Effect of the Distal Residues on the Vibrational Modes of the Fe-CO Bond in Hemoglobin Studied by Protein Engineering†

Shun-Hua Lin,‡ Nai-Teng Yu,‡ Jeremy Tame,§ Daniel Shih,§,∥ Jean-Paul Renaud,§,⊥ Josée Pagnier,§,# and Kiyoshi Nagai*,§

School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332, and Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

Received September 18, 1989; Revised Manuscript Received January 30, 1990

ABSTRACT: Using an *Escherichia coli* gene expression system, we have engineered human hemoglobin (Hb) mutants having the distal histidine (E7) and valine (E11) residues replaced by other amino acids. The interaction between the mutated distal residues and bound carbon monoxide has been studied by Soret-excited resonance Raman spectroscopy. The replacement of Val-E11 by Ala, Leu, Ile, and Met has no effect on the ν(C-O), ν(Fe-CO) stretching or δ(Fe-C-O) bending frequencies in both the α and β subunits of Hb, although some of these mutations affect the CO affinity as much as 40-fold. The strain imposed on the protein by the binding of CO is not localized in the Fe-CO bond and is probably distributed among many bonds in the globin. The replacement of His-E7 by Val or Gly brings the stretching frequencies ν(Fe-CO) and ν(C-O) close to those of free heme complexes. In contrast, the substitution of His-E7 by Gln, which is flexible and polar, produces no effects on the resonance Raman spectrum of either α- or β-globin. The replacement of His-E7 of β-globin by Phe shows the same effect as replacement by Gly or Val. Therefore, the steric bulk of the distal residues is not the primary determinant of the Fe-CO ligand vibrational frequencies. The ability of both histidine and glutamine to alter the ν(C-O), ν(Fe-CO), or δ(Fe-C-O) frequencies may be attributed to the polar nature of their side chains which can interact with bound CO in a similar manner.

Resonance Raman spectroscopy has proved an invaluable technique for studying iron-ligand bonds and the effects of the ligand environment on them (Yu & Kerr, 1988; Asher, 1981). The stretching vibration of the covalent bond between the heme and the globin, ν(Fe-N_ϵ), has been clearly identified (Nagai et al., 1980; Nagai & Kitagawa, 1980), and the regulation of heme reactivity (Perutz, 1970) by strain in this bond has been assessed by many investigators (Ondrias et al., 1982; Rousseau et al., 1983; Kerr et al., 1985). Vibrational modes involving the Fe-ligand bond have been assigned in Soret-excited resonance Raman spectra for Fe-CO (Tsubaki et al., 1982), Fe-O_2 (Brunner, 1974), Fe-NO (Chottard & Mansuy, 1977), Fe-CN (Yu et al., 1984), and Fe-N_3 (Tsubaki et al., 1981). The frequencies of these vibrational modes are sensitive to variations in the bonding strength and geometry; any significant changes in these parameters caused by mutations of the distal residues are expected to show clear changes in the resonance Raman spectra. Of particular interest are the ligand vibrational modes in carbonmonoxy-Hb. Unlike oxygen, which binds to heme in a bent fashion (Collman et al., 1974; Phillips, 1980; Shaanan, 1983), carbon monoxide binds to the heme iron of unhindered model hemes perpendicular to the heme plane (Peng & Ibers, 1976). However, X-ray crystallography and neutron diffraction have shown the Fe-CO bond is tilted or bent in carbonmonoxy-Mb (Kuriyan et al., 1986; Hanson & Schoenborn, 1981); in carbonmonoxy-Hb, the ligand lies much closer to the heme normal (Baldwin, 1980; Heidner et al., 1976; Derewenda et al., 1989). The tilting or bending has generally been interpreted as steric hindrance by the distal residues. Several groups have argued that the ligand binding pocket of the globins is tailored to fit a ligand which binds to the heme iron with a bent conformation and that this reduces the CO affinity compared to the oxygen affinity. The exact nature of the interaction between the distal residues and the bound CO is not known. Tucker et al. (1978) proposed that the distal histidine may weaken CO binding by $sp^2 \rightarrow \pi^*$ donation from the N_ϵ atom to the bound CO. This hypothesis predicts that both the ν(Fe-CO) and ν(C-O) stretching frequencies will be lower in carbonmonoxy-Hb than in free heme complexes, contrary to what is observed (Yu & Kerr, 1988). In the crystal structure of carbonmonoxy-Mb, the N_ϵ atom lies too far away from the ligand for effective electron donation.

We have produced human β-globin (Nagai & Thøgersen, 1984; Nagai et al., 1985) and α-globin (Tame, Pagnier, Shih, and Nagai, unpublished results) in *Escherichia coli*. The bacterial gene expression system has enabled us to replace the distal residues of Hb with other amino acids and to compare the functional properties of the mutants. His-E7 and Val-E11 are both highly conserved among globin sequences, but the exact geometry of these residues differs significantly with respect to the heme in α- and β-globins, suggesting that they may play different functional roles. Using engineered Hb and Mb in which the distal histidine has been replaced by glycine, it has been shown that His-E7 of α-globin and Mb increases the oxygen affinity by forming a hydrogen bond with bound oxygen and decreases the CO affinity by steric hindrance (Olson et al., 1988; Springer et al., 1989). In β-globin, the distal histidine plays a much smaller role in the control of ligand affinity.

We have replaced the distal histidine and valine of both α- and β-globins with residues of different sizes and polarity to

† This work was supported by the MRC and by NIH Grants GM-18894 and HL-31461. J.P. received support from the Foundation Recherche Medicale.

‡ Georgia Institute of Technology.

§ Medical Research Council Laboratory of Molecular Biology.

∥ Present address: Department of Biochemistry, Oregon Health Sciences University, Portland, OR 97201.

⊥ Present address: Laboratoire de Chimie et Biochimie, Pharmacologiques et Toxicologiques, CN7S UA 400, 45 rue des Saints-Pères, 75270 Paris, Cedex 06, France.

Present address: INSERM Unité 299, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre, France.

0006-2960/90/0429-5562$02.50/0 © 1990 American Chemical Society

study the effects of these replacements on the vibrational modes of bound CO by resonance Raman spectroscopy. In an earlier paper (Nagai et al., 1987), we showed that the Val-E11β → Ala, Leu, and Ile mutations have no effect on the ν(Fe–CO), ν(C–O), and δ(Fe–C–O) vibration frequencies. In contrast, the replacement of His-E7β by Gly or Val causes the ν(Fe–CO) mode to shift to lower frequency and the ν(C–O) mode to shift to higher frequency. These frequencies are unchanged by the His-E7 → Gln mutation, and we therefore proposed that they are primarily determined by the polarity of the distal residues. Morikis et al. (1989) and Braunstein et al. (1988) have performed similar experiments using engineered mutant Mb, and noted a similar effect. They did not attribute the changes in ν(Fe–CO) and ν(C–O) to polarity of the E7 residue since the peak seen in the resonance Raman spectrum of Mb–CO appears to split rather than shift. Here we present additional experiments carried out using artificial Hbs with mutated distal residues in either the α or β subunits. By examining a variety of mutants, we have shown that the vibrational modes of bound CO are sensitive to the polarity of the heme pocket, but not its size.

Materials and Methods

Wild-type and mutant β-globins were produced in *E. coli* by using the cleavable fusion protein expression vector pLcIIFXβ-globin (Nagai & Thøgersen, 1987). The β-globin polypeptide was liberated from the fusion protein with blood coagulation factor X_a and reconstituted to form functional $\alpha_2\beta_2$ tetramers. The reconstituted Hb was reduced in the presence of CO and purified by ion-exchange chromatography on CM-cellulose and DEAE-Sepharose columns (Nagai et al., 1985).

Hbs containing wild-type and mutant α-globins were prepared by the method of Tame et al. (unpublished results). The ligand binding properties of the wild-type Hb prepared with *E. coli* synthesized α- and β-globins were identical with those of Hb A prepared from human blood (Nagai et al., 1987; Mathews et al., 1989; Olson et al., 1988).

The samples of Hb mutants were stored in the concentrated carbonmonoxy form (~30 mg/mL, 0.05 M Tris-HCl buffer, pH 7.5) at 0 °C. Prior to resonance Raman experiments, the samples were brought to room temperature and diluted to approximately 80 μM (heme) with 0.05 M Tris-HCl buffer, pH 7.5. The solutions (~0.2 mL) were centrifuged at 12000 rpm to remove any insoluble material and then transferred by syringe to a CO-filled Raman cell. Resonance Raman spectra were recorded at room temperature; throughout the measurement, the Raman cell was kept spinning at about 1500 rpm to minimize photodissociation and local heating.

All resonance Raman spectra were obtained by a highly sensitive multichannel Raman system (Yu & Srivastava, 1981) which was designed specifically for optimal performance with Soret excitation. It consists of a dry ice cooled SIT (silicon-intensified target) detector with 500 channels of 12.5-mm width, a detector controller (Princeton Applied Research Model 1260), a microprocessor-based console (PAR OM3), and a Spex 1402 0.85-m Czerny–Turner double-grating spectrometer. The 406.7-nm line of a Krypton ion laser (Spectra-Physics Model 171-01) was used as the excitation source for all the spectra. The laser power as the samples was ~15 mW. The reported wavenumbers are accurate to within 1 cm^{-1} for sharp lines and 2 cm^{-1} for broad ones.

Results

Figure 1 shows the resonance Raman spectra of CO-Hbs in which the distal His of the α subunit has been replaced with other amino acid residues. Human Hb A and Hb A reconstituted with α subunit produced in *E. coli* exhibit a Fe–CO stretching mode [ν(Fe–CO)] at 507 cm^{-1}, a Fe–C–O bending mode [δ(Fe–C–O)] at 578 cm^{-1}, and the CO stretching mode [ν(C–O)] at 1951 cm^{-1}. As shown in Figure 1b, the His-E7α to Gln substitution produces no effect on these vibrational modes. In contrast, the replacement of His-E7α by Gly decreases the ν(Fe–CO) and increases the ν(C–O) for the α subunits to 494 and 1970 cm^{-1}, respectively (Figure 1a). The peaks with a reduced intensity at 505 and 1951 cm^{-1} are assigned to the ν(Fe–CO) and ν(C–O) of the native β subunits, respectively. The resonance Raman spectra of the corresponding E7β mutants are shown in Figure 2. Again, no spectral changes are observed when His-E7β is replaced by Gln. However, the substitution of His-E7β by Gly, Val, or Phe produces a shoulder at ~493 cm^{-1}, which is due to the ν(Fe–CO) mode of the mutated β subunits. These mutations shift the ν(C–O) mode to higher frequencies: 1965 and 1971 cm^{-1} (Gly-E7β), 1971 cm^{-1} (Val-E7β), and 1968 cm^{-1} (Phe-E7β). Comparison of the E7 Gly α and β mutants (Figures

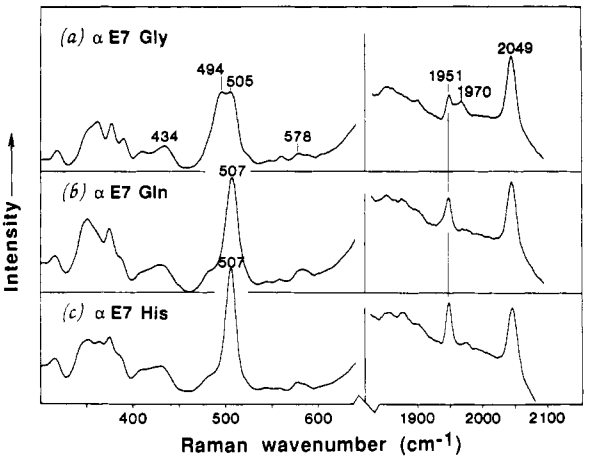

FIGURE 1: Resonance Raman spectra (300–650 cm^{-1}; 1850–2100 cm^{-1}) of αE7 mutant Hbs and *E. coli* wild-type (αE7 His) Hb in the carbonmonoxy form. (a) α(His-E7 → Gly)$_2\beta_2$; (b) α(His-E7 → Gln)$_2\beta_2$; (c) α(*E. coli*)$_2\beta_2$.

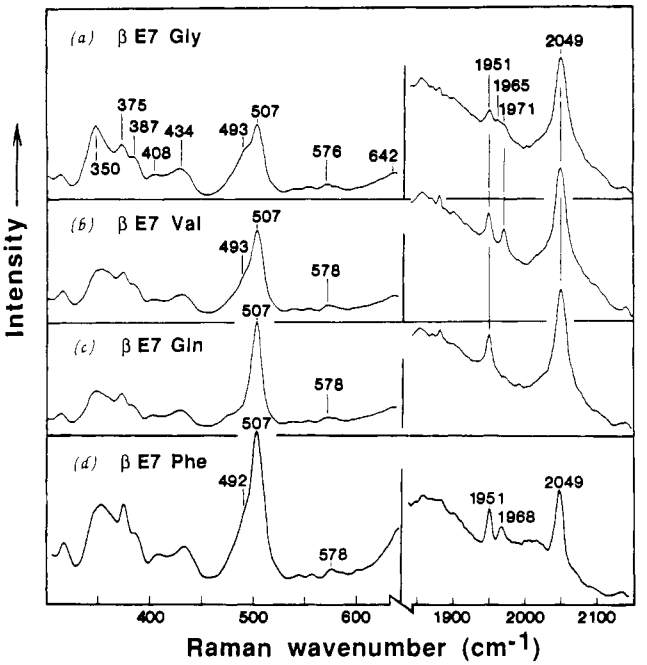

FIGURE 2: Resonance Raman spectra (300–650 cm^{-1}; 1850–2100 cm^{-1}) of βE7 mutant Hbs in the carbonmonoxy form. (a) $\alpha_2\beta$(His-E7 → Gly)$_2$; (b) $\alpha_2\beta$(His-E7 → Val)$_2$; (c) $\alpha_2\beta$(His-E7 → Phe)$_2$.

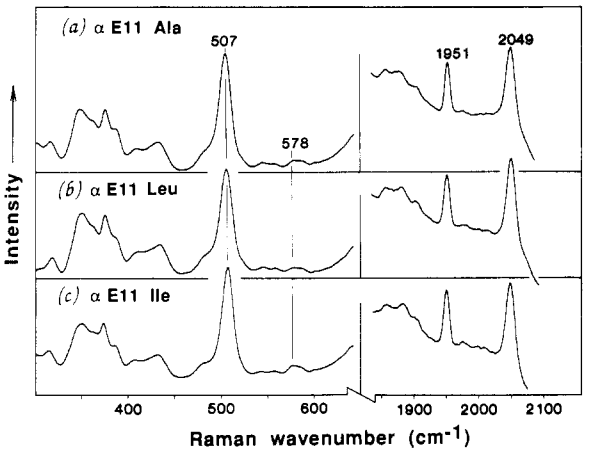

FIGURE 3: Resonance Raman spectra (300–650 cm^{-1}; 1850–2100 cm^{-1}) of αE11 mutant Hbs in the carbonmonoxy form. (a) α(Val-E11 → Ala)$_2\beta_2$; (b) α(Val-E11 → Leu)$_2\beta_2$; (c) α(Val-E11 → Ile)$_2\beta_2$.

1a and 2a) reveals differences between the α and β subunits; the intensity at 494 cm^{-1} for the Gly-E7α mutant is greater than that at 493 cm^{-1} for the Gly-E7β mutant. Furthermore, the Fe–CO stretching frequency in the native β subunit now appears at 505 cm^{-1}, 2 cm^{-1} lower than that for the native α subunits. These two peaks are not resolved in the spectrum of carbomonoxy-Hb A. It should be noted that some significant spectral differences are also apparent in the 350–400 cm^{-1} region.

The replacement of Val-E11α by Ala, Leu, or Ile produces no effect on the three vibrational modes of the Fe–C–O moiety; the ν(Fe–CO) at 507 cm^{-1}, the δ(Fe–C–O) at 578 cm^{-1}, and the ν(C–O) at 1951 cm^{-1} (Figure 3) are identical with those of native carbonmonoxy-Hb A. There are no spectral changes in the region (1200–1700 cm^{-1}) where the porphyrin ring modes appear (spectra not shown). Interestingly, we also observed no changes in the three spectral regions (300–650, 1200–1700, and 1800–2150 cm^{-1}) upon substitution of Val-E11β by Ala, Met, Leu, or Ile (Figure 4).

Discussion

The X-ray crystallographic structure of the unhindered heme–CO complex, (pyridine)(carbonyl)(5,10,15,20-tetraphenylporphinato)iron(II), Fe^{II}(TPP)(Py)(CO), was solved by Peng and Ibers (1976), who showed the Fe–C–O bond to be linear ($\theta \sim 179°$) and perpendicular to the heme plane. Hb and Mb cannot accommodate a CO ligand lying along the heme axis without the distal residues moving aside. Therefore, when CO binds, either the protein or the ligand has to move from its preferred conformation. Although it is difficult to determine the precise geometry of the CO ligand in large protein complexes, X-ray and neutron diffraction studies of carbonmonoxy-Mb (Kuriyan et al., 1986; Hanson & Schoenborn, 1981) have shown the oxygen atom of the bound CO is definitely displaced away from the heme normal. In contrast, the crystal structure of carbonmonoxy-Hb at 2.7 Å shows the ligand to be nearly perpendicular to the heme plane (Baldwin, 1980; Heidner et al., 1976), and the recent 2.1-Å resolution map confirms that the Fe–CO bond is not significantly distorted (Derewenda et al., 1990).

The expression of the α and β subunits of Hb in *E. coli* has enabled us to replace the distal residues with any other amino acids; in this way, we can assess the effect of individual amino acids on ligand binding. For example, if the Val-E11 residue significantly distorts the Fe–CO bond by steric hindrance, then its replacement with alanine should relieve that strain, and its replacement with isoleucine should increase it. Table I summarizes the ν(Fe–CO) and ν(C–O) frequencies which we have obtained for various engineered Hb mutants, as well as the kinetic and equilibrium CO binding parameters obtained by Mathews et al. (1989). Replacement of Val-E11α with isoleucine or leucine has no effect on the affinity or kinetic parameters of CO binding, but changing this residue to alanine causes a 10-fold increase in the association rate constant. In contrast to the α subunit, the CO affinity of the β subunit is reduced 40-fold by replacing the distal valine with isoleucine but is unchanged when this residue is replaced with alanine or leucine (Mathews et al., 1989). None of these mutations alters the ν(Fe–CO) or ν(C–O) stretching frequencies, however.

The effects of steric hindrance on the vibrational modes of CO bound to model Fe(II) hemes were clearly demonstrated by Yu et al. (1983) using a series of three "strapped" heme compounds. These have a 13-, 14-, or 15-atom hydrocarbon chain across one face of the heme and an imidazole ligand

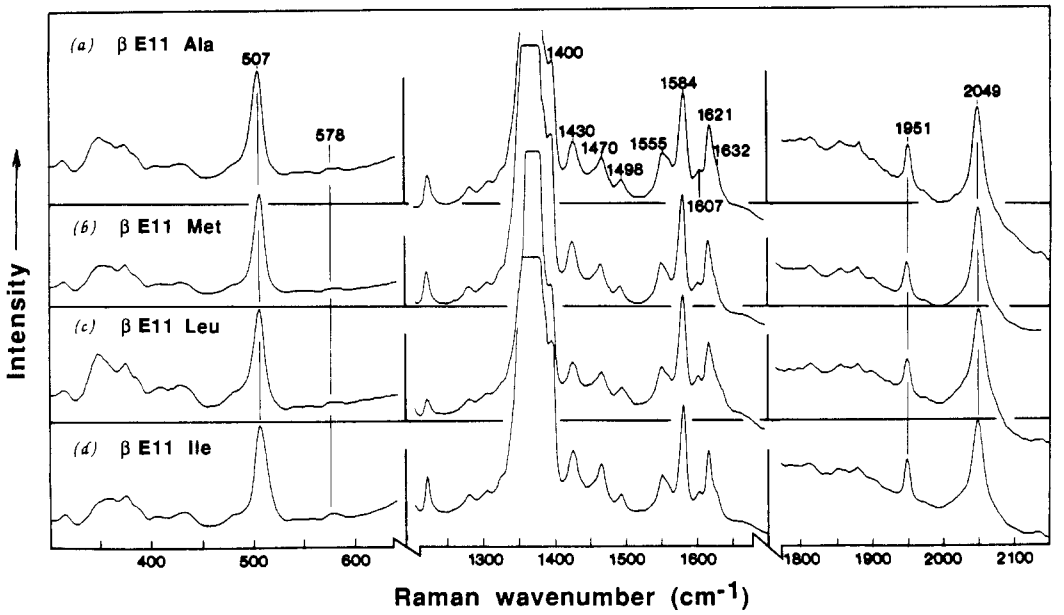

FIGURE 4: Resonance Raman spectra (300–650 cm^{-1}; 1200–1700 cm^{-1}; 1850–2100 cm^{-1}) of βE11 mutant Hbs in the carbonmonoxy form. (a) $\alpha_2\beta$(Val-E11 → Ala)$_2$; (b) 18 $\alpha_2\beta$(Val-E11 → Met)$_2$; (c) $\alpha_2\beta$(Val-E11 → Leu)$_2$; (d) $\alpha_2\beta$(Val-E11 → Ile)$_2$.

Table I: Vibrational Frequencies of the Fe–CO Bond and Kinetic and Equilibrium CO Binding Parameters

mutants	ν(Fe–CO) (cm^{-1})	ν(C–O) (cm^{-1})	k'^a ($\times 10^{-6}$ M^{-1} s^{-1})	k^b (s^{-1})	K^c ($\times 10^{-6}$ M^{-1})
α(native)	507	1951	3.2 ± 0.4	0.0047 ± 0.0005	6.8 ± 1.1
α(Gly-E7)	494	1970	19.2	0.0067	28.7
α(Gln-E7)	507	1951	13.2	0.0044	30.0
α(Ala-E11)	507	1951	32.3	0.0038	85.0
α(Ile-E11)	507	1951	0.9	0.0045	2.0
α(Leu-E11)	507	1951	2.5	0.0030	8.3
β(native)	507	1951	9.8 ± 1.9	0.0086 ± 0.003	11.4 ± 4.5
β(Gly-E7)	493	1971 (1965)	5.0	0.0130	3.9
β(Gln-E7)	507	1951	7.1	0.0098	7.2
β(Phe-E7)	492	1968	5.9	0.0143	4.1
β(Val-E7)	493	1971	nd[e]	nd	nd
β(Ala-E11)	507	1951	7.0	0.0115	6.1
β(Ile-E11)	507	1951	0.30	0.0117	0.26
β(Leu-E11)	507	1951	6.6	0.0114	5.8
heme-5[d]	495	1954	nd	nd	2.0×10^{4}[f]
FeSP-15[d]	509	1945	nd	nd	5.0×10^{-2}[f]
FeSP-14[d]	512	1939	nd	nd	5.0×10^{-1}[f]
FeSP-13[d]	514	1932	nd	nd	1.2×10[f]

[a] Kinetic association constant. [b] Kinetic dissociation constant. [c] Equilibrium binding constant (Mathews et al., 1989). [d] Yu et al. (1983). [e] nd, not determined. [f] P_{50} (torr).

bound to the free side (Ward et al., 1981). The CO binding affinity decreases with decreasing strap length due to the increased steric hindrance to ligand binding. Greater steric hindrance is accompanied by a decrease in ν(C–O) stretching frequency and an increase in ν(Fe–CO) stretching frequency. A similar steric effect was observed on the CO affinity and ν(C–O) stretching frequency of a "pocket" porphyrin in which the ligand binding site is covered with a benzene ring (Collman et al., 1983). Kim et al. (1989) solved the crystal structure of this complex and found the Fe–C–O bond angle to be 172.5(6)° and the off-axis displacement of the ligand C and O atoms to be 0.18 and 0.38 Å, respectively. These results clearly show that steric hindrance by a short, rigid hydrocarbon chain or benzene ring distorts the Fe–CO bond, and this distortion is reflected in the ν(Fe–CO) and ν(C–O) stretching frequencies. Why then are the vibrational properties of the Fe–CO bond in carbonmonoxy-Hb unchanged by amino acid changes which increase steric hindrance to ligand binding? Unlike the model heme compounds, Hb is flexible enough to accommodate bound CO without significant distortion of the ligand, and the steric strain in the protein–ligand complex is distributed over many bonds. In the crystal structure of the pocket porphyrin, Kim et al. (1989) also found the porphrin to be ruffled and the benzene cap shifted away from the CO ligand. This clearly shows that the strain is not localized in the Fe–CO bond, but is spread throughout the molecule.

In contrast to the E11 mutations, the replacement of His-E7 with Gly or Val causes striking changes in the ν(Fe–CO) and ν(C–O) frequencies. By comparing all of the mutant Hbs studied, these frequencies are shown to be unrelated to the CO affinity (Table I). As we have shown previously (Olson et al., 1988; Mathews et al., 1989), His-E7 of Mb and α-globin increases the oxygen affinity of the protein by forming a hydrogen bond with bound oxygen and decreases the affinity for CO and methyl isocyanide by steric hindrance. In β-globin, His-E7 does not form a hydrogen bond to bound oxygen (Shaanan, 1983) and plays a smaller role in controlling ligand affinity. Replacement of His-E7α with Gly increases the CO affinity due to the removal of steric hindrance; the stretching frequency ν(C–O) rises to 1970 cm^{-1} and the ν(Fe–CO) frequency falls to 494 cm^{-1}. Similar changes are observed in the β-subunit ν(C–O) and ν(Fe–CO) frequencies when His-E7 is replaced by Gly or Val. However, these mutations have no effect on the CO affinity. In the α subunit, the replacement of His-E7 with a more flexible Gln residue increases the CO affinity, probably due to the relief of steric hindrance, although the ν(C–O) and ν(Fe–CO) frequencies remain unchanged. This clearly shows that steric hindrance is not the primary determinant of the ν(C–O) and ν(Fe–CO) frequencies in the case of carbonmonoxy-Hb, quite different behavior from that found for model hemes. We previously proposed that the ν(C–O) and ν(Fe–CO) frequencies of carbonmonoxy-Hb may depend primarily on the polarity of the E7 residue (Nagai et al., 1987). In order to confirm this, we replaced His-E7β with Phe, which is hydrophobic and slightly larger than His. If the ν(C–O) and ν(Fe–CO) frequencies depend on the polarity of the E7 residue rather than its size, then they should be similar for the Phe-E7, Gly-E7, and Val-E7 mutants. This is in fact what we observed; the Val-E11β → Phe mutation has no effect on the CO affinity, but the ν(C–O) band was observed at 1968 cm^{-1} and ν(Fe–CO) at 492 cm^{-1}. It is clear that the ν(C–O) and ν(Fe–CO) frequencies depend primarily on the polarity of the E7 residue. By what mechanism do His-E7 and Gln-E7 affect these stretching vibrations? Both histidine and glutamine are good hydrogen bond donors, but neutron diffraction studies of CO-Mb showed unambiguously that there is no hydrogen bond between CO and His-E7 (Hanson & Schoenborn, 1981). Instead of the inward-facing N_ϵ being protonated, a hydrogen atom was found on the N_δ atom of the imidazole ring pointing to the solvent. Thus, the negative pole of the His dipole is directed toward the CO ligand. The E7 residue of opossum α-globin is glutamine instead of histidine (Stenzel et al., 1975); in the oxy form, the N_ϵ of this Gln side chain is probably pointing toward the oxygen ligand so that it can form a hydrogen bond as does N_ϵ of His-E7 in human α globin. In the X-ray crystal structure of deoxy-Hb (His-E7β → Gln), the Gln side chain is well localized, but we cannot distinguish between N_ϵ and O_ϵ (Nagai et al., 1987). Perutz suggested to us that in the CO form the atom nearer to the CO ligand may be O_ϵ so that the negative end of the side chain dipole is directed at the carbon monoxide, as in native human β-globin. This dipole appears to be the primary determinant of the ν(C–O) or ν(Fe–CO) frequencies. Li and Spiro (1988) have suggested that the polarity of the E7 residue may also allow the δ(Fe–C–O) bending frequency to become enhanced on Soret excitation. This mode is seen in the resonance Raman spectra of sterically hindered carbonmonoxy model hemes, but not unhindered ones, suggesting that bending or tilting of the ligand allows this vibration to become enhanced (Yu et al., 1983). The polarity of the heme pocket may therefore explain

why this mode is visible in the spectrum of carbonmonoxy-Hb when the ligand is not significantly distorted from the heme normal (Derewenda et al., 1990). When His-E7 is replaced by Gly and Val, the optical absorption spectrum of the deoxy form is anomalous (Shih and Nagai, unpublished results). We conclude that His-E7 has an important effect on the electronic state of the ligand and porphyrin π system. X-ray crystallographic studies of these mutants in the carbonmonoxy form are underway to examine the structural perturbation of the protein–ligand complex.

ACKNOWLEDGMENTS

We thank Max Perutz and Giulio Fermi for their helpful comments, Peter Esnouf for his generous gift of factor X, and Colin Young for growing cells.

Registry No. His, 71-00-1; Val, 72-18-4; Gly, 56-40-6; Phe, 63-91-2; Fe, 7439-89-6; CO, 630-08-0.

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