Modification of α-Chain or β-Chain Heme Pocket Polarity by Val(E11) → Thr Substitution Has Different Effects on the Steric, Dynamic, and Functional Properties of Human Recombinant Hemoglobin

DEOXY DERIVATIVES*

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The dynamic and functional properties of mutant deoxyhemoglobins in which either the β-globin Val^E11^ or the α-globin Val^E11^ is replaced by threonine have been investigated through the thermal evolution of the Soret absorption band in the temperature range 300 to 20 K and through the kinetics of CO rebinding after flash photolysis at room temperature. The conformational properties of the modified α chain and β chain distal heme pockets were also studied through x-ray crystallography and molecular modeling. The data obtained with the various techniques consistently indicate that the polar isosteric mutation in the distal side of the α chain heme pocket has a larger effect on the investigated properties than the analogous mutation on the β chain. We attribute the observed differences to the presence of a water molecule in the distal heme pocket of the modified β chain, interacting with the hydroxyl of the threonine side chain. This is indicated by molecular modeling which showed that the water molecule present in the α chain distal heme pocket can bridge by H bonding between Thr^E11^ and His^E7^ without introducing any unfavorable steric interactions. Consistent with the dynamic and functional data, the presence of a water molecule in the distal heme pocket of the modified β chains is not observed by x-ray crystallography.

The mechanism of hemoglobin cooperativity requires the concerted action of the constituent α and β subunits. The presence of conformational differences between the α and β heme pockets is well recognized (1–3). This is reflected in different functional characteristics of the α and β subunits, as higher oxygen affinity of the α subunits with respect to β subunits (4), and increased affinity of the α heme pocket for the heme (5). An approach to a better understanding of the factors regulating the functional properties of the heme pockets is to investigate the effect of the same mutation on the dynamics and ligand accessibility of the α and β pockets of mutant hemoglobins carrying the same amino acid substitution and to relate these effects with the conformational properties of the heme pockets. The latter can be characterized by techniques such as crystallographic analysis or molecular modeling. This approach is now feasible owing to the recent development of recombinant techniques for the expression and production of mutant hemoglobins (6–8).

Access of ligand to the heme group is hindered by the presence of the polypeptide chain which buries the heme in a hydrophobic crevice in the protein interior. Iron-ligand combination must therefore be accompanied by conformational fluctuations in the protein which create a pathway for ligand entry. It has been proposed that such a pathway involves concerted movements of the side chains of His^E7^ and Val^E11^ (9, 10). Several mutant hemoglobins carrying the same mutation in the α and β heme pocket have been obtained, and the association and dissociation rate constants for O2 and CO binding to R-state derivatives have been measured (11). Replacement of His^E7^ with Gly and Gln affected the kinetic parameters of O2...
and CO binding to the α heme but did not affect the kinetic parameters for the binding of these ligands to the β heme. Similarly, replacement of Val(E11) with Ala and Leu affected the kinetic parameters of O₂ and CO binding to the α heme but not to the β heme; conversely, replacement of Val(E11) with Ile produced smaller changes in the rate of ligands binding to α than to β heme pocket mutants. These data point to a different role of His(E7) and Val(E11) in the α and β pockets of R state hemoglobin.

The α and β heme pockets are hydrophobic, as the only two polar residues present are the proximal and distal histidines. In our laboratory we have extensively investigated the effect of polar residues present are the proximal and distal histidines.

In the α-globin the Thr(E11) was introduced using the Promega pAlteR Mutagenesis System. The mutagenic oligonucleotide employed changed the Val codon GTC to the Thr codon ACC. The Ala(E12) codon (GCC) was also changed to GCT which created a MspAI site to facilitate screening for the mutation.

Spectra of recombinant hemoglobins were stored in the CO form at −80 °C or in liquid nitrogen. The proteins were thawed immediately before use and diluted with the appropriate buffer.

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Spectroscopic Measurements—Portions of the stock solutions, thawed immediately before use and suitably diluted, were converted to the oxy form by photolysis under a flux of oxygen. Oxygen was then removed by flushing with nitrogen, and the sample was reduced by adding sodium dithionate; all steps were performed in the cold and, when dithionite was present, under anaerobic conditions. The final samples for spectroscopic measurements were 65% (v/v) glycerol/water solutions containing 0.1 M potassium phosphate buffer (pH 7 in water, at room temperature) and ~3·10⁻³ M sodium dithionite; the final protein concentration was ~10⁻⁴ M in heme.

Optical spectra in the spectral region 500 to 730 nm and in the temperature range 300 to 20 K were recorded in digital form at 0.4-nm intervals using a Cary 2300 spectrophotometer set at 0.4-nm bandwidth, 1-s time constant, and 40 nm/min scan speed; under these conditions the spectral resolution is about 20 cm⁻¹ at 450 nm. The base line (cuvette + solvent) was measured at room temperature and subtracted from each spectrum (in this spectral range, the base line does not depend on temperature, and absorption due to dithionite is negligible); moreover, we stress that our samples remained homogeneous and transparent at all temperatures. All other experimental details were as described previously (16, 17).

Spectral Data Analysis—The spectral deconvolution used in this paper yields information on the different contributions to the line width and on the various parameters that characterize the vibrational coupling. An analytical expression for the Soret band profile at various temperatures is obtained by considering a single electronic transition coupled to Franck-Condon active vibrational modes within the adiabatic and harmonic approximation. Details on the theoretical approach used have been given in previous publications (17, 18), where it has been shown that the absorbance at frequency ν can be written as a progression of Voigtians (i.e. Gaussian convolutions of Lorentzians) (Equation 1).

\[ A(λ) = Mν \left( \sum_{i=1}^{N} \frac{S_i ν_0}{λ - ν_0 + G_i} \right) \oplus \frac{1}{σ(T)} \]  

(Eq. 1)

where \( M \) is a constant proportional to the square of the electric dipole moment, and \( Γ \) is a damping factor related to the finite lifetime of the excited state (homogeneous broadening); the product extends to all high frequency vibrational modes (i.e. with \( hν > k_BT \) coupled to the electronic transition, the summations to their occupation numbers, while \( ν_0 \) and \( S_i \) are, respectively, the frequency and the linear coupling constant for the \( i \)th high frequency mode; \( ν_0 \) is the frequency of the fundamental. In Equation 1 quadratic coupling of the high frequency vibrational modes is neglected. The symbol \( \oplus \) indicates the convolution operator. Coupling of the electronic transition with low frequency modes (i.e. with modes having frequency smaller than the observed bandwidth) is treated within the "short times approximation" (19); this brings about the convolution with a gaussian line shape and contributes the temperature-dependent terms \( σ(T) \) and \( ν_0(T) \) to the line width and peak position of the band, respectively.

Further contributions to the spectral line width are inhomogeneous broadening arising from different conformational substates and heme environments (20). For the deoxy derivatives, it has been suggested (18, 21–23) that the energy of the \( σ → π^* \) electronic transition responsible for the Soret band depends upon the position of the iron atom relative to the heme plane (see Equation 2), and therefore,

\[ ν_0 = ν_0(Q = 0) + b Q^2 \]  

(Eq. 2)

where \( Q \) is a generalized iron coordinate representing both the out-of-plane displacement and other angular coordinates such as the histidine tilt and the azimuthal orientation of the vector connecting the iron with the histidine nitrogen, and \( b \) is a proportionality constant that reflects the electronic properties of the iron-porphyrin system. The coordinate \( Q \) is assumed to have a statistical distribution of width \( b \) around the mean coordinate position \( Q_0 \) (see Equation 3).

MATERIALS AND METHODS

Sample Preparations—Growth, expression, and purification of the recombinant α- and β-chains, reconstitution, and assembly into tetrameric hemoglobin followed the protocols previously described (7, 8). In the tetrameric hemoglobin obtained with this type of expression system one type of subunit is native and the other is recombinant (αHbA and βHbA). This allows the unambiguous assignment of observed differences to the recombinant subunit. The expression and purification of the β(E11)T mutant has been previously described (12).

The abbreviations used are: β(E11)T, human hemoglobin with a Thr at position E11 in the recombinant β chains; α(E11)T, human hemoglobin with a Thr at position E11 in the recombinant α chains; HbA, native human hemoglobin; αHbA, human hemoglobin with recombinant α chains; βHbA, human hemoglobin with recombinant β chains; Bis-Tris, 2-[bis(2-hydroxyethyl)aminol-2-(hydroxymethyl)-propane-1,3-diol.
Substitution of Equation 3 into Equation 2 yields a non-gaussian distribution of transition frequencies. The analytical expression that describes the Soret band line shape for the deoxy derivatives (18, 21, 22) is shown in Equation 4.

\[
P(Q) \propto \exp\left(-\frac{(Q - Q_0)^2}{2\sigma^2}\right)
\]  

(Eq. 3)

The fitting parameters are \( M, \Gamma, S, \alpha, \nu, Q_0, \sqrt{\delta}, \) and \( \delta \sqrt{\Gamma} \); the values relative to high frequency modes are taken from resonance Raman spectra of human deoxyhemoglobin reported in the literature (25). As in previous work (14), we assume that the mutations investigated do not alter the frequency of these modes; this assumption is supported by the excellent quality of the fittings obtained (see Fig. 2 and Table I). The most coupled modes are those centered at 570, 674, and 1357 cm\(^{-1}\) whereas other less coupled modes do not contribute significantly to the line shapes and are therefore neglected. It is worth mentioning that in the resonance Raman spectra of hemoglobin the frequencies 674 cm\(^{-1}\) and 1357 cm\(^{-1}\) correspond to very sharp lines and are thought to arise from in-plane vibrational modes of the heme group (the well known \( v_1 \) and \( v_3 \) respectively; Ref. 26). On the contrary, 370 cm\(^{-1}\) is the “average effective” frequency accounting for a spectral region characterized by several quasi-degenerate peaks; both in-plane and out-of-plane modes contribute to these spectral regions. \( \Sigma \) parameters are fixed at the low temperature values determined from the clearly resolved vibronic structure of the band; in this way we avoid fitting ambiguities arising from broadening of the band and consequent lack of a clearly resolved vibronic structure at high temperature.

The second step includes the temperature dependence of parameter \( \sigma^2 \) (i.e. the gaussian width of the band) in Equation 4. This is done within the Einstein harmonic oscillator approximation and considering the coupling of the Soret band with a “bath” of low frequency modes (18); within this model (Equation 5) one has

\[
\sigma^2 = N S < v > \times \frac{2 \text{coth}(h\nu/kT)}{2kT}
\]  

(Eq. 5)

where \( < v > \) and \( S \) are the effective frequency and linear coupling constants of the low frequency bath, and \( N \) is the number of soft modes. The \( \sigma^2 \) increase with temperature predicted by Equation 5 simply reflects the well known amplitude increase of harmonic nuclear motions as the temperature is increased. As mentioned above, the effect of inhomogeneous broadening is taken into account by the parameter \( \delta \sqrt{\Gamma} \). The \( \sigma^2 \) thermal behavior therefore gives information on dynamic properties (linear coupling with the low frequency bath of the system) that involve motions not only of the chromophore but also of the heme pocket and of larger parts of the protein.

Complementary information on the stereodynamic properties of the heme pocket is also obtained from the parameter \( \nu_b \) (i.e. the peak frequency of the band); in fact, its temperature dependence reflects not only the quadratic coupling of the electronic transition with the bath of soft modes but also the local electric field experienced by the chromophore within its surroundings.

**CO Binding Kinetics**—Flash photolysis was carried out in solutions containing 5 \( \mu \)m heme and 50 \( \mu \)m CO at 23 °C in 0.1 \( \mu \)m Bis-Tris (pH 7.0) containing 0.1 M KCl. Approximately 0.5 mg of sodium dithionite was added to reduce any ferric heme to the ferrous state. The instrumentation and experimental details for laser flash photolysis were essentially as described previously (27). A pulse (0.6 \( \mu \)s) from a dye laser disrupted the photolabile heme Fe–CO bond, and the recombination of CO with the hemeprotein was then monitored by following the absorbance change at 436 nm. Data were transmitted to a microcomputer for processing and analysis.

Standard multiexponential analysis of the kinetic data was performed according to Equation 6.

\[
\Delta A(t) = \sum_{i=1}^{n} a_i \exp(-kt)
\]  

(Eq. 6)

\( \Delta A(t) \) is the total absorbance change observed at time \( t \), \( a_i \) is the absorbance change for component \( i \) at \( t = 0 \), \( k \) is the observed pseudo-

**RESULTS AND DISCUSSION**

**Optical Spectroscopy**—Fig. 1 shows the spectra of deoxy-\( \beta \)VE11/T at various temperatures (from top to bottom) at the peak wavelength: \( T = 30 \) K, \( T = 120 \) K, \( T = 180 \) K, \( T = 240 \) K, and \( T = 298 \) K. The arrows indicate the direction of spectral changes observed on lowering the temperature. first order rate constant for component \( i \), and \( n \) is the number of independent components. Least squares analysis was performed with RS/1 software (BBN software products, Cambridge, MA) on a Dell 450/ME microcomputer. Statistical significance (\( p < 0.05 \)) was evaluated using the Student’s t test, assuming equal variances for all the experimental points.

**Molecular Modeling**—Molecular modeling was carried out with the program O (28). Comparison of the heme pockets of deoxymyoglobin with a Thr at position E11 (molecule B of the structure 1YCB) and of deoxyhemoglobin (molecule a\(_2\) of the structure 2HHD) was done using the program ALIGN (29).

**FIG. 1. Absorption spectra of deoxy-\( \beta \)VE11/T at various temperatures (from top to bottom) at the peak wavelength: \( T = 30 \) K, \( T = 120 \) K, \( T = 180 \) K, \( T = 240 \) K, and \( T = 298 \) K. The arrows indicate the direction of spectral changes observed on lowering the temperature.**

As can be seen, the band is markedly asymmetric at all temperatures; this asymmetry is accounted for by the asymmetric distribution of transition frequencies in Equation 4. The bandwidth of the Soret band decreases as the temperature is lowered, and the asymmetry is almost constant; moreover, a slight blue shift of the band position upon lowering the temperature is also present. Fig. 2 shows the deconvolution of the 70 K spectrum of \( \alpha \)VE11/T in terms of Equation 4, together with the residuals on an expanded scale. A fitting of analogous quality is obtained also for \( \beta \)VE11/T; for both proteins the fitting quality improves at higher temperatures. The values of the linear coupling constants for the high frequency modes (\( S_i \)) and of the Lorentzian width (\( \Gamma \)) are listed in Table I, in comparison with the values obtained for the recombinant wild type proteins \( \alpha \)HbA and \( \beta \)HbA, for native human hemoglobin (HbA), and for sperm whale myoglobin (SwMb). As can be seen, no relevant effect on these parameters is observed; this indicates that the mutations, both in the \( \alpha \) and in the \( \beta \) chain, do not affect the local electronic and vibrational properties of the chromophore. In particular, parameters \( Q_0 \sqrt{\delta} \) and \( \delta \sqrt{\Gamma} \) are unaffected, thus suggesting that the iron-heme-proximal histidine geometry is also unaffected.

The temperature dependence of parameter \( \nu_b \) for the mutants \( \alpha \)VE11/T and \( \beta \)VE11/T is reported in the left panels of Fig. 3, together with the analogous quantities relative to the recombinant wild type proteins. Values relative to HbA and to SwMb are reported in the right panels of the same figure, for the sake of comparison. From Fig. 3 it can be seen that both the HbA recombinant proteins exhibit the same \( \nu_b \) behavior as HbA; for the mutant \( \beta \)VE11/T there is a very small effect while, for \( \alpha \)VE11/T, the \( \nu_b \) behavior is markedly different and
rather similar to that exhibited by SwMb. The difference is clearly evident at low temperatures, where a blue shift of \( n_0 \) values upon lowering the temperature is observed for HbA, \( aV(E11)/T \), \( bV(E11)/T \), while SwMb is characterized by a red shift; \( n_0 \) values relative to \( aV(E11)/T \) have an intermediate behavior and remain approximately constant.

The temperature dependence of parameter \( \sigma^2 \) for the investigated proteins is reported in Fig. 4. As can be seen, both mutants exhibit a sharper \( \sigma^2 \) temperature dependence with respect to the wild types and to HbA; moreover, the \( a \) chain mutant has lower \( \sigma^2 \) values at low temperatures. The continuous lines represent the fittings of the data in the temperature range 20–120 K in terms of Equation 5; values of the parameters \( NS \) and \( \Gamma \) obtained from the fittings are reported in Table II. The two mutants are characterized by lower values of the average frequency of the soft modes, and the \( a \) chain mutant exhibits also a lower value of parameter \( NS \). At temperatures higher than 120 K an increase of \( \sigma^2 \) values well above the predictions of Equation 5 is observed. Since Equation 5 has been derived within the harmonic approximation, we attribute these deviations to the onset of large amplitude non-harmonic nuclear motions (17, 18). An increase of average atomic fluctuations well above the prediction of the harmonic behavior and occurring at high temperatures has been observed, for hemeproteins, with a variety of experimental techniques (30–33) and has been attributed to a transition in protein mobility from the low temperature “solid like” behavior characterized by essentially harmonic oscillations of the nuclei around their equilibrium positions, to a high temperature “liquid like” behavior characterized by the jumping among different conformational substates of the biomolecule. It should be noted that, for deoxyhemoglobin and myoglobin, the onset of anharmonic

### Table I

|          | \( S_{370} \) | \( S_{674} \) | \( S_{L357} \) | \( \Gamma \) | \( Q \), \( \sqrt{\delta} \) | \( \delta \), \( \sqrt{\delta} \) |
|----------|---------------|---------------|---------------|------------|-----------------|-----------------|
| HbA\(^a\) | 0.11 ± 0.02   | 0.21 ± 0.02   | 0.05 ± 0.01   | 175 ± 9    | 0.18 ± 0.03     | 0.20 ± 0.03     |
| \( \beta V(E11)/T \) | 0.04 ± 0.02   | 0.18 ± 0.03   | 0.04 ± 0.02   | 184 ± 10   | 0.22 ± 0.03     | 0.19 ± 0.03     |
| \( b\beta HbA \) | 0.09 ± 0.02   | 0.16 ± 0.03   | 0.06 ± 0.02   | 190 ± 10   | 0.19 ± 0.03     | 0.19 ± 0.03     |
| \( aV(E11)/T \) | 0.05 ± 0.02   | 0.21 ± 0.02   | 0.06 ± 0.02   | 190 ± 10   | 0.19 ± 0.03     | 0.14 ± 0.03     |
| \( arHbA \) | 0.05 ± 0.02   | 0.27 ± 0.03   | 0.03 ± 0.01   | 186 ± 10   | 0.19 ± 0.03     | 0.20 ± 0.03     |
| SwMb\(^a\) | 0.32 ± 0.02   | 0.24 ± 0.02   | 0.09 ± 0.01   | 180 ± 10   | 0.17 ± 0.01     | 0.16 ± 0.02     |

\(^a\) Data relative to HbA and to SwMb have been taken from Ref. 23.
motions occurs at about 120 K, i.e. well before the temperature at which the glass transition of the glycerol/water solvent mixture occurs (~180 K) (34–36). Anharmonic motions can be characterized by the quantity $D_s^2$, i.e. by the difference between the experimental $s^2$ values and the theoretical harmonic behavior given by the continuous lines in Fig. 4.

$D_s^2$ values relative to the two mutants investigated, as well as to the wild type and native hemoglobin, are reported in Fig. 5. As can be seen, the non-harmonic motions of the two HbA recombinant hemoglobins are very similar to those observed for native HbA, whereas the two mutant hemoglobins show a more pronounced effect and, in agreement with the effect on parameter $n_0$ reported in Fig. 3, the temperature dependence of the mutant $aV(E11)T$ is steeper than the other. In previous works (33, 36) it has been shown that $D_s^2$ values reflect non-harmonic relative local motions of the porphyrin-iron atom system; these motions are governed by the protein matrix either directly via the iron-proximal histidine bond or indirectly via the (eventually) bound exogenous ligand and/or the residues in the distal heme pocket. Evidence for the modulation of $D_s^2$ values by different bound ligands and by the steric crowding of the distal heme pocket has been presented previously (36–38); the present results point out the relevance of the distal heme pocket polarity in the regulation of iron-porphyrin motions.

All together, the low temperature optical absorption data indicate that the polar isosteric mutation in the $\alpha$ chain has a larger effect on the steric and dynamic properties of the heme pocket than the mutation in the $\beta$ chain.

Kinetics of CO Rebinding—To investigate the functional relevance of the steric and dynamic alterations introduced by the Val $\rightarrow$ Thr mutation in the heme pocket of the $\alpha$ and $\beta$ chains, we have studied the kinetics of CO rebinding after flash photolysis at room temperature. In Fig. 6 we report typical observed time courses; the continuous lines in Fig. 6 represent fittings using a multiexponential model (Equation 6). Each binding curve is well represented by two or three components whose rates vary by about 30-fold (Table III). The slowest component originates from CO binding to the tetramers, whereas the faster ones originate from CO binding to the $\alpha$ and $\beta$ chains within the dimers (39).

It should be noted that the rebinding time scale is on the order of $10^{-1}$ s, indicating that rebinding of CO molecules from...
the solvent to the deoxy proteins is being observed; in fact, the
time scale of geminate rebinding is, at room temperature, on
the order of 50 ns, while tertiary and quaternary conforma-
tional relaxations occur in time scales ranging from 1 to 200 μs
(40).

Comparison of the rate constants (Table III) shows that in
βHbA and in βVE11T they are not significantly different,
both for dimers and tetramers (the rate constant for the β
chains within the dimers is, for βVE11T, slightly smaller than
for βHbA; the effect is, however, at the limit of experimental
error). In contrast, a 2-fold decrease in the rate of CO binding
to tetramers is observed for αVE11T as compared with
αHbA; the effect is even larger (almost a 4-fold decrease) for
the rate of CO binding to the α chains within the dimers. No
significant differences are observed between the native HbA
and the recombinant HbA hemoglobins αHbA and βHbA.

The results in Table III show that the polar isosteric muta-
tion in the β chain slightly decreases the CO rebinding rate to
the modified β chain and unmodified α chain in the deoxy
dimer (although the effect is at the limit of experimental error); however, this smaller effect is abolished by the constraints
imposed by the T quaternary conformation in the deoxy tet-
ramer. In contrast highly significant change is observed when
the polar isosteric mutation is introduced in the heme pocket of
the α chain. Although in the deoxy dimer the CO rebinding rate
to the modified α chain is decreased by more than a factor of 3,
the effect is not propagated to the β in the dimer but is also
present in the T conformation deoxy tetramer.

The kinetic analysis also yields absorbance changes (α, val-
ues) that reflect the amounts of tetramers and dimers in the
original carbon monoxyhemoglobin solutions; this is due to the
fact that the experimental time scale (0.1 s) is much smaller
than the dimer association time constant at the protein con-
centrations used (≥2 s) or than the deoxy tetramer dissociation
time constant (~5×10^4 s) (41). Absorbance values are used to
calculate the dimer-tetramer association constants (Kα) and
the accompanying ΔG values for this transition (see Table III). Comparing the results for βHbA and βVE11T shows that the
mutation in the β chains significantly (p = 0.008) increases the
amount of dimers. In contrast the Kα values of αHbA and
αVE11T indicate that the mutation in the α chains only
slightly increases the dimer content, but this difference is less
significant (p = 0.054). The polar isosteric mutation in the β
chain thus has a greater effect on the energetic of dimer-
tetramer association, which presumably derives from a confor-
mational perturbation transmitted to the αβ interface. As the
corresponding mutation in the α chain has little effect on the
dimer-tetramer association, its effect is more confined to the
heme region.

The functional results are in full agreement with the spec-
rosopic data reported in the preceding section. Both sets of
data indicate that the Val → Thr substitution introduces a local
structural perturbation in the deoxymyoglobin pocket; this
perturbation, small for the β subunit and rather large for the α,
alters the local electric field at the chromophore (parameter ν3),
increases the anharmonic motions within the heme pocket (pa-
rameter Δσp), and slows down the rate of CO rebinding after
photolysis. We suggest that the effect is due to the presence of a
water molecule in the distal heme pocket of the mutant deoxy
α subunit, stabilized by hydrogen bonding not only to the
His(E7) (like in the native deoxy α chains or in sperm whale
myoglobin) but also to the OH group of the substituted threo-
ine. Such an hypothesis would explain the similar ν3 tempera-
ture dependence of SwMb and of the mutant αVE11T and
the decreased rate of CO rebinding; the mobility of this water
molecule could also contribute to the increased anharmonic
motions evidenced in Fig. 5. It should be stressed that, for
the deoxy derivatives, the presence of a water molecule in the
distal heme pocket has been detected by x-ray crystallography
in the Thr68(E11) mutant of porcine myoglobin (15, 44). In

### Table III

**Functional properties of the investigated proteins**

|        | k (×10^-5 M^-1 s^-1) | kα (×10^-5 M^-1 s^-1) | kβ (×10^-5 M^-1 s^-1) | Kα (Kcal/mol) | ΔG (Kcal/mol) |
|--------|----------------------|----------------------|----------------------|---------------|--------------|
| HbA    | 0.62 ± 0.10          | 20.52 ± 3.77         | 20.52 ± 3.77         | 2.93 ± 1.02   | -7.34 ± 0.23 |
| βVE11T | 0.78 ± 0.10          | 20.15 ± 2.35         | 9.93 ± 1.19          | 0.66 ± 0.04   | -6.51 ± 0.04 |
| βHbA   | 0.80 ± 0.19          | 26.6 ± 4.98          | 13.58 ± 2.42         | 1.18 ± 0.32   | -6.85 ± 0.16 |
| αVE11T | 0.39 ± 0.04          | 5.82 ± 0.52          | 21.9 ± 1.81          | 1.47 ± 0.12   | -6.97 ± 0.05 |
| αHbA   | 0.72 ± 0.13          | 19.0 ± 2.54          | 19.0 ± 2.54          | 2.0 ± 0.63    | -7.12 ± 0.22 |

**FIG. 7. Stereo view of human deoxy-
hemoglobin (2HHD) (thin lines, light-
face numbers): α-heme aligned with
the heme of the mutant deoxymyog-
oglobin VE11T (1YCB) (thick lines,
bold numbers) showing the relative
position of the water molecule pres-
ent at their active site. Distances are in
angstroms.**
contrast, a water molecule was not detected in the crystal structure of deoxy-βV(E11)T (13). The increased anharmonicity observed in the β chain mutant with respect to the wild type (see Fig. 5) could be related to an increased mobility of the heme pocket induced by the increased polarity of the distal side.

Molecular Modeling—The above data indicate that the dynamics of the heme pocket and heme accessibility is modified by the Val → Thr substitution to a larger extent in the α subunits. This must represent differences between the heme pockets of the α and β subunits that are introduced by the mutation. As previously reported the Val → Thr substitution does not affect the conformation of the heme pocket both in tetrameric and dimeric form of the α mutant hemoglobin.

Conclusions—In this work the steric, dynamic, and functional effects of the polar isosteric substitution Val → Thr in the distal heme pocket of the α and β chains of recombinant human deoxyhemoglobin have been investigated by means of optical absorption spectroscopy, kinetics of CO rebinding, and molecular modeling. The whole body of experimental evidence clearly shows that the effect of the same mutation in the different subunits is different. In particular, the investigated polar isosteric mutation affects the stereodynamic and functional properties of the deoxy α chains much more than those of the deoxy β chains. We attribute the observed differences to the presence of a water molecule in the distal heme pocket of the mutant α subunits, stabilized by H bonding to the Nε of His(E7) and to the Oδ of the substituted ThrE11. The presence of a stable polar water molecule slows down the rate of CO rebinding, alters the electric field at the chromophore (and therefore the temperature dependence of the parameter νs), and introduces a larger mobility in the heme pocket. The polarity increase, and therefore the observed effects, is much smaller in the mutant deoxy β chains since in these subunits the presence of an extra water molecule in the distal side of the heme pocket is possibly hindered by the different geometry of the pocket.

As previously mentioned, differences in the ligands’ accessibility between R state α and β heme pockets have been observed in mutant hemoglobinins in which the stereochemistry of the heme pocket was, however, modified by the mutations (11). Our data indicate that these differences are also present in T state hemoglobinins, despite the fact that the isosteric amino acid substitution does not modify the stereochemistry of the heme pocket, as indicated by x-ray crystallography of βV(E11)T (13) and molecular modeling of αV(E11)T. The similarity of νs in myoglobin and αV(E11)T and the large decrease in CO affinity measured in a mutant myoglobin with a Thr at position E11 (15) suggest similarity of the deoxy α pocket with that of myoglobin, and for the βV(E11)T mutant the perturbation is sensed by α1β2 interface, which is destabilized. An analogous destabilization, however, is not observed in the αV(E11)T mutant, despite the larger modification of the heme pocket dynamics. This suggests a more direct correlation between the β heme pocket and the α1β2 interface than between the α pocket and the same interface. Similar conclusions have been drawn from heme transfer measurements indicating that β-heme-globin linkage is stabilized by interactions at the α1β2 interface (47, 48).

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