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Structure and Function of Hemoglobin Variants at an Internal Hydrophobic Site: Consequences of Mutations at the β 27 (B9) Position†

Yuê Huang,‡ Josée Pagnier,*,‡ Philippe Magne,‡ Faouzi Baklouti,§ Jean Kister,‡ Jean Delaunay,§ Claude Poyart,‡ Giulio Fermi,∥ and Max F. Perutz∥

Institut National de la Santé et de la Recherche Médicale, Unité 299, Hôpital de Bicêtre, 94275 Le Kremlin-Bicêtre, France, Centre National de la Recherche Scientifique, Unité de Recherche Associée 1171, Faculté de Médecine Grange-Blanche, 69373 Lyon Cedex 08, France, and MRC Laboratory for Molecular Biology, Hills Road, Cambridge CB2 2QH, England

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ABSTRACT: We have studied the structure–function relationships in newly discovered hemoglobin (Hb) mutants with substitutions occurring at the tight and highly hydrophobic cluster between the B and G helices in the β chains, namely, Hb Knossos or β A27S and Hb Grange-Blanche or β A27V. The β A27S mutant has a 50% decrease in oxygen affinity relative to native human Hb A, while the β A27V mutant has an increased oxygen affinity. We have also engineered the artificial β A27T mutation through site-directed mutagenesis. This new mutant exhibits functional properties similar to those of Hb A. None of these mutants is unstable. X-ray analyses show that the substitution of Val for Ala may reduce the relative stability of the T structure of the molecule through packing effects in the β chains; for the β A27S mutant a new hydrogen bond between serine and the carbonyl O at β 23 (B5) Val is observed and is likely to increase the relative stability of the T structure in the mutant hemoglobin. However, no significant changes in the crystals were observed for these mutants between the quaternary R and T structures relative to native Hb A. We conclude that small tertiary structural changes in the tight hydrophobic B–G helix interface are sufficient to induce functional abnormalities resulting in either low or high intrinsic oxygen affinities.

Two hemoglobin (Hb)[1] natural variants have been reported recently at the β 27 (B9) site, namely Hb Knossos (A27S) (Fessas et al., 1982; Baklouti et al., 1986) and Hb Grange-Blanche (A27V) (Baklouti et al., 1987). These mutants are of particular interest as they occur at the tight and highly hydrophobic cluster between the B and G helices in the β chains. X-ray analyses (Baldwin & Chothia, 1979; Shanaan, 1983; Fermi et al., 1984) have shown that in native Hb A this portion of the β chains does not undergo significant structural changes on oxygen binding. Substitution of the polar Ser residue for Ala in Hb Knossos leads to a 50% decrease in oxygen affinity; substitution of the more hydrophobic Val for Ala in Hb Grange-Blanche leads to an increased oxygen affinity. To understand more precisely the reasons for the abnormal oxygen affinities of these natural mutants, we have

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* To whom correspondence should be addressed.
‡ INSERM U 299.
§ CNRS URA 1171.
∥ MRC Laboratory for Molecular Biology.

[1] Abbreviations: Hb, hemoglobin; DPG, 2,3-diphosphoglycerate; P_{50}, oxygen partial pressure at half-saturation; n_{50}, Hill coefficient at half-saturation; Bistris, bis(2-hydroxyethyl)aminotris(hydroxymethyl)-methane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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engineered a third mutant through site-directed mutagenesis with Thr for Ala (β A27T) whose side chains carries both a methyl and a hydroxyl group. We report below results of equilibrium oxygen-binding properties and X-ray analyses for the two natural mutants. The results indicate that β 27 Val may destabilize the subunit structure due to packing effects, while Ser stabilizes it through the formation of an additional intrahelix hydrogen bond. In the A27T mutant the two effects may be present and probably compensate each other, leading to an oxygen affinity similar to that of normal Hb A.

Materials and Methods

Hb A was obtained from healthy nonsmoking donors at the blood bank of the hospital and purified by DEAE-Sephadex chromatography. The β A27V Hb was isolated from Hb A by preparative isoelectric focusing. The β A27S Hb was prepared from the hemolysate of a patient homozygous for Hb Knossos (Baklouti et al., 1986). Identification of these two natural variants was also obtained by DNA restriction mapping, by use of a 1.9-kb *Bam*HI β-globin gene probe kindly provided by Prof. J. Godet. In particular, *Ava*II restriction mapping ascertained the GCC → G*T*C mutation in Hb β A27V through the creation of a new *Ava*II cleavage site (fragment at 1.6 kb versus 1.75 kb in controls). The Hbs were stripped of anions and other contaminants after anion-exchange chromatography and kept in the CO form in liquid nitrogen until use. Before the experiments CO was removed by illumination under a flow of oxygen. The β A27T mutant was obtained after site-directed mutagenesis as described by Nackamaye and Eckstein (1986), and the mutated β chains were produced in *Escherichia coli* (QY13 strain) by using the expression vector pLcIIFXβ described by Nagai and Thøgersen (1984). The mutated β chains were then mixed with native α chains to obtain the tetrameric hemoglobin. In order to estimate differences in the preparation procedures, Hb A and the β A27S mutant were also synthetized in *E. coli*. The mutations in the β chains were verified by HPLC.

Oxygen binding curves were recorded with a continuous method (Kister et al., 1987) in 50–70 μM heme solutions with and without 2,3-diphosphoglycerate (DPG) and at different pH's. The effect of pH (Bohr effect) was calculated as $\Delta \log P_{50}/\Delta pH$ and the effect of DPG as $\Delta \log P_{50}$ (±1 mM DPG) at pH 7.2. Equilibrium curves were fitted to the equation of the two-state allosteric model (Monod et al., 1965) to obtain the values of K_T and K_R, the oxygen dissociation constants for the T and R states, respectively (Kister et al., 1987). Temperature and 2-propanol stability tests were performed according to current methods.

X-ray data were collected on a Hilger four-circle diffractometer. Data between 10- and 2.5-Å resolution were collected from a single crystal of each mutant on a FAST area detector with MADNES software, with a rotating anode X-ray source. Atomic models were constructed from the native model and the difference maps and were difference-refined (Fermi et al., 1982), varying the mutant coordinates by the method of Jack and Levitt (1978) to minimize $F_{cm} - F_{om} + F_{on} - F_{cn}$, where the F's are structure amplitudes, the subscripts o and c represent observed and calculated, and m and n represent mutant and native hemoglobins, respectively. The weight of X-ray terms relative to restraints was varied so as to maintain the root mean square C–C bond length between 0.02 and 0.03 Å. Details of data processing and refinement are given in Table I. The accuracy of the refinement was checked by comparing the observed difference maps ($F_{on} - F_{om}$) to the calculated difference maps ($F_{cn} - F_{cm}$), both phased with the same native phases. Good agreement between the observed and calculated difference maps indicated that the differences between mutant and native models adequately represent the differences in the underlying structures.

Table I: Data Processing and Refinement for the β A27S and β A27V Mutants

	β A27S	β A27V
no. of unique *hkl*	17833	16743
R factor		
internal on I (R_{symm})	0.082	0.046
versus native (on *F*)	0.064	0.058
cycles of refinement		
position	4	4
B factor[a]	2	5
difference *R* factor[b]		
initial	0.075	0.085
final	0.044	0.052

[a] Atomic *B* averaged within main- and side-chain groups. [b] $\sum |F_{cm} - F_{om} + F_{on} - F_{cn}| / \sum |F_{od}|$. *F*'s are structure amplitudes, subscripts o and c represent observed and calculated, and m and n represent mutant and native hemoglobin, respectively.

Table II: Oxygen-Binding Parameters for Hb Mutants at the β (B9) 27 Site

Hb	P_{50}[a]	$P_{50}X/P_{50}A$[b]	n_{50}	Bohr effect[c]	DPG effect[d]
		Native Hb			
A[e]	5.3	1.00	2.8	−0.50	0.48
A27V	3.9	0.74	2.6	−0.42	0.44
A27S	7.7	1.46	2.7	−0.48	0.48
		E. coli Hb			
A[e]	4.3	1.00	2.3		0.44
A27S	6.4	1.49	2.5		0.38
A27T	4.5	1.05	2.3	−0.49	0.33

[a] P_{50} in mmHg (1 mmHg = 0.133 kPa). [b] $P_{50}X/P_{50}A$ is the ratio of the P_{50} mutant (X) to normal Hb (A). [c] The Bohr effect was estimated from the $\Delta \log P_{50}/\Delta pH$ between 6.8 and 8. [d] The DPG effect was calculated as $\Delta \log P_{50}$ ± 1 mM DPG at pH 7.2. [e] pH 7.2, 100 mM NaCl, 50 mM Bistris buffer, 50 μM EDTA, 20 μg/mL catalase, 25 °C, and 60–80 μM heme.

Results

The oxygen-binding properties for the various Hb's are shown in Table II. The native mutant β A27V has a *reduced* P_{50} relative to Hb A, with a left shift of the bottom portion of the curve that indicates a destabilized deoxy structure (Figure 1). On the other hand, P_{50} of Hb β A27S is *increased* by 50%; its entire oxygen equilibrium curve is right-shifted relative to Hb A. The Hill coefficients and alkaline Bohr and DPG effects of the two mutant Hb's are similar to those of Hb A (Table II). Temperature and 2-propanol tests showed that their stabilities are normal.

Table II also compares the oxygen-binding parameters for the mutant β A27T synthesized in *E. coli* with those of Hb β A27S and of Hb A synthesized in *E. coli*. It has nearly the same P_{50} and Hill coefficient but a slightly diminished DPG effect relative to Hb A synthesized in *E. coli*.

Table III gives the parameters of the two-state allosteric model calculated for the three mutants. The high oxygen affinity for Hb β A27V is accounted for by reductions of K_T and of the allosteric constant *L*. K_R and K_T of Hb β A27S are increased and *L* is decreased relative to the values for Hb A. In both mutants, P_{50} is altered by a combination of affinity and allosteric parameters. The parameters of the synthetic mutant β A27T are nearly identical with those of Hb A.

Figure 2 shows symmetry-averaged difference electron density maps of the mutants Hb β A27S and A27V minus native deoxyhemoglobin. They show the positions of the mutant side chains and indicate that they leave the surrounding

Table III: Calculated Parameters of the Two-State Allosteric Model[a]

Hb	K_R	K_T	L	c	σ
A	0.21 (1.8%)[b]	23.7	3×10^5 (5.8%)	0.0088 (1.7%)	0.0031
A27V	0.21 (1.1%)	15.0	9×10^4 (4.4%)	0.0138 (1.4%)	0.0057
A27S	0.46 (1.4%)	34.3	6×10^4 (5.3%)	0.0134 (1.2%)	0.0024
A27T	0.22 (1.4%)	20.7	3×10^5 (5.5%)	0.0108 (1.6%)	0.0052

[a] The parameters were obtained after fitting to the equation of the two-state allosteric model by using a nonlinear least-squares procedure according to Bevington (1969). K_R and K_T (mmHg) are the oxygen dissociation constants for the R and T states, respectively; L is the allosteric constant ($=T_0/R_0$); c is K_R/K_T; and σ is the standard error per point (referred to Y values) between observed and calculated values (number of data points: 150–200). [b] Percent error for the parameters relative to the best fit. Typically a 2% variation of K_R, 7% variation of L, and 15% variation of c (K_T) produces a change in σ by a factor of 2.

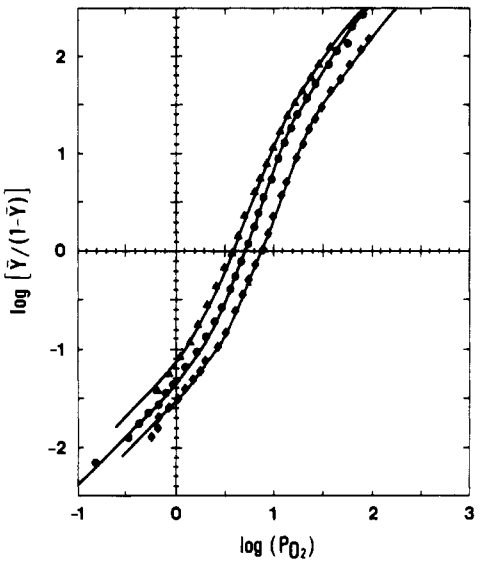

FIGURE 1: Oxygen binding curves for Hb A (●), Hb β A27V (▲), and Hb β A27S (◆). Experimental conditions were pH 7.2, 100 mM NaCl, and 50 mM Bistris buffer at 25 °C. EDTA (50 μM) and catalase (20 μg/mL) were added to avoid excess MetHb formation. Heme concentrations were typically 60 μM. Each symbol represents one of every six data experimental points. The curves are calculated after fitting to the two-state allosteric model. Values of the allosteric parameters for these curves are given in Table III.

globin unperturbed. This was confirmed when the structures were refined (maximum shift ~0.2 Å). The Oγ of β_1 27 (B9) Ser lies at a distance of 2.8 ± 0.1 Å from the carbonyl oxygen of β_1 23 (B5) Val, ideal for a hydrogen bond. On the other hand, the γ-methyl of Val β A27V is at a distance of only 3.2 ± 0.2 Å from that carbonyl oxygen, which is about 0.3 Å short of the minimal van der Waals distance. The valine also makes close contacts with helix G, which are avoided by the serine. In Figure 3 the atomic model of oxyhemoglobin is superimposed upon that of native deoxyhemoglobin. The region illustrated is closely packed, with Ala 27 (B9) functioning as a knob that fills the hole between the two valines and the two leucines on helix G. The differences between the B helices in the R and T structures are barely significant. In the deoxy model, the distance from Cγ2 of Val 27 (B9) (or Oγ of a Ser or Thr) to the carbonyl oxygen of Val 23 (B5) is 2.8 Å, correct for a hydrogen bond but too short for a van der Waals contact; in the oxy model it is 3.5 Å, correct for a van der Waals contact but too long for a hydrogen bond. This difference could explain why the valine mutant destabilizes the T state relative to the R state and the serine mutant does the reverse. However, the contacts between the side chains of Val 27 (B9) and Val 113 (G15) Cβ and Leu 110 Cα are worse in HbO_2 than in deoxyHb, which weakens the argument. Besides, the Cα–Cα distances between the residue B9 and its neighbors in helix G are the same in the two structures. Finally, the serine side chain fits into either the R or T structure with no apparent distortion.

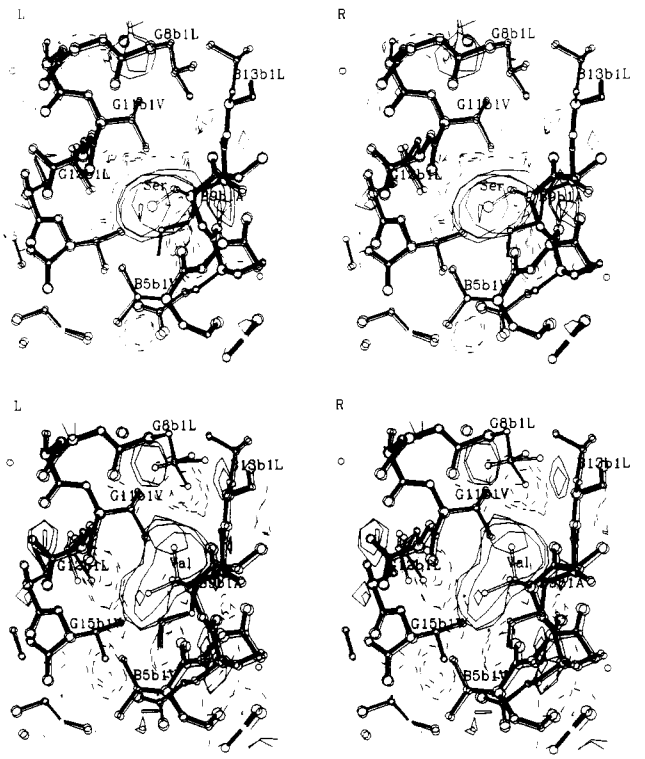

FIGURE 2: Symmetry-averaged difference electron density maps of the deoxy mutants β A27S (top) and β A27V (bottom) minus native deoxyhemoglobin, superimposed on the atomic models of native (filled bonds) and mutants (open bonds). The contour levels are plus (filled contours) and minus (broken contours) 0.04 e/Å^3. Details of data processing and refinement are given in Table I.

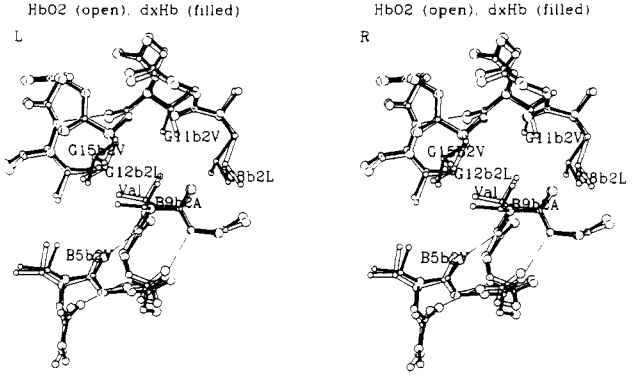

FIGURE 3: The atomic model of oxyhemoglobin (open bonds) superimposed upon that of native deoxyhemoglobin (filled bonds). Both models have a valine residue introduced at position β 27 (B9) with $\chi_1 = 180°$.

Discussion

Experience has shown low oxygen affinity to be always associated with mutations that strengthen the deoxy relative to the oxy structure, for example, by the loss of a hydrogen bond specific for the quaternary oxy structure. High oxygen affinity may arise from mutations doing the reverse, such as

the breaking of a hydrogen bond that is specific for the quaternary deoxy structure, but it also arises from mutations that weakens *both* structures. This happens because such mutations relax the tension of the T structure, which lowers K_T, but since the R structure is already fully relaxed, they cannot relax it any further and therefore leave K_R unchanged (Fermi & Perutz, 1981).

For reasons that are not clear, in A27S the introduction of a hydrogen bond from the OH of serine B9 to the carbonyl oxygen of valine B5 stabilizes the tertiary deoxy structure of the β chains and thus raises both K_T and K_R. In Hb A27V, on the other hand, the γ-methyls of the valine are misfits in both the T and R structures. They thus weaken the T structure, which lowers K_T; they also weaken the R structure, but this has no effect on K_R, since K_R can drop no further than in Hb A. In Hb A27T, the two effects appear to compensate, leaving the oxygen affinity unchanged, because the hydrogen bond from the threonine OH to the carbonyl oxygen of valine B5 strengthens the tertiary deoxy structure, but the γ-methyl is a misfit and weakens it.

Another mutant, Hb Volga or β (B9) A27D, has been described (Idelson et al., 1975; Blanke et al., 1989). The introduction of a polar Asp residue in the hydrophobic B–G interface should create an uncompensated negative charge that would have a disruptive effect and result in the observed high oxygen affinity and instability.

In conclusion, the present study demonstrates that very small changes in the structure of the hemoglobin molecule distant from the heme groups may slightly alter their ligand affinities—slightly, because the changes in free energy equivalent to those in K_T amount to no more than 270 cal/mol in A27V, 220 cal/mol in A27S, and 80 cal/mol in A27T. It is difficult to rationalize such energetic changes stereochemically.

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