Probing the $\alpha_1\beta_2$ Interface of Human Hemoglobin by Mutagenesis

ROLE OF THE FG-C CONTACT REGIONS*

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The allosteric transition of hemoglobin involves an extensive reorganization of the $\alpha_1\beta_2$ interface, in which two contact regions have been identified. This paper concerns the effect of two mutations located in the “switch” (C3 Thr $\rightarrow$ Trp) and the “flexible joint” (B37 Trp $\rightarrow$ Thr). We have expressed and characterized one double and two single mutants: Hb $\alpha$T38W/$\beta$W37T, Hb $\beta$W37T, and Hb $\alpha$T38W, whose structure has been determined by crystallography.

We present data on: (i) the interface structure in the two contact regions, (ii) oxygen and CO binding kinetics and cooperativity, (iii) dissociation rates of deoxy tetramers and association rates of deoxy dimers, and (iv) the effect of Nai on deoxy tetramer dissociation rate constant.

All the mutants are tetrameric and T-state in the deoxygenated derivative. Reassociation of deoxygenated dimers is not modified by interface mutations. DeoxyHb $\alpha$T38W dimerizes 30% slower than HbA; Hb $\beta$W37T and Hb $\alpha$T38W/$\beta$W37T dissociate much faster. We propose a binding site for I$^-$ at the switch region.

The single mutants bind O$_2$ cooperatively; the double one is almost non-cooperative, a feature confirmed by CO binding. The functional data, analyzed with the two-state model, indicate that these mutations reduce the value of the allosteric constant L$_D$.

The three-dimensional structure of liganded and deoxyhemoglobin (Baldwin and Chothia, 1979; Shaanan, 1983; Perutz et al., 1987) indicates that the allosteric transition can be described topographically as a change in the relative orientation of the two dimers identified as $\alpha_1\beta_2$ and $\alpha_2\beta_1$, which rotate with respect to each other and slide along the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces. Therefore, the amino acid residues that contribute to the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interface (Fig. 1) play a major role in controlling the relative stability of the allosteric states and, as a consequence, cooperativity. This interface contains residues of helix C and the FG corner of both chains. In total 17 residues establish interactions across the $\alpha$F-$\beta$C and the $\alpha$C-$\beta$FG contacts with a pseudo-symmetric arrangement (Fig. 1). Other interactions of minor significance are present across the $\alpha$F-$\beta$G and $\alpha$C-$\beta$C contacts (Baldwin and Chothia, 1979).

The $\alpha$F-$\beta$C contact is extensive and forms a network of weak bonds, which is largely maintained in the allosteric transition, in spite of some changes in the orientation of specific amino acid side chains. B37 (C3) Trp in particular makes contacts with $\alpha$92(FG4) Arg, $\alpha$94(G1) Asp, and $\alpha$95(G2) Pro in both oxy- and deoxyhemoglobin; nevertheless, it is a good probe of the allosteric transition because its optical spectrum is perturbed upon ligand binding (Briehl and Hobbs, 1970; Perutz et al., 1974). The amino acid residues at the $\alpha$C-$\beta$FG contact region of the $\alpha_1\beta_2$ interface undergo an important reorganization in the course of the T-R transition. In deoxyhemoglobin the side chain of B97(FG4) His lies between $\alpha$44(CD2) Pro and $\alpha$41(C6) Thr, while in oxyhemoglobin this same His settles between $\alpha$41(C6) Thr and $\alpha$38(C3) Thr. Thus, this region of the $\alpha_1\beta_2$ interface, which seems to be compatible with only two states, plays a key role in determining the allosteric transition (see Fermi and Perutz (1981)).

Baldwin and Chothia (1979) summarize the function of the $\alpha_1\beta_2$ interface with the definition of the $\alpha$FG-$\beta$C contact region as a “flexible joint” and the $\alpha$C-$\beta$FG as a “switch.” Hence the amino acid residue at position C3 has a different role in the $\alpha$ and $\beta$ chains; B37(C3) Trp is involved in a series of contacts, which stabilize the tetramer in both the oxy and deoxy derivatives, while $\alpha$38(C3) Thr participates in the reorganization of the $\alpha_1\beta_2$ interface associated with the allosteric transition.

Human Hb has been expressed in Esherichia coli and yeast, and several interesting mutants have been prepared to test specific hypotheses (Nagai et al., 1985; Martin de Llano et al., 1993; Komiyama et al., 1995). In view of the considerations reported above, we have investigated the role of the residue at C3 in both chains of human hemoglobin by synthesizing the site-directed mutants bearing in the $\beta$ chains the residue found in the $\alpha$ chains and vice versa. The type of substitution to be inserted at C3 was chosen to probe the effect of changes in the number of atomic contacts (Schaad et al., 1993), avoiding the introduction of charged residues. Three hemoglobins were therefore expressed in E. coli: the single mutants $\alpha$38 Thr $\rightarrow$ Trp ($\alpha$T38W)1 and $\beta$37 Trp $\rightarrow$ Thr ($\beta$W37T) and the double mutant $\alpha$38 Thr $\rightarrow$ Trp/$\beta$37 Thr $\rightarrow$ Trp ($\alpha$T38W/$\beta$W37T, called herein the double mutant). It may be recalled that there are no known natural mutants of $\alpha$38 Thr, while the two natural mutants of $\beta$37 Trp, Hb Hirose and Hb Rothschild, have Ser and Arg, respectively (Yamaoka, 1971; Gacon et al., 1977; Sasaki et al., 1978) (see also Huisman (1992)).

Our three mutants were compared to wild type HbA with respect to the reaction with oxygen and carbon monoxide, the

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The atomic coordinates (1GLI) and structure factors (R1GLISF) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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The abbreviations used are: $\alpha$T38W, mutant Hb $\alpha$38 Thr $\rightarrow$ Trp; $\beta$W37T, mutant Hb $\beta$37 Thr $\rightarrow$ Trp; $\alpha$T38W/$\beta$W37T, mutant Hb $\alpha$38 Thr $\rightarrow$ Trp/$\beta$37 Thr $\rightarrow$ Thr; SVD, singular value deconvolution algorithm.

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stability of liganded and unliganded tetramers, the kinetics of association of deoxy dimers and the dissociation of deoxy tetramers, the effect of sodium iodide, and the changes in the molecular contacts at the interface by x-ray crystallography of deoxy-Hb α38 Thr → Trp by modeling.

Structural analysis leads to simple predictions on the role of the α₁β₂ interface, suggesting that (a) semi-conservative mutations at topological position C3 affect tetramer stability and cooperativity in a simple manner, without loss of allosteric behavior; (b) substitutions at equivalent positions of the α and β chains should yield different functional effects, in spite of the pseudo-symmetry of that interface. By-and-large these predictions are fulfilled by our results.

EXPERIMENTAL PROCEDURES

Materials—The site-directed mutant hemoglobin was produced in E. coli and purified as described previously (Nagai et al., 1985; Hoffman et al., 1990; Vallone et al., 1993). Reagents were of analytical grade.

Functional Properties—The oxygen binding isotherms were determined using the tonometric method (Rossi Fanelli and Antonini, 1958). The time course of O₂ binding to deoxyhemoglobin was followed using either the Applied Photophysics stopped flow apparatus (Leatherhead, United Kingdom) or the flash photolysis apparatus described by Brunori and Giacometti (1981).

The time course of dissociation of deoxyhemoglobin into dimers was monitored by recording the optical spectrum (in the Soret region) after mixing deoxyhemoglobin with a stoichiometric amount of haptoglobin (isoform 1.1 purchased from Sigma), as described by Ip et al. (1984). The map was symmetry-averaged about the molecular dyad to improve accuracy.

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Oxygen Equilibrium Experiments—The oxygen binding properties of the three site-directed mutants and HbA, depicted in Fig. 2, show that both single mutants are cooperative and display Hill coefficients larger than 2, while the double mutant is almost non-cooperative (n = 1.3).

In spite of its limitations (see, for example, Ackers et al. (1992)), the analysis of the oxygen binding isotherms was carried out according to the two-state allosteric model (Monod et al., 1965). Fit of data is often beset by uncertainties in Kᵣ and L₀. Nonetheless, since kinetic data on O₂ and CO have provided independent support that Kᵣ is identical or very close to that of the isolated α and β chains and of the αβ dimer (Edelstein and Gibson, 1987; Szabo and Karplus, 1972), we fitted the data in Fig. 2 assuming Kᵣ to be the same for HbA and all the mutants, and thus similar to the oxygen affinity of the isolated chains. This assumption proved compatible with a good fit, with the parameters presented in Table I. By contrast, very poor fits were obtained if Kᵣ of the mutants was imposed to be the same as that of HbA, which is not surprising and agrees with the theory of Szabo and Karplus (1972). Within the limitations of the two-state model, the numerical values of the allosteric
The values of $p_{50}$, $K_a$, and $K_T$ are expressed in mmHg; swp is the calculated switch-over point (i.e. the oxygen saturation at which the concentration of T state Hb equals that of the R state); experimental conditions are as described in the legend of Fig. 2.

| Hemoglobin | $p_{50}$ | n | $K_a$ | $K_T$ | $L_0$ | swp |
|------------|---------|---|-------|-------|-------|-----|
| A          | 7.1     | 2.9 | 0.2   | 27.4 ± 2.0 | 1.3 ± 0.5 × 10^6 | 2.9 |
| αT38W      | 5.6     | 2.5 | 0.2   | 15.4 ± 1.0 | 4.2 ± 1.0 × 10^5 | 3.0 |
| βW37T      | 3.7     | 2.1 | 0.2   | 12.2 ± 1.0 | 1.9 ± 0.6 × 10^5 | 3.0 |
| αT38W/βW37T| 3.3     | 1.3 | 0.2   | 4.2 ± 0.5 | 8.9 ± 2.0 × 10^4 | 3.7 |

parameters indicate that mutations at C3 (i) affect the equilibrium constant between the two quaternary states, reducing the difference in stability between T and R state Hb at each ligation state were taken from Table I; the values of $k_9$ were obtained by partial flash photolysis, which is known to populate partially liganded species that recombine as fast as the static clusters (Sawicki and Gibson, 1976; Vandegriff et al., 1991; Jones et al., 1992). The CO combination data in Fig. 3 are satisfactorily fitted with this model, regardless of the presence of evident kinetic cooperativity, and the rate constants are given in Table II. Regarding Hb βW37T, an autocatalytic time course may have been expected. However, it is known that speeding up the CO combination is very sensitive to the value of c, which may not be identical for O2 and CO, even though this difference is sometimes very difficult to demonstrate. Under these conditions the estimate of $T_{1/2}$ can differ from 0, which introduces some uncertainties, and the overall time course is essentially exponential.

Even complete photolysis of dilute solutions of HbCO usually follows a biphasic rebinding time course (Antonini and Brunori, 1971). This has been attributed to the presence of rapidly reacting dimers in equilibrium with the slowly reacting tetramers (Gibson and Antonini, 1967; Edelstein et al., 1970). Thus flash photolysis has also been used to probe the extent of dissociation of HbCO into dimers. Employing this technique, Vallone et al. (1993) have shown that Hb αT38W CO is a more stable tetramer than HbA CO, by approximately 0.6 kcal/mol. Similar experiments carried out as a function of Hb concentration (data not shown) have demonstrated that the CO derivative of the two βW37T mutants is almost completely dissociated into dimers at [Hb] = 15 μM, and thus a lower limit of 40 μM can be set to the value of the tetramer-dimer dissociation constant of these mutants.

Since O2 and CO exhibit nearly parallel equilibrium curves, the data in Tables I and II may be considered together even though most of the cooperativity is expressed in the dissociation rate constants in the case of O2 and in the association rate constants in the case of CO (Antonini and Brunori, 1971; Szabo, 1978).

A significant conclusion is that all our mutants display a ligand affinity higher than HbA in the T-state. However, this increase is smaller for the two single mutants, and substantial for the double mutant, in keeping with the reduced number of contacts at the α1β2 interface (see below). More interestingly, the value of $L_0$ decreases significantly in the mutants, being, in the double mutant, -15-fold smaller than in HbA.

Recombination of Dimers following Oxygen Dissociation—The time course of the recombination of deoxygenated αβ dimers from HbA and the mutant Hbs was studied by recording the slow optical transitions that follow the rapid deoxydiation by dithionite of a dilute solution of HbO2 (Antonini et al., 1968; Keletti and Gutfriend, 1970), given that, at micromolar concentration, HbO2 is an equilibrium mixture of tetramers and dimers. This is possible because (a) the optical spectrum of deoxyhemoglobin in the T-state differs from that in the R-state (the transient R0 Hb, as described by Sawicki and Gibson (1976); the deoxy dimers and the deoxy chains, as reviewed by Bellini and Brunori (1994)), and (b) the T0-R0 difference spectrum is, within errors, the same for HbA and our three mutants.
The experiment is described in the following scheme (Scheme 1).2

\[
2^a(\alpha\beta)(O_2)_2 \rightarrow 2^b(\alpha\beta) \rightarrow T^c(\alpha\beta)
\]

**Scheme 1**

An example of this type of experiment for HbA and the three mutants is reported in Fig. 4. The plot shows the second column of \(V\) (from the SVD analysis), which contains most of the slow optical transition. The faster phase seen in this figure is the time course of \(O_2\) dissociation (upon mixing with dithionite), and is described by steps 1 and 3 of Scheme 1. The slower phase (step 2 in the same scheme) relates to the time course of association of deoxy dimers. The relative amplitude of this phase is a measure of the fraction of oxy dimers in equilibrium with oxy tetramers. Since addition of 0.5 M NaI promotes complete dissociation of the liganded tetramers but does not prevent the reassociation of unliganded dimers (Kellett and Gutfreund, 1970), experiments carried out under these experimental conditions yield the full amplitude of the slow phase. Estimates for the rate constant of deoxy dimers association for HbA and the three mutants (in the absence and in the presence of 0.5 M NaI) are reported in Table III.

**Rate of Dissociation of Unliganded Tetramers into Dimers**—

The dissociation of deoxyhemoglobin was followed by the absorbance changes at 430 nm after reaction with haptoglobin, recorded either statically or kinetically (Ip et al., 1976). This method relies on the essentially irreversible binding of Hp to free \(\alpha\beta\) dimers, which forces the tetramer-dimer equilibrium toward dissociation, the rate-limiting step being dimerization (see also Nagel and Gibson (1972)). In view of the results reported above, the effect of NaI has also been tested on unliganded Hb.

The static difference spectra obtained upon binding to Hp are very similar to those obtained by the kinetics of dimer recombination (data not shown). The total absorbance change for complete dissociation of oxy tetramers into dimer changes and to be 13% of the total absorbance of the sample at 430 nm. This is in good agreement with the value expected for the \(T_{R_2}\) spectral change (for a review, see Bellieli and Brunori (1994)).

Fig. 5 shows the observed time courses of dissociation. This experimental approach suffers from the extremely slow time course, which, at lower temperatures, extends over several days; thus, for some proteins and under some conditions, the optical transition could not be followed to completion. Nonetheless, the time course was fitted in all cases to a single exponential following Ip et al. (1976). In addition, the asymptotic value was independently estimated from the total optical density change (see above).

**Table IV** reports the first order rate constants determined from the data in Fig. 5, HbA and Hb \(\alpha\text{T38W}\) behave similarly, while both Hb \(\beta\text{W37T}\) and the double mutant dissociate faster than HbA (in spite of being tetrameric in the deoxygenated derivative, as judged by the recovery of the expected optical transition). The effect of NaI on the dissociation of HbA and Hb \(\beta\text{W37T}\) is the same (see the ratio \(k/\text{NaI}\) in Table IV). However, quite unexpectedly, this effect is considerably reduced in the two mutants in which \(\alpha\text{B}38\) (C3) is mutated to Trp; this point will be discussed below, with reference to the structure of the interface.
Mutants of the $\alpha_1\beta_2$ Interface of Hb

Crystallographic Structure of Deoxy-Hb: $\alpha38$ Thr → Trp — Fig. 6 (panel A) shows the difference map $F(Hb_{\alpha T38W}) - F(HbA)$ in the deoxy state; in panel B only the negative values of the difference map (red contours) are shown, and the HbA structure is displayed. The excellent fit of the Trp side chain to the main positive peak may be seen. The largest negative peak represents the absence in this mutant of Thr $\alpha38$ O-$\gamma$-1 and a bound water molecule, seen in the structure of HbA; a second less intense negative peak may correspond to another water molecule. In the mutant, Trp C7 overlaps with the native Thr C7-2 of HbA and thus no difference peak is observed. It may be clearly seen that there is no evidence of any change in position of neighboring residues of the $\alpha_1\beta_2$ interface.

Molecular Modeling — Modeling of the T and R structures of our mutants (over and above the crystallographic structure of deoxy-Hb $\alpha T38W$) was limited to simple geometric computations, such as the number of atoms in contact with the side chain at position C3, and the number of side chains interactions lost or established after amino acid substitutions. Nevertheless even this simple analysis yielded information useful for the interpretation of functional experiments.

As shown in Table V, upon introduction of a Trp at $\alpha38$ (C3) the increase in the number of interface contacts between $\alpha_1$ and $\beta_2$ is negligible in deoxy, but considerable in oxy-Hb. Conversely the substitution Trp $\rightarrow$ Thr at $\beta37$ (C3) leads to a substantial decrease in the number of contacts in both oxy- and deoxy-Hb, with loss of a hydrogen bond between $\beta37$ Trp and $\alpha94$ Asp in the deoxy-Hb (see Fig. 7, panel C). Hence one could deduce that compared to HbA, the $\alpha T38W$ mutant has a more compact and extensive $\alpha_1\beta_2$ interface especially in the R-state; on the other hand, Hb $\beta W37T$ has a looser interface, especially in the T-state, with loss of a hydrogen bond.

Assuming that these effects are additive in the double mutant, one may expect that in this hemoglobin cooperativity will be severely impaired, since the R-state would be favored with a concomitant destabilization of the T-state; this prediction agrees well with the experimental data reported above.

In order to compare the data obtained on Hb $\beta W37T$ with observations reported in the literature on Hb Hirose (which bears the substitution $\beta W37S$, Sasaki et al., 1978), we have measured the atoms in a sphere of 4 Å to a Ser in $\beta C3$. As may be seen in Table V, the number of contacts in Hb Hirose is reduced, in both oxy and deoxy state, compared to our mutant $\beta W37T$, in agreement with the experimental data (see “Discussion”).

Inspection of the water accessible surface in the proximity of position C3 allows evaluation of the cavities at the edge of the $\alpha_1\beta_2$ interface, and how the shape of the surface is affected by the mutations. The crystallographic structure of deoxy-Hb $\alpha T38W$ shows that the Trp in $\alpha C3$ lies flat on the edge of the interface, excluding $\beta 145$ Tyr and $\beta 100$ Pro from contact with the solvent, and thus seems to act as a “hydrophobic plug” (Fig. 7, panel F). Modeling the oxygenated state of the same mutant shows that the side chain of the Trp introduced in $\alpha C3$ could also fit nicely in a cavity, in contact with $\beta 145$ Tyr.

The substitution $\beta W37T$, on the other hand, seems to deepen a cavity at the edge of the opposite side of the $\alpha_1\beta_2$ interface, possibly allowing to $\alpha 141$ Arg greater accessibility to the external medium.

**TABLE IV**

| Hemoglobin | $k$ | $k'$ (0.5 M NaI) | $k'/k$ |
|------------|-----|-----------------|--------|
| A          | 0.03| 0.4             | 13.3   |
| $\alpha T38W$ | 0.02| 0.06            | 3.0    |
| $\beta W37T$ | 0.1 | 1.3             | 13.0   |
| $\alpha T38W/\beta W37T$ | 0.8 | 2.9             | 3.6    |

**DISCUSSION**

Allosteric Properties of the Mutants — This paper presents a correlation between structural and functional properties of two

![Symmetry averaged difference map $F(Hb_{\alpha T38W}) - F(HbA)$. The map is contoured at +0.15 (green contours) and −0.15 (red contours), or approximately 3 times the root-mean-square density value of the unaveraged map. Panel a, the superimposed model is that of wild type HbA with $\alpha38$ Thr replaced by Trp with γ1 angle −60°. Residues of the $\alpha_1$ chain are labeled with their ordinary position numbers and the letter A, those of the $\beta_2$ chain with position numbers increased by 600 and the letter D. Panel b, only the negative values of the difference map are shown (red contours) and the HbA structure is displayed. The dashed lines represent hydrogen bonds. Labels as in panel a.](image-url)
single mutants and one double mutant in the \(\alpha_3\beta_2\) interface of human hemoglobin. The mutations are in the so-called "flexible joint" and "switch" regions, which are pseudo-symmetric, \(\alpha T38W\) being in the switch region, and \(\beta W37T\) in the flexible joint; the double mutant contains both mutations. The first mutation (Thr \(\rightarrow\) Trp) might be expected to affect primarily the allosteric properties, while the second (Trp \(\rightarrow\) Thr) might also affect the stability of liganded and unliganded tetramers. Most, but not all, of the results presented above bear out these predictions. The properties of these new mutants are discussed in the framework of the two-state MWC model (Monod et al., 1965), in spite of the limitations that have been discussed in the literature (see, for example, Ackers et al. (1992)). The model provides a convenient analytical description of the data and allows discussion of the more interesting findings with a nomenclature known to most.

Hb \(\alpha T38W\), which bears two Trp residues in the \(\alpha_3\beta_2\) interface, is a slightly more stable tetramer than HbA, especially in the liganded form where the tetramer dissociation constant is decreased 6-fold (Vallone et al., 1993). The three-dimensional structure of this mutant in the deoxy state shows that the indole side chain of Trp \(\alpha 38\) has been accommodated by expulsion of two water molecules hydrogen-bonded to \(\alpha 38\) Thr in HbA, without significant changes of the adjacent residues (Fig. 6). Thus, the enhanced stability of the oxy tetramer is consistent with the increased hydrophobic character of the interface (which is not perturbed by the bulkier side chain), with the more extensive contacts of the indole (Table V), and with the hypothesis that the most significant contribution to the stability of the tetramer resides in the pseudo symmetric \(\alpha F G-\beta C\) contact, which is unmodified. The slightly increased affinity of the T-state observed for Hb \(\alpha T38W\) as compared to HbA is not easily accounted for, given that the constraints in the T-state are not decreased. On the other hand, the lower value of \(L_o\) is consistent with the increased stability of R and thus with a (slightly) reduced cooperativity.

Hb \(\beta W37T\), which has no Trp residues in the \(\alpha_3\beta_2\) interface, dissociates into dimers more readily than HbA, in both the unliganded and liganded derivatives, as shown by results from analytical ultracentrifuge and flash photolysis to be published elsewhere, indicating that Trp at \(\beta 3\) contributes to the stability of the \(\alpha_3\beta_2\) interface in both quaternary structures. In spite of the destabilization of this interface, cooperativity of \(\beta W37T\) is reduced but preserved, not only because the contacts in the \(\alpha C-\beta F G\) switch region (\(\beta 97\) His, \(\alpha 42\) Thr, \(\alpha 44\) Pro, and \(\alpha 38\) Thr) are preserved, but also because Thr at \(\beta 37(C3)\) can to some extent fulfill the role of Trp in the flexible joint, as indicated by modeling (Table V). The remarkable but limited competence of Thr at \(\beta 37(0)\) residue is reflected in a small increase of the \(O_2\) affinity of the T-state (Table I) and a somewhat greater increase of the rate constant for CO binding to deoxy-Hb (Table II); thus, the constraints of the T-state are partially released by mutation of \(\beta C3\) and essentially maintained by mutation of \(\alpha C3\).

The interesting behavior of the double mutant Hb \(\alpha T38W/\beta W37T\) may be understood qualitatively on the basis of the properties of the two single mutants. Although the double mutant, like HbA, has only one Trp residue at the \(\alpha_3\beta_2\) interface, ligand binding and tetramer dissociation are both remarkably different from wild type. This is the best evidence that the effect of mutations at topological position C3 is asymmetric and that perturbing the flexible joint and the switch indeed has different consequences. This double mutant dissociates more readily into dimers in both the liganded and unliganded derivatives, which we attribute largely to the flexible joint mutation \(\beta W37T\). However, cooperativity is also reduced, even when compared to that of the two single mutants, as shown by the increased \(L_o\) affinity of the T-state (Table I). These properties may be understood on the basis of two synergistic effects, i.e. a significant release of the interface constraints due to \(\beta W37T\), leading to a more relaxed T-state (hence the smaller \(K_r\)), and an increase in the stability of the R-state tetramer, related to mutation \(\alpha T38W\). As a result the energy difference between the two allostERIC states is reduced, as indicated by the value of the allostERIC constant \(L_0\), which is smaller than HbA by 15-fold.

As already stated, there is no known natural mutant of position \(\alpha 38\) (Huisman, 1992); however, two site-directed mutants of this residue, namely Hb \(\alpha T38S\) and \(\alpha T38V\), have been expressed and characterized by Hashimoto et al. (1993). The functional properties of both these Hbs are by and large similar to those of HbA. However, the substitutions in these cases are semiconservative, and it is easily conceivable that the residues effectively replace the wild type Thr.

As to position \(\beta 37\), our data may be compared with those of the natural mutants Hb Hirose and Hb Rothschild. Comparison of Hb \(\beta W37T\) with Hb Rothschild (\(\beta 37\) Trp \(\rightarrow\) Arg; Gacon et al. (1977)) is complex, because of arginine’s positive charge. Nonetheless, consistent with the other \(\beta 37\) mutants, Hb Rothschild displays reduced cooperativity and an increased tendency to dissociate into dimers. However, the structure of the deoxygenated derivative (Kavanaugh et al., 1992b) shows a novel and strong chloride binding site, which affects the functional properties of this Hb.

Hb Hirose (\(\beta 37\) Trp \(\rightarrow\) Ser) displays extremely low (if any) cooperativity, very rapid CO binding by flow, and a high tendency to dissociate into dimers in both the presence and the absence of oxygen (Sasaki et al., 1978). Hb \(\beta W37T\), on the other hand, although extensively dissociated into dimers when liganded, is fully associated as the deoxy derivative (Fig. 5) and maintains a higher cooperativity than Hb Hirose, confirming that Thr but not Ser is a partially competent substitute for \(\beta 37\) Trp at the flexible joint. The computer-simulated structures of oxy- and deoxy-Hb \(\beta W37T\) (Fig. 7) indicate that the methyl group of Thr \(\beta 37\) makes contacts with \(\alpha 95\) Pro and \(\alpha 140\) Tyr in the oxy derivative and with \(\alpha 140\) Tyr in the deoxy derivative; these contacts are lost or less extensive with Ser (Table V).

A site-directed mutant of position \(\beta 37\), in which a Phe substitutes the Trp, has been obtained by Ishimori et al. (1992). In this case the Phe would be expected to provide a more effective

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**Table V**

| Residue | Deoxyhemoglobin Contacts | No. of atoms | Oxyhemoglobin Contacts | No. of atoms |
|---------|--------------------------|--------------|------------------------|--------------|
| \(\alpha 38\) Thr | \(\beta 100\) Pro | 7 | \(\beta 97\) His | 4 |
| \(\alpha 38\) Trp | \(\beta 100\) Pro | 3 | \(\beta 97\) His | 6 |
| \(\alpha 38\) Thr | \(\beta 145\) Tyr | 3 | \(\beta 145\) Tyr | 4 |
| \(\beta 37\) Trp | \(\alpha 92\) Arg | 2 | \(\alpha 92\) Arg | 4 |
| \(\beta 37\) Thr | \(\alpha 92\) Arg | 2 | \(\alpha 92\) Arg | 4 |
| \(\beta 37\) Ser | \(\alpha 92\) Arg | 3 | \(\alpha 92\) Arg | 4 |
Mutants of the $\alpha_1\beta_2$ Interface of Hb
replacement for Trp than either Hb Hirose or our mutant. Unfortunately the authors did not measure the tetramer-dimer equilibrium and kinetics on their mutant Hb. As with βW37T, Hb ββ37F displays high oxygen affinity and low cooperativity, even though the 1H NMR spectrum of this mutant is consistent with that typical of T-state HbA.

Rates of Dimer Reassociation and Tetramer Dissociation in Deoxyhemoglobin—Fitting the time course of association of deoxy αβ dimer to a second order reaction (as reported in Fig. 4) shows small but systematic deviations, which are more marked for the mutants than for HbA. Nevertheless, the time course of reassociation of deoxy αβ dimers of HbA, when fitted to the simplest possible scheme (see Scheme 1, under “Results”), yields a rate constant at low salt concentration slightly larger than, but not inconsistent with, that reported in the literature (Gray, 1974; Wiedermann and Olson, 1975). Small differences may be due either to the experimental conditions or the analysis. Among the rate constants reported in Table III, those determined for Hb βW37T and for the double mutant are more reliable because these Hbs are completely dissociated into dimers as HbO2, and thus the total signal is larger and the initial dimer concentration does not need to be fitted.

Interestingly, Table III shows that the rate constant of reassociation of unliganded dimers and the effect of NaI are the same (within a factor of two) for all four hemoglobin. Thus, the effect of the amino acid residue at position C3. This is evident comparing the ratio of the rate constants k+ and k− reported in Table IV. With a Trp in the switch region as in Hb αT38W, I− accelerates dissociation only 3-fold compared to 13-fold in HbA. We interpret the reduced effect as resulting from the more hydrophobic character of the interface and from the position of Trp, which seems to act as a hydrophobic plug, shielding from contact with the solvent β145 Tyr and β100 Pro and forming a barrier to the penetration of this ion.

When Trp is removed from the flexible joint as in Hb βW37T, I− accelerates dissociation approximately 13-fold, just as in HbA. In deoxy-Hb, β37 Trp is on the edge of the αβ37 interface (Figs. 1 and 7), and its orientation is such that α95 Pro and α140 Tyr are also exposed to the external medium; mutation of Trp with Thr therefore does not result in exposure to the solvent of side chains previously buried in the interface. Thus, the similarity in the effect of I− on the dissociation rate constants of HbA and Hb βW37T mutant is fully consistent with the proposal that access of the anion to the αβ37 interface is toward the switch region, near position α38, and not affected by substitution in the flexible joint region. In support of this hypothesis, the effect of I− on the dissociation rate constants of HbA and Hb βW37T mutant is fully consistent with the proposal that access of the anion to the αβ37 interface is toward the switch region, near position α38, and therefore not affected by substitution in the flexible joint region. In support of this hypothesis, the effect of I− on the dissociation rate constants of HbA and Hb βW37T mutant is fully consistent with the proposal that access of the anion to the αβ37 interface is toward the switch region, near position α38, and therefore not affected by substitution in the flexible joint region.
Mutants of the $\alpha_1\beta_2$ Interface of Hb

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