Sireesha Ratakonda,^a
Arvind Anand,^b Kanak Dikshit,^b
Benjamin C. Stark^a and
Andrew J. Howard^{a*}

^aBiology Division, Department of Biological and
Chemical Sciences, Illinois Institute of
Technology, Chicago, IL 60616, USA, and

^bInstitute of Microbial Technology, Sector 39A,
Chandigarh 160 036, India

Correspondence e-mail: howard@iit.edu

Received 15 September 2012

Accepted 29 October 2012

PDB References: *Vitreoscilla* hemoglobin,
3tm3; Tyr29Phe mutant, 3tld; Tyr29Ala mutant,
3tm9

Crystallographic structure determination of B10 mutants of *Vitreoscilla* hemoglobin: role of Tyr29 (B10) in the structure of the ligand-binding site

Site-directed mutants of the gene encoding wild-type *Vitreoscilla* hemoglobin were made that changed Tyr29 (B10) of the wild-type *Vitreoscilla* hemoglobin (VHb) to either Phe or Ala. The wild-type and the two mutant hemoglobins were expressed in *Escherichia coli* and purified to homogeneity. The binding of the two mutants to CO was essentially identical to that of wild-type VHb as determined by CO-difference spectra. Circular-dichroism spectra also showed the two mutants to be essentially the same as wild-type VHb regarding overall helicity. All three VHbs were crystallized and their structures were determined at resolutions of 1.7–1.9 Å, which are similar to that of the original wild-type structure determination. The Tyr29Phe mutant has a structure that is essentially indistinguishable from that of the wild type. However, the structure of the Tyr29Ala mutant has significant differences from that of the wild type. In addition, for the Tyr29Ala mutant it was possible to determine the positions of most of the residues in the D region, which was disordered in the originally reported structure of wild-type VHb as well as in the wild-type VHb structure reported here. In the Tyr29Ala mutant, the five-membered ring of proline E8 (Pro54) occupies the space occupied by the aromatic ring of Tyr29 in the wild-type structure. These results are discussed in the context of the proposed role of Tyr29 in the structure of the oxygen-binding pocket.

1. Introduction

The hemoglobin from the bacterium *Vitreoscilla* sp. C2 (VHb) was the first to be discovered and is probably the best characterized of the many known microbial hemoglobins. It is a homodimer, the monomers of which are single-domain proteins that contain a single heme molecule to which the usual hemoglobin ligands can bind (Webster, 1987). VHb can bind oxygen reversibly, which is in line with one of its best characterized functions, namely to bind oxygen and deliver it to the terminal respiratory oxidase, particularly under hypoxic conditions (Webster, 1987; Ramandeep *et al.*, 2000; Park *et al.*, 2002). VHb also appears to have a number of other functions, including delivery of oxygen to oxygenases (Lin *et al.*, 2003), detoxification of NO (Kaur *et al.*, 2002) and the activation of at least one oxygen-responsive transcription factor (Anand *et al.*, 2010). It is possible that VHb has many effects on cell physiology, as its expression in *Escherichia coli* results in substantial changes in the expression of hundreds of genes (Roos *et al.*, 2004).

The first X-ray crystallographic determination of the structure of VHb was made by Tarricone and coworkers (Tarricone, Calogero *et al.*, 1997; Tarricone, Galizzi *et al.*, 1997). It showed that each monomer of the dimer assumes a classic globin fold. Within this basic structure, however, VHb has some unique features. One of these is that the region which is the (ordered) D helix in the standard globin fold is disordered, with little or no electron density visible for amino acids Asp44–Glu52. The second is the absence of a histidine as a ligand in the distal site for bound oxygen. In its place at the E7 position is a glutamine, which could substitute for histidine, except that its side

chain points away from the oxygen-binding site. The lack of a role for GlnE7 in oxygen binding was confirmed by functional studies of site-directed mutants in this and the adjacent position (Dikshit *et al.*, 1998; Verma *et al.*, 2005). A mutational study of the D region suggests that it may be involved in heme binding or the binding of partner proteins (Lee *et al.*, 2004).

Apparently related to the absence of an oxygen-binding role for GlnE7 is a network of hydrogen-bonded residues in the distal (oxygen-binding) pocket of VHb, in which Tyr29 (B10) is thought to play a prominent role. Given the attention paid to this particular residue by previous authors, we chose to investigate the role of Tyr29 in more detail. Accordingly, Tyr29Ala and Tyr29Phe mutant VHb genes were constructed and the corresponding mutant VHbs were expressed. Spectrometric and crystallographic studies of both mutants as well as of wild-type VHb provided interesting information about the function of Tyr29 as well as the structure of the D region. In this study, we report the structural and functional consequences of these mutations.

2. Experimental

2.1. Site-directed mutagenesis

Site-directed mutagenesis of wild-type *vgb* was accomplished as described by Dikshit *et al.* (1998) by using gene-specific primers to amplify two portions of *vgb* (one primer containing the necessary mutation) and then joining them together by PCR overlap. The Tyr29Ala mutant gene was cloned into the *Sma*I site of pBluescript 11KS+ (Stratagene) under the control of the native *vgb* gene promoter to form pY29A, whereas the Tyr29Phe mutant gene was cloned in pET-28c(+) (Novagen) at the *Nde*I and *Bam*HI sites to form pY29F. Wild-type *vgb* was cloned into pBluescript 11KS+ along with its native promoter at its *Sma*I site to form pNKD1, as described previously (Dikshit *et al.*, 1998). The purified plasmids (pNKD1, pY29A and pY29F) were sequenced at the Northwestern University Departmental Genomics Core Facility. In all cases the sequences confirmed the expected amino-acid sequence of the VHb encoded by each plasmid, with the wild-type amino-acid sequence for all three VHbs apart from the expected single-amino-acid changes noted above for each of the two mutants.

2.2. Purification of wild-type and mutant VHbs

Each of the three plasmids noted in §2.1 were transformed into *E. coli* DH5a and the resulting strains were grown in 2 l Fernbach flasks containing 1.5 l Terrific Broth (Sambrook & Russell, 2001) for 18–20 h. Cells were harvested by centrifugation and lysed for 30 min using 330 µg ml⁻¹ egg-white lysozyme in a lysis buffer consisting of 50 mM Na₂HPO₄ pH 8.0, 300 mM NaCl using 3 ml buffer per gram of cell pellet. The cell lysates were incubated with DNase I (20 µl of 1 mg ml⁻¹ solution per gram of cell material) at room temperature for 30 min. The lysate was then centrifuged at 13 000 rev min⁻¹ for 15 min and the supernatant was filtered and concentrated by ammonium sulfate precipitation at 277 K. The pellet, which was distinctly red in color, was collected and redissolved in 50 mM sodium phosphate pH 8.0. The redissolved ammonium sulfate precipitate was subjected to gel-filtration chromatography on a 75 × 1.5 cm Sephadex G-100 column equilibrated and eluted with 50 mM sodium phosphate pH 8.0. Fractions were monitored for absorbance at both 280 nm (total protein) and 410 nm (heme-containing protein). Fractions from the second 410 nm peak (containing VHb) were pooled and saved. Off-peak fractions from the second peak were pooled and

rerun on the G-100 column and fractions from the resulting peak were combined with those from the first run.

The combined G-100 peak pool was loaded onto a 16 × 1.0 cm DEAE Sephadex A-50 column equilibrated in the G-100 running buffer. The column was eluted stepwise with 1.5 column volumes of 0.05, 0.1, 0.2 and (in some cases) 0.35 M NaCl in the same buffer. The fractions were again monitored at both 280 and 410 nm and the 410 nm peak fractions were pooled. This pool was then chromatographed on a 5 ml Octyl Sepharose Fast Flow hydrophobic column (GE Healthcare). The wash buffer used was 25 mM sodium phosphate, 1 M ammonium sulfate pH 6.0. Elution took place using 25 mM sodium phosphate, 30% acetonitrile pH 6.0.

The Tyr29Phe mutant, constructed with a hexahistidine tag and a thrombin cleavage site at the N-terminal end, required an additional purification step after DEAE Sephadex A-50 chromatography. The His tag was removed from partially purified VHb by thrombin cleavage according to the instructions of the supplier of the vector (Novagen). It was observed that 0.02 units of enzyme cleaved 10 µg protein sample in approximately 3 h at room temperature. The cleaved VHb protein contained two extra amino acids, namely serine and histidine, at the N-terminus. The thrombin was removed by chromatography on a 1 ml HiTrap Benzamidine FF (high sub) column (GE Healthcare, Uppsala, Sweden) according to the instructions of the supplier. An additional step of chromatography on an NTA–nickel column was used to remove traces of the cleaved His tag as well as uncleaved VHb. At each purification step samples were analyzed on SDS–PAGE (see below).

2.3. Protein-concentration determinations

Protein concentrations were determined by both the Bradford method (Bradford, 1976) using BSA as the standard and from the absorbance at 280 nm assuming that 1.0 A_{280 nm} units is equivalent to a protein concentration of 1 mg ml⁻¹. For SDS–PAGE analysis, individual lanes of commercially available 4–20% SDS gels (Pierce) were loaded with 0.1–10 µg of protein along with an equal volume of 2× loading dye [375 mM Tris pH 6.8, 60% glycerol, 6% (w/v) SDS, 0.003% bromophenol blue and one quarter final volume of β-mercaptoethanol]. Samples of 10–220 kDa size standards (Benchmark Protein Ladder, Invitrogen) were prepared in the same way.

2.4. Molecular-size estimation of VHbs

Each of the purified VHbs was chromatographed on a 140 × 1.5 cm Sephadex G-100 column that had been standardized with blue dextran to measure the void volume, chymotrypsinogen A (25 kDa) and lysozyme (14.7 kDa). *K*_{av} values (Stark, 1977) were calculated for each of the two standard proteins and plotted as log molecular weight versus *K*_{av}, which allowed estimation of the molecular sizes for each of the three VHbs after calculation of the *K*_{av} values.

2.5. Analytical methods

CO-difference spectra were determined as described by Dikshit & Webster (1988). Circular-dichroism (CD) spectra were determined on a Jasco J715 spectrometer in a 1 mm path-length quartz cell. Spectra were collected from 260 to 190 nm with a data pitch of 1 nm and a scan speed of 50 nm min⁻¹ from 200 µl samples with a protein concentration of 1 mg ml⁻¹. Circular-dichroism data were analyzed with specialized *Microsoft Excel* tools. Thermal denaturation was probed by CD by measuring spectra from 250 to 200 nm as a function of temperature, scanning from 286 to 368 K using a 1 mm quartz cuvette and a Peltier cell holder. Since these proteins are known to be

primarily α -helical, analysis of the denaturation data was limited to calculation of the molar ellipticity at 220 nm, which is known to be the minimum of the helical trough. CD spectra were also obtained from carbonmonoxy adducts of each of the three VHb forms; these adducts were produced by reducing the proteins with dithionite and exposing the reduced proteins to CO.

2.6. Crystallization of the three VHbs

Conditions for crystallization of the wild-type and mutant hemoglobins were based on those used in previous studies (Tarricone, Calogero *et al.*, 1997) but were optimized for each specific VHb. Crystals of the wild-type protein and the Y29F mutant were grown to full size using streak-seeding (Stura, 1999). Crystals were harvested from the hanging drops, cryoprotected with either 10%(v/v) glycerol or 10%(v/v) ethylene glycol and flash-cooled in liquid nitrogen prior to mounting for crystallographic data collection.

2.7. X-ray crystallography and data analysis

Diffraction data were obtained on beamline 22-BM (SER-CAT) of the Advanced Photon Source, Argonne National Laboratory, Argonne, Illinois, USA. Data were collected at an X-ray wavelength of 1 Å by the rotation method using a MAR 225 fiber-optic coupled CCD detector system (Rayonix Inc.). For each data set, 200–240 images were collected with a step size between images of 0.75°. Control of the SER-CAT beamline was accomplished *via* the *SERGUI* software package (Howard *et al.*, 2006) and data were acquired using the *marccd* software package (Rayonix Inc.) modified for compatibility with *SERGUI*. Crystallographic data processing was accomplished using *X-GEN* (Howard, 1996). Determination of solvent content based on unit-cell parameters was carried out using the *Matthews Probability Calculator* (Matthews, 1968; Kantardjieff & Rupp, 2003).

Structure determinations for the wild-type and both mutant hemoglobins were carried out by molecular replacement using *Phaser* v.2.1 (McCoy *et al.*, 2007) as implemented in *CCP4* v.6.1.3 (Winn *et al.*, 2011) or *PHENIX* v.1.8-1069 (Adams *et al.*, 2010). The molecular-replacement models were derived from the structure of the azide derivative of wild-type VHb (PDB entry 2vhb; Tarricone, Galizzi *et al.*, 1997), with the heme group included but with other ligands and solvent removed. Structure refinement was initially accomplished using *REFMAC5* (Murshudov *et al.*, 2011) as implemented in *CCP4* and subsequently using *phenix.refine* (Afonine *et al.*, 2012) as implemented in *PHENIX*. All of the results reported here are based on the refinements using *PHENIX*. *Coot* (Emsley & Cowtan, 2004) was used for manual structure manipulations and to produce the electron-density images presented here. Water molecules were added to these structures either through *phenix.refine* or manually with *Coot*; cryoprotectant molecules were added manually in *Coot* and were only retained if they remained well ordered in subsequent refinements. Structure improvement was accomplished through alternating rounds of structure refinement with *REFMAC5* or *phenix.refine* and manual manipulation with *Coot*. Images of the structures suitable for publication were obtained using *MacPyMOL* (Schrödinger LLC).

al., 1997), with the heme group included but with other ligands and solvent removed. Structure refinement was initially accomplished using *REFMAC5* (Murshudov *et al.*, 2011) as implemented in *CCP4* and subsequently using *phenix.refine* (Afonine *et al.*, 2012) as implemented in *PHENIX*. All of the results reported here are based on the refinements using *PHENIX*. *Coot* (Emsley & Cowtan, 2004) was used for manual structure manipulations and to produce the electron-density images presented here. Water molecules were added to these structures either through *phenix.refine* or manually with *Coot*; cryoprotectant molecules were added manually in *Coot* and were only retained if they remained well ordered in subsequent refinements. Structure improvement was accomplished through alternating rounds of structure refinement with *REFMAC5* or *phenix.refine* and manual manipulation with *Coot*. Images of the structures suitable for publication were obtained using *MacPyMOL* (Schrödinger LLC).

3. Results

3.1. VHb purification

In all three cases, VHb was expressed at a high level which allowed the three-step column-chromatography protocol to produce essentially homogenous protein (Figs. 1*a*, 1*b* and 1*c*). In the case of the Tyr29Phe mutant VHb, thrombin effectively removed the His tag from the protein (Fig. 1*d*).

3.2. Initial VHb analysis

Each of the three purified VHbs was chromatographed on a calibrated G-100 column as described in §2 and each protein eluted as a single symmetrical peak. The molecular sizes for the three proteins were determined to range from 30.3 to 30.7 kDa. These values agreed well with the calculated molecular weight of 16 406 for the wild-type VHb monomer (PDB entry 1vhb; *cf.* Webster, 1987). Thus, we can assume that all three VHbs exist, as expected, in the dimeric form. The CO-difference spectra of the three purified proteins were essentially identical to each other and to the known CO-difference spectrum of wild-type VHb (Fig. 2); this showed that both mutant VHbs were able to bind CO. Quantification of the spectra showed

Figure 1

The purity of the VHbs, as determined by SDS-PAGE, following the three-column purification procedure. (*a*) Tyr29Ala mutant; (*b*) Tyr29Phe mutant after thrombin cleavage; (*c*) wild type. The lane containing the size markers, as well as the position of the 15 kDa standard, are indicated in each panel. Thrombin cleavage of the Tyr29Phe mutant effectively removes the His tag. The left lane in (*d*) shows the Tyr29Phe mutant after thrombin cleavage and the right lane shows the Tyr29Phe mutant before thrombin cleavage. The position of the 15 kDa standard in the middle lane (size markers) is indicated.

that there was no substantial difference in the CO-binding abilities of the two mutants compared with wild-type VHb. The $\mu\text{mol CO}:\mu\text{mol VHb}$ ratios for the wild-type, Tyr29Ala and Tyr29Phe VHbs were 0.92, 1.21 and 0.90, respectively, normalized using protein concentrations determined by the Bradford assay.

Figure 2
CO-difference spectra of (a) wild-type VHb, (b) Tyr29Phe VHb and (c) Tyr29Ala VHb.

3.3. CD spectral analysis

Both mutants (Tyr29Phe and Tyr29Ala) exhibited an α -helical spectrum similar to that of wild-type VHb (Fig. 3). The percentage helicity of the wild-type, Tyr29Phe and Tyr29Ala proteins was 96, 92 and 96%, respectively. The T_m values were 354, 356 and 359 K and the thermal reversibilities were 60, 68 and 62% for the wild-type, Tyr29Phe and Tyr29Ala VHbs, respectively. The thermal denaturation of each of the three VHbs is shown in Fig. 4. The carbonmonoxy forms of the three VHb proteins yielded similar results (data not shown).

3.4. Crystallization and crystallography

Crystals of the three VHb proteins were obtained by salting out in ammonium sulfate near pH 6 using sodium pyrophosphate as buffer. The wild-type and Tyr29Phe crystals were very small, and streak-seeding was used to grow larger crystals. The streak-seeded crystals tended to grow in rosettes, and individual crystals ($\sim 60 \times 100 \times 240 \mu\text{m}$) had to be separated out from the rosettes. The Tyr29Ala crystals did not require streak-seeding and grew to similar sizes to the

Figure 3
CD spectra of VHb: wild type (triangles), Tyr29Phe (squares) and Tyr29Ala (diamonds) VHbs. Spectra were taken at 277 K.

Figure 4
Thermal denaturation curves obtained by CD at 222 nm for wild-type VHb (triangles), Tyr29Phe (squares) and Tyr29Ala (diamonds). Each curve represents the ratio of the CD_{222} value at the represented temperature to the CD_{222} value at $T = 294 \text{ K}$.

Table 1

Crystallization conditions and crystallographic parameters.

Values in parentheses are for the outermost resolution shell.

	Wild type	Tyr29Phe	Tyr29Ala
Precipitant	1.6 M ammonium sulfate	1.2 M ammonium sulfate	1.3 M ammonium sulfate
Buffer	0.05 M Na ₂ HP ₂ O ₇	0.05 M Na ₂ HP ₂ O ₇	0.05 M Na ₂ HP ₂ O ₇
pH	6.0	6.0	6.2
Cryoprotectant	3% glycerol	3% glycerol	3% ethylene glycol
Protein concentration (mg ml ⁻¹)	25	30	27
Streak-seeded?	Yes	Yes	No
Crystal dimensions (mm)	0.2 × 0.07 × 0.04	0.2 × 0.07 × 0.04	0.2 × 0.07 × 0.04
Resolution range (Å)	30.16–1.75 (1.81–1.75)	31.20–1.90 (1.97–1.90)	30.95–1.72 (1.78–1.72)
Space group	C222 ₁	P2 ₁	C2
Unit-cell parameters			
<i>a</i> (Å)	74.9	61.6	84.9
<i>b</i> (Å)	98.9	41.1	46.3
<i>c</i> (Å)	41.0	62.4	62.5
β (°)	90	105.6	128.6
No. of reflections	15731 (1561)	23653 (2082)	22086
Completeness (%)	99.87 (100)	98.39 (86.97)	98.72 (95.95)
<i>I</i> / <i>σ</i> (<i>I</i>)	22.53 (3.97)	24.13 (7.84)	15.12 (3.06)
<i>R</i> _{merge}	0.0564 (0.3741)	0.0471 (0.1516)	0.0730 (0.3768)
<i>R</i>	0.1739 (0.2495)	0.1571 (0.1749)	0.1712 (0.3143)
<i>R</i> _{free}	0.2092 (0.2561)	0.1916 (0.2245)	0.2040 (0.3347)
Protein atoms per monomer	1147	1122	1161
Waters per monomer	72	80	86
Cryoprotectant molecules per monomer	6	5.5	14
Alternate conformers†	14	10	13
R.m.s.d., bond lengths (Å)	0.004	0.013	0.018
R.m.s.d., bond angles (°)	1.06	1.56	1.51
Ramachandran favored‡ (%)	98	97	99
Ramachandran outliers‡ (%)	0.72	0	0
Solvent content (%)	48.5	48.7	55.6
⟨ <i>B</i> ⟩, main chain (Å ²)	33.9	24.8	23.0
⟨ <i>B</i> ⟩, side chains (Å ²)	41.8	32.3	28.8
⟨ <i>B</i> ⟩, solvent§ (Å ²)	41.2	30.3	39.9
No. of residues in dimer interface	n.a.	~22	n.a.
PDB code	3tm3	3tld	3tm9

† Number of protein residues per monomer that were built in two or more conformations. ‡ Number of residues per monomer in the favored or allowed region of the Ramachandran plot. § Including glycerol or ethylene glycol atoms.

streak-seeded crystals. Details of the crystallization conditions are presented in Table 1.

Examination of the diffraction patterns of the wild-type and mutant VHB protein crystals showed that they all diffracted to resolutions of 1.8 Å or better and were reasonably radiation-resistant: for one of the Tyr29Ala mutant crystals the intensity scale factors at $\varphi = 0^\circ$ and $\varphi = 180^\circ$, collected 35 min apart, differed by less than 5% on an intense bending-magnet beamline, and the Tyr29Phe mutant showed no degradation in scale factor throughout a similar exposure. The wild-type protein crystallized in space group C222₁, with one VHB monomer in the asymmetric unit. This C-centered orthorhombic form is almost a perfect re-indexing of the primitive monoclinic (P2₁) form found by Tarricone, Galizzi *et al.* (1997), except that the molecular twofold axis found in that study becomes a true crystallographic twofold axis in the current crystal. The Tyr29Phe mutant crystallized in the same space group (P2₁), with unit-cell parameters similar to those found by Tarricone, Galizzi *et al.* (1997) and with two VHB monomers in the asymmetric unit. The Tyr29Ala mutant crystallized in space group C2 with one VHB monomer per asymmetric unit. Crystallographic parameters for the three structures are provided in Table 1.

Both the wild-type and Tyr29Phe data can be successfully indexed in either the original P2₁ unit cell or the C222₁ unit cell. However, the scaling results unambiguously distinguish the two arrangements. For the wild-type crystals the *R*_{merge} values determined in the P2₁ unit cell are no better than in the C222₁ unit cell; for example, for one data run the *R*_{merge} to a resolution limit of 1.7525 Å was 0.0622 weighted and 0.0560 unweighted in the P2₁ unit cell and was 0.0605 weighted and 0.0536 unweighted in the C222₁ cell. In contrast, the *R*_{merge} values for

the Tyr29Phe mutant calculated in the C222₁ unit cell are considerably higher than in the P2₁ unit cell. Of course, the *R*_{merge} value is not an absolute indicator of data quality, but in comparing identical data processed in two different space groups it offers significant evidence. Refinement of the wild-type structure in the P2₁ unit cell yields results that offer no additional information beyond what is observed in the C222₁ structure (data not shown).

Figure 5

Overlay of wild-type VHB (red) and the Tyr29Ala mutant (green) in a ribbon representation. The E region through Pro54 is on the right and the B helix is on the left. Tyr29 and its counterpart Ala29 are at the bottom of the B helix. The overlay in the E helix is close from residue 56 onwards, beyond the bottom of the displayed helix. The two structures diverge significantly near the top of the picture, which corresponds to residues 50–55. The Pro54 ring in the Ala mutant occupies the space in which the Tyr29 aromatic ring appears in the wild-type structure.

Determination of the three structures by molecular replacement proceeded uneventfully and refinement required no unusual approaches. A number of residues required refitting relative to their positions in the 1997 structure, even in the wild type; whether this is because our crystallization conditions were slightly different or whether we were analyzing the data differently cannot be determined since the structure factors for the structures of Tarricone and coworkers are not available in the PDB.

All three structures display the traditional globin fold, apart from the disorder in region D. The wild-type and Tyr29Phe structures are only partially helical in the E region, although the φ - ψ angles satisfy the standard criteria for α -helical structures beginning at residue 55. The E region of the Tyr29Ala structure was fully helical from residue 51 onwards (Fig. 5). The helix is mostly α -helical, although the segment from Leu57 to Val61 has some 3_{10} -helix character: the O—N distances from each carbonyl to the main-chain N atom of its neighbour at position ($n + 3$) are almost as short (3.05–3.28 Å) as the distances to that at position ($n + 4$) (2.75–3.1 Å).

As in the original structure determination (Tarricone, Galizzi *et al.*, 1997), we found that residues in the D region were disordered. We were able to build somewhat more residues in this region for wild-type Vhb than had been accomplished in the earlier determination; only residues 47–49 are omitted from the current model, although the fit to the electron density of residues 44–46 and 50–52 is poorer than in other parts of the structure. The Tyr29Phe structure is somewhat more disordered than the wild type: we were able to build residues 44–46 and 51–52 but not residues 47–50. Interpretable density was visible throughout the D region in the Tyr29Ala mutant, so the final model for this mutant includes all atoms; but again the fit of the model to the density is poorer in this region than in other parts of the structure.

As noted in Table 1, 8–14 amino-acid residues per monomer were modeled in two or more conformations in these structures. Residue Glu19 appears in two conformations in each of the four monomers

(one each for the wild-type protein and the Tyr29Ala mutant and two for the Tyr29Phe mutant); residues Lys11, Gln66, Asn70, Lys79, Lys84, Gln100 and Gln143 appear in two conformations in three of the four structures. As is typical, the residues that appear in multiple conformations are polar and are near the surface. Only one nonpolar residue (Met59) appears in two conformations and it does so only in the Tyr29Ala mutant.

Few important differences are observed between the wild-type protein and the Tyr29Phe mutant. Of course, the absence of a hydroxyl group at the end of the side chain of residue 29 in the Phe mutant means that the hydrogen bond cannot be made between this hydroxyl and the main-chain N atom of residue Pro54 (E8). The list of surface residues that appear in two conformations is different for the wild type than for either monomer of the Tyr29Phe mutant, perhaps because the packing arrangements are slightly different.

Much more significant differences appear in the Tyr29Ala mutant. Of course, the aromatic side chain of residue 29 itself is absent, but the main chain of residue 29 aligns very closely with its position in the wild-type structure (Fig. 5). As noted above, the entire D region can be modeled in the Tyr29Ala structure; the D-region residues are less helical than other portions of the structure, although residues 42, 43, 47, 48 and 50 still have helix-like Ramachandran angles. Fig. 6 shows the geometry of this region for the Tyr29Ala mutant. The E region is more fully helical in the Tyr29Ala mutant than in either the wild-type or the Tyr29Phe structures.

One important difference between the wild-type and Tyr29Ala structures involves Met45. This residue is barely visualized in our wild-type structure and the Tyr29Phe mutant, whereas in the Tyr29Ala mutant it is readily observable (Fig. 7). In the Tyr29Ala structure the main-chain amide of Met45 makes a hydrogen bond to the terminal carboxylate of Asn67 of a neighboring molecule. The increased ordering of this methionine probably arises at least in part from this inter-chain contact. There are, however, other inter-chain contacts in this region of the wild-type structure, such as a hydrogen bond between the carbonyl O atom of Asp44 and the NE2 atom of Gln66 in a neighboring molecule (Fig. 8).

The side chain of Pro54 occupies a different position in the Ala mutant from its position in the wild-type and Phe structures; the five-membered ring of the proline moves toward residue 29. One leg of the ring and the C α —C bond occupy much of the space vacated by the aromatic ring of residue 29 found in the other structures and can be

Figure 6
Ramachandran plot of residues 43–59 of Tyr29Ala Vhb. The only residues that lie outside the preferred α -helical region are Asp44 ($\varphi = -96^\circ$, $\psi = 114^\circ$), Met45 ($\varphi = -100^\circ$, $\psi = 35^\circ$), Gly46 ($\varphi = -60^\circ$, $\psi = 140^\circ$), Glu49 ($\varphi = -130^\circ$, $\psi = 40^\circ$) and Leu51 ($\varphi = -100^\circ$, $\psi = 0^\circ$).

Figure 7
Residue Met45 in the Tyr29Ala mutant. The main-chain N atom (right center in the figure) forms a hydrogen bond to ND2 of Asn67 of a neighboring molecule.

considered to form part of the ligand-binding site. Gln53 (E7) is in a significantly different position in the Tyr49Ala mutant. Its main-chain atoms move towards the position vacated by the movement of Pro54 (Fig. 9) and the side chain occupies the space between this location and the surface. This is in contrast to the orientation of this residue in the wild-type and Phe structures, in which the side-chain points directly towards the solvent.

4. Discussion

4.1. Comparisons between the original structure and the current wild-type structure

It is unlikely that the differences between the wild-type structure reported here and that reported by Tarricone, Galizzi *et al.* (1997) constitute meaningful changes in the protein. The fact that more residues are visualized is likely to reflect (i) slightly better analytical tools for handling the diffraction data, (ii) the inclusion of more solvent molecules, including explicitly modeled cryosolvent molecules, which probably provide an overall improvement in phase quality, and (iii) a somewhat more aggressive approach to electron-density fitting.

4.2. Comparisons between the current wild-type structure and that of the Phe mutant

The fact that the structure of the Phe mutant is similar to that of the wild type is not surprising, given that the change in overall architecture is small and the crystal contacts do not depend on the residue that has been changed. It is also unsurprising that the helicity and thermal stability of the Phe mutant are similar to those of the wild type, since neither the helicity nor the folding equilibrium is likely to be influenced by an internal contact such as that provided by the hydrogen bond between Tyr29 OH and the main-chain N atom of Pro54. What is less intuitive is that the ligand-binding properties of this mutant, as represented by its CO-difference spectrum, are identical to within experimental error to those of the wild type. This identity suggests that the Tyr29–Pro54 hydrogen bond either has less significance than has been suggested (Tarricone, Galizzi *et al.*, 1997)

Figure 8

Residue Asp44 in wild-type Vhb. Its carbonyl O atom (upper right) hydrogen bonds to NE2 of Gln66 in a neighboring molecule and its side-chain OD2 hydrogen bonds to ND2 of Asn67 of the same neighbor (right center).

or that some other solvent restructuring in the vicinity of the ligand-binding site has compensated for the lack of this particular hydrogen bond. There are few water molecules in the ligand-binding domain of this protein in both the previously published structure and in all of our structures, so we are inclined to believe that the hydrogen bond is in fact fairly unimportant to ligand binding.

4.3. Comparisons between the current wild-type structure and that of the Ala mutant

As noted above, the differences between the wild-type and Ala-mutant structures are more substantial, involving greater ordering of the D region, major movement of both the main chain and the side chain of E7 (Gln53; Fig. 9) and, most importantly, occupation of the aromatic ring space in the ligand-binding region by the aliphatic ring of E8 (Pro).

Since the Ala mutant crystallizes in a different space group and has very distinct crystal-packing interactions, it is natural to inquire whether the differences between the mutant structure and the wild type arise from the difference in crystal packing. The stabilization of Met45 is indeed probably attributable to the hydrogen bond between its main-chain N atom and the side chain of Asn67 in a neighboring molecule. However, the other residues that have distinctly different positions are mostly far from the nearest crystal contact point and as such are unlikely to be influenced by packing. Even an indirect effect on important residues such as Gln53 and Pro54 is hard to imagine given their centrality in ligand binding. Accordingly, we would argue that, apart from the stabilization of Met45, the observed differences between the wild-type and Ala-mutant structures are real and of relevance to the soluble protein. Nevertheless, both the CD and CO-difference spectral data indicate that these changes do not

Figure 9

Overlay of wild-type Vhb (green) and the Tyr29Ala mutant (red) in the vicinity of residues 53 and 29. Tyr29 points towards the heme in the wild type, while Pro54 and Gln53 of the Tyr29Ala mutant point towards the heme. Residue Glu52 in the mutant is unlabeled because it is behind Pro54 in this view. The two structures were aligned in *MacPyMOL* on the basis of the C α atoms of residues 56–146, with an r.m.s. fit of 0.823 Å.

greatly (if at all) alter the overall ligand-binding ability and stability of the protein.

We would argue then that the movement of Pro54 into the position occupied by the aromatic ring in the wild type, and the attendant adjustments of Gln53 and Glu52, are instances in which the overall architecture of the ligand-binding site is recreated in the mutant using slightly different starting materials. We suggest that this preservation of the overall active-site architecture might be an instance of 'compensatory rearrangement', a phenomenon that probably occurs with considerable frequency in other situations in structural biology.

Data were collected on the Southeast Regional Collaborative Access Team (SER-CAT) 22-ID (or 22-BM) beamline at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at <http://www.ser-cat.org/members.html>. Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. W-31-109-Eng-38. The authors would like to thank Professor Joseph Orgel, Dr Olga Antipova, Professor Nick Menhart and Neha Sahni for discussions and for help in carrying out the circular-dichroism studies.

References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H. & Adams, P. D. (2012). *Acta Cryst.* **D68**, 352–367.
- Anand, A., Duk, B. T., Singh, S., Akbas, M. Y., Webster, D. A., Stark, B. C. & Dikshit, K. L. (2010). *Biochem. J.* **426**, 271–280.
- Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
- Dikshit, K. L., Orii, Y., Navani, N., Patel, S., Huang, H.-Y., Stark, B. C. & Webster, D. A. (1998). *Arch. Biochem. Biophys.* **349**, 161–166.
- Dikshit, K. L. & Webster, D. A. (1988). *Gene*, **70**, 377–386.
- Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* **D60**, 2126–2132.
- Howard, A. J. (1996). In *Crystallographic Computing 7*, edited by P. E. Bourne & K. D. Watenpugh. Oxford University Press.
- Howard, A. J., Fait, J. F., Fu, Z.-Q., Lavender, W. M. & Chrzas, J. J. (2006). *NOBUGS 2006 Conference*, contribution 24. <http://nobugs2006.lbl.gov/abstracts.php>.
- Kantardjieff, K. A. & Rupp, B. (2003). *Protein Sci.* **12**, 1865–1871.
- Kaur, R., Pathania, R., Sharma, V., Mande, S. C. & Dikshit, K. L. (2002). *Appl. Environ. Microbiol.* **68**, 152–160.
- Lee, S. Y., Stark, B. C. & Webster, D. A. (2004). *Biochem. Biophys. Res. Commun.* **316**, 1101–1106.
- Lin, J.-M., Stark, B. C. & Webster, D. A. (2003). *J. Ind. Microbiol. Biotechnol.* **30**, 362–368.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Park, K.-W., Kim, K.-J., Howard, A. J., Stark, B. C. & Webster, D. A. (2002). *J. Biol. Chem.* **277**, 33334–33337.
- Ramandeep, Hwang, K. W., Raje, M., Kim, K.-J., Stark, B. C., Dikshit, K. L. & Webster, D. A. (2001). *J. Biol. Chem.* **276**, 24781–24789.
- Roos, V., Andersson, C. I. J. & Bülow, L. (2004). *J. Biotechnol.* **114**, 107–120.
- Sambrook, J. & Russell, D. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd ed. New York: Cold Spring Harbor Laboratory Press.
- Stark, B. C. (1977). PhD dissertation. Yale University.
- Stura, E. (1999). *Protein Crystallization: Techniques, Strategies, and Tips*, edited by T. M. Bergfors, pp. 141–153. La Jolla: International University Line.
- Tarricone, C., Calogero, S., Galizzi, A., Coda, A., Ascenzi, P. & Bolognesi, M. (1997). *Proteins*, **27**, 154–156.
- Tarricone, C., Galizzi, A., Coda, A., Ascenzi, P. & Bolognesi, M. (1997). *Structure*, **5**, 497507.
- Verma, S., Patel, S., Kaur, R., Chung, Y.-T., Duk, B. T., Dikshit, K. L., Stark, B. C. & Webster, D. A. (2005). *Biochem. Biophys. Res. Commun.* **326**, 290–297.
- Webster, D. A. (1987). *Adv. Inorg. Biochem.* **7**, 245–265.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.