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Structure of a Third Cooperativity State of Hemoglobin: Ultraviolet Resonance Raman Spectroscopy of Cyanomethemoglobin Ligation Microstates†

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Received December 20, 1994; Revised Manuscript Received February 15, 1995⊗

ABSTRACT: Ultraviolet resonance Raman (UVRR) spectra have been obtained for cyanometHb (Hb = hemoglobin) hybrid tetramers representing several ligation microstates, using dimer interchange techniques and spectral subtraction. Relative to fully ligated tetramers, $Hb(CN)_4$, the monoligated hybrids and deoxyHb all show fully developed T/R difference UVRR bands which are associated with T state quaternary contacts across the $\alpha_1\beta_2$ interface, involving the Trp β37 and Tyr α42 residues. Triligated species show quite different signals, arising from the interior residues Trp α14 and/or β15. From earlier studies, these R_{deoxy} signals are attributed to E helix displacement toward the heme in deoxy subunits within R state tetramers, resulting in weakened Trp H-bonds. Asymmetric diligated hybrids, containing both ligands in the same dimer unit, show signals characteristic of the T quaternary contacts, but they are attenuated by 40%. An equilibrium mixture of T and R state molecules is ruled out by the absence of significantly strong R_{deoxy} difference bands. Rather, the spectral attenuation is attributed to weakening of the T state contacts at the $\alpha_1\beta_2$ interface. This interpretation is supported by previous observations that the mutational pattern of free energy perturbations for the asymmetric hybrid is T-like and not that of a T/R equilibrium or an R-like state. The asymmetric hybrid represents a third cooperativity state, T′, having a T quaternary arrangement of the subunits but a deformed $\alpha_1\beta_2$ interface, with weakened contacts.

There is continuing interest in the molecular mechanism of cooperativity in hemoglobin (Hb), as a paradigm for allostery in proteins. The enormously successful two-state model of Hb cooperativity (MWC) has guided research in the field for over 30 years and has shaped our thinking about other allosteric proteins (Perutz, 1990). In this model, the low-affinity T state of the $(\alpha\beta)_2$ tetramer has a lower free energy than the high-affinity R state, but the free energy gained upon successive ligand binding overcomes this difference and switches Hb from T to R, thereby producing cooperative binding. Analyses of the binding data indicate that the switch occurs when two or more ligands are bound. While the two-state model explains a vast body of equilibrium and kinetic data on ligand binding (Sawicki & Gibson, 1976; Shulman et al., 1975), a truly searching examination of its premises has only recently become possible with the development of methodology for characterizing all ten ligation microstates of Hb (Figure 1) (Ackers et al., 1992). Normally these microstates cannot be distinguished because of rapid ligand exchange among subunits and because of rapid interchange of $\alpha\beta$ dimers. The ligand exchange limitation can be overcome by substituting nonlabile heme mimics, e.g., cyanomet heme for oxygenated heme or Co(II) protoporphyrin for deoxy heme, to produce hybrid hemoglobin molecules (Ackers et al., 1992). Dimer interchange can be prevented by chemically cross-linking the dimers (Miura & Ho, 1982; Miura et al., 1987) or by inhibiting the interchange at sufficiently low temperature (Perrella & Bernardi, 1981). In the latter case, the protein molecules corresponding to the ligation microstates can be separated and quantitated, permitting a direct determination of their free energies (LiCata et al., 1990).

Using these hybrids it has been possible to determine the free energy of dimer association to tetramers for all ten ligation microstates in several hybrid systems (Ackers et al., 1992). The values for the cyanomet hybrid system are given in Figure 1. The two-state model predicts simple relationships among these values, based on the free energy difference between the T and R states and on the ratio of their ligand affinities (Ackers, 1990). These predictions are not met in any of the systems so far examined (Ackers, 1990). At least one additional cooperativity state of the tetramers is required to explain the data.

The requirement for a third state is seen most clearly in comparing the tetramer free energy for the asymmetrically diligated cyanomet species, −11.4 kcal/mol, with those of the remaining diligated species, all of which are within experimental error at a value of −8.3 kcal/mol (see Figure 1). Thus the tetramer is fully 3 kcal/mol more stable when two ligands are bound to the same $\alpha\beta$ dimer within the tetramer than when they are bound to opposite dimers, regardless of which chain is occupied. A separate cooperativity state is therefore implied for the asymmetrically diligated species (Ackers, 1990).

What is the nature of this third state? Experiments in which the effect of mutations on the tetramer free energy are examined show the pattern for the asymmetrically

† This work was supported by NIH Grant GM 25158.
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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1995.

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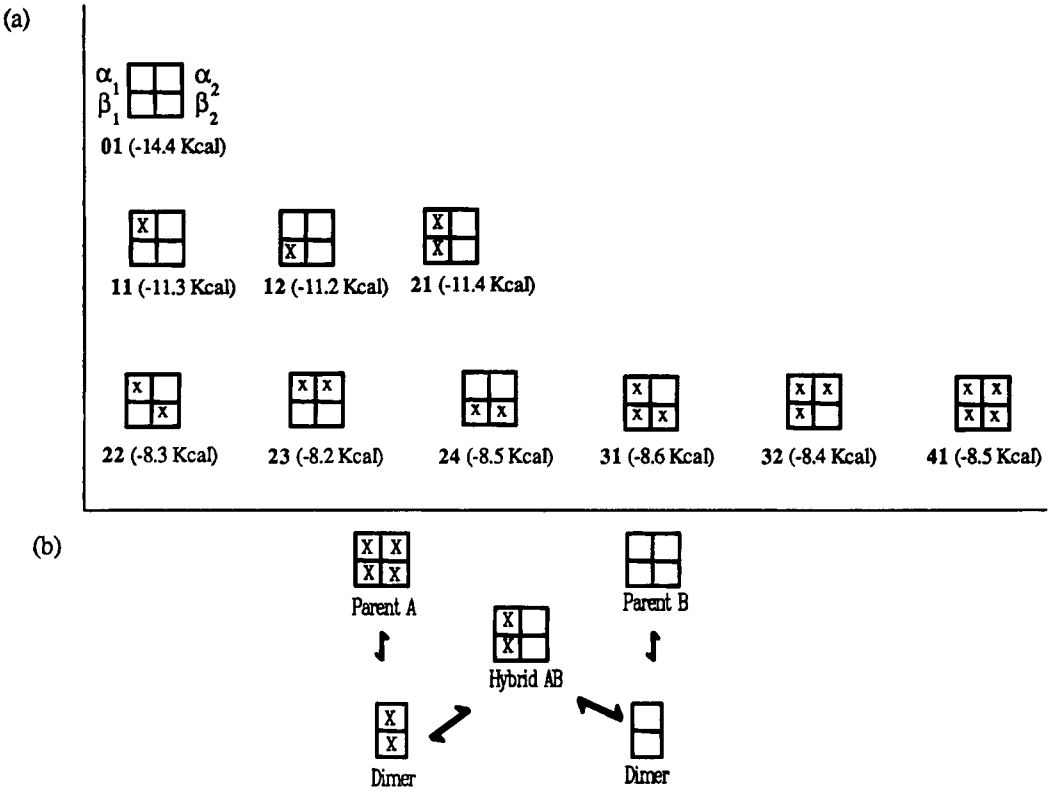

FIGURE 1: (a) The ten ligation microstates of tetrameric Hb. Ligated subunits are denoted as X. The orientation of the α and β subunits within the tetramer is shown in deoxyHb. The free energies of tetramer assembly from dimers, ΔG, are listed for the cyanomet system. (b) Diagram illustrating the equilibration of parent tetramers A and B to form the hybrid AB through dimer interchange.

Table 1: Compositions of the Equilibrium Mixtures Used To Examine CyanometHb Hybrids

	parents	
hybrid AB (% in the mixture)[a]	A (% in the mixture)[a]	B (% in the mixture)[a]
$\alpha_1^+CN\alpha_2\beta_1\beta^2$ (47.6%)[a]	$\alpha_1^+CN\alpha_2^+CN\beta_1\beta_2$ (25.6%)[a]	deoxyHb (26.8%)[a]
$\alpha_1\alpha_2\beta_1^+CN\beta_2$ (45.9%)[a]	$\alpha_1\alpha_2\beta_1^+CN\beta_2^+CN$ (27.4%)[a]	deoxyHb (26.6%)[a]
$\alpha_1^+CN\alpha_2\beta_1^+CN\beta_2$ (45%)[a]	cyanometHb (26.3%)[a]	deoxyHb (28.6%)[a]
$\alpha_1^+CN\alpha_2^+CN\beta_1^+CN\beta_2$ (48%)[a]	cyanometHb (24.4%)[a]	$\alpha_1^+CN\alpha_2^+CN\beta_1\beta_2$ (26.4%)[a]
$\alpha_1^+CN\alpha_2\beta_1^+CN\beta_2^+CN$ (49%)[a]	cyanometHb (24.5%)[a]	$\alpha_1\alpha_2\beta_1^+CN\beta_2^+CN$ (27.5%)[a]

[a] Perrella et al. (1990).

diligated species to resemble that of unligated molecules, suggesting a T-like structure (LiCata et al., 1993). It is possible to assess this suggestion using ultraviolet resonance Raman (UVRR) spectroscopy, which probes the environment of aromatic residues in the protein (Asher et al., 1983; Copeland & Spiro, 1985; Su et al., 1989; Kitagawa, 1992) and has been shown to be responsive to changes in tertiary and quaternary structure (Rodgers et al., 1992). In this study we have determined UVRR difference spectra for the asymmetric diligated cyanomet hybrid [designated as 21], as well as for the mono- [11,12] and triligated species [31, 32]. The results strongly indicate that the asymmetric hybrid has a T quaternary structure in which critical $\alpha_1\beta_2$ contacts have been weakened. The view that emerges is that the second ligand weakens but does not break the T state restraints, provided that it binds in the same dimer as the first ligand.

MATERIALS AND METHODS

Hb was prepared from fresh human blood (Antonini & Brunori, 1971). CyanometHb was prepared from Hb by oxidation with potassium ferricyanide followed by reaction with potassium cyanide. The resulting ferrocyanide and excess ferricyanide were removed by gel chromatography using a Sephadex G-25 column. Symmetrically diligated species, [23] and [24], were prepared as described elsewhere (Mukerji & Spiro, 1994).

Preparation of Hybrid Hemoglobins. Six of the ten ligation microstates in Table 1 cannot be studied in isolation due to the presence of $\alpha\beta$ dimer rearrangement reactions (Perrella et al., 1990; Ackers et al., 1992). These six hybrids can be obtained by equilibrating two parents, as illustrated by the diagram in Figure 1 (Doyle & Ackers, 1992). The parents required for preparing the various hybrids, and also the percentages of the species present at equilibrium are shown in Table 1. These percentages are close to the statistical expectation, 50% hybrid and 25% parents. The UVRR spectra were obtained for these equilibrium mixtures, and the intensities of the UVRR spectra were normalized with respect to the 934 cm^{-1} band of ClO_4^- present as an internal standard. The spectra of the hybrid species were obtained by subtracting the spectra of the parent species, obtained in separate runs, using the known equilibrium concentrations. At the hemoglobin concentrations used, 1 mM in heme, the concentration of dissociated dimers is

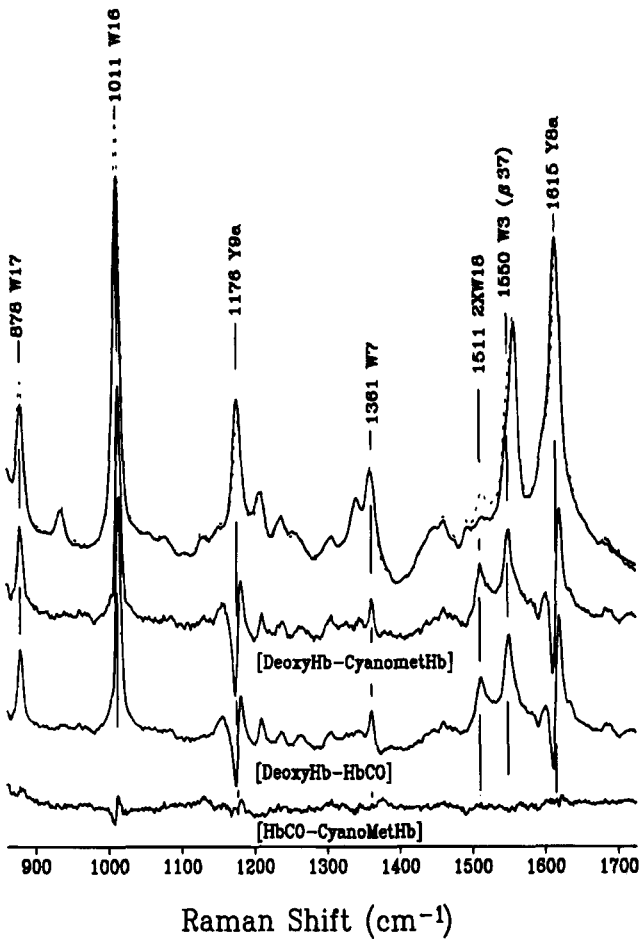

FIGURE 2: 230-nm excited UVRR spectrum of cyanometHb (—) and deoxyHb (- - -) and the difference spectrum, multiplied by a y scale factor of 3. Shown for comparison is the difference spectrum between deoxyHb and HbCO, with the same scale factor. The bottom trace is the difference spectrum between cyanometHb and HbCO.

negligible. The hemoglobin samples were prepared in 25 mM phosphate buffer at pH 7.4 with 100 μM KCN and 0.2 M ClO_4^-. The parents were incubated for a time sufficient to achieve equilibrium, as determined by Perrella et al. (1990).

The UVRR spectrometer used to obtain the UVRR spectra has been described in a previous report from this laboratory (Rodgers et al., 1992). Sample volumes of 0.5 mL were contained in a quartz NMR tube, which was spun around a stationary helical wire. A nitrogen atmosphere was maintained over the deoxy samples by delivering the gas into the NMR tube through a thin stainless steel tube. Spectral acquisitions were carried out in 1-h increments. The final spectrum was obtained by coadding five or six of the 1-h spectra. Before addition, each spectrum was subtracted from the first spectrum taken, for a given sample. If there were difference features, the spectrum was discarded.

RESULTS AND DISCUSSION

UVRR spectra with 230-nm excitation are shown in Figure 2 for deoxy- and cyanometHb. At this wavelength, the resonance-enhanced bands arise from tyrosine and tryptophan residues, labeled Y and W in the figure. The mode assignments have been discussed elsewhere (Rodgers et al., 1992). When the spectra are subtracted, using the 934 cm^{-1} band of the ClO_4^- internal standard for normalization, the

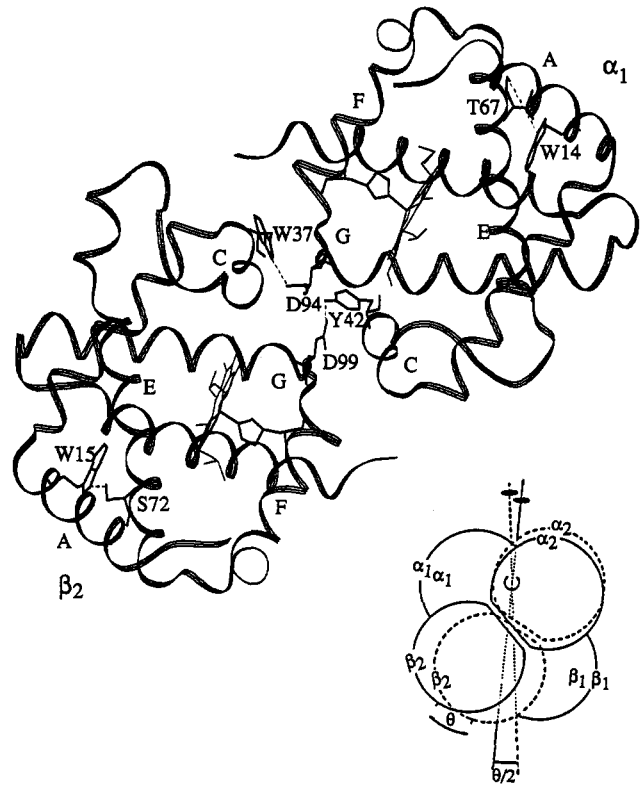

FIGURE 3: Ribbon diagram (from Brookhaven Protein Data Bank coordinates) of the α_1 and β_2 subunits of deoxyHb showing the interface contacts involving Tyr α42 and Trp β37 and the H-bonding interactions of the interior Trp residues α14 and β15. Also shown is a diagram of the interdimer rotation between the T and R quaternary structures (Perutz, 1990).

difference spectrum is the same as the deoxyHb/HbCO difference spectrum, which arises from altered molecular interactions between the T and R structures. Subtraction of the cyanomet-Hb spectrum from that of HbCO leaves a trace (bottom of Figure 2) with no difference features above the noise level. Thus the same T/R difference spectrum is obtained whether the tetraligated molecules contain CO heme or cyanomet heme.

The T/R difference features are believed to arise mainly from H-bond changes of the Trp β37 and Tyr α42 residues, which are found at the "flexible joint" and "switch" regions, respectively, of the $\alpha_1\beta_2$ subunit interface (Figure 3) (Baldwin & Chothia, 1979). The contribution of Trp β37 is particularly clear-cut, because the position of the W3 mode is different for Trp β37 than for the two interior tryptophan residues α14 and β15 (Rodgers et al., 1992), because of different dihedral angles about the bonds connecting the indole rings to the C_β atoms (Miura et al., 1989). The W3 band of Trp β37 occurs at a lower frequency, 1550 cm^{-1}, and is seen as a shoulder on the main W3 band, centered at 1558 cm^{-1}. The difference band, however, is found at 1550 cm^{-1}, the Trp β37 position. There is no corresponding dispersion of tyrosine residue frequencies, but the bisignate difference signals of the Y8a and Y8b bands are most likely due to the T state H-bond interaction between Tyr α42 and Asp β99 (Rodgers et al., 1992), which is absent in the R state (Baldwin & Chothia, 1979).

The same T/R difference spectra are found (Figure 4) when the spectrum of cyanometHb is subtracted from the spectra of monoligated hybrids [species 11 and 12], whether the cyanomet heme is in an α or a β chain. Because these

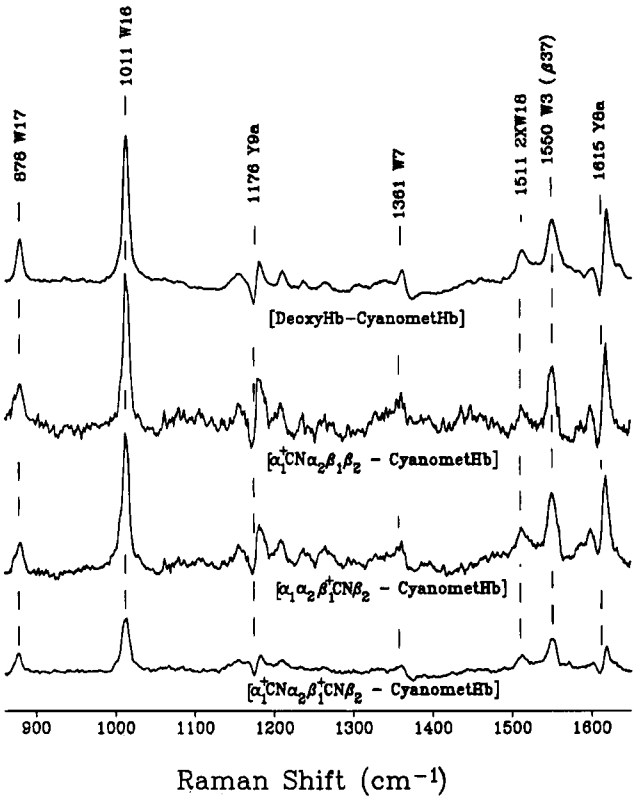

FIGURE 4: Comparison of 230-nm excited UVRR difference spectra relative to cyanometHb for (top to bottom) deoxyHb, $\alpha_1^+CN\alpha_2\beta_1\beta_2$ [11], $\alpha_1\alpha_2\beta_1^+CN\beta_2$ [12], and the asymmetric diligated species $\alpha_1^+CN\alpha_2\beta_1^+CN\beta_2$ [21].

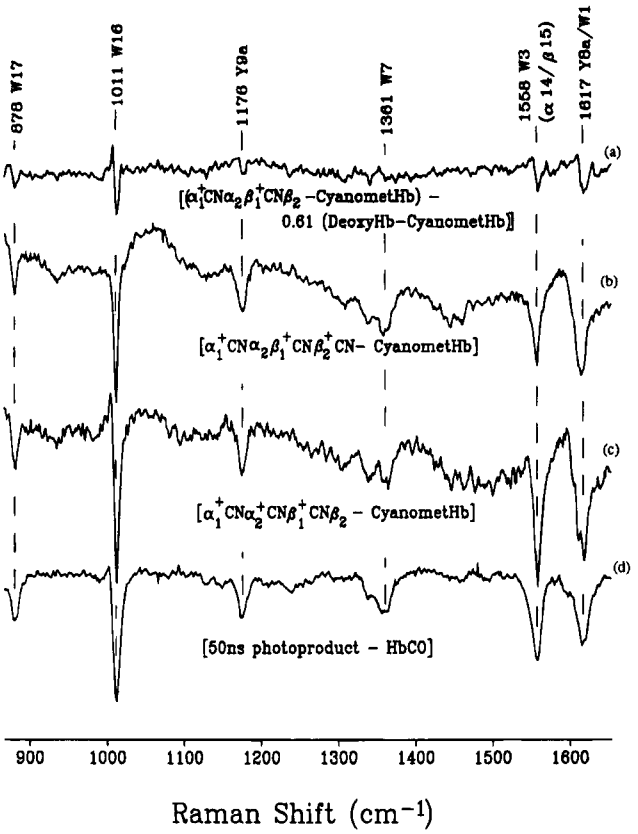

FIGURE 5: Comparison of (top to bottom) the double difference spectrum obtained by subtracting the T/R features from the difference UVRR spectrum (Figure 3) of the asymmetric hybrid, the difference spectra relative to cyanometHb for the triply ligated species $\alpha_1^+CN\alpha_2^+CN\beta_1^+CN\beta_2$ [31] and $\alpha_1\alpha_2^+CN\beta_1^+CN\beta_2^+CN$ [32], and the difference spectrum relative to HbCO of the 50-ns HbCO photoproduct spectrum from Rodgers and Spiro (1994).

difference spectra are linear combinations of several parent spectra (see Table 1), the noise level is appreciably higher than in the simple deoxyHb/cyanometHb difference spectrum, but all the R/T features are recognizable, and they are present at full amplitude. This experiment establishes that the monoligated Hb molecules are in the T state, as expected.

Very different results are obtained for the triligated hybrids (Figure 5), which are expected to be in the R state. For these molecules, subtraction of the cyanometHb spectrum leaves a series of strong negative bands; as seen in the figure, these are the same bands detected in the earliest phase of protein relaxation following ligand photodissociation in HbCO (Rodgers & Spiro, 1994). Likewise, these bands are observed in the difference UVRR spectra of di- minus tetraligated species in the Co,Fe hybrid Hb system (Rodgers & Spiro, 1994). The W3 band falls at the position assigned to the interior residues Trp α14 and β15. These residues are located on the A helices but form H-bonds to the OH groups of Ser α72 and Thr β67 on the E helices, which line the distal side of the heme binding pockets (Figure 3). The UVRR negative bands are proposed to result from weakening of these H-bonds due to displacement of the E helix toward the heme in those subunits bearing no exogenous ligand within R state Hb molecules (Rodgers & Spiro, 1994). The difference spectrum is therefore assigned to deoxy- vs ligated subunits within the R state, i.e., a R_{deoxy} signature. As seen in Figure 4, the same spectrum is observed whether the deoxy heme is in an α or a β subunit; this equivalence was also observed in Co, Fe hybrids (Rodgers & Spiro, 1994).

Turning to the asymmetric diligated cyanomet hybrid, in which the two ligands are in the same αβ dimer (species 21), we see (Figure 4) that subtracting the cyanometHb spectrum leaves a difference spectrum containing all the T/R difference signals, with the proper relative intensities. The overall amplitude, however, is significantly lower, by 40%, than in the deoxyHb or the monoligated hybrid difference spectra. This loss in amplitude has two alternative explanations: (1) The T state contacts involving Trp β37 and Tyr α42 are weakened and produce weaker signals or (2) only a fraction of the molecules are in the T state.

In a recent study of the symmetrically diligated cyanometHb hybrids, in which the cyanomet hemes are in either the two α or the two β subunits [species 23 and 24 in Figure 1], the second explanation was invoked to explain even lower amplitude T/R difference signals, because the percentages of T state molecules calculated on this assumption (43% and 30% when the ligands were in the α or in the β subunits, respectively) (Mukerji & Spiro, 1994) were in quantitative agreement with the percentages which had earlier been reported from kinetic measurements (Cassoly & Gibson, 1972). Moreover, the symmetric hybrid difference spectra had additional features, which were revealed by subtracting out the T/R difference bands to be the same R_{deoxy} negative bands as observed here for triligated hybrids. This is just the behavior expected if the symmetric hybrids contain an equilibrium mixture of R and T state molecules.

This behavior is not seen for the asymmetrically diligated hybrids, however. The T/R difference features are attenuated by 40%, but a compensating R_{deoxy} spectrum is not observed. Since the asymmetric hybrid molecules have two deoxy hemes, the R_{deoxy} signal should be 80% as intense as the

spectrum obtained for the triply ligated state, where there is one heme deligated. However, when the T/R bands are subtracted out of the asymmetrically diligated hybrid difference spectrum, using the deoxyHb difference spectrum with a 60% weighting factor, the resulting trace (top of Figure 5) contains only weak negative features, amounting to no more than 20% of the negative band intensities observed for the triligated hybrids. This weak residual spectrum can be attributed to a small fraction of R state molecules present in the hybrid solutions, as revealed by kinetic studies on this hybrid (Doyle & Ackers, 1992).

We therefore conclude that the asymmetric diligated hybrid molecules do not exist in a T/R equilibrium mixture but rather in a structure, T′, which has the T state quaternary arrangement of the subunits but with significantly altered contacts at the $\alpha_1\beta_2$ interface. The alteration results in weakening the intersubunit H-bonds involving the Trp β37 and Tyr α42 side chains, accounting for the observed 40% attenuation of the difference UVRR signals. This inference is entirely consistent with the mutational pattern of free energy perturbations, showing the asymmetric hybrid to be T-like rather than R-like (LiCata et al., 1993).

The weakening of the $\alpha_1\beta_2$ interface implied by the UVRR spectra suggests a physical mechanism for the symmetry rule (Ackers et al., 1992) derived from the tetramer assembly free energy measurements. The T state contacts are strong enough to accommodate one ligand without significant perturbation. If a second ligand binds to the second subunit of the same dimer, the resulting tertiary forces result in a plastic deformation of the $\alpha_1\beta_2$ interface, with attendant weakening of the contacts. However, if the second ligand binds to a subunit on the other side of the interdimer interface, then the $\alpha_1\beta_2$ contacts break, resulting in rearrangement to the R state. This happens to most (60−70% for the symmetric cyanomet hybrids) but not all of the molecules, since the T/R equilibrium is set by the overall binding energy. When three or four ligands bind, the molecules are essentially all in the R state.

ACKNOWLEDGMENT

We thank Prof. Gary Ackers for helpful discussions and Dr. Ishita Mukerji for her help in sample preparation.

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BI942927F