Properties of Human Hemoglobins with Increased Polarity in the α- or β-Heme Pocket

CARBONMONOXY DERIVATIVES*

(Received for publication, April 22, 1998, and in revised form, July 1, 1998)

Michael Karavitis and Clara Fronticelli‡
From the Department of Biochemistry and Molecular Biology, University of Maryland Medical School, Baltimore, Maryland 21201

William S. Bringar
From the Department of Chemistry, Temple University, Philadelphia, Pennsylvania 19122

Gregory B. Vasquez
From the Center for Advanced Research in Biotechnology, University of Maryland, Rockville, Maryland 20850

Valeria Militello, Maurizio Leone, and Antonio Cupane§
From the Istituto Nazionale di Fisica della Materia and Dipartimento di Scienze Fisiche ed Astronomiche, University of Palermo, 90123 Palermo, Italy

The spectroscopic, conformational, and functional properties of mutant carbonmonoxy hemoglobin in which either the β-globin Val<sup>67</sup>(E11) or the α-globin Val<sup>62</sup>(E11) is replaced by threonine have been investigated. The thermal evolution of the Soret absorption band and the stretching frequency of the bound CO were used to probe the stereodynamic properties of the heme pocket. The functional properties were investigated by kinetic measurements. The spectroscopic and functional data were related to the conformational properties through molecular analysis. The effects of this nonpolar-to-polar isosteric mutation are: (i) increase of heme pocket anharmonic motions, (ii) stabilization of the A<sub>0</sub> conformer in the IR spectrum, (iii) increased CO dissociation rates. The spectroscopic data indicate that for the carbonmonoxy derivatives, the Val → Thr mutation has a larger conformational effect on the β-subunits than on the α-subunits. This is at variance with the deoxy derivatives where the conformational modification was larger in the heme pocket of the α-subunit (Cupane, A., Leone, M., Militello, V., Friedman, R. K., Koley, A. P., Vasquez, G. P., Bringar, W. S., Karavitis, M., and Fronticelli, C. (1997) J. Biol. Chem. 272, 26271–26278). These effects are attributed to a different electrostatic interaction between O<sup>•</sup> of Thr(E11) and the bound CO molecule. Molecular analysis indicates a more favorable interaction of the bound CO with Thr<sup>•</sup> in the β-subunit heme pocket.

* This work was supported by United States Public Health Service Grant HBLBI-48517 (to C. F.) from the National Institutes of Health and by Italian National Council Grant 97.043550 (to A. C. and C. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Maryland Medical School, 108 North Greene St., Baltimore, MD 21201. Tel.: 410-706-7672; Fax: 410-706-7390; E-mail: cfronte@umaryland.edu.

§ To whom correspondence should be addressed: Dipartimento di Scienze Fisiche ed Astronomiche, University of Palermo, Via Archirafi 36, 90123 Palermo, Italy. Tel.: 39-91-6234220; Fax: 39-91-6162461; E-mail: cupane@fisica.unipa.it.

Hemoglobin is a tetrameric protein composed of two structurally similar subunits assembled through two interfaces. Each subunit contains eight α-helices, designated A–H, that form an amphipathic pocket containing the heme. On the proximal side of the pocket, the heme iron coordinates with a histidine at position 8 in the F-helix. The exogenous ligand binds to the heme iron on the distal side of the pocket. Upon ligand binding, the iron moves into the plane of the heme, whereas upon release of the ligand, the heme domes and the iron moves out of the mean heme plane. This movement is transmitted to the proximal histidine, initiating a cascade of events leading to the quaternary transition of the protein from the R- to T-state. Access of ligands to the heme is hindered by the presence of the polypeptide chain that buries the heme in a largely hydrophobic crevice in the protein interior. The iron-ligand binding is therefore associated with conformational fluctuations in the protein creating a pathway for ligand entry (1).

The structure and dynamics of the heme pocket govern the functionality of the protein using mechanisms such as steric hindrance, electrostatic interactions, and hydrogen bonding as well as anharmonic motions. The relative weights of the various contributions and the interplay among structure, dynamics, and function in different hemoproteins (in particular in the α- and β-subunits of hemoglobin) are, however, not fully understood. The above issues have been extensively investigated using site-directed mutagenesis, especially in myoglobin, which can be considered a prototype of oxygen transport proteins (2). On the distal side, the role of Val(E11), His(E7), Leu(10), Phe(CD1), and Phe(CD4) has been investigated by replacing these residues primarily with amino acids of different size to investigate the role of steric hindrance in ligand binding (3–6). These studies indicate that, although steric hindrance is, in certain cases, an effective means of regulating ligand affinity, it is not the major determinant. The results suggest that ligand affinity in myoglobin is greatly dependent on the necessity to displace the distal pocket water molecule, which is within hydrogen-bonding distance of N<sup>ε</sup> of His(E7) (6), and on electrostatic interactions between the bound ligand and adjacent amino acid residues (7). On the back of the distal side of the heme pocket, Leu (B10) regulates the affinity of the heme for ligands. Its replacement with less hydrophobic amino acids

23740 This paper is available on line at http://www.jbc.org
(Ala, Gly, Ser) decreases the rate of CO binding (8). On the proximal side Ser(F7) is within hydrogen-bonding distance of N\(^\text{e}\) of His(F8); replacement of this residue with a less polar residue, such as Ala, Val, or Leu, affects the reactivity of the iron toward ligands (9). These results are a further illustration of how ligand affinity is sensitive to the polar nature of the heme pocket.

Mutational studies also have been applied to the study of ligand affinity regulation in the \(\alpha\) - and \(\beta\)-subunits (2). The \(\beta\)-subunits show a relative insensitivity to apolar substitutions, with the only exception being the Val(E11)I mutation, in which the larger sec-butyl group sterically hinders access of ligands to the heme iron. In contrast, the ligand affinity of the \(\alpha\)-chain is more comparable to that of myoglobin, indicating a similar molecular mechanism of ligand regulation in these two globins. Thus, ligand affinity appears to be regulated by different mechanisms in the \(\alpha\) - and \(\beta\)-heme pockets.

Our aim is to gain a better understanding of the role electrostatic interactions play in the regulation of ligand affinity. Toward this end, Val(E11)I was substituted with the isosteric polar Thr in the \(\alpha\) - or \(\beta\)-heme pockets. In a previous paper (10), we have shown that in the deoxy derivatives, this mutation stabilizes the water molecule in the distal heme pocket of the \(\alpha\)-subunit by hydrogen bonding between O\(^\text{e}\) of Thr(E11) and N\(^\text{e}\) of His(E7). A water molecule in the \(\beta\)-heme pocket of the deoxy Val(E11)I mutant was not observed in the x-ray structure (11).

Stabilization of the water molecule slows down the rate of CO binding, alters the electric field, and increases the mobility in the heme pocket of the \(\alpha\)-subunit.

In this paper, we report the effects of the Val(E11)I/T substitution on the carbonmonoxy derivatives, in which the ligand displaces the \(\alpha\)-pocket water molecule. In view of the polar character of the liganded CO molecule, this study allows the specific investigation into the role of ligand-heme pocket electrostatic interactions in the conformational and functional properties of these proteins. The heme pocket dynamics and the environment of the bound CO molecule have been monitored by low-temperature optical absorption and FT-IR\(^3\) spectroscopies, whereas molecular analysis has provided a structural basis for interpretation of the observed spectroscopic effects. Functional properties have been investigated through the kinetics of CO replacement by NO.

Our data indicate a Thr-CO electrostatic interaction in both the \(\alpha\) - and \(\beta\)-chain mutants; however, the effects are different in the two subunits. On the basis of molecular analysis, we attribute the observed spectroscopic and functional differences to the respective Thr-CO distances and orientations. Comparison of functional and spectroscopic data shows that a good correlation is present between the average weighted CO stretching frequency and the ligand binding parameters of R-state HbA, \(\alpha\)V(E11)I, and \(\beta\)V(E11)I.

**MATERIALS AND METHODS**

Proteins and experimental methods were nearly the same as those described by Cupane et al. (10). The following differences are noted.

**Samples for Spectrophotometric Measurements**—Concentrated protein stocks were stored under liquid nitrogen in the CO form; they were thawed immediately before the measurements were made and diluted in the appropriate water/cosolvent/buffer mixtures, previously saturated with CO. Approximately \(3 \times 10^{-4}\) M sodium dithionite was also added to ensure full reduction of the sample; the final protein concentration was \(\sim 10^{-3}\) M in heme.

**Analysis of Soret Bands**—The analytical expression used to fit the Soret band of the CO derivatives at various temperatures is as follows (Equation 1),

\[
A(v) = M^v \nu L(v) \otimes G(v)
\]  

where \(M\) is a constant proportional to the electronic dipole matrix element, \(\nu\) denotes the frequency, and \(\otimes\) denotes the convolution operator. \(L(v)\) is the temperature-independent lorentzian part of the band shape. It arises from the Franck-Condon-type coupling of the electronic transition with the high-frequency vibrational modes of the heme (i.e. with modes having vibrational frequency \(\nu\)), such that \(h\nu \gg k_BT\) and can be written as Equation 2,

\[
L(v) = \sum_{n_l} \frac{\Gamma}{(v - \nu_l)^2 + \Gamma^2}
\]

where \(\Gamma\) is a damping factor that depends on the finite lifetime and the dephasing of the excited state, \(\nu_l\) is the frequency of the pure electronic (0–0) transition, and \(N_l\) denotes the number of high-frequency modes vibronically coupled to the electronic transition. The product extends to all high-frequency heme vibrations, whereas the sum runs over their occupation numbers \(m_{nl}\). Vibronic coupling of the "high-frequency" heme modes to the electronic transition is described by the linear coupling constant \(S_l\). The high-frequency modes considered are those centered at 350, 676, 1100, and 1374 \(\text{cm}^{-1}\) as determined by resonance Raman spectroscopy. Bands at 676 and 1374 \(\text{cm}^{-1}\) correspond to the well known \(\nu_2\) and \(\nu_4\), whereas those at 350 and 1100 cm\(^{-1}\) are "average effective" frequencies accounting for several peaks in the Raman spectra.

The second term, \(G(v)\), in Equation 1 is the temperature-dependent gaussian part of the band shape. It arises from the coupling of the electronic transition with a "bath" of low-frequency modes of the iron-heme-protein system; such coupling is described by an Einstein harmonic oscillator model that, in the short time limit (12), yields a temperature-dependent gaussian broadening of the Soret band whose analytical expression is as follows (Equation 3),

\[
G(v) = \frac{1}{\sigma(T)^2} e^{-v^2/(2\sigma(T)^2)}
\]

where \(\sigma(T)\) is the temperature-dependent half-width. "Low-frequency modes" are those whose vibrational frequency is of the same order of, or smaller than, \(h\nu_g T\). If one considers the above low-frequency bath as a set of \(N\) degenerate harmonic oscillators with an average coupling constant \(S\) and an average frequency \(\nu_g\) (harmonic Einstein approximation), the temperature dependence of the gaussian width (parameter \(\sigma(T)\) in Equation 3) is given by Equation 4.

\[
\sigma(T)^2 = NS\nu_g^2 \coth \left( \frac{h\nu_g}{2k_BT} \right) + \sigma_m^2
\]

The subscript \(h\) indicates that Equation 4 is only valid in the harmonic regime. Contrary to the deoxy derivatives, in which the equilibrium position of the iron atom out of the mean plane gives rise to a nonsymmetric inhomogeneous broadening of the Soret band (see Cupane et al. (10) for an in-depth discussion), in the CO derivatives, the effect of conformational heterogeneity can be modeled as a gaussian distribution of 0–0 transition frequencies and gives rise to the additional term \(\sigma_m^2\) in Equation 4.

**IR Spectra**—Infrared spectra were recorded at room temperature using a Perkin-Elmer 1600 series FT-IR spectrometer. Samples were dialyzed against 100 mM sodium phosphate buffer, pH 7.0, concentrated to between 6 and 16 mM in heme and then placed between two CaF\(_2\) windows separated using a 50-\(\mu\)m Teflon spacer. Between 64 and 256 interferograms were recorded for each sample with a resolution of 2.0 \(\text{cm}^{-1}\). Absorbance spectra were calculated from the ratio of the sample spectrum to the spectrum of the appropriate buffer taken just prior to sample data collection. These absorbance spectra were then decomposed, using Peakfit (Jandel Scientific), into one or two voigtians corresponding to the respective Thr-CO distances and orientations. Comparison of functional and spectroscopic data shows that a good correlation is present between the average weighted CO stretching frequency and the ligand binding parameters of R-state HbA, \(\alpha\)V(E11)I, and \(\beta\)V(E11)I.
ance spectrum making the following approximations. 1) The extinction coefficients of $A_1$ and $A_2$ are identical. 2) The absorbance of the $A_1$ band is entirely due to the mutant subunits. 3) The contributions from the $\alpha$- and $\beta$-subunits to the $A_2$ band are identical. From this it follows that for the mutant subunits (Equation 5),

$$ \bar{r}_{CO} = (\text{area}_{A_1} \times r_{COA_1} + \text{area}_{A_2} \times r_{COA_2}) \times \text{area}_{\text{total}} \quad (\text{Eq. 5}) $$

The $\bar{r}_{CO}$ of $A_1$ is used as the $\bar{r}_{CO}$ for the native subunits without any correction.

**CO Dissociation Kinetics**—CO dissociation rates were measured using a Hewlett-Packard 8452A diode array spectrophotometer coupled to an Applied Photophysics RX 2000 rapid mixing apparatus with a dead time of <10 ms. Temperature was controlled at 20 °C using a NESLAB water bath and monitored on the RX 2000 apparatus. For these experiments, the ligand replacement reaction developed by Moore and Gibson (13) was used in which NO replaces bound CO. Buffer equilibrated with NO at atmospheric pressure (−2 mTorr) was placed in one syringe of the rapid mixing device while the protein (−6 μM/heme), equilibrated with 5% CO at atmospheric pressure (−50 μM), was placed in the other.

The contents of the two syringes were mixed into a micro-flow cuvette with a 1-cm path length. Absorbance changes were monitored at 538 nm every 0.2 s using a 0.2-s integration time.

In this case, where both ligands are in excess of the protein, the reaction exhibits first-order kinetics. Also, under these conditions, the apparent rate constant closely approximates the CO dissociation constant; thus, these experiments can be described by a linear combination of two first-order rate equations of equal amplitude corresponding to both the $\alpha$- and $\beta$-subunits (Equation 6),

$$ A_{\text{total}} = (A_{\text{HbCO}} - A_{\text{HbNO}}) \times \left( \frac{1}{k_1} \right) + A_{\text{HbNO}} \quad (\text{Eq. 6}) $$

where $A_{\text{total}}$ is the total absorbance; $A_{\text{HbCO}}$ is the absorbance of the carbonmonoxy derivative of the protein at 538 nm; $A_{\text{HbNO}}$ is the absorbance of the nitrosyl derivative of the protein at 538 nm; $k_1$ and $k_2$ are the observed rate constants for the $\alpha$- and $\beta$-subunits, respectively; and $t$ is the time. The observed rate constant described here is a function of the on- and off-rate constants for CO as well as NO. Since the dissociation rate of NO is negligible and a much higher concentration of NO over CO is used, the observed rate constant approximates the CO dissociation constant (14). Three to five measurements were recorded for each protein and analyzed simultaneously, sharing parameters $k_1$ and $k_2$ (Equation 6). Standard errors for these analyses were below 5%.

**Molecular Analysis**—Visualization of the structures was carried out using the program O (15). The hemes of the carbonmonoxy MbV(E11)T molecule B (1YCA) (7) and the $\alpha$- and $\beta$-hemes of carbonmonoxy HbA (1AJ9) (16) were superimposed, without contributions from the E11 residues, propionates, vinyl groups, or ligands, using the program ALIGN (17).

**RESULTS AND DISCUSSION**

**Optical Absorption Spectroscopy**—Fig. 1 shows the spectra of carbonmonoxy αV(E11)T at various temperatures between 300 and 20 K. As can be seen, the band was almost symmetric at all temperatures; the two small shoulders at lower wavelength, clearly visible in the low-temperature spectra, represent the vibronic side bands due to the coupling with vibrational modes at 676 and 1374 cm$^{-1}$, respectively. The bandwidth decreased as the temperature was lowered, and a clear blue shift of the peak frequency was also present.

Fig. 2 shows the deconvolution of the 20 K spectrum of carbonmonoxy αV(E11)T in terms of Equations 1–3, together with the residuals on an expanded scale. Fittings of analogous quality were obtained for all the proteins investigated; moreover, the fitting quality improved at higher temperatures. The values of the linear coupling constants for the high-frequency modes ($S_0$) and of the lorentzian width (Γ) are listed in Table I, in comparison with the analogous values obtained for the carbonmonoxy recombinant wild-type hemoglobin, native human hemoglobin, and sperm whale myoglobin. No appreciable effect on these parameters was observed, demonstrating that like the deoxy derivatives, these mutations in the CO derivatives do not affect the local electronic and vibrational properties of the chromophore.

The temperature dependence of parameter $\nu_0$ for the carbonmonoxy mutants αV(E11)T and βV(E11)T is reported in Fig. 3 (left panels), together with the analogous data for the carbonmonoxy recombinant wild-type proteins; for comparative purposes, values obtained for HbA and SwMb are also reported (right panels). From Fig. 3, it can be seen that, at variance with the deoxy derivatives (10), in the carbonmonoxy derivatives, a remarkable effect (red shift) was observed for the βV(E11)T mutant, whereas the recombinant wild-type proteins and the αV(E11)T mutant exhibited almost the same behavior as HbA. This shows that, for the CO derivatives, the Val(E11)T substitution in the $\beta$-chain considerably alters the local electric field at the chromophore (and therefore the pure electronic transi-
tion frequency $v_{\alpha}$, whereas the effect is smaller for the $\alpha$-chain mutation.

The temperature dependence of parameter $\sigma^2$ for the investigated proteins is reported in Fig. 4. The continuous lines represent the fittings of the data in the temperature range 20–160 K in terms of Equation 4. Values of the parameters $N_S$, $s_n$, and $s_{m}$ are reported in Table II. For all the proteins investigated, the onset of anharmonic motions occurred at temperatures higher than $\sim$180 K, in close proximity to the glass transition temperature of the solvent. The different behavior of CO derivatives with respect to the deoxy ones (where the onset of anharmonicity has taken place already at 120 K (Fig. 4 of Ref. 10)) has already been noted for HbA and SwMb (18, 19) and has been attributed to a stabilizing effect of the ligand on the protein-driven anharmonic motions to the chromophore.

In the case of the $\alpha$-chain mutant, both the $\beta$-chain and the native hemoglobins was approximately the same, whereas the anharmonicity of the mutants was increased. However, anharmonic motions were larger for the $\beta$-chain than for the $\alpha$-chain mutant, a result just the opposite from that found for the deoxy derivatives. As described previously by Militello et al. (20), the effects observed in the $\beta$-chain mutant could be attributed to a dipolar interaction between the bound CO molecule and the OH group of the threonine side chain; this interaction is responsible for the peak frequency shift reported in Fig. 3, for the decreased coupling to the low-frequency bath, as well as for the increased anharmonicity. In the case of the $\alpha$-chain mutant, this dipolar interaction was smaller, probably due to a larger distance between the bound CO and the threonine OH and/or a less favorable mutual orientation.

**Infrared Spectroscopy**—Fig. 6 shows the spectra at 296 K of both the $\alpha$VE11/T and $\beta$VE11/T mutants in comparison with those of HbA. The measured spectra can be fitted in terms of voigtian components; the parameters obtained from the deconvolution (peak frequencies, bandwidths at half-maximum, and areas of the single components in percent of the total) are listed in Table III. These components represent the CO stretching band of conformers that do not interconvert within the time window of the experimental technique ($\sim 10^{-12}$ s); the frequency of the CO stretch ($v_{1/2}$) is dependent on the electronic environment of the heme-carbonyl complex and, to a lesser extent, on the steric properties of the heme pocket (21), whereas the bandwidth at half-maximum ($\Delta E_{1/2}$) reflects band broadening, which in turn can be related to the flexibility of the ligand in its local environment (22).

The band at $\sim$1950 cm$^{-1}$, termed $A_1$, is the major conformer found in HbA under physiological conditions; it contains overlapping contributions from the $\alpha$- and $\beta$-subunits that differ by $\sim$2 cm$^{-1}$ and are centered at $\sim 1950$ and 1952 cm$^{-1}$, respectively (23). For HbA, the $A_1$ conformer represents $\sim 96\%$ of the

![Figure 2: Deconvolution of the 20 K spectrum of carbonmonoxy $\alpha$VE11/T. Circles are the experimental points; dotted and continuous lines represent the Soret band fitted in terms of Equation 1, the contributions from the higher frequency $N$ band, and the overall synthesized band profile, respectively. For figure readability, not all the experimental points are included. The residuals are also reported in the upper panel, on an expanded scale.](image)

**Table I**

| Linear coupling constants of the high-frequency modes and $\Gamma$ values for the carbonmonoxy derivatives of the mutant hemoglobins investigated |
|---------------------------------------------------------------|
| $S_{300}$ | $S_{176}$ | $S_{1100}$ | $S_{1174}$ | $\Gamma$ |
|-----------|-----------|-----------|-----------|---------|
| HbA       | 0.05 ± 0.01 | 0.07 ± 0.01 | 0.016 ± 0.005 | 0.08 ± 0.01 | 215 ± 4  |
| $\beta$VE11/T | 0.06 ± 0.02 | 0.06 ± 0.01 | 0.015 ± 0.006 | 0.07 ± 0.01 | 221 ± 10 |
| $\beta$HbA     | 0.02 ± 0.02 | 0.07 ± 0.01 | 0.013 ± 0.005 | 0.07 ± 0.01 | 230 ± 10 |
| $\alpha$VE11/T  | 0.05 ± 0.01 | 0.08 ± 0.01 | 0.016 ± 0.005 | 0.07 ± 0.01 | 225 ± 10 |
| $\alpha$HbA     | 0.05 ± 0.01 | 0.08 ± 0.01 | 0.016 ± 0.005 | 0.08 ± 0.01 | 217 ± 10 |
| SwMb        | 0.12 ± 0.02 | 0.06 ± 0.01 | 0.020 ± 0.008 | 0.09 ± 0.01 | 211 ± 7  |
total absorbance and has a peak frequency of 1951.8 cm$^{-1}$. For both the V(E11)T mutants, $A_1$ represents ~75% of the total absorbance.

For native HbA and SwMb, the band centered at ~1968 cm$^{-1}$, termed $A_0$, is formed in significant quantities only at cryogenic temperatures or upon acidification at pH <5 (24–26). It is usually attributed to an “open pocket” conformer in which the distal histidine has rotated away from the ligand enough to reduce the electrostatic interaction with its positively charged imidazole side chain (27, 28). For both the V(E11)T mutants,
the $A_0$ conformer accounts for $\sim 25\%$ of the total intensity; it appears at 1968.2 cm$^{-1}$ for $\beta$V(E11)$^T$ and at 1964.7 cm$^{-1}$ for $\alpha$V(E11)$^T$.

We attribute the $A_1$ conformer essentially to the native chains; indeed, for the $\alpha$V(E11)$^T$ mutant, the $A_1$ peak frequency is positioned at 1952 cm$^{-1}$, where the $A_1$ band of the native $\beta$-chains is typically found. For the $\beta$V(E11)$^T$ mutant, the $A_1$ peak frequency is positioned at 1950.3 cm$^{-1}$, where the $A_1$ band of the native $\alpha$-subunits is typically found. Having assigned the $A_1$ conformer primarily to the native chains, it follows that the $A_0$ conformer has to be assigned to the mutant chain. Following Li et al. (21) and Cameron et al. (7), we attribute the stabilization of the $A_0$ conformer found in the mutants to an electrostatic interaction similar to the one proposed for pig myoglobin, MbV(E11)$^T$, containing an analogous Val $\rightarrow$ Thr mutation at position E11. In fact, the room temperature IR spectra of this pig myoglobin mutant show a qualitatively similar stabilization of the $A_0$ conformer. On the basis of the crystal structure of the pig myoglobin mutant (7), it has been suggested that this interaction arises from the fact that the substituted threonine places the negative portion of its hydroxyl dipole adjacent to the bound ligand. According to the interpretation of CO stretching frequencies by Li and Spiro (29), the presence of a negative electrostatic field inhibits the formation of the $A_1$ peak frequency. In our case, the mutant $\alpha$- and $\beta$-subunits contribute to both the $A_0$ and $A_1$ bands. This last possibility is supported by the data of Cameron et al. (7) indicating that in MbV(E11)$^T$, $\sim 80\%$ of the total intensity is observed at 1966 cm$^{-1}$ ($A_0$ band) and $\sim 20\%$ at 1944 cm$^{-1}$ ($A_1$ band). In our case, the mutant $\alpha$- and $\beta$-subunits would contribute $\sim 50\%$ to the $A_1$ conformer and $\sim 50\%$ to the $A_0$ conformer.

It should also be stressed that the stabilization of the $A_0$ conformer observed in the present hemoglobin mutants and in MbV(E11)$^T$ arises from an entirely different mechanism than that observed in HbA or SwMb at low temperatures or acidic pH. For native proteins, an increase in the size of the heme pocket with a concomitant reduction of the electrostatic interaction between the bound CO and the positively charged imidazole side chain of the distal histidine is thought to be ultimately responsible for the formation of the $A_0$ band. This is evidenced by an increased half-bandwidth ($\Delta v_{1/2}$), which is related to the flexibility of the ligand in its local environment. However, for the V(E11)$^T$ mutants, the $\Delta v_{1/2}$ for the $A_0$ band is similar to that of the $A_1$ band, suggesting that the blue shift of the CO stretching frequency arises from a more specific event such as the electrostatic interaction of the partially negative charge of Thr O$^\ominus$ and the bound CO.

Overall, the IR data reported in Fig. 6 and Table III are in good agreement with the results from optical absorption spectroscopy in that they are suggestive of an electrostatic interac-

---

**Table II**

Values of the parameters obtained by fitting the low-temperature $\sigma^2$ behavior with Equation 4

|       | $\sigma^2$ | $\sigma_{\text{ns}}$ |
|-------|-----------|---------------------|
| HbA   | $0.50 \pm 0.10$ | $165 \pm 10$ |
| $\beta$V(E11)$^T$ | $0.43 \pm 0.03$ | $135 \pm 10$ |
| $\beta$HbA | $0.54 \pm 0.03$ | $135 \pm 10$ |
| $\alpha$V(E11)$^T$ | $0.55 \pm 0.05$ | $135 \pm 10$ |
| $\alpha$HbA | $0.48 \pm 0.05$ | $135 \pm 10$ |
| SwMb* | $0.30 \pm 0.10$ | $180 \pm 20$ |

* Value taken from Ref. 19.
Molecular Analysis—On the basis of the IR spectra reported in Fig. 6, we have proposed an electrostatic interaction between the Thr OH and the bound CO ligand. This interaction is stronger in the mutant \( \alpha \)-subunit than in the mutant \( \beta \)-subunit, likely due to a reduced distance and/or to a more favorable mutual orientation in the former. We have analyzed the structures to see whether a conformational basis for the above hypothesis can be found. We started from the recent crystallographic structure of HbA-CO at 2.2-Å resolution (1AJ9) (16), and more recently published crystallographic structures of MbV(E11)T (7) and MbV(E11)E (1YCA). Comparison of the CO ligand positions shows that in MbV(E11)T, the CO molecule resides in a position intermediate between those observed in the \( \alpha \)- and \( \beta \)-pockets of hemoglobin; the ligand-heme geometry in MbV(E11)T is, however, more similar to that of the \( \beta \)-chains of HbA than to that of the \( \alpha \)-chains, both with respect to the CO position over the heme (Fig. 7, left column) and to the Fe-C-O angle (Fig. 7, right column). In particular, the distances between CO and the oxygen atom of the bound CO are 3.2, 3.6, and 3.4 Å for myoglobin, the \( \alpha \)-subunit, and the \( \beta \)-subunit, respectively. The angles formed by the CO dipole and the segment connecting Thr O\(^{\gamma} \) and the oxygen atom of the bound CO are 98.5°, 83.4°, and 96.3°, respectively.

The structural superimposition shows that the heme pocket geometry of carbonmonoxy MbV(E11)T is more similar to the \( \beta \)-subunit than to the \( \alpha \)-subunit of carbonmonoxy HbA. This is in agreement with the finding that the Val \( \rightarrow \) Thr substitution in the \( \beta \)-subunits causes large spectroscopic changes (both in the IR CO stretching frequency and in the Soret absorption band) similar to those observed for MbV(E11)T. In particular, the relative blue shift of the \( A_0 \) conformer of carbonmonoxy MbV(E11)T is, being 17.9 and 17.0 wave numbers, respectively, whereas it is shifted 12.7 wave numbers in \( \alpha \)Ve(E11)T. In concordance, structural analysis shows that both the distance and orientation of the bound CO molecule in the \( \beta \)-subunits of carboxymyoglobin are more favorable for establishing an electrostatic interaction between the CO dipole moment and the negatively charged O\(^{\gamma} \) of the substituted Thr than in the \( \alpha \)-subunits, again in full agreement with results from low-temperature optical absorption and FT-IR spectroscopies.

Kinetics Analysis—Measurements of CO replacement by NO show that the Val \( \rightarrow \) Thr mutation increases the rate of CO release. The effect is similar for both mutants, slightly larger for the mutant \( \alpha \)-subunit. This result is in overall agreement with the data from IR and optical absorption spectroscopies. Indeed, the stabilization of the \( A_0 \) conformer detected by IR spectroscopy indicates a decreased back-bonding interaction between the iron and the CO ligand and consequently an increase in the C-O bond order. In view of the negative correlation between \( v_{\text{CO}} \) and \( v_{\text{Fe-CO}} \) (29), this implies a weakening of the Fe–CO bond and therefore an increased rate of CO dissociation. Moreover, the increased anharmonic motions detected by optical absorption spectroscopy for both mutants are also suggestive of a greater mobility within the heme pocket and are therefore in agreement with an increased rate of CO release.

Although IR spectroscopy indicates a shift of the \( A_0 \) conformer toward higher frequencies for the mutant \( \beta \)-subunits than for the mutant \( \alpha \)-subunits (1968.2 cm\(^{-1} \) versus 1964.6 cm\(^{-1} \)), the mean weighted frequencies are identical (1958.6 and 1958.7 for \( \alpha \)Ve(E11)T and \( \beta \)Ve(E11)T, respectively), this is in good agreement with the similar rates of CO release measured for the two E11 mutants.

As previously discussed, the Val \( \rightarrow \) Thr substitution does not

---

**TABLE III**

Parameters of the \( A_0 \) and \( A_1 \) conformers obtained by fitting the spectra in Fig. 6 in terms of two voigtian components.

|       | \( A_0 \) | \( A_1 \) |
|-------|-----------|-----------|
|       | Area      | \( v_{\text{CO}} \) | \( \Delta v_{1/2} \) | Area      | \( v_{\text{CO}} \) | \( \Delta v_{1/2} \) |
| HbA   | 96% 1951.7 9.4 | 4 1966.3 13.9 |
| \( \alpha \)Ve(E11)T | 74% 1952.0 9.6 | 26 1964.6 10.7 |
| \( \beta \)Ve(E11)T | 76% 1950.3 8.5 | 24 1968.2 10.3 |

---

**Fig. 6.** Infrared spectra at room temperature of the carbonmonoxy \( b \), \( \alpha \)Ve(E11)T and \( c \), \( \beta \)Ve(E11)T mutants in comparison with \( a \), carbonmonoxy HbA.
modify the steric characteristics of the heme pocket (7, 10, 11); therefore, this system represents an excellent model to investigate the effect of modified heme pocket polarity on the ligand binding parameters and their correlation with IR spectroscopy. For this analysis, the kinetic curves have been analyzed in terms of two exponentials of equal amplitudes (describing the contributions from α- and β-subunits). Since this is a replacement reaction, the protein remains in the R-state throughout the course of the reaction. The rates of CO binding to the dimers have been taken from a previous paper (10); these rates approximate those of the R-state, avoid complications arising from the R-T transition, and are in good agreement with the rate of CO binding to dimers reported by Doyle et al. (31) These two quantities were used to determine the CO affinity constants for the R-state. These data are reported in Table IV and show that in the R-state, the αVE11/T and βVE11/T mutant subunits have a CO affinity decreased by a factor of 7.0 and 4.0, respectively, as compared with native HbA. This indicates that the modified electrostatic interactions in the distal heme pocket destabilize the bound CO, the effect being larger in the

![FIG. 7. Plots comparing the hemes and E11 residues of the carbonmonoxy derivatives of HbA (1AJ9) and of the B molecule of MbVE11/T (1YCA). The left column shows a view of the hemes from the top, looking down at the heme from the perspective of the ligand. The right column shows a skewed side view of the hemes looking from the A pyrrole ring in the front to the C pyrrole ring in the back. The heme propionate groups have been removed for clarity. In the upper panel, the HbA α-heme and the Val(E11) residue (thin lines) are compared with the HbA β-heme and the Val(E11) residue (heavy lines). In the center panel, the HbA β-heme and the Val(E11) residue (thin lines) are compared with MbVE11/T and the Thr(E11) residue (heavy lines). In the lower panel, the HbA β-heme and the Val(E11) residue (thin lines) are compared with MbVE11/T and the Thr(E11) residue (heavy lines).]

| Table IV | Rate and equilibrium constants for CO binding to HbA, αVE11/T, and βVE11/T at pH 7 (0.1 M bis-Tris + 0.1 M KCl) and T = 23 °C | Values k’CO are taken from Ref. 10; they refer to the association constants of the dimers for CO. |
|----------|----------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|
|          | kCO                                                  | kCO                                                   | KCO                                                      |
|          | μ⁻¹ s⁻¹          | s⁻¹          | μ⁻¹                                                      |
| HbAα     | 2.05 × 10⁶       | 1.55 × 10⁻²  | 1.32 × 10⁸                                              |
| βVE11/T α-chains | 2.01 × 10⁶   | 1.47 × 10⁻²  | 1.36 × 10⁸                                              |
| βVE11/T β-chains | 0.99 × 10⁶   | 3.12 × 10⁻²  | 0.32 × 10⁸                                              |
| αVE11/T α-chains | 0.55 × 10⁶   | 2.93 × 10⁻³  | 0.20 × 10⁸                                              |
| αVE11/T β-chains | 2.19 × 10⁶   | 1.48 × 10⁻²  | 1.48 × 10⁸                                              |

a The CO binding parameters of the α- and β-chains could not be resolved.
alpha-subunits than in the beta-subunits. An additional factor, which modifies the affinity for CO, is the water molecule, which appears to be stabilized in the heme pocket of the mutated alpha-subunits.

Fig. 8 shows the correlation between the CO binding parameters and the CO stretching frequency for HbA, $\alpha$VE11/T, and $\beta$VE11/T. Fig. 8 (left panel) is a plot of the off-rate, $K_{CO}$ (s$^{-1}$), against $\nu_{CO}$. The best straight line through the points has a positive slope and a correlation coefficient of 0.99, indicating that thermal disruption (directly proportional to the strength of the Fe–CO bond) governs CO dissociation (21). Fig. 8 (center panel) is a plot of the equilibrium constant $K_{CO}$ (M$^{-1}$) against $\nu_{CO}$. The best straight line through the points has a negative slope and a rather high correlation coefficient of 0.96. The negative slope together with a high correlation coefficient indicate that the strength of the Fe–CO bond is a primary factor in regulating ligand affinity for both the alpha- and beta-heme pockets in R-state hemoglobin. Fig. 8 (right panel) is a plot of the on-rate, $k_{on}$ (M$^{-1}$ s$^{-1}$), versus $\nu_{CO}$. The best line through the points has a negative slope and a correlation coefficient of 0.91. The correlation between the rate of CO binding and $\nu_{CO}$, although fairly good, is not as precise as that seen for the rate of CO release and for the equilibrium constant. This suggests that other processes, such as the stabilization of the water molecule in the alpha-heme pocket of $\alpha$VE11/T (10), not directly correlated with the Fe–CO bond order, play an important role in this phenomenon.

Conclusions—In this work, we have investigated the steric, dynamic, and functional effects of the polar isosteric substitutions Val$\rightarrow$Thr in the distal heme pocket of the alpha- and beta-subunits of carbonmonoxy recombinant human hemoglobin using optical absorption spectroscopy, FT-IR spectroscopy, kinetics of CO release, and structural analysis. The results obtained with the different experimental approaches consistently indicate that the VE11/T mutation alters the electrostatic interactions within the alpha- and beta-heme pockets. In the carboxy derivative, the modification was more evident in the beta-subunits, whereas in the deoxy derivative, it had a larger effect in the alpha-subunits. For the deoxy derivatives, the observed differences were attributed to the presence of a water molecule in the distal heme pocket of the mutant alpha-subunit, stabilized by hydrogen bonding to $N^\alpha$ of His(E7) and to $O^\gamma$ of the substituted Thr(E11). In the case of the CO derivatives, we attribute the observed effects to the electrostatic interaction between the (partially) negatively charged $O^\gamma$ of Thr(E11) and the dipole moment of the bound CO molecule. Such an electrostatic interaction has a larger effect in the mutant beta-heme pocket, likely due to a more favorable distance and/or orientation of the bound CO. The electrostatic interactions also cause alterations in the local electric field at the chromophore, as evidenced by low-temperature optical absorption spectroscopy, and in the CO stretching frequency, as observed by FT-IR spectroscopy. Our attribution is supported by the structural analysis results indicating that the heme-CO geometry in the beta-heme pocket is such that both the Thr(O$^\gamma$)-CO(O) distance ($r = 3.4$ Å) and the orientation of the CO dipole with respect to $\varphi = 96.3^\circ$ are more favorable for establishing electrostatic interaction than the analogous quantities in the alpha-heme pocket ($r = 3.6$ Å and $\varphi = 83.4^\circ$).

We have also been able to show a correlation between the carbonyl stretching frequency and the ligand binding parameters. This indicates that the strength of the Fe–CO bond (and thus the polarity of the heme pocket) is a primary parameter in regulating the ligand affinity in R-state hemoglobin, pointing to the relevance of electrostatic interactions to the modulation of ligand affinity.

The increase in heme pocket mobility evidenced by the temperature dependence of the gaussian width of the Soret band is also in qualitative agreement with the increased rates of CO release reported in Table IV. However, our dynamic measurements reflect the properties of the entire heme-CO-heme pocket complex, and therefore, direct correlations with the specific kinetic processes are, at present, premature.

Acknowledgments—The technical help of W. Nie for the recombinant protein preparations and G. Lapis and F. D’Anca (of the cryogenic laboratory) is gratefully acknowledged.

REFERENCES
1. Perutz, M. F. (1990) Annu. Rev. Physiol. 52, 1–25
2. Springer, B. A., Sligar, S. G., Olson, J. S., and Phillips, G. N., Jr. (1994) Chem. Rev. 94, 699–714
3. Rohlf, R. J., Mathews, A. J., Carver, T. E., Olson, J. S., Springer, B. A., Egeberg, K. D., and Sligar, S. G. (1990) J. Biol. Chem. 265, 3168–3176
4. Egeberg, D. D., Springer, B. A., Martinis, S. A., Sligar, S. G., Morikis, D., and Champion, O. M. (1996) Biochemistry 35, 9783–9791
5. Smerdon, S. G., Dodson, G. G., Wilkinson, A. J., Gibson, Q. H., Blackmore, R. S., Carver, T. E., and Olson J. S. (1991) Biochemistry 30, 6252–6260
6. Quillin, M. L., Arduini, R. M., Olson, J. S., and Phillips, G. N., Jr. (1990) J. Mol. Biol. 234, 140–155
7. Cameron, A. D., Smerdon, S. J., Wilkinson, A. J., Habash, J., Hellwell, J. R., Li, T., and Olson, J. S. (1993) Biochemistry 32, 13061–13070
8. Uchida, T., Ishimori, K., and Morishima, T. (1997) J. Biol. Chem. 272, 30108–30114
9. Smerdon, S. J., Krayweda, S., Wilkinson, A. J., Brantley, B. E., Carver, T. E., Hargreave, M. S., and Olson, J. S. (1993) Biochemistry 32, 5132–5138
10. Cupane, A., Leone, M., Milioti, V., Friedman, R. K., Koley, A. P., Vasquez, G. P., Brinigar, W. S., Karavitis, M., and Fronticelli, C. (1997) J. Biol. Chem. 272, 26271–26278
11. Pechek, L. J., Pidelis, K., Karavitis, M., Moul, J., Brinigar, W. S., Fronticelli, C., and Gilliland, G. L. (1996) Biochemistry 35, 1935–1945
12. Chan, C. K., and Page, J. B. (1983) J. Chem. Phys. 79, 5234–5250
13. Moore, E. G., and Gibson, Q. H. (1976) J. Biol. Chem. 251, 2768–2784
14. Olson, J. S. (1981) Methods Enzymol. 63, 625–636
15. Jones, T. A., Zou, J.-V., Cowan, S. W., and Kjeldaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
16. Vasquez, G. B., Ji, X., Fronticelli, C., and Gilliland, G. L. (1996) Acta Crystallogr.
Increased α/β-Heme Pocket Polarity Dynamic/Functional Effects

17. Satow, Y., Cohen, G., Padlan, E. A., and Davies, D. R. (1986) J. Mol. Biol. 190, 593–604
18. Cupane, A., Leone, M., and Vitrano, E. (1993) Eur. Biophys. J. 21, 385–391
19. Cupane, A., Leone, M., Vitrano, E., and Cordone, L. (1995) Eur. Biophys. J. 23, 385–398
20. Militello, V., Leone, M., Cupane, A., Brinigar, W. S., Lu, A. L., and Fronticelli, C. (1995) Proteins Struct. Funct. Genet. 22, 12–19
21. Li, T., Quillin, M. L., Phillips, G. N., Jr., and Olson, J. S. (1994) Biochemistry 33, 1433–1446
22. Potter, W. T., Hazzard, J. H., Choc, M. G., Tucker, M. P., and Caughey, W. S. (1990) Biochemistry 29, 6283–6295
23. Potter, W. T., Hazzard, J. H., Kawanishi, S., and Caughey, W. S. (1983) Biochem. Biophys. Res. Commun. 116, 719–724
24. Shimandu, H., and Caughey, W. S. (1982) J. Biol. Chem. 257, 11893–11900
25. Balasubramanian, S., Lambright, D. G., and Boxer, S. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4718–4722
26. Braunstein, D. P., Chu, K., Egeberg, K. D., Frauenfelder, H., Meurant, J. R., Nienhaus, G. U., Ormos, P., Slijgar, S. G., Springer, B. A., and Young, R. D. (1993) Biophys. J. 65, 2447–2454
27. Morikis, D., Champion, P. M., Springer, B. A., and Slijgar, S. G. (1989) Biochemistry 28, 4791–4800
28. Braunstein, D., Ansari, A., Berendzen, J., Cowen, B. R., Egeberg, K. D., Frauenfelder, H., Hong, M. K., Ormos, P., Sauke, T. B., Scholl, R., Schulte, A., Sligar, S. G., Springer, B. A., Steinbach, P. J., and Young, R. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8497–8501
29. Li, X. Y., and Spiro, T. G. (1988) J. Am. Chem. Soc. 110, 6024–6033
30. Kushkuley, B., and Stavrov, S. S. (1996) Biophys J. 70, 1214–1229
31. Doyle, M. L., Lew, G., De Young, A., Kwiatkowski, L., Wierzba, A., Noble, R. W., and Ackers, G. K. (1992) Biochemistry 31, 8629–8639