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Modeling the Hemoglobin Switchpoint with Cyanomet Valency Hybrids: Raman Spectroscopic Probes of Tertiary and Quaternary Structure[†]

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ABSTRACT: Hybrid hemoglobins with cyanomet hemes in the α or in the β chains have been investigated by resonance Raman (RR) spectroscopy, using ultraviolet (230 nm) and visible (441.6 nm) excitation. For the CO adducts, the UVRR spectra are identical with that of native HbCO, showing the tyrosine and tryptophan signals to be insensitive to ligand substitution within the R state. In the absence of CO, the doubly ligated hybrids show differences in the UVRR spectra, relative to the CO adducts, which are the superposition of two difference spectra: (1) the T–R difference spectrum obtained by subtracting the spectrum of HbCO from that of deoxyHb and (2) a perturbed R state spectrum, characteristic of deligated chains within the R state. These spectra arise from alterations, respectively, in the quaternary contacts of interface aromatic residues and in the tertiary contacts of interior aromatic residues. From the amplitudes of the difference spectra, the T state population was determined to be 30% for $(\alpha^{\mathrm{Fe^{II}}}\beta^{\mathrm{Fe^{III}}}\mathrm{CN})_2$ and 43% for $(\alpha^{\mathrm{Fe^{III}}}\mathrm{CN}\beta^{\mathrm{Fe^{II}}})_2$, in good agreement with the kinetic analysis of Cassoly and Gibson [Cassoly, R., & Gibson, Q. H. (1972) *J. Biol. Chem. 247*, 7332]. Addition of inositol hexaphosphate (IHP) increased the T state population, but only by a modest amount, to 40 and 53%, respectively, in contrast to the frequent assumption that the T state conversion is quantitative in the presence of IHP. Since current understanding of the quaternary state dependence of the Fe–histidine stretching frequency is based on that assumption, the RR band envelope for this vibration was reexamined. For $(\alpha^{\mathrm{Fe^{II}}}\beta^{\mathrm{Fe^{III}}}\mathrm{CN})_2$ the band is readily resolvable into two components, whose areas yield the same T/R population ratios as do the UVRR difference spectra, in the presence and in the absence of IHP. The resolved components yield 223 and 210 cm^{-1} as the R and T state frequencies; they both shift significantly upon IHP binding, to 219 and 203 cm^{-1}, revealing an IHP effect on tertiary as well as quaternary structure. For $(\alpha^{\mathrm{Fe^{III}}}\mathrm{CN}\beta^{\mathrm{Fe^{II}}})_2$, the components are not independently resolvable, but fixing their areas to correspond to the UVRR-derived T/R ratios yielded R and T state frequencies of 222 and 213 cm^{-1}, with no perceptible shift upon IHP binding. The T/R ratios are much lower than those derived from oxygenation curves for CN$^-$ adducts of partially oxidized Hb [Marden, M. C., Kiger, L., Kister, J., Bohn, B., & Poyart, C. (1991) *Biophys. J. 60*, 770]. This apparent discrepancy is ascribed to a preponderance of asymmetric hybrids $(\alpha_1^{\mathrm{Fe^{II}}}\beta_1^{\mathrm{Fe^{III}}}\alpha_2^{\mathrm{Fe^{III}}}\mathrm{CN}\beta_2^{\mathrm{Fe^{III}}}\mathrm{CN})$, which Ackers et al. [Ackers, G. K., Doyle, M. L., Myers, D., & Daugherty, M. A. (1992) *Science 255*, 54] have shown to be thermodynamically more stable than the symmetric hybrids. The R state perturbation for unligated chains which is revealed in the UVRR spectra is believed to involve collapse of the E helix toward the heme and weakening of a tryptophan hydrogen bond between the E and A helices. This perturbation is suggested to account for the phenomenon of quaternary enhancement, an R state augmentation of ligand affinity for unligated chains.

The molecular mechanism of hemoglobin cooperativity is of great importance to current thinking about protein dynamics and allostery. The classical two-state model of Monod et al. (1965) captures the main features of Hb[1] cooperativity, although additional states are clearly available to Hb under various conditions (Ackers et al., 1992; Smith et al., 1991; Silva et al., 1992). The crystal structures of deoxyHb, and of various ligated Hb's (Fermi & Perutz, 1977; Ladner et al., 1977; Baldwin & Chothia, 1979; Shanaan, 1983; Fermi et al., 1984), are widely accepted to represent the low- and high-affinity quaternary states, T and R. The reaction coordinate between the R and T states remains largely undefined, however, and is the object of much current research. Because of cooperativity, intermediate states are sparsely populated at equilibrium and must be studied with kinetic techniques (Sawicki & Gibson, 1976, 1979; Hofrichter et al., 1983) or by chemical modification of the protein. A particularly useful modification is to oxidize two of the four hemes to the "met" form and bind the Fe^{3+} ions with CN$^-$ ligands, producing "valency hybrids". Hb molecules which contain two cyanomet hemes resemble the diligated state of native Hb, which is difficult to isolate because of ligand redistribution and cooperativity.

Symmetrical valency hybrids, in which the cyanomet hemes are located exclusively in the α or in the β chains, are readily produced by selective chemical treatment of separated chains, which are then recombined to form holoprotein. Ogawa and Shulman (1972) used this construct to demonstrate a direct connection between affinity state and heme structure by showing that the chemical shifts of the heme NMR peaks are altered by the addition of inositol hexaphosphate (IHP), an effector molecule known to stabilize the T state. This work and that of Cassoly and Gibson (1972) gave rise to the view

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[1] Abbreviations: DPG, 2,3-diphosphoglycerate; CNmetHb, cyanomethemoglobin; FmetHb, fluoromethemoglobin; Hb, hemoglobin; IHP, inositol hexaphosphate; UVRR, ultraviolet resonance Raman spectroscopy; $(\alpha^{+}\mathrm{CN}\beta)_2$, $(\alpha^{\mathrm{Fe^{III}}}\mathrm{CN}\beta^{\mathrm{Fe^{II}}})_2$; $(\alpha\beta^{+}\mathrm{CN})_2$, $(\alpha^{\mathrm{Fe^{II}}}\beta^{\mathrm{Fe^{III}}}\mathrm{CN})_2$.

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that the symmetrical valency hybrids are in the R state but can be switched to T by IHP addition. This view was reinforced by the recent work of Ackers and co-workers (1992), who have been able to measure the dimer–tetramer assembly free energies for all the ligation states accessible to the cyanomet hybrid system, using kinetic methods. For the symmetrical dicyanomet hybrids, they found the assembly free energy to be almost the same as for tetracyanometHb, −8.2 and −8.5 kcal/mol, respectively, consistent with the view that the symmetrical hybrids are predominantly R. They discovered, however, that the unsymmetrical hybrids, in which two cyanomet hemes are located in one $\alpha\beta$ dimer, have a much larger assembly free energy, −11.4 kcal/mol, halfway between the values for the symmetrical hybrids and deoxyHb (−14.4 kcal/mol). Thus, the free energy surface for ligand binding must depend upon the order in which the chains are filled. Ackers et al. (1992) suggested that the unsymmetrical diligated species remains in the T quaternary state.

In this study, we provide new information on the tertiary and quaternary structures of the symmetrical cyanomet valency hybrids from resonance Raman spectroscopy. Laser excitation at 230 nm has been employed to enhance the vibrational bands of tyrosine and tryptophan residues, which have been shown (Rodgers et al., 1992) to monitor tertiary and quaternary interactions. In addition, the dependence on quaternary state of the important Fe–histidine stretching frequency has been reexamined with excitation in the visible region. The quaternary equilibrium is found to be nearly evenly balanced between the R and T structures, consistent with a diligand switch point when the ligands are bound symmetrically. On the other hand, IHP is found to stabilize the T state to only a modest extent for the valency hybrids, implying comparable affinity for both quaternary structures. In addition, IHP binding is found to influence the Fe–histidine stretching frequency of α subunits in both T and R states. In all cases, R state molecules containing deoxyhemes display a perturbed UVRR spectrum which resembles that of the recently discovered early transient of photodeligation (Rodgers & Spiro, 1994). This perturbation is believed to reflect motions of the helices lining the heme pocket, which lead to the quaternary rearrangement.

MATERIALS AND METHODS

HbA was prepared from fresh human blood by standard procedures (Antonini & Brunori, 1971). The α and β subunits were separated following the procedure of Bucci and Fronticelli (1965) as modified by Yip et al. (1977). The purity of the subunits was determined by nondenaturing gel electrophoresis (Riggs, 1981). Full regeneration of the sulfhydryl groups was assessed using the reagent *N*-ethylmaleimide. Isolated α and β subunits were exposed to potassium ferricyanide and potassium cyanide to generate the cyanomet derivative. The cyanomet chains were then passed through a Sephadex G-25 column to remove potassium ferrocyanide and excess potassium cyanide. The presence of cyanide ligand bound to the heme was checked using both absorption and visible resonance Raman spectroscopy. The cyanomet chains were combined with complementary chains containing ferrous heme CO adducts to create the symmetrical hybrids $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}}\mathrm{CO})_2$ and $(\alpha^{\mathrm{FeII}}\mathrm{CO}\beta^{\mathrm{FeIII}}\mathrm{CN})_2$. The hybrid tetramers were isolated using a CM-52 (Whatman) ion exchange chromatography column as previously described (Yip et al., 1977). All manipulations were performed at 4 °C. Photolysis of these CO adducts in an N_2 atmosphere resulted in the formation of the half-ligated forms. The concentration of the hybrids for spectroscopy was approximately 0.21 mM for $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}}\mathrm{CO})_2$ and 0.15 mM for $(\alpha^{\mathrm{FeII}}\mathrm{CO}\beta^{\mathrm{FeIII}}\mathrm{CN})_2$ per tetramer. At these concentrations, the fraction of dimeric Hb is less than 5% based upon the dimer–tetramer association rate constants determined by Smith and Ackers (1985). Spectroscopic measurements were done in a 0.01 M potassium phosphate, pH 7.0 buffer containing 0.5 mM Na_2EDTA and 10 μM KCN. In experiments performed with IHP, the effector was present in a 10-fold molar excess with respect to the Hb tetramers.

UVRR spectroscopy was carried out as previously described (Rodgers et al., 1992). Sample volumes were approximately 0.5 mL and were contained in a suprasil quartz NMR tube. The sample was rotated about a stationary helical coil to ensure complete mixing of the sample during spectroscopy. The temperature was kept between 7–10 °C by flowing cooled N_2 gas across the sample tube. Samples were maintained as fully-ligated or half-ligated forms by flowing CO or N_2 gas, bubbled through H_2O, across the sample surface.

Spectra result from 4 h of data collection. Each sample was in the laser beam for no longer than 2.5 h. Absorption spectra were measured before and after UVRR spectroscopy to check sample integrity. The quality of each UVRR spectrum, acquired over the period of an hour, was assessed by subtracting it from the first one-hour spectrum obtained with a fresh sample. If any discrepancies were observed the spectrum was discarded. Spectra were corrected for the response of the monochromator and the detector using a deuterium lamp. Difference spectra were generated using the internal intensity standard sodium perchlorate, which was present at a concentration of 0.2 M.

Visible RR spectroscopy was performed using the 441.6-nm line from a HeCd laser. Spectra were collected using a scanning double monochromator and detected with a photomultiplier tube. Sample conditions were the same as those used for UVRR spectroscopy. Curve fitting of the data was accomplished using 50% Lorentzian and 50% Gaussian line shapes with fixed widths of 15–17 cm^{-1}. The adequacy of the fit was checked by examining χ^2 values resulting from the deviations between the simulations and the actual data.

RESULTS

Fully-Ligated Hybrids Have R State UVRR Spectra. Figure 1 compares UVRR spectra for the CO adducts of the valency hybrids and of native hemoglobin, HbA. In all three Hb species each heme bears a ligand, and the quaternary state is therefore expected to be R. Excitation at 230 nm enhances Raman modes associated with tyrosine and tryptophan ring vibrations. These are labeled Y and W, respectively, in Figure 1, with the mode numbers as assigned previously (Harada & Takeuchi, 1986; Rava & Spiro, 1985). Many of these bands show altered intensities and/or frequencies in deoxyHb; the deoxyHb minus HbCO difference spectrum is shown at the bottom of Figure 2. This difference spectrum is specifically associated with R → T quaternary structure changes (Rodgers et al., 1992). Arguments have been given for assigning the difference features mainly to the Tyr α42 and Trp β37 residues, which are at the $\alpha_1\beta_2$ interface, and which alter their hydrogen-bond status in the R–T transition (Baldwin & Chothia, 1979).

When UVRR spectra of the hybrid CO adducts are subtracted from the HbCO spectrum, however, there are no spectral features above the noise level. Only the hybrid with cyanomet hemes in the β chains, $(\alpha\mathrm{CO}\beta^{+}\mathrm{CN})_2$ is shown in Figure 1, but the same result was obtained with $(\alpha^{+}\mathrm{CN}\beta\mathrm{CO})_2$. This control experiment confirms that hybrid CO adducts are

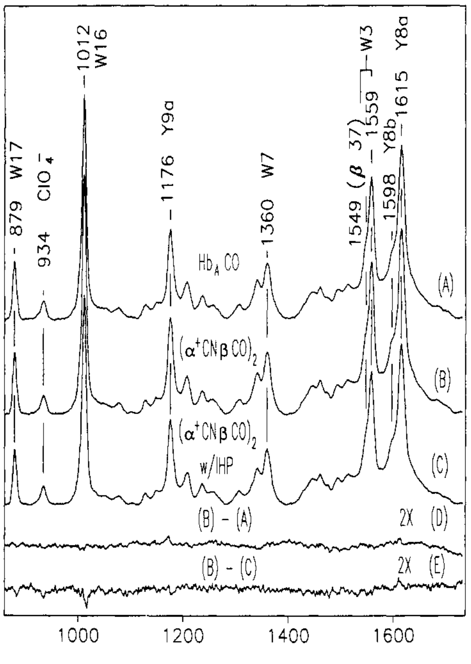

FIGURE 1: Comparison of UVRR spectra for fully ligated tetramers. The 230-nm excited RR spectrum of (A) HbCO, (B) $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}})_2$, (C) $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}})_2$ with 2.1 mM IHP, and (D) $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}}\mathrm{CO})_2$ minus HbCO. The difference spectrum is multiplied by a y-scale factor of 2. (E) $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}}\mathrm{CO})_2$ with 2.1 mM IHP minus $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}}\mathrm{CO})_2$. The difference spectrum is multiplied by a y-scale factor of 2. Similar data were obtained for $(\alpha^{\mathrm{FeII}}\mathrm{CO}\beta^{\mathrm{FeIII}}\mathrm{CN})_2$. At the Hb concentrations used, 0.15–0.25 mM, the degree of dissociation into dimers is <3–5% (Smith & Ackers, 1985). The solutions contained 0.2 M $\mathrm{ClO_4^-}$ as an intensity standard ($\mathrm{ClO_4^-}$ peak at 934 cm^{-1}). The bands are assigned to ring vibrations of tyrosine (Y) and tryptophan (W) (Rodgers et al., 1992).

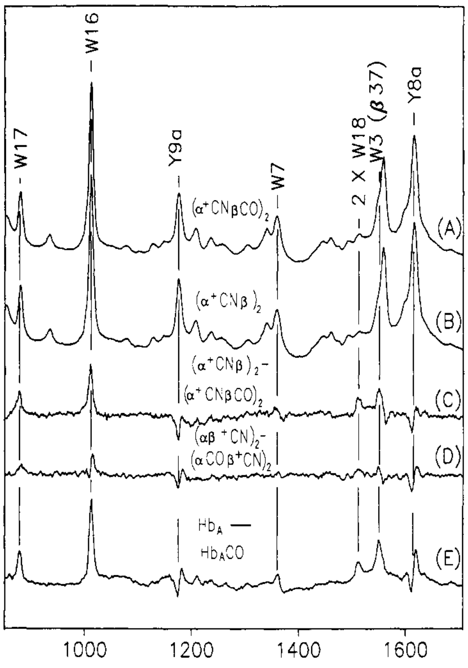

FIGURE 2: 230-nm excited UVRR spectra of (A) $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}}\mathrm{CO})_2$ and (B) $(\alpha^{\mathrm{FeIII}}\mathrm{CN}\beta^{\mathrm{FeII}})_2$ and (C) the difference spectrum, multiplied by a y-scale factor of 5. (D) The $(\alpha^{\mathrm{FeII}}\beta^{\mathrm{FeIII}}\mathrm{CN})_2 - (\alpha^{\mathrm{FeII}}\mathrm{CO}\beta^{\mathrm{FeIII}}\mathrm{CN})_2$ difference spectrum, multiplied by a y-scale factor of 5. (E) The deoxyHb – HbCO difference spectrum, multiplied by a y-scale factor of 2.

in the R state, and that the UVRR spectrum is insensitive to the difference between a CO or a CN$^-$ ligand, provided all four hemes are ligated. An additional control was carried out by examining the effect on the UVRR spectrum of adding IHP to the hybrid CO adducts (spectrum C in Figure 1). Again, there are no significant features in the difference spectrum (E), although IHP does bind to the protein (vide infra). Thus, neither IHP itself nor its binding to the protein affects the UVRR spectrum, provided the R state is maintained. It has been shown elsewhere (Jayaraman et al., 1993) that adding IHP to deoxyHb likewise has no effect on the UVRR spectrum.

Half-Ligated Hybrids Are Partly in the T-State. Figure 2 shows that when the UVRR spectrum of $(\alpha^+\mathrm{CN}\beta)_2$, in which the β chains contain deoxyheme, is compared to that of its CO adduct, the difference spectrum (C) contains features resembling quite closely those of the deoxyHb/HbCO difference spectrum (E), but with smaller amplitudes. A similar result is obtained for $(\alpha\beta^+\mathrm{CN})_2$ relative to its CO adduct (spectrum D). Since the CO adducts are in the R state, we infer that a substantial fraction of doubly ligated hybrids, in which half the hemes are unligated, exists in the T state. This fraction can be estimated to be 30% for $(\alpha\beta^+\mathrm{CN})_2$ and 43% for $(\alpha^+\mathrm{CN}\beta)_2$ by comparing the peak height of the 2xW18 band (1512 cm^{-1}) with that measured in the deoxyHb/HbCO difference spectrum. This band was chosen because it is well isolated from other bands and has no contribution from the perturbed R state spectrum that accounts for part of the hybrid difference spectrum (vide infra).

These estimates are in excellent agreement with the fractions of slow-binding hybrid molecules, 32 and 39%, respectively, for $(\alpha\beta^+\mathrm{CN})_2$ and $(\alpha^+\mathrm{CN}\beta)_2$, determined by Cassoly and Gibson (1972) from the biphasic time course of CO binding. Moreover, the NMR spectra of Ogawa and Shulman (1972) under certain conditions showed a superposition of two sets of heme resonances, which were interpreted as arising from T, as well as R state molecules, although the populations were not quantitated.

T-State Conversion Is Incomplete with IHP Addition. When IHP is added to the doubly ligated hybrids, the difference spectra, relative to those of the tetraligated hybrids, again show features characteristic of T–R differences. As seen in Figure 3, the amplitudes of these features are increased by IHP, consistent with its expected stabilization of the T state. The effect is modest, however, and the amplitudes remain well below that found in the deoxyHb/HbCO difference spectrum. Again using the 2xW18 peak height, we estimate that adding IHP increases the fraction of T state molecules from 30% to 40% in the case of $(\alpha\beta^+\mathrm{CN})_2$, and from 43 to 53% in the case of $(\alpha^+\mathrm{CN}\beta)_2$. Thus, the [T]/[R] ratio increases from 0.43 to 0.67 for $(\alpha\beta^+\mathrm{CN})_2$, and from 0.75 to 1.13 for $(\alpha^+\mathrm{CN}\beta)_2$, upon IHP binding. These increases translate to 0.30 and 0.24 kcal/mol additional stabilization of the T state by IHP for $(\alpha\beta^+\mathrm{CN})_2$ and $(\alpha^+\mathrm{CN}\beta)_2$, respectively. The excess energies represent the ratio of IHP binding affinities to T state relative to R state molecules; under the conditions of the experiment, IHP is bound to both sets of molecules. The IHP concentration was 1.5–2.1 mM, ensuring saturation of the binding sites in both states, since the dissociation constants are unlikely to exceed 1.9 μM, the value reported for CNmetHb (Perutz et al., 1978).

It has generally been held that IHP switches the doubly ligated cyanomet hybrids entirely to the T state. This impression has been based in part on the report by Ogawa and Shulman (1972) of a single set of heme resonances in the presence of IHP, presumably characteristic of the T state. It

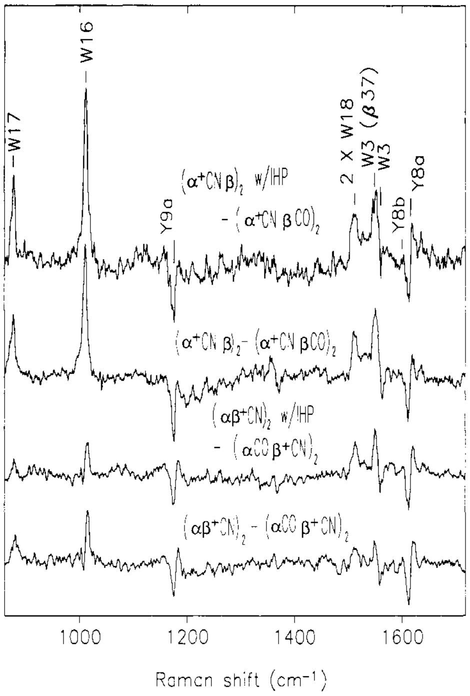

FIGURE 3: 230-nm excited UVRR difference spectra of the indicated hybrids, with and without added IHP [2.1 mM, $(\alpha\beta^{+}CN)_2$; 1.5 mM, $(\alpha^{+}CN\beta)_2$]. The amplitude multiplication factor was 5 in each case.

is possible, however, that overlap of resonances arising from the deoxy and cyanomet hemes obscure the quaternary assignment (Ho, 1992). The +9.2 ppm resonance, seen in deoxyHb and assigned to the Tyr α42–Asp β99 hydrogen bond in the T state, is exchangeable only in the R state and is not observed when IHP is added to the doubly ligated hybrids (Ho, 1992), implying that the R and T state molecules are in rapid equilibrium. Another reason for thinking that IHP shifts all the molecules to the T state was the observation by Cassoly and Gibson (1972) that the CO rebinding curve is no longer biphasic when IHP is added to the hybrids but shows single-exponential behavior. It is notable, however, that biphasic behavior *was* maintained when a weaker effector, 2,3-diphosphoglycerate was added to the hybrids, although the rates were altered for both the fast and slow components. Thus, the recombination rates are sensitive to effector binding within the R and T states. It seems possible that a further reduction in the rate constant for the fast component when IHP is added could make it impossible to distinguish the two components, even if a large fraction of the molecules are in the R state.

Deoxyheme Perturbs Tertiary Structure in the R State. Although the UVRR difference spectra of the hybrids show clear signatures of the T–R quaternary structure difference, there are additional features which are not present in the deoxyHb/HbCO difference spectrum. Figure 4 shows the effect of scaling the deoxyHb/HbCO difference spectrum to the fraction of T state molecules in each hybrid difference spectra using the 2xW18 band and subtracting these scaled spectra from the difference spectra of the hybrids. These double difference spectra all show negative peaks, mostly for tryptophan modes, although a tyrosine peak, Y9a, can also be identified. The spectra all bear a striking resemblance to other difference UVRR spectra discovered recently (Rodgers & Spiro, 1994) and shown in Figure 5. They include the difference spectra of diligated minus tetraligated Co, Fe hybrid Hb's, in which Co replaces Fe in either the α or the β chains; they also include the difference spectrum of the HbCO photoproduct minus HbCO, obtained after a 50-ns delay.

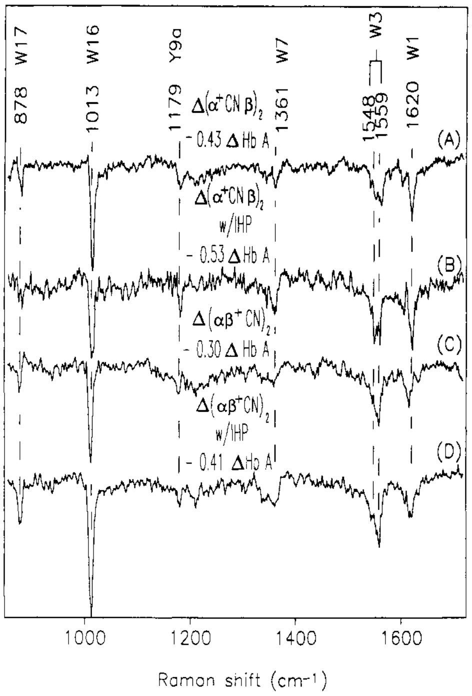

FIGURE 4: Double difference UVRR spectra intended to expose tertiary changes within the R quaternary state. The difference spectra in Figure 3 were corrected for T – R differences by subtracting the deoxyHb/HbCO difference spectrum with a scaling factor representing the fraction of T molecules, as determined from the intensity of the 2xW18 difference band (see text).

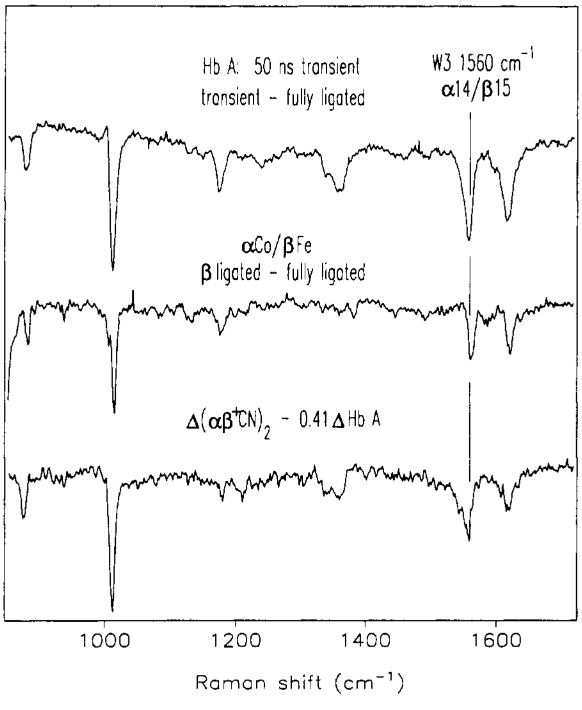

FIGURE 5: Comparison of the double difference spectra (Figure 4D) for $(\alpha^{FeII}\beta^{FeIII}CN)_2$ plus 1.5 mM IHP (bottom) with spectra of the 50-ns HbCO phototransient (top) and of the αCo/βFe Hb hybrid: β ligated – fully ligated (middle). The reference spectra are from Rodgers and Spiro (1994).

These difference spectra have been interpreted (Rodgers & Spiro, 1994) as arising from a perturbation in tertiary structure of R state molecules, illustrated in Figure 6, when the heme pocket is emptied of ligand. The key to this interpretation lies in the frequency of the tryptophan W3 peak, 1560 cm^{-1}. This frequency is associated with the two

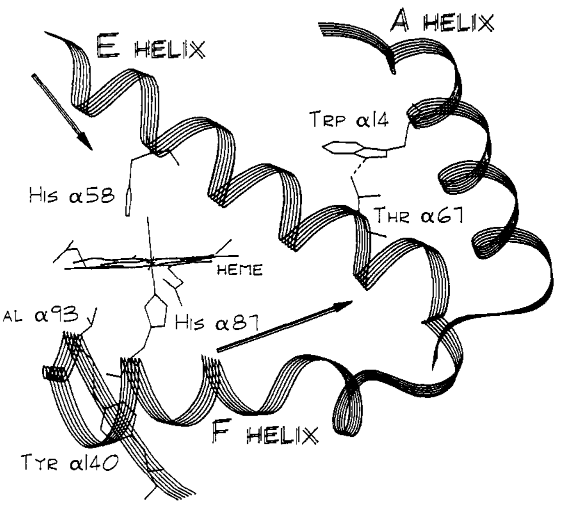

FIGURE 6: Diagram of the helix arrangement around the heme pocket of the α chains of HbCO, showing (a) the hydrogen bond between Trp α14 and Thr α67 that bridges the A and E helices and (b) the hydrogen bond between Val α93 and Tyr α140, which bridges the FG corner and the carboxy terminus. The arrangement is similar in the β chains, except that Trp β15 and Ser β72 form the A–E helix hydrogen bond bridge, and Val β98 and Tyr β145 form the bridge between the FG corner and the carboxy terminus. The arrows indicate the direction and relative magnitudes of the helix displacements in deoxyHb, relative to HbCO (Baldwin & Chothia, 1979). It is proposed that departure of the ligand from the heme pocket is concerted with E helix motion toward the heme, together with the indicated F helix motion [from Rodgers & Spiro (1994)].

tryptophan residues, α14 and β15, which are located in the interior of the chains. The third tryptophan, β37, located at the $\alpha_1\beta_2$ intersubunit interface, has a distinctly lower frequency, 1549 cm^{-1}, because of a lower dihedral angle between the indole ring and the C_β atom. A model compound study has revealed a direct correlation between this angle and the W3 frequency (Miura et al., 1989). The T–R difference spectrum (Figure 2) contains a W3 peak at 1549 cm^{-1}, consistent with a change in Trp β37 environment in the two quaternary states, while the difference spectra associated with the R state perturbation contain a W3 peak at 1560 cm^{-1}, reflecting a change monitored by the interior tryptophans. These are located in the A helices, but the indole protons form hydrogen bonds with the OH groups of the E helix residues Thr α67 and Ser β72 (Baldwin & Chothia, 1979; Fermi et al., 1984). The E helix lines the distal side of the heme pocket, and it seems likely that escape of exogenous ligands from the heme pocket is concerted with motion of the E helix toward the heme, with attendant weakening of the Trp α14 and β15 hydrogen bonds (Figure 6). Hydrogen bond weakening is known to shift the tryptophan excitation profiles to higher energies, thereby diminishing the RR intensities at 230 nm (Rodgers et al., 1992). The several tryptophan peaks in the difference spectra of Figures 4 and 5 are all negative, consistent with this interpretation. The negative Y9a peak has been suggested to arise from alteration in the strength of the hydrogen bonds from the Tyr α140 and β145 side chains to the carbonyl oxygens of the F helix residues Val α93 and β98 (Rodgers & Spiro, 1994), reflecting a shift in the proximal F helix in concert with the distal E helix.

The species represented in these spectra are all R state molecules which lack ligands in at least half of the chains, the early photoproduct because of ligand diffusion out of the heme pocket after photolysis, and the hybrids because of the chemical differences in the two kinds of hemes. The present analysis establishes that the R state cyanomet hybrid molecules display the same perturbation as is seen for the Co, Fe hybrids and for the early HbCO photoproduct.

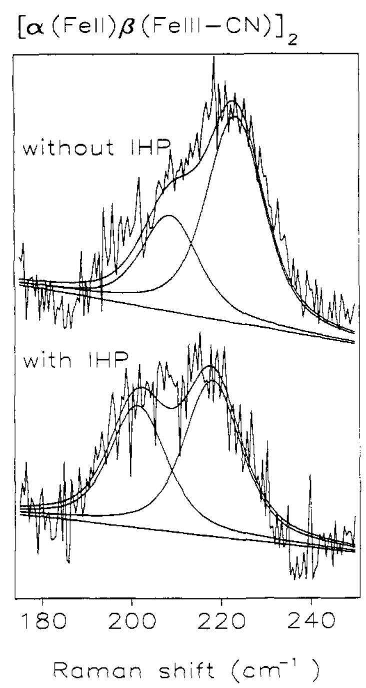

FIGURE 7: 441.6-nm excited visible RR spectra of $(\alpha\beta^{+}CN)_2$ and $(\alpha\beta^{+}CN)_2$ with 1.5 mM IHP. The smooth curves are obtained from curve fitting using a fixed width of 15 and 17 cm^{-1} for the two components.

The Fe–His Bond Responds to IHP Binding in Both Quaternary States. Because IHP addition to cyanomet hybrids has been used to assign the frequency of the important Fe–histidine stretching vibration in the R and T states, we have reexamined the relevant RR data in the light of the current results regarding the extent of R–T interconversion. Exploiting the fact that excitation near the Soret band of the heme group enhances the Fe–His Raman mode for deoxyhemes, but not for ligated hemes, Nagai and Kitagawa (1980) used the symmetrical cyanomet hybrids to assign the α and β chain Fe–His frequencies separately. The reported values for the R and T states were 220 and 207 cm^{-1} for the α chains, and 222 and 218 cm^{-1} in the β chains, on the assumption that the molecules were in the R state, but switched to T upon IHP addition. Similar results were obtained by Ondrias et al. (1982), using Co, Fe hybrids and by Jeyarajah and Kincaid (1990), with the cyanomet hybrids. In addition to the downshift, the Fe–His RR band was observed to broaden substantially, especially for the α chains, upon IHP addition. This broadening was suggested to reflect differences between the two α subunits (Ondrias et al., 1982), although, similar broad features have also been observed in RR studies of singly α(Fe)-ligated cross-linked Fe–Co hybrids (Kaminaka et al., 1989).

Figure 7 shows the Fe–His RR band profile, obtained with 441.6-nm excitation, for $(\alpha\beta^{+}CN)_2$, with and without added IHP. Clearly, there is a shift to lower frequency upon IHP addition, as reported previously. It is also evident, however, that more than one band contributes significantly to the profile. As demonstrated in Figure 7, the data can be fit satisfactorily with two bands of fixed width (15 and 17 cm^{-1}). Moreover, the peak area ratios are in excellent agreement (Table 1) with the [T]/[R] ratios obtained from the UVRR difference spectra, if the low frequency Fe–His peak is assigned to the T state and the high frequency peak to the R state. Thus, the Fe–His RR band profile is entirely consistent with the view

Table 1: Estimation of T-State Populations (%) Using Ultraviolet and Visible RR Spectroscopy

hybrid	from 2xW18 peak height[a]	from Fe–His band deconvolution[b]	from CO binding kinetics[d]
$(\alpha^{II}\beta^{III}CN)_2$			
−IHP	30 ± 10	32 ± 4	32
+IHP[c]	40 ± 10	42 ± 4	
$(\alpha^{III}CN\beta^{II})_2$			
−IHP	43 ± 10		39
+IHP[c]	53 ± 10		

[a] Percentage as determined from comparison of difference spectra with that of Hb_A as described in the text. [b] Percentages based upon two-peak curve fitting of visible RR spectra. Values reported are the averages of at least 10 fits using a fixed peak width of 15–17 cm^{-1}. The T state percentage is the ratio of the area of the lower frequency peak to the total area. [c] IHP present in a 10-fold molar excess with respect to Hb tetramer concentration. [d] Results from Cassoly and Gibson (1972); tetramer concentration 15 μM, measured at 445 nm.

Table 2: Fe–His Stretching Frequencies for Hybrid Deoxy Hemes from Band Deconvolution via T State Populations

hybrid	R (cm^{-1})	T (cm^{-1})
$(\alpha^{II}\beta^{III}CN)_2$		
−IHP	223	210
+IHP	219	203
$(\alpha^{III}CN\beta^{II})_2$[a]		
−IHP	222	212
+IHP	222	212

[a] Component band area ratios were fixed to the values determined by UVRR spectroscopy (Table 1).

that the doubly ligated hybrid contains a significant fraction of T state molecules, and that the fraction is augmented only modestly by IHP addition.

The frequencies of the bands assigned to the R and T states are not, however, independent of IHP. The R state frequency is 223 cm^{-1}, but shifts to 219 cm^{-1} when IHP is bound, while the T state frequency is shifted from 210 to 203 cm^{-1} by IHP. We note that the averages of these frequencies are in quite good agreement with the values reported by Nagai and Kitagawa (1980) for the R and T states, from the peak frequencies determined in the absence and presence of IHP. But the influence of IHP on each of the quaternary state frequencies is not insignificant. It is apparent that IHP binding lowers the Fe–His frequency via a tertiary change in both the R and the T states.

In the case of $(\alpha^+CN\beta)_2$, the R and T contributions to the Fe–His band profile, shown in Figure 8, are not sufficiently resolved to permit an independent estimate of the [T]/[R] ratio. Adopting the ratio obtained from the UVRR data, however, and fixing the bandwidths at 17 cm^{-1}, we determined R and T frequencies of 222 and 212 cm^{-1} with or without IHP. In this case IHP does not shift the frequencies but only the T and R populations. Although the shift in the band profile upon IHP addition is in agreement with that reported by Nagai and Kitagawa (1980), the present interpretation is quite different. We conclude that the R and T state frequencies are almost the same for the α and β chains in the absence of IHP. The major difference between the α and β chains lies in the additional effect of IHP on both frequencies of the α chains.

It should be noted that in the earlier studies, IHP was added at lower pH, generally ~6, while the "R state" spectra were recorded at higher pH, generally ~9. Since the T state is stabilized at low pH and destabilized at high pH, this procedure produced greater spectral changes than are observed in our study. We used a pH of 7 for consistency with the previous NMR (Ogawa & Shulman, 1972) and kinetic (Cassoly & Gibson, 1972) studies. As it happens, the significant proportions of coexisting R and T state molecules and the modest population shift induced by IHP at this pH make it easier to deconvolute the Fe–His band at pH 7 than at the pH extremes.

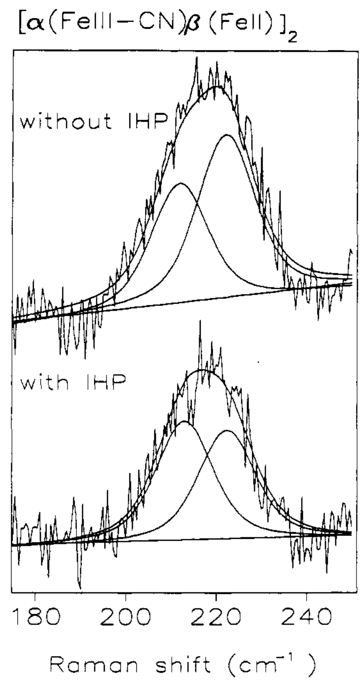

FIGURE 8: 441.6-nm excited visible RR spectra of $(\alpha^+CN\beta)_2$ and $(\alpha^+CN\beta)_2$ with 2.1 mM IHP. The smooth curves result from curvefitting the spectra to fixed band widths (17 cm^{-1}) component band area ratios determined by UVRR spectroscopy (see text).

DISCUSSION

Cyanomet hybrids are useful models for intermediates in the ligation pathway of hemoglobin. Like the $Fe^{2+}O_2$ adduct, the $Fe^{3+}CN^-$ adduct is low-spin, with the Fe atom firmly anchored in the heme plane by strong bonds to the pyrrole N atoms. Because of backbonding to O_2, the charge distribution is essentially the same in the two adducts, as evidenced by similar porphyrin skeletal mode RR frequencies (Yu et al., 1984). Consistent with the chemical similarity of the $Fe^{3+}CN^-$ and $Fe^{2+}O_2$ adducts, Marden et al. (1991) were able to fit oxygenation curves for Hb preparations having variable content of cyanomet heme with the same T/R affinity ratio for CN^- as for O_2. They used the classical two-state equilibrium model, in which the protein shifts from the low-affinity T state to the high-affinity R state when the average number of ligands reaches a certain value. For O_2, this number is close to 2.5 (Baldwin, 1975). Thus, Hb tetramers with two ligands are expected to be in the T state, predominantly, while those with three ligands are expected to be mainly R. Consistent with this view, the fitting parameters of Marden et al. (1991) give T state population estimates of 98% and 27% for Hb molecules with two and three cyanomet hemes on average.

There are, however, four different ways of arranging two cyanomet hemes in the Hb tetramer: both in the α chains ($\alpha_1\alpha_2$), both in the β chains ($\beta_1\beta_2$), one in the α and β chains of the same dimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$), or one in an α and a β chain of opposite dimers ($\alpha_1\beta_2$ or $\alpha_2\beta_1$). The first two of these can be separately prepared via chain assembly; they are the species examined in this study by RR spectroscopy. The UVRR estimates of T state content, 43 and 30% [which are in excellent agreement with those obtained from the CO binding analysis of Cassoly and Gibson (1972)], differ sharply from the 98% value obtained by Marden et al. (1991) for Hb's with an average of two cyanomet hemes. The discrepancy must reflect a greater T state propensity for one or both of the remaining doubly ligated isomers.

These two isomers cannot be isolated because the dimer–tetramer equilibrium leads to dimer scrambling and to a distribution of species. Their thermodynamics have been studied by Ackers and co-workers (1992), however, using kinetic methods. Remarkably, they found that the asymmetric hybrid in which the two ligands are on the same dimer has a much larger free energy for dimer dissociation than the other isomers. This free energy, −11.4 kcal/mol, is halfway between the values for tetraligated (R state) molecules, −8.5 kcal/mol, and unligated (T state) molecules, −14.4 kcal/mol. In contrast, the free energies for the other three isomers, −8.2 to −8.5 kcal/mol, are close to the R state value. This unique stability of the asymmetric dimer was observed by Ackers and co-workers in other hybrid systems as well and led them to the conclusion that there are three cooperative states of importance in Hb, and that the binding of ligands follows a symmetry rule: binding two ligands is less disruptive to the interfacial forces holding the tetramer together if they bind to the same dimer than to opposite dimers. From a comparison of mutational effects and proton coupling, Daugherty et al. (1991) inferred that the asymmetric doubly ligated hybrid is a T state molecule while the other isomers are largely in the R state. This inference is consistent with the 98% T state estimate of Marden et al. (1991) for Hb molecules with an average of two cyanomet hemes, since they should mostly (ca. 93%) consist of asymmetric hybrids, judging from the thermodynamic data of Ackers et al. (1992). The present results confirm that doubly ligated symmetric hybrids are largely (60–70%) in the R state; they represent microstates which are at low abundance on the equilibrium binding pathway.

The UVRR difference spectra provide a direct monitor of the T state population because they respond to changes in the environment of the Tyr α42 and Trp β37 residues at the $\alpha_1\beta_2$ subunit interface. Signals from these residues do not respond to tertiary changes associated with ligand or effector binding but only to the quaternary rearrangement. Most other physical measurements respond to both effects. This ambiguity can explain the previous impression that IHP converts the symmetric hybrids entirely to the T state. Thus, conversion of slow and fast binding components to a single slow phase by IHP (Cassoly & Gibson, 1972) can be explained by a slowing of the faster rate by R state binding of IHP, causing the two phases to become kinetically indistinguishable; a slowing of the fast phase is actually seen in the case of DPG binding, where the two phases remain distinguishable. The apparent collapse of the heme NMR resonances from two sets into one (Ogawa & Shulman, 1972) may have a similar explanation, since the heme environment may be influenced by effector binding to the R state. Indeed, Perutz and co-workers (1974) observed changes in histidine and aromatic resonances when IHP binds to cyanometHb, which remains in the R state. Significantly, the proton resonance assigned to the Tyr α42–Asp β99 quaternary hydrogen bond does not appear in the NMR spectra of the symmetric hybrids (Ho, 1992), even when IHP is added, presumably due to rapid exchange between the T and R molecules present in solution. The UVRR spectra establish that IHP induces only a modest increase in the T state population to 40 and 50% for the two hybrids.

From their modeling of oxygenation curves of partially oxidized Hb's, Marden et al. (1991) concluded that the influence of effectors on the Hb quaternary state has frequently been overestimated. For example, they calculated that IHP shifts the T state population of fluorometHb from 29 to 72%, although quantitative conversion has often been assumed. UVRR measurements have recently confirmed that the conversion is only partial (Jayaraman et al., 1993). In the case of aquometHb, Marden et al. (1991) calculated 18% T state molecules, increasing to 52% upon IHP addition. When these populations were used to reinterpret the magnetic data of Philo and Deyer (1985), the free energy change associated with the spin equilibrium difference between the R and T states was found to be in agreement with the ca. 1.2 kcal/mol predicted by Perutz et al. (1974), although a much smaller value had been calculated on the assumption of quantitative conversion from R to T upon IHP addition.

In the same vein, we find that the RR data on the Fe–His vibrational band of the symmetric cyanomet hybrids requires reinterpretation because of the earlier assumption of quantitative R–T conversion by IHP. The present results show that the R and T state frequencies of this vibration are 222 and 211 cm^{-1} (± 1 cm^{-1}) for *both* α and β chains, although the β chain difference had been thought to be much smaller (Nagai & Kitagawa, 1980). In addition, IHP binding shifts the α chain frequencies, by 4 cm^{-1} for the R state and by 7 cm^{-1} for the T state, but the β chain frequencies do not shift.

Nagai and Kitagawa's (1980) observation implied substantial weakening of the α chain Fe–His bond in the T relative to the R state, and the present reanalysis extends this conclusion to the β chain as well. There has been much speculation about the mechanism of this weakening, including (1) mechanical tension on the bond due to the displacement of the F helix relative to the heme, (2) weakening of the hydrogen bond from the proximal imidazole ring to a backbone carbonyl group, (3) tilting of the imidazole away from the heme normal, (4) rotation of the imidazole relative to the heme pyrrole rings, and (5) displacement of the Fe atom from the heme plane. Recent discussion and modeling efforts have focused on 3–5 (Friedman et al., 1990; Ahmed et al., 1991; Gilch et al., 1993; Stavrov, 1993), although all five effects may contribute to different extents. Our finding that IHP shifts both frequencies for the α but not the β chains is consistent with other evidence for α chain deformability. For example, in the Hb adduct with NO, a ligand with a strong trans effect, IHP is known to break the Fe–His bonds in the α but not in the β chains (Nagai et al., 1980).

Moreover, the present results require that the Fe–His frequency be responsive to the state of ligation of neighboring chains, even within a quaternary state. Thus, 211 cm^{-1} cannot be the frequency in all T state molecules, since in that case the Fe–His band maximum in native deoxyHb would be 211 cm^{-1}, whereas it is known to be 215 cm^{-1}. We conclude that the cyanomet hemes induce a tertiary change within the T state, which extends to neighboring chains with unligated hemes, lowering their Fe–His frequencies by 3–5 cm^{-1}. The effect is reciprocal between α and β chains and has a nearly equal influence in each of them.

Recently, Gilch et al. (1993) deconvolved the Fe–His band of deoxyHb into five components, which vary with pH and among different modified Hb's. They ascribed these components to substates having differing tertiary conformations. Our deconvolution is not so fine-grained, but it captures the T and R state quaternary populations as revealed by UVRR spectroscopy. Nevertheless, the results do imply tertiary changes within the T-state induced by the presence of the cyanomet hemes, which extends to neighboring unligated chains, leading to a redistribution of the intensities and consequently, to lower Fe–His frequencies. It is possible that the intensity redistribution reflects a variety of conformational changes leading to the spectral heterogeneity analyzed by Gilch et al. (1993) and by Stavrov (1993).

The UVRR difference spectra of the hybrids are found to be superpositions of two spectra, one characteristic of the T/R differences, and one characteristic of perturbations in the R state associated with loss of ligand from the heme pocket. The latter spectrum has previously been obtained from the 50-ns HbCO photoproduct and also from the doubly ligated Co, Fe hybrids. The R state is more stable for the Co, Fe hybrids than for the cyanomet hybrids because in the unligated hemes the Co atom is displaced toward the proximal histidine to a smaller extent than is the Fe atom (Scheidt, 1974; Dwyer et al., 1974; Little & Ibers, 1974). Nevertheless, the spectral comparison shows the same perturbation to the structure of those cyanomet hybrid molecules which are in the R state (Figure 5). This perturbation has been inferred to be a displacement of the E helix toward the unligated heme (Figure 6) and a resultant weakening of a hydrogen bond between the A and E helices (Trp α14 and β15 to the OH group of Thr α67 or Ser β72) (Rodgers and Spiro, 1994). The E helix displacement may be responsible for the phenomenon of "quaternary enhancement", manifested as a higher ligand affinity at the last binding step of the tetramer, relative to isolated dimers (Doyle & Ackers, 1992). Alternatively, the effect is seen in a less favorable free energy of dimer association for triligated relative to tetraligated tetramers. This free energy deficit can also be seen at the diligation stage for symmetric hybrids. For example, the symmetric Co, FeCO hybrids have dimer association free energies of –7.5 and –7.6 kcal/mol, compared to –8.0 kcal/mol for the tetraligated, form (Ackers et al., 1992), giving a quaternary enhancement of 0.5 kcal/mol. For the symmetric cyanomet hybrids, the corresponding values are –8.2 and –8.5 kcal/mol compared to –8.5 kcal/mol for the tetraligated species. The apparently low quaternary enhancement for these species should be corrected for the 30 and 40% population of T state molecules, whose dissociation into dimers is negligible. Thus, the free energies of association for the diligated cyanomet hybrid molecules which are in the R state are calculated to be –8.7 and –8.8 kcal/mol, giving quaternary enhancements within experimental error of those found for the Co,FeCO hybrids.

We associate the destabilization of these partially ligated R state molecules with the E helix displacement in unligated chains. The weakening of the A–E helix hydrogen bond can account for part or all of this destabilization. The effect of the destabilization is to augment ligand binding, which restores the helix position, and the interhelix hydrogen bond. Alternatively, the destabilization is a source of excess energy which promotes the transition to the T state upon deligation of Hb.

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