collected for a flash-frozen crystal of the met-azido form as described in Kidd et al. (2001). For the carbonmonoxy form, 30% glycerol was added to the reservoir solution as a cryoprotectant. X-ray data to 1.7 Å were collected at 110 K using a MAR Research 345 image plate detector with synchrotron radiation from beamline 7-1 at the Stanford Synchrotron Radiation Laboratory. Data were collected as series of 0.5° oscillation frames, each of 10-s exposure, at a crystal to detector distance of 200 mm. Image data were processed and scaled using the DENZO and SCALEPACK (Otwinowski and Minor 1997) software. Data collection statistics for both crystal forms are summarized in Table 4.

Structure determination and refinement

Both structures were solved by molecular replacement. Because the crystals grew from solutions of either CO- or NNN- $\zeta_2\gamma_2$, we assumed that a $\zeta\gamma$ dimer was present in the crystal asymmetric unit. An $\alpha\gamma$ dimer from the 2.5 Å structure of deoxy fetal Hb (PDB code 1FDH; Frier and Perutz 1977), with side chains that differed between α and ζ truncated to Ala or Gly, was therefore taken as an initial search model. Molecular replacement using AMoRe (Navaza 1994), with data in the resolution range 10.0 to 3.5 Å failed to give a clear rotation solution, however. Assuming the failure was due to use of a T-state, unliganded, Hb as search model, we next used a liganded $\alpha\beta$ dimer from the 1.7 Å structure of carbonmonoxy adult Hb (PDB code 1BBB; Silva et al. 1992) with side chains that differed between α and ζ chains and between β and γ chains truncated to their common atoms. Using CNS (Brünger et al. 1999), rotation and translation function calculations based on data between 15 and 4 Å gave a single unambiguous solution. After rigid-body refinement in which each subunit was treated as a separate rigid body the R -factor was 45.4% and R_{free} was 47.2%. For the R_{free} calculation (Brünger 1992) in this and subsequent refinement steps, 10% of the reflections were randomly selected. The model was then built into $2F_o - F_c$ and $F_o - F_c$ electron density maps using O (Jones et al. 1991). The γ -chain side chains were straightforward to build; however, the side chains for a number of the ζ chain residues did not fit the density. Model building from a "generic" globin poly-alanine dimer model yielded the same result: 100% of the γ chain residues fit their density but only about 60% of the ζ chain residues fit well. Interestingly, the ζ chain had extra density around residue 50, at the site of a six-residue insertion that is found in β -type but not α -type globins. At this point, we suspected that we had a gamma dimer in the asymmetric unit and had actually crystallized γ_4 rather than $\zeta_2\gamma_2$. Sequencing via density revealed that over 95% of the structure could be built as gamma. This was confirmed by the automatic model-building program wARP (Lamzin and Wilson 1997). Biochemical analysis (Kidd et al. 2001) then confirmed that the crystals contained only γ chains, implying that we had crystallized γ_4 , Hb Bart's.

Molecular replacement calculations using CNS were repeated using a β_2 dimer from the 1.8 Å structure of CO- β_4 (PDB code 1CBM; Borgstahl et al. 1994a), with CO removed and side chains that differed between β and γ truncated to their common atoms. The R -factor after rigid-body refinement was 27.6% and the R_{free} 27.6%. The structure was refined by alternating cycles of CNS refinement (using data between 25 and 1.7 Å) with model rebuilding using O. Typically, CNS refinement included the following steps (in order): simulated annealing, individual B-factor refinement, water picking, and conjugate gradient minimization refinement. To check that the planar heme and the long Fe-C bond were not artefacts of the refinement protocols, the final stages of refine-

ment were repeated using REFMAC (Murshudov et al. 1997), with no restraints on the Fe-C bond length or Fe-C-O bond angle. This resulted in minimal change. The final R (R_{free}) values were 21.1% (24.4%).

NNN- γ_4 was built directly from CO- γ_4 (CO atoms removed) and after refinement has a final R (R_{free}) value of 18.4% (22.0%). The refinement protocol followed that for CO- γ_4 . Full refinement statistics for both structures are given in Table 1. Structural superpositions were carried out with the program LSQKAB from the CCP4 program suite (Collaborative Computing Project Number 4 1994), and the quality of the final model was analyzed with PROCHECK (Laskowski et al. 1993). RCSB Protein Data Bank accession numbers for CO- γ_4 and NNN- γ_4 are 1I3D and 1I3E, respectively.

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References

- Adachi, K., Zhao, Y., Yamaguchi, T., and Surrey, S. 2000. Assembly of γ - with α -globin chains to form human fetal hemoglobin *in vitro* and *in vivo*. *J. Biol. Chem.* **275**: 12424-12429.
- Ager, J.A.M. and Lehmann, H. 1958. Observations on some "fast" haemoglobins: K, J, N, and "Bart's." *Br. Med. J.* **1**: 929-931.
- Allen, D.W., Wyman Jr., J., and Smith, C.A. 1953. The oxygen equilibrium of fetal and adult human hemoglobin. *J. Biol. Chem.* **203**: 81-87.
- Benesch, R. and Benesch, R.E. 1974. Homos and heteros among the hemos. *Science* **185**: 905-908.
- Benesch, R., Benesch, R.E., and Enoki, Y. 1968. The interaction of hemoglobin and its subunits with 2,3-diphosphoglycerate. *Proc. Natl. Acad. Sci.* **61**: 1102-1106.
- Borgstahl, G.E., Rogers, P.H., and Arnone, A. 1994a. The 1.8 Å structure of carbonmonoxy- β_4 hemoglobin. Analysis of a homotetramer with the R quaternary structure of liganded $\alpha_2\beta_2$ hemoglobin. *J. Mol. Biol.* **236**: 817-830.
- . 1994b. The 1.9 Å structure of deoxy- β_4 hemoglobin. Analysis of the partitioning of quaternary-associated and ligand-induced changes in tertiary structure. *J. Mol. Biol.* **236**: 831-843.
- Brünger, A.T. 1992. Free R-value: A novel statistical quantity for assessing the accuracy of crystal structures. *Nature* **355**: 472-475.
- Brünger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N.S., et al. G.L. 1999. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr.* **D54**: 905-921.
- Bunn, H.F. and Forget, B.G. 1986. *Hemoglobin: Molecular, genetic and clinical aspects*. W. B. Saunders, Philadelphia.
- Collaborative Computational Project, Number 4. 1994. The CCP4 suite: Programs for protein crystallography. *Acta Crystallogr.* **D50**: 760-763.
- Czerwinski, E.W., Risk, M., and Matustik, M.C. 1981. Crystallization and preliminary X-ray diffraction studies of methemoglobin Bart's. *J. Biol. Chem.* **256**: 13128-13129.
- Dance, N. and Huehns, E.R. 1962. A haemoglobin containing only δ -chains. *Biochem. Biophys. Res. Commun.* **7**: 444-447.
- Dance, N., Huehns, E.R., and Beaven, G.H. 1963. The abnormal haemoglobins in haemoglobin-H disease. *Biochem. J.* **87**: 240-248.
- Derewenda, Z., Dodson, G., Emsley, P., Harris, D., Nagai, K., Perutz, M., and Reynaud, J.-P. 1990. Stereochemistry of carbon monoxide binding to normal human adult and Cowtown haemoglobins. *J. Mol. Biol.* **211**: 515-519.

- Dickerson, R.E. and Geis, I. 1983. *Hemoglobin: Structure, function, evolution, and pathology*. Benjamin/Cummings, Menlo Park.
- Esnouf, R.M. 1997. An extensively modified version of molscript that includes generally enhanced colouring capabilities. *J. Mol. Graph. Model.* **15**: 132–134.
- Fermi, G., Perutz, M.F., Shaanan, B., and Fourme, R. 1984. The crystal structure of human deoxyhaemoglobin at 1.74 Å resolution. *J. Mol. Biol.* **175**: 159–174.
- Frier, J.A. and Perutz, M.F. 1977. Structure of human foetal deoxyhaemoglobin. *J. Mol. Biol.* **112**: 97–112.
- Hofmann, O., Mould, R., and Brittain, T. 1995. Allosteric modulation of oxygen binding to the three human embryonic haemoglobins. *Biochem. J.* **306**: 367–370.
- Huehns, E.R. and Beavan, G.H. 1962. The reaction of haemoglobin α^A with haemoglobin H and haemoglobin "Bart's." *Biochem. J.* **40**: 40P.
- Huisman, T.H.J. 1981. The human fetal hemoglobins. *Tex. Rep. Biol. Med.* **40**: 29–42.
- Jones, T.A., Zou, J.Y., Cowan, S.W., and Kjeldgaard, M. 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr.* **A47**: 110–119.
- Kavanaugh, J.S., Rogers, P.H., and Arnone, A. 1992. High resolution X-ray study of deoxy recombinant human hemoglobins synthesized from β -globins having mutated amino termini. *Biochemistry* **31**: 8640–8647.
- Kidd, R.D., Mathews, A.J., Baker, H.M., Brittain, T., and Baker, E.N. 2001. Subunit dissociation and reassociation leads to preferential crystallization of hemoglobin Bart's (γ_4) from solutions of human embryonic hemoglobin Portland ($\zeta_2\gamma_2$) at low pH. *Acta Crystallogr.* **D57**: 921–924.
- Kingston, R.L., Baker, H.M., and Baker, E.N. 1994. Search designs for protein crystallization based on orthogonal arrays. *Acta Crystallogr.* **D50**: 429–440.
- Kraulis, P.J. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**: 946–950.
- Kurtz, A., Rollem, H.S., and Baurer, C. 1981. Heterotropic interactions in monomeric β^{SH} chains from human hemoglobin. *Arch. Biochem. Biophys.* **210**: 200–203.
- Kushkuley, B. and Stavrov, S.S. 1997. Theoretical study of the electrostatic and steric effects on the spectroscopic characteristics of the metal–ligand unit of heme proteins. *Biophys. J.* **72**: 899–912.
- Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**: 283–291.
- Lamzin, V.S. and Wilson, K.S. 1997. Automated refinement for protein crystallography. *Methods Enzymol.* **277**: 269–305.
- Lehmann, H. and Huntsman, R.G. 1974. *Man's Haemoglobins* (2nd ed.). North-Holland Publishing, Amsterdam.
- Li, X.-Y. and Spiro, T. G. 1988. Is bound carbonyl linear or bent in heme proteins? Evidence from resonance Raman spectroscopic data. *J. Am. Chem. Soc.* **110**: 6024–6033.
- Manning, J.M., Dumoulin, A., Manning, L.R., Chen, W., Padovan, J.C., Chait, B.T., and Popowicz, A. 1999. Remote contributions to subunit interactions: Lessons from adult and fetal hemoglobins. *Trends Biochem. Sci.* **24**: 211–212.
- Matthews, B.W. 1968. Solvent content of protein crystals. *J. Mol. Biol.* **33**: 491–497.
- Maurus, R., Bogumil, R., Nguyen, N.T., Mauk, A.G., and Brayer, G. 1998. Structural and spectroscopic studies of azide complexes of horse heart myoglobin and the His-64 $\rightarrow$ Thr variant. *Biochem. J.* **332**: 67–74.
- Merritt, E.A. and Murphy, M.E.P. 1994. Raster3D version 2.0. A program for photorealistic molecular graphics. *Acta Crystallogr.* **D50**: 869–873.
- Mould, R.M., Hofmann, O.M., and Brittain, T. 1994. Production of human embryonic haemoglobin (Gower II) in a yeast expression system. *Biochem. J.* **298**: 619–622.
- Murshudov, G.N., Vagin, A.A., and Dodson, E. J. 1997. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr.* **D53**: 240–255.
- Navaza, J. 1994. AMoRe: An automated package for molecular replacement. *Acta Crystallogr.* **A50**: 157–163.
- Otwinowski, Z. and Minor, W. 1997. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**: 307–326.
- Peng, S.M. and Ibers, J.A. 1976. Stereochemistry of carbonylmetalloporphyrins. The structure of (pyridine) (carbonyl) (5, 10, 15, 20-tetraphenylprophinate) iron(II). *J. Am. Chem. Soc.* **98**: 682–689.
- Philo, J.S., Lary, J.W., and Schuster, T.M. 1988. Quaternary interactions in hemoglobin β subunit tetramers. *J. Biol. Chem.* **263**: 682–689.
- Ray, G.B., Li, X.-Y., Ibers, J.A., Sessler, J.L., and Spiro, T.G. 1994. How far can proteins bend the FeCO unit? Distal polar and steric effects in heme proteins and models. *J. Am. Chem. Soc.* **116**: 162–176.
- Rigas, D.A., Koler, R.D., and Osgood, E.E. 1956. Hemoglobin H. Clinical, laboratory, and genetic studies of a family with a previously undescribed hemoglobin. *J. Lab. Clin. Med.* **47**: 51–64.
- Rizzi, M., Ascenzi, P., Coda, A., Brunori, M., and Bolognesi, M. 1993. Molecular bases for heme:ligand recognition in sperm whale (*Physeter catodon*) and *Aplysia limacina* myoglobin. *Rend. Fis. Acc. Lincei IX* **4**: 65–73.
- Shaanan, B. 1983. Structure of oxyhaemoglobin at 2.1 Å resolution. *J. Mol. Biol.* **171**: 31–59.
- Silva, M.M., Rogers, P.H., and Arnone, A. 1992. A third quaternary structure of human hemoglobin A at 1.7 Å resolution. *J. Biol. Chem.* **267**: 17248–17256.
- Smith, F.R. and Simmons, K.C. 1994. Cyanomet human hemoglobin crystallized under physiological conditions exhibits the Y quaternary state. *Proteins Struct. Funct. Genet.* **18**: 295–300.
- Smith, F.R., Lattman, E.E., and Carter, C.W. 1991. The mutation $\beta 99$ Asp-Tyr stabilizes Y, a new composite quaternary state of human hemoglobin. *Proteins Struct. Funct. Genet.* **10**: 81–91.
- Tame, J.R.H. 1999. What is the true structure of liganded haemoglobin? *Trends Biochem. Sci.* **24**: 372–377.
- Tarricone, C., Galizzi, A., Coda, A., Ascenzi, P., and Bolognesi, M. 1997. Unusual structure of the oxygen-binding site in the dimeric bacterial hemoglobin from *Vitreoscilla* sp. *Structure* **5**: 497–507.
- Valdes, R.J. and Ackers, G.K. 1977. Thermodynamic studies on subunit assembly in human hemoglobin: Self-association of oxygenated chains (α^{SH} and β^{SH}): Determination of stoichiometries and equilibrium constants as a function of temperature. *J. Biol. Chem.* **252**: 74–81.
- Wasi, P. 1986. Geographic distribution of hemoglobin variants in Southeast Asia. In *Haemoglobin variants in human populations, Vol II* (ed. W.P. Winter), pp. 111–127. CRC Press, Boca Raton, FL.
- Weatherall, D.J., Pembrey, M.E., and Prichard, J. 1974. Fetal haemoglobin. *Clin. Hematol.* **3**: 467–508.
- Yang, F. and Phillips Jr., G.N. 1996. Crystal structures of CO-, deoxy- and met-myoglobins at various pH values. *J. Mol. Biol.* **256**: 762–774.