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The Mutation β99 Asp-Tyr Stabilizes Y—A New, Composite Quaternary State of Human Hemoglobin

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ABSTRACT **Carbonmonoxy hemoglobin Ypsilanti (β99 Asp-Tyr) exhibits a quaternary form distinctly different from any structures previously observed for human hemoglobins. The relative orientation of αβ dimers in the new quaternary form lies well outside the range of values observed for normal unliganded and liganded tetramers (Baldwin, J., Chothia, C., J. Mol. Biol. 129:175–220, 1979). Despite this large quaternary structural difference between carbonmonoxy hemoglobin Ypsilanti and the two canonical structures, the new quaternary structure's hydrogen bonding interactions in the "switch" region, and packing interactions in the "flexible joint" region, show noncovalent interactions characteristic of the $\alpha^1\beta^2$ contacts of both unliganded and liganded normal hemoglobins. In contrast to both canonical structures, the β97 histidine residue in carbonmonoxy hemoglobin Ypsilanti is disengaged from quaternary packing interactions that are generally believed to enforce two-state behavior in ligand binding. These features of the new quaternary structure, denoted Y, may therefore be representative of quaternary states that occur transiently along pathways between the normal unliganded, T, and liganded, R, hemoglobin structures.**

Key words: mutant hemoglobin, cooperativity, protein structure, conformational change, quaternary structure, allosteric proteins

INTRODUCTION

The hemoglobin system is a classic prototype for studies of the molecular mechanisms of intersubunit communication in allosteric proteins. The allosteric model of Monod et al.[1] specifies that cooperativity arises from the coupling between ligand binding and intersubunit interactions via equilibria between two or more conformational forms of the hemoglobin molecule, each with a different affinity for heme-site ligands. Previous crystallographic studies have been consistent with the simplest such model: two quaternary states of the hemoglobin tetramer have been characterized, corresponding to the deoxygenated (deoxy)[2] and liganded[3] forms, and these have been identified with the canonical T and R states in the thermodynamic theory.[4] However, recent thermodynamic studies[5] indicate that the two-state model is an oversimplification. The mutation β99 Asp-Tyr, which occurs in hemoglobin Ypsilanti,[6] is located in an intersubunit region of the molecule where previous studies have identified noncovalent interactions that most clearly differentiate between the deoxy and liganded quaternary forms.[7] Moreover, this mutation has a profound effect on the thermodynamics of intersubunit interactions, inverting the stability of the deoxy and oxy forms.[6] As we will describe below, this mutation also dramatically alters the liganded state quaternary structure from that observed in either T or R state hemoglobins. The altered quaternary state nevertheless exhibits features previously thought unique to each canonical structure. We will call this new quaternary state Y.

Human hemoglobin is a tetrameric structure comprised of two α and two β chains, each of which contains a heme group where a ligand such as oxygen is reversibly bound. Oxygen binding to the $\alpha_2\beta_2$ tetramer leads to structure changes at the heme itself and tertiary changes within the oxygenated (oxy) subunit. The principal effect of these tertiary changes is to modify contacts within the $\alpha^1\beta^2$ interface region connecting the two dimer pairs, thereby promoting quaternary structure changes.[7] Thus, the $\alpha_2\beta_2$ tetramer acts essentially as a system of interacting dimers, $\alpha^1\beta^1$ and $\alpha^2\beta^2$, related by a 2-fold rotation axis.

Interactions within the $\alpha^1\beta^2$ interface region are of two distinct types. Packing contacts between the α^1FG corner* (residues $\alpha^1$89 through $\alpha^1$95) and the

*The tertiary structure of the α and β subunits are generally described by designating the helical segments with the letters A through H and denoting connecting loops as corners (e.g., FG corner). Eight helical segments are present in the β chains, while the α chains do not contain a D helix. The superscripts to α and β identify relative positions in the hemoglobin tetramer.[8]

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β^2C helix (residues $\beta^2$35 through $\beta^2$41) principally involve the same side chains in deoxy and liganded forms, leading to the description of this region as a "flexible joint."[7] The principal distinction between the dimer–dimer contacts in deoxy and liganded quaternary structures involves interactions between residues $\beta^2$94 through $\beta^2$99, designated the β^2FG corner, and the α^1C helix (residues $\alpha^1$36 through $\alpha^1$44), along with symmetry-related contacts.[7] Protruding side chains of residues $\alpha^1$38Thr, $\alpha^1$41Thr and $\alpha^1$44Pro of the α^1C helix create two mutually exclusive packing environments for $\beta^2$97His. In deoxy hemoglobin $\beta^2$97His packs between $\alpha^1$44 and $\alpha^1$41; in liganded hemoglobin it packs between $\alpha^1$41 and $\alpha^1$38. This situation has been described as a two-state "switch," and has led to the conclusion that any intermediate quaternary structures would be unstable.[7] For this reason, the contacts between βFG corners and αC helices have been called the "switch" region.

Based on these structural observations, the deoxy and liganded structures are thought to be the only two stable quaternary forms accessible to the hemoglobin tetramer. This conclusion has been supported by the fact that hemoglobin molecules have always crystallized with either deoxy-like or oxy-like quaternary structures, although with significant variations in subunit tertiary structure.[9–18] These and other studies[19] have clarified the chemical events involved in the coupling between ligand binding and tertiary structure changes that trigger the conversion of one quaternary form to the other. However, they provide little information regarding how the pathway from the deoxy to liganded quaternary structures can overcome the stereochemical barrier presented by the packing of β97His in the switch region without destabilizing the $\alpha^1\beta^2$ interface. We report here the crystal structure of mutant hemoglobin Ypsilanti (β99 Asp-Tyr), whose liganded (carbonmonoxy) state assumes a novel quaternary form in which this barrier is greatly diminished. The details of the packing of β97His coupled with the composite nature of the $\alpha^1\beta^2$ interface in carbonmonoxy hemoglobin Ypsilanti strongly suggest that the β99Tyr may stabilize a quaternary state, the Y-state, that occurs transiently along the pathway from the normal deoxy to oxy forms.

MATERIALS AND METHODS

Crystallization and Data Collection

Carbonmonoxy (CO) hemoglobin Ypsilanti (CO-YPS), obtained from Dr. Gary K. Ackers (Washington University, St. Louis, MO), was crystallized using a modification of the conditions described by Perutz[20] for liganded forms of human hemoglobin. Crystallization experiments were carried out at room temperature under an atmosphere of carbon monoxide.[21] Crystals grew from solutions containing 2.25–2.30 M NaH_2PO_4/K_2HPO_4 at pH 6.7, and were stabilized by equilibration with 3.5 M NaH_2PO_4/K_2HPO_4 prior to X-ray exposure. These crystals were bipyramidal with typical dimensions of 300 × 300 × 700 μm, and diffracted to at least 2.3 Å. The crystals were characterized by precession photography and showed the symmetry of space group $P3_221$ or its enantiomorph, with unit cell dimensions $a = b = 93.1$ Å, $c = 144.6$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$. Estimation of the fraction of crystal volume occupied by solvent[22] was consistent with either two or three dimers in the asymmetric unit. This represents a new crystal form of human hemoglobin, which has not previously been observed to crystallize in a trigonal space group.

Three-dimensional data to 3.0 Å resolution were collected using a Xuong-Hamlin multiwire area detector (San Diego Multiwire Systems, San Diego, CA), and images were integrated using the program PROFL (developed by Frank Hage, University of North Carolina at Chapel Hill, Chapel Hill, NC). Virtual films were scaled using the fourier surface scaling program FS.[23] The 3.0 Å data set contained 88% of all unique reflections, with equivalent reflections merging with a residual $(R_{symm})^\dagger$ of 0.088. A limited set of Friedel related reflections were measured from another crystal ($R_{symm} = 0.062$) by collecting h,k,l at (ω,χ,ϕ) and $-h, -k, -l$ at $(\omega,-\chi,\phi+180)$ in alternating 10° wedges of data, in order to minimize systematic errors between Freidel mates.

Structure Solution

The trigonal crystal form of CO-YPS was solved by molecular replacement methods.[24] Calculations were carried out using the MERLOT[25] and CCP4[26] program packages, for reflections with Bragg spacings from 8.0 to 4.0 Å, and a 23 Å radius of integration. The molecular replacement calculations were consistent only with a tetramer in the asymmetric unit. The direction of the two-fold axis relating dimers in this tetramer ($\alpha = 14°$, $\beta = 84°$, $\gamma = 180°$)[‡] was determined by a self-rotation function[27] and is shown relative to the crystallographic axes in Fig-

† $$R_{symm} = \frac{\sum_{\mathbf{h}} \sum_{j} |I(\mathbf{h})_j - \bar{I}(\mathbf{h})|}{\sum_{\mathbf{h}} \sum_{j} I(\mathbf{h})_j}$$

where $I(\mathbf{h})_j$ represents the j^{th} observation of the intensity at reflection $\mathbf{h}$.

‡ The Euler angles (α,β,γ) denote rotations about the z, new y, and new z axes, respectively. The molecular x, y, and z axes coincide with the crystallographic a^*, b, and c axes when $\alpha = \beta = \gamma = 0.0$.

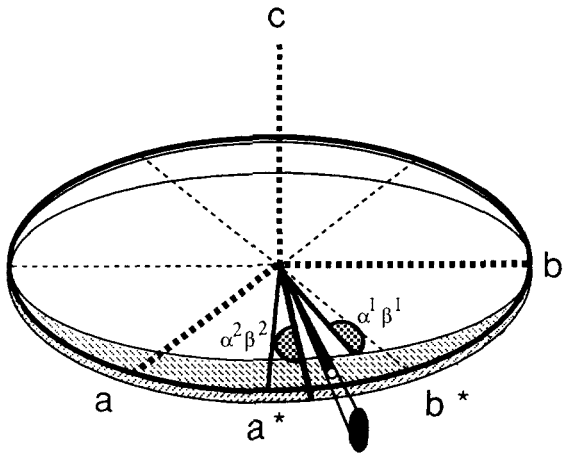

Fig. 1. Orientations of the two dimers in CO-YPS. Oxy hemoglobin dimers (shaded semicircles) are drawn in a crystallographic coordinate system (a,b,c) as oriented according to the two major peaks of the cross-rotation function between CO-YPS and an oxy hemoglobin dimer. The molecular dyad (thick axis) was determined from the Ypsilanti self-rotation function.

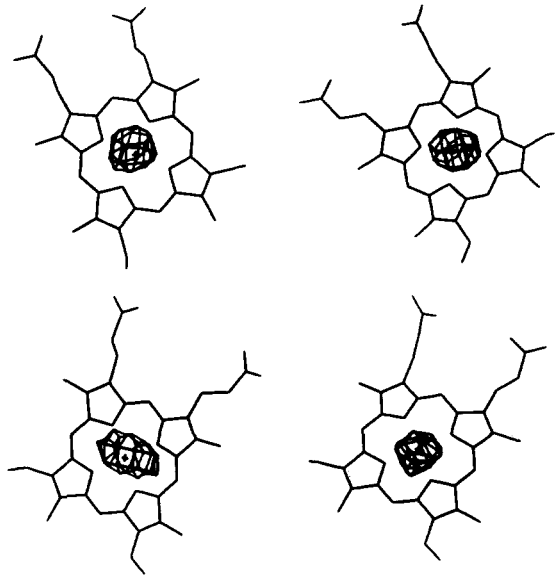

Fig. 2. Bijvoet difference Fourier maps for CO-YPS. The electron density was calculated using $[F(h,k,l) - F(-h,-k,-l)]\exp(i\alpha_c + \frac{\pi}{2})$ as the Fourier coefficient,[29] using data from 10.0 to 3.0 Å with intensity $(I) > 2.0\sigma$ and anomalous difference $[F(h,k,l) - F(-h,-k,-l)] > 1.0\sigma$. The electron density, contoured at 4.0σ, is superimposed upon coordinates for the four heme groups (placed in arbitrary positions) and associated iron atoms (indicated by +) determined from molecular replacement calculations.

ure 1. Cross-rotation function[27] searches using either deoxy[2] (Protein Data Bank file 2HHB) or oxy tetramer[3] (Protein Data Bank file 1HHO) models failed to produce consistent solutions. The reason for this failure became clear when searches using an oxy dimer[3] produced strong cross-rotation peaks, with values of 6.6 and 5.2 standard deviations above the mean value of the map, at Euler angles $\alpha = 9°$, $\beta = 95°$, $\gamma = 2°$ and $\alpha = 19°$, $\beta = 72°$, $\gamma = 181°$. The striking feature of these two peaks is that they are canted in opposite directions away from the molecular 2-fold axis by more than 5° (Figure 1).

The translation function solution subsequently proved that the peaks illustrated in Figure 1 correctly define the orientations of the two dimers in one hemoglobin Ypsilanti tetramer. Translation searches, using the Crowther–Blow translation function,[28] were initially carried out for distances between symmetry-related dimers in both enantiomeric space groups consistent with the 3m Laue symmetry and systematic extinctions of the diffraction pattern. These gave consistent dimer positions for only one enantiomer, $P3_221$. The origin of the oxy dimer used as the molecular replacement model was defined to be at the center of the oxy tetramer. Translation peaks, typically 8 to 11 standard deviations above the mean, consistently placed this origin at the same point along the molecular 2-fold axis for both dimers. Optimization of orientation angles to maximize the signal to noise ratio of the translation function peaks gave an initial residual (R-value)[§] between observed (F_o) and calculated (F_c) structure factor amplitudes of 0.371, and a correlation coefficient of 0.655 for all data between 10.0 and 4.0 Å.

§

$$R = \frac{\sum_{h=0}^{N} |F_o| - |F_c|}{\sum_{h=0}^{N} |F_o|}$$

Anomalous Dispersion Calculations

The unusual quaternary structure exhibited by CO-YPS results in an altered location of the heme groups and iron atoms relative to their positions in normal deoxy or oxy hemoglobin. Verification of the iron atom positions in CO-YPS was carried out using Bijvoet difference Fourier maps (Fig. 2), which provide an image of the anomalous or resonantly scattering electrons associated with iron.[29] The location of the iron atoms indicated from the anomalous dispersion Fourier synthesis were coincident with the location predicted by the molecular replacement solution. An anomalous dispersion Patterson synthesis[30] gave peaks consistent with these locations, but the latter maps were of limited quality due to an incomplete set of anomalous differences coupled with a low signal to noise ratio of the anomalous differences.

Refinement of the Structure

The starting model for refinement was a tetramer comprised of oxy hemoglobin dimers oriented and

TABLE I. Root Mean Square Differences Between CO-YPS and the Two Canonical Structures

Region	Deoxy–Oxy	Deoxy–CO-YPS	CO-YPS–Oxy
$\alpha^1\beta^1$ dimer contact*	0.28 Å†	0.28 Å	0.19 Å
Overall $\alpha^1\beta^1$ dimer‡	0.92 Å	0.96 Å	0.30 Å

*Main chain atoms of residues in the B, G, and H helices involved in the $\alpha^1\beta^1$ contact region were used in these calculations.
†The rms error in the atomic coordinates is estimated to be 0.4 Å.[33]
‡All main chain atoms in an αβ dimer were used in these calculations.

positioned as determined from the molecular replacement solution and with the β99Asp side chains removed. Five cycles of stereochemically restrained least-squares refinement, using the PROTIN/PROLSQ package,[31,32] were carried out using the 11,006 reflections ($I > 2.0\sigma$) between 10.0 and 3.0 Å in a merged data set with data from both crystals. The Tyr side chains at the $\beta^1$99 and $\beta^2$99 positions were then added to the model, based on examination of the residual, $(F_o - F_c)$ difference electron density. An additional 19 cycles of refinement were then carried out, using the 8,307 reflections ($I > 2.0\sigma$) between 5.0 and 3.0 Å, resulting in an R-value of 0.246. At the current state of refinement, the root mean square (rms) deviation from ideal bond lengths and angles are 0.008 Å and 1.2°, respectively, and the rms difference between main chain atoms related by noncrystallographic symmetry is 0.031 Å for α chains and 0.029 Å for β chains. The upper limit of the rms error in the atomic coordinates is approximately 0.4 Å, based on the method of Luzzati.[33]

RESULTS

A New Quaternary Structure

Protein conformational changes are difficult to analyze because they involve both tertiary structure changes and quaternary rearrangements. One effective procedure for resolving quaternary changes from tertiary changes was developed to analyze different hemoglobin structures by Baldwin and Chothia.[7] This procedure involves identifying reference regions that are relatively unaffected by tertiary structure changes between the two structures. Superposition of related structures via these reference regions permits a nearly independent analysis of tertiary changes and rigid body motions of protein subunits.

The molecular replacement solution (Fig. 1) suggests three important features of the CO-YPS tetramer. (1) The $\alpha^1\beta^1$ contact region in CO-YPS is comparable to that of normal hemoglobin. (2) The tertiary structures of both α and β subunits in CO-YPS are very similar to those found in other liganded hemoglobins. (3) The relative disposition of αβ dimers in CO-YPS is quite distinct from that found in either deoxy or liganded hemoglobins. Analysis of refined coordinates by superposition of αβ dimers at the $\alpha^1\beta^1$ contact reference region verified these conclusions in detail (Table I). The dimer contact of CO-YPS is indistinguishable from the corresponding region in either normal deoxy or oxy hemoglobin (Table I, row 1), and CO-YPS exhibits a liganded tertiary structure (Table I, row 2).

As with the comparison between normal deoxy and CO hemoglobins,[7] the tertiary structure changes that accompany ligand binding leave the $\alpha^1\beta^1$ contact region as the only valid reference point for comparing all three structures. Moreover, comparisons between the quaternary structures of all three molecules can be described quantitatively in terms of the rigid body motions of the $\alpha^2\beta^2$ contact regions. This analysis is summarized in Figure 3. Anticipating conclusions elaborated below, we have represented these comparisons by showing how the transition from deoxy (T-state) to oxy (R-state) hemoglobins (Fig. 3a) might proceed via an intermediate quaternary state, Y. Thus, the quaternary changes that occur in going from normal deoxy hemoglobin to the Y-state are shown in Figure 3b, and from the Y-state to normal oxy hemoglobin in Figure 3c. The dramatic difference between the Y-state and the two canonical hemoglobin quaternary structures can be assessed from the fact that the rotation axis directions (P, P'', P') of the three different rigid body motions differ by up to 56° around the molecular dyad y. Thus, the two views down the rotation axes presented in Figure 3b and 3c for the Y-state are quite different from the conventional view presented in Figure 3a for the transition between deoxy and oxy normal hemoglobin. Curiously, the quaternary changes between the canonical structures and the Y-state involve rotations about axes (P'' and P') that pass very close to the flexible joint region, where similar $\alpha^1\beta^2$ contacts are maintained in the canonical T- and R-state structures.

For reference, the transition from deoxy to oxy hemoglobin involves a screw displacement of corresponding $\alpha^2\beta^2$ dimers by a rotation of −13.0° about, and a translation of 1.4 Å along, a rotation axis P (Fig. 3a). In the transition from normal deoxy hemoglobin to the Y-state, the $\alpha^2\beta^2$ dimers differ from one another by −21.7° and +3.1 Å relative to the axis P'' (Fig. 3b). This rotation (arrow) results in an orien-

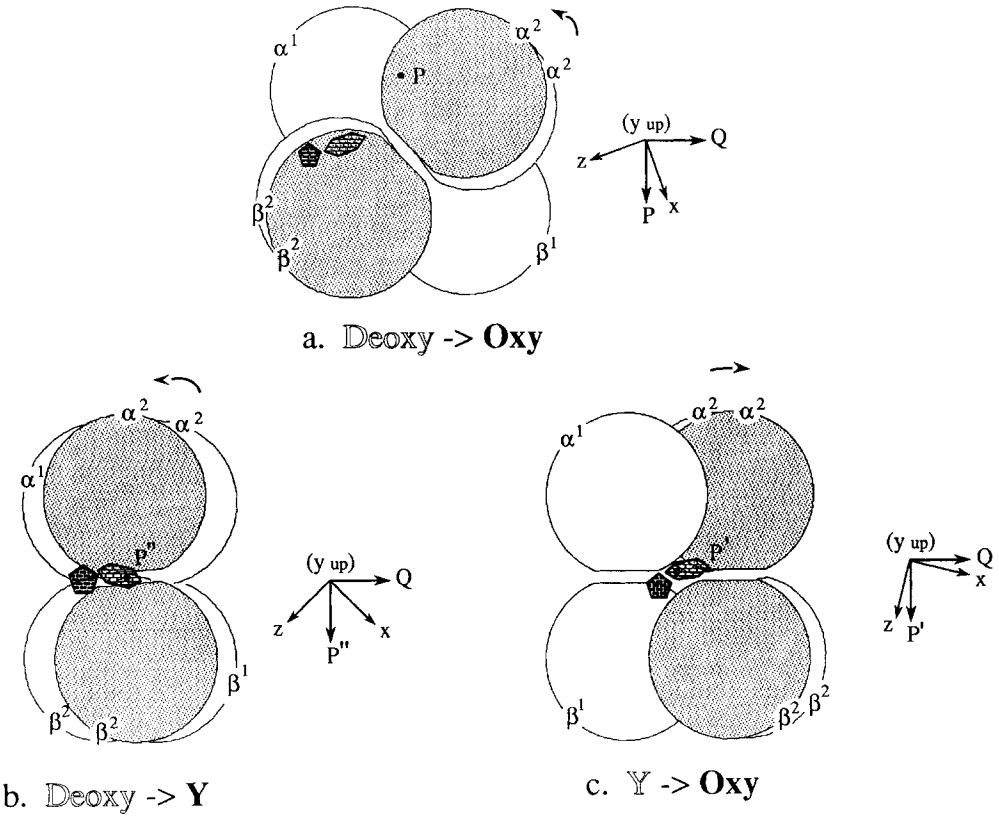

Fig. 3. Rigid body representation of the quaternary differences between CO-YPS (the Y-state) and normal deoxy and oxy human hemoglobins. All of the pairwise comparisons illustrated are based on superposition of corresponding dimers at the $\alpha^1\beta^1$ interface, as described in the text. The molecules are viewed along the rotation axes *P*, *P"*, *P'* relating corresponding dimers. The local coordinate systems *P(P",P')QR*, with *R* coincident with the molecular dyad *y* of the unshaded tetramer, are related to conventional axes *x*, *y*, and *z* as shown to the right of each diagram. The molecular dyad of the unshaded tetramer is vertical in each diagram and perpendicular to the figure in the small schematics to the right of each diagram. The rotation direction about the appropriate axis *(P, P", or P')* is indicated by an arrow. Approximate locations of the switch and flexible joint regions are indicated by the symbols ⬟ and ⬭, respectively. **(a)** The quaternary structure change in the transition from normal deoxy (unshaded) to oxy (shaded) human hemoglobin. The rotation axis *P* is 20° from the *x* axis and 70° from the *z* axis, and intersects the molecular dyad of deoxy hemoglobin at a point 12 Å from the molecular center, between the α subunits.[7] **(b)** The quaternary change in the transition from normal deoxy (unshaded) to the Y-state (shaded). The rotation axis *P"* is 45° from both the *x* and *z* axes, and intersects the molecular dyad of deoxy hemoglobin at a point near the flexible joint region. **(c)** The quaternary change in the transition from the Y-state (unshaded) to normal oxy (shaded) hemoglobin. The rotation axis *P'* is 76° from the *x* axis and 14° from the *z* axis, and intersects the molecular dyad of CO-YPS in the flexible joint region of the molecule.

tation of αβ dimers in the Y-state that is outside the limits observed in normal deoxy and oxy hemoglobins. A retrograde rotation of $\alpha^2\beta^2$ dimers by +11.2° coupled with a translation of −1.2 Å relative to the axis *P'* is necessary to achieve the quaternary structure of normal oxy hemoglobin (Fig. 3c). Thus, in a sense the structure of normal oxy hemoglobin lies in between those of normal deoxy and CO Ypsilanti hemoglobin.

Figure 4 shows ribbon and α-carbon backbone representations of CO-YPS superimposed on the structures of normal deoxy and oxy hemoglobin. In these comparisons all $\alpha^1\beta^1$ interface regions are superimposed on the $\alpha^1\beta^1$ interface of oxy hemoglobin and the molecular dyad of the reference oxy molecule is oriented horizontally. As a consequence, the structural comparisons are illustrated for the $\alpha^2\beta^2$ subunits relative to this reference $\alpha^1\beta^1$ interface, and the CO-YPS structure (solid line) is the same in both views, whereas the oxy and deoxy structures (dashed lines) differ.

It is apparent that CO-YPS exhibits a quaternary structure dramatically different from that observed in either normal deoxy or oxy hemoglobin. Several features of this new quaternary structure, Y, which will not be modified by refinement of the model to higher resolution, should be noted. (1) The $\alpha^2\beta^2$ dimer of CO-YPS shows large displacements from the corresponding dimer of either deoxy or oxy hemoglobin. The overall rms difference, for α-carbon atoms only, between corresponding $\alpha^2\beta^2$ dimers of the new quaternary structure and deoxy hemoglobin is 7.9 Å, while the corresponding difference for oxy hemoglobin is 3.9 Å. For comparison, the rms difference between $\alpha^2\beta^2$ dimers of the two canonical structures is 4.8 Å (Fig. 4a,b). (2) The relative ori-

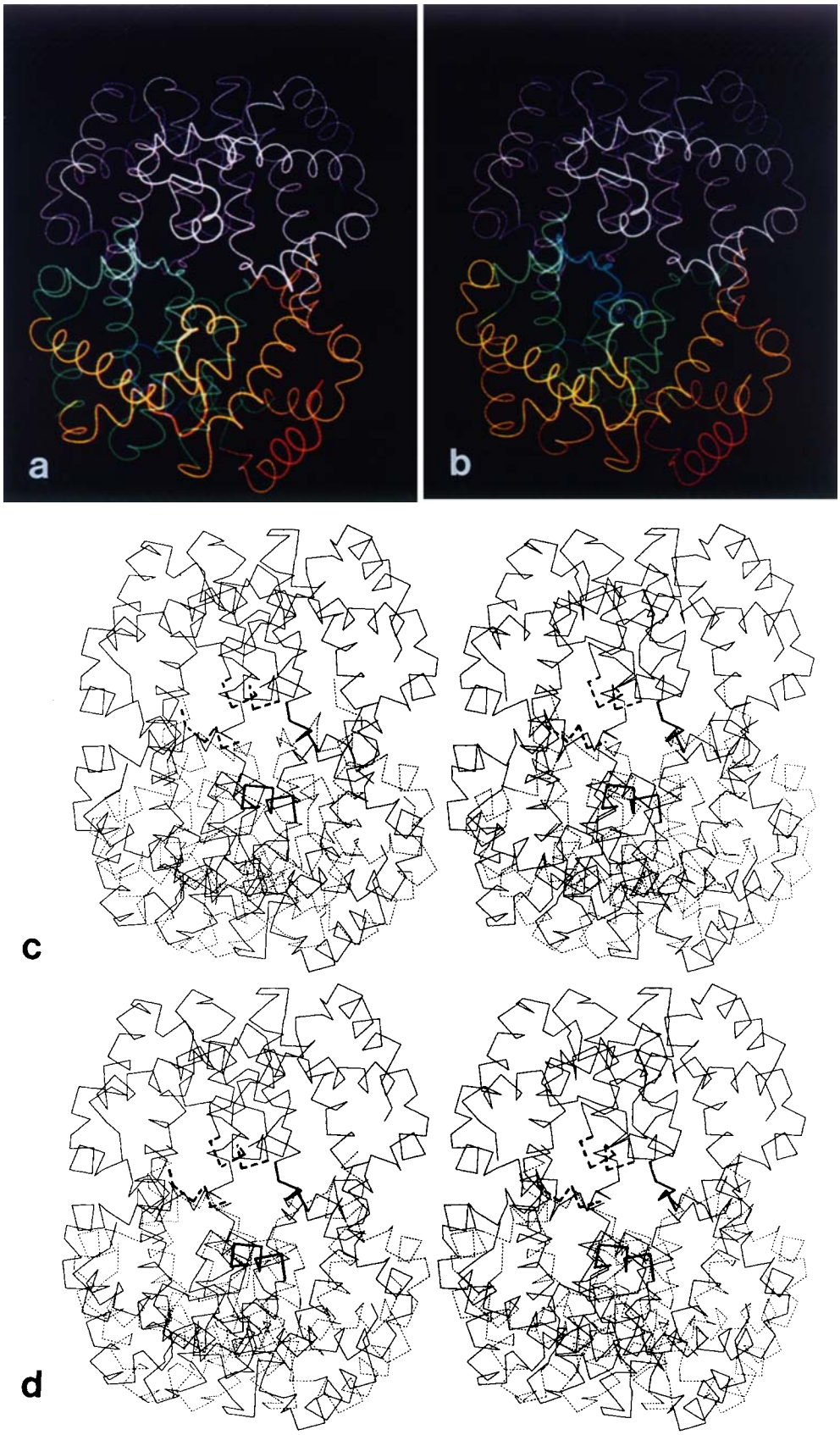

Fig. 4.

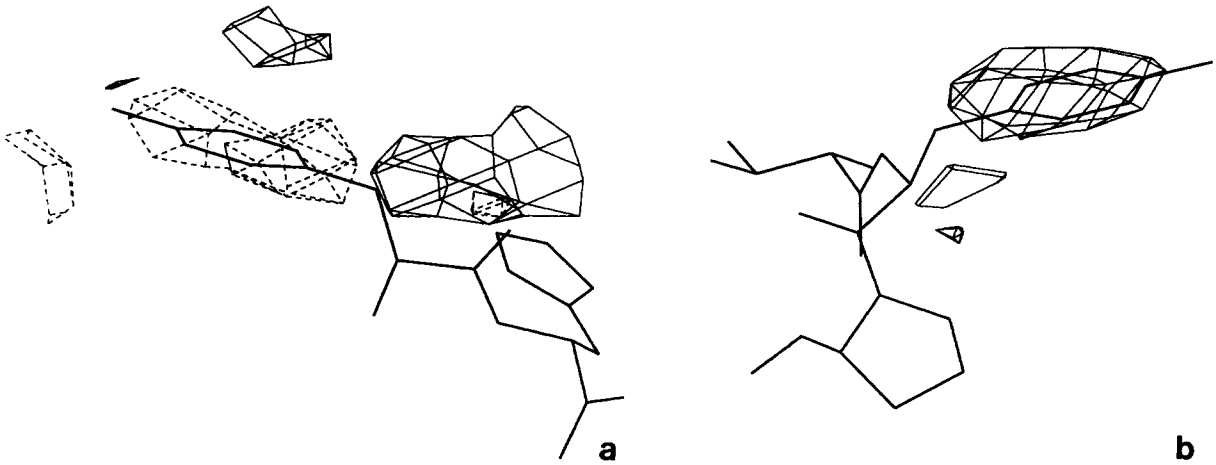

Fig. 5. Difference density maps for CO-YPS. **(a)** $F_o - F_c$ residual difference map, contoured at 2.0σ, with the coordinates for β145Tyr from the model of normal oxy hemoglobin. Positive contours are solid, negative contours are dashed. **(b)** $F_o - F_c$ fragment difference map for the β99 side chain, contoured at 2.0σ, with the coordinates for the β99Tyr in CO-YPS. Structure factors (F_c) were calculated by omitting all atoms of the β99Tyr residue after Cβ from the model of CO-YPS.

entation of αβ dimers in CO-YPS leads to a larger cavity between the β subunits, and a smaller cavity between the α subunits, than is observed in normal deoxy or oxy hemoglobin (Fig. 4c,d). (3) Different patterns of displacements differentiate CO-YPS relative to deoxy and oxy hemoglobins. The flexible joint region of the new quaternary structure shows the smallest overall change (1.4 Å average rms difference) relative to oxy hemoglobin. This same region shows larger changes (4.3 Å average rms difference) relative to deoxy hemoglobin (Fig. 4a,b). The corresponding rms difference between the flexible joint regions of normal deoxy and oxy hemoglobins is 3.3 Å. (4) The switch region of CO-YPS shows significant changes relative to both deoxy (8.8 Å average rms difference) and oxy (3.0 Å average rms difference) hemoglobins (Fig. 4a,b). This region also shows large changes between the two canonical structures (6.2 Å average rms difference). These important modifications of the $\alpha^1\beta^2$ contact region result directly from the replacement of β99Asp with Tyr in the βFG corner, as described in more detail below.

Fig. 4. Backbone representations of CO-YPS compared to the two canonical structures. All $\alpha^1\beta^1$ interface regions are superimposed on the $\alpha^1\beta^1$ interface of oxy hemoglobin and the molecular dyad of the reference oxy molecule is oriented horizontally. The $\alpha^1\beta^1$ dimer is uppermost in each figure, with the α chain on the left and β chain on the right. **(a)** CO-YPS molecule color-coded to reveal differences from the structure of normal deoxy hemoglobin. The $\alpha^1\beta^1$ dimer is shown in white, while colors in the $\alpha^2\beta^2$ dimers represent increasing rms differences from the corresponding region in the deoxy structure (blue < green < orange < red), in increments of 4 Å. **(b)** CO-YPS molecule color-coded to reveal differences from the structure of normal oxy hemoglobin. Color schemes are as described above, except that each color now represents an increment of 2 Å rms difference. **(c)** Stereo view of the superposition of the structures of CO-YPS (solid line) and normal deoxy hemoglobin (dashed line). The flexible joint region of CO-YPS is indicated by thick dashed lines and the switch region by thick solid lines. Only one of the two symmetry-related sets of flexible joint and switch regions is highlighted. **(d)** Stereo view of the superposition of the structures of CO-YPS (solid line) and normal oxy hemoglobin (dashed line). The flexible joint and switch regions of CO-YPS are indicated as described in Figure 4c.

The $\alpha^1\beta^2$ Contact Region

Extensive thermodynamic studies have shown that mutations and chemical modifications to the hemoglobin tetramer that alter the cooperative free energy change map to the $\alpha^1\beta^2$ contact region.[6,34,35] Details of this region in CO-YPS emerge from examination of the residual, ($F_o - F_c$) difference electron density map, and ($F_o - F_c$) "fragment" maps.[36] The residual difference electron density map shows paired positive and negative electron density at the location of the β145Tyr, the penultimate tyrosine[4] (Fig. 5a). These peaks clearly show that in CO-YPS, the β145Tyr side chain is rotated approximately 180° about the torsion angle χ_1 from its orientation in oxy hemoglobin.[3] Comparison of the structures of horse deoxy and CO hemoglobins originally led to the proposal that the tertiary structure changes upon ligation lead to rupture of an intrachain hydrogen bond between β145Tyr and the carbonyl oxygen of β98Val.[37] More recent work indicates that this hydrogen bond is maintained in both deoxy and oxy human hemoglobins.[2,3] Clearly, the altered conformation of β145Tyr observed in CO-YPS precludes hydrogen bonding between β145Tyr and β98Val, and resembles the conformation originally proposed for horse CO hemoglobin.

The fragment difference electron density map in Figure 5b shows one of the two β99Tyr side chains. The β99Tyr residue is in a different location from the β99Asp residue in normal deoxy hemoglobin, with a difference in torsion angle χ_1 of 100° for the

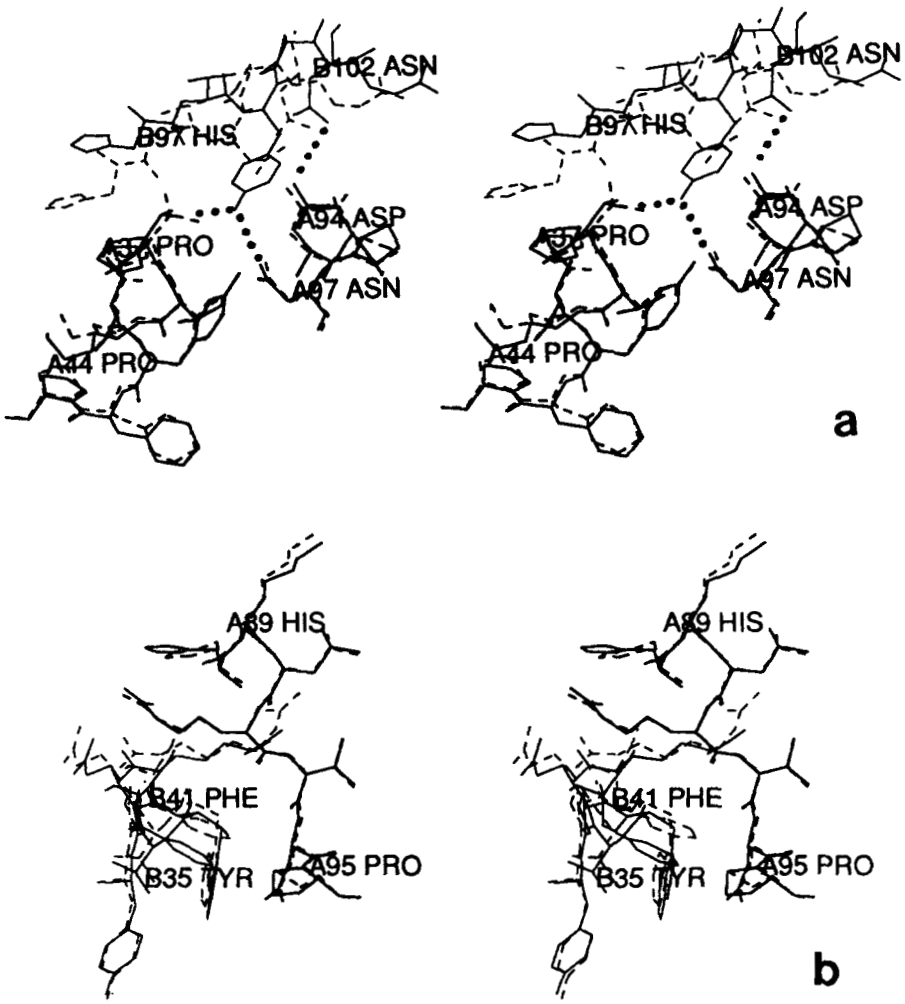

Fig. 6. The switch and flexible joint regions of CO-YPS and the corresponding regions of normal oxy hemoglobin. The two structures were superimposed at the $\alpha^1\beta^1$ interface, as described in the text. Coordinates for CO-YPS are shown with solid lines and those for oxy hemoglobin with dashed lines, with α chains drawn in thick lines. β-carbons of beginning and ending residues within each helical segment are labeled. **(a)** Stereo view of the switch region. Coordinates are shown for residues in the αC helix (α37–α44), αG helix (α94–α97), and βFG corner/G helix (β97–β102). Hydrogen bonding interactions are indicated by dotted lines. **(b)** Stereo view of the flexible joint regions. Coordinates are shown for the αFG corner (α89–α95) and βC helix (β35–β41).

two conformations. New noncovalent interactions between residues in the switch region of CO-YPS result from the presence of β99Tyr in this altered conformation. In normal deoxy hemoglobin, $\beta^2$99Asp is hydrogen bonded to both $\alpha^1$97Asn and $\alpha^1$42Tyr.[7] The altered disposition of dimers in the new quaternary structure relative to the canonical structures, coupled with the size of the Tyr side chain, permit hydrogen bonding interactions between $\beta^2$99Tyr and $\alpha^1$97Asn (Fig. 6a). Thus, the switch region of the Y-state shows some features characteristic of the deoxy quaternary structure.

In normal liganded hemoglobins, the residues $\alpha^1$92Arg, $\alpha^1$94Asp, and $\alpha^1$95Pro of the αFG corner form packing contacts with residues $\beta^2$37Trp and $\beta^2$40Arg of the βC helix, forming what has been called the flexible joint region of the $\alpha^1\beta^2$ contact. Contacts between these residues are maintained in deoxy hemoglobin, although the detailed atomic interactions differ.[7] In CO-YPS, the packing contacts in this flexible joint region are nearly identical to those observed in normal oxy hemoglobin (Fig. 6b). Interactions between the G helices of α and β chains in CO-YPS are also similar to those observed in normal oxy hemoglobin, as the intersubunit hydrogen bond between $\beta^2$102Asn and $\alpha^1$94Asp is maintained in CO-YPS (Fig. 6a). Despite the large quaternary structure change of CO-YPS relative to the deoxy and oxy quaternary structures, the hydrogen bonding interactions between $\beta^2$99Tyr and $\alpha^1$97Asn, $\beta^2$102Asn and $\alpha^1$94Asp, coupled with the packing interactions in the flexible joint region, indicate that the local noncovalent interactions in the Y-state show features of both the deoxy and oxy $\alpha^1\beta^2$

contacts. In summary, in terms of the $\alpha^1\beta^2$ contact region, the Y-state lies in between the two canonical structures of normal deoxy and liganded hemoglobins.

A striking difference in the $\alpha^1\beta^2$ contact region of the Y-state relative to the two canonical structures is the environment of β97His (Fig. 6a). In normal hemoglobin, the relative rotation of αβ dimers that occurs in the transition from the deoxy to liganded states alters the position of $\beta^2$97His such that in deoxy hemoglobin it packs between $\alpha^1$44Pro and $\alpha^1$41Thr, and in liganded hemoglobin it nests between $\alpha^1$41Thr and $\alpha^1$38Thr. This requirement for the relocation of β97His acts as a steric barrier to a direct transition between the deoxy and liganded quaternary structures.[7] In CO-YPS, the orientation of the αβ dimers is such that $\beta^2$97His no longer nests between residues of the α^1C helix. Rather, the rigid body rotation disengages the steric barrier by lifting β97His directly out of the pocket it occupies in the normal liganded quaternary structure (Fig. 6a). This new position of β97His is accompanied by a new hydrogen bonding interaction between the side chains of $\beta^2$99Tyr and $\alpha^1$38Thr. Together with the previously identified hydrogen bond between $\beta^2$99Tyr and $\alpha^1$97Asn, this preserves for $\beta^2$99Tyr an interaction with both the α^1G and α^1C helices, as is observed in normal deoxy hemoglobin for the $\beta^2$99Asp residue.

Additional distinctions between the structures of normal deoxy and oxy hemoglobin are electrostatic and side chain packing interactions involving the carboxy termini of both α and β chains. Principal among these are ion pairs formed in deoxy hemoglobin between $\alpha^1$141Arg and $\alpha^2$126Asp, and between $\beta^2$146His and $\alpha^1$40Lys.[7] These interactions are ruptured in both the normal oxy and CO-YPS structures. In addition, a second specific switching interaction[38] involving $\alpha^1$140Tyr and $\beta^2$37Trp assumes different configurations in normal deoxy and oxy hemoglobins.[3] The arrangement of these two side chains in CO-YPS closely resembles that in the normal oxy structure. Therefore, the large quaternary change characteristic of the Y-state does not greatly alter these carboxy terminal side chain interactions, which assume the configuration found in normal R-state hemoglobins.

It is interesting to note that, consistent with the other composite features of the Y-state, the $\alpha^1\alpha^2$ contacts in both deoxy and CO-YPS hemoglobins are missing a set of electrostatic interactions found in oxy hemoglobin. Recent analysis of refined coordinates for oxy hemoglobin[39] identified a salt bridge between the carboxylate of $\alpha^1$141Arg and the amino group of $\alpha^2$1Val, and an ion-mediated contact between $\alpha^1$141Arg and $\alpha^2$141Arg. These pairwise interactions are ruptured in both deoxy and CO-YPS hemoglobins. Only the electrostatic interaction between the carboxylate of $\alpha^1$141Arg and $\alpha^2$127Lys, and a hydrogen bonding interaction between $\alpha^1$140Tyr and $\alpha^2$127Lys are maintained in CO-YPS.

DISCUSSION

This crystallographic investigation was motivated by thermodynamic measurement of the differential stability of deoxy and oxy forms of over 50 modified human hemoglobins.[6,34,35] This stability difference is the energy available to alter the intrinsic ligand binding constants of the α and β subunits in a hemoglobin tetramer as a function of ligation state, and has been called the cooperative free energy.[5] Results of these studies demonstrated that the $\alpha^1\beta^2$ contact region is the principal site of interactions that govern the cooperative free energy.[34] Of particular interest are mutations of β99Asp, which lies in the switch region. Four of these mutants (Gly, Ala, Asn, His) have the equivalent effect of equalizing the stability of deoxy and oxy forms, thus abolishing the cooperative free energy.[6,34,35] In contrast, the modification in hemoglobin Ypsilanti (β99Asp-Tyr) actually inverts the stability of the deoxy and oxy forms, resulting in a negative cooperative free energy,[6] extremely high affinity, and greatly reduced cooperativity of oxygen binding (M. Doyle and G.K. Ackers, personal communication).

The structure of CO-YPS demonstrates that the hemoglobin tetramer has considerably greater plasticity than previously recognized. From detailed analyses of the deoxy, carbonmonoxy, and met hemoglobin structures, Baldwin and Chothia[7] concluded that there were only two stable packing arrangements in the switch region of the hemoglobin tetramer, formed by interactions between residues of βFG corners and αC helices. Yet CO-YPS illustrates an alternative packing arrangement of side chains in the switch region associated with a quaternary structure, Y, that is thermodynamically quite stable. In normal hemoglobin A, the Gibbs free energy for dimer–tetramer assembly of the liganded (oxy) molecule is −8.0 kcal/mol, while the value for the deoxy molecule is −14.3 kcal/mol.[34] In hemoglobin Ypsilanti this situation is reversed, with a dimer–tetramer assembly free energy of −11.3 kcal/mol for the oxy molecule, and by implication, the CO tetramer, and −8.0 kcal/mol for the deoxy molecule.[6] This distinctive pattern of Gibbs free energies for dimer–tetramer assembly, with the oxy molecule more stable than the deoxy molecule, remains constant over a wide range of temperature and pH values.[6] The liganded form of hemoglobin Ypsilanti is 100-fold more stable with respect to tetramer dissociation than normal oxy hemoglobin.

The $\alpha^1\beta^2$ interface observed in the Y-state, which contains features of both deoxy-like and oxy-like interfaces, should be contrasted with previous structural observations on another β99 mutant, hemoglobin Yakima (β99Asp-His).[40] Crystals of deoxy

hemoglobin Yakima are isomorphous with crystals of deoxy hemoglobin A, while no crystals of the liganded form have been investigated. Analysis of a 3.5 Å difference electron density map between deoxy Yakima and normal deoxy hemoglobin demonstrates significant alterations of tertiary and quaternary structure in this mutant.[40] The greatest changes are in the $\alpha^1\beta^2$ contact region, where replacement of β99His for Asp results in loss of the hydrogen bonds characteristic of the deoxy quaternary form ($\beta^2$99Asp with $\alpha^1$97Asn and $\alpha^1$42Tyr). A new hydrogen bond, usually associated with the oxy quaternary structure, is formed between $\alpha^1$94Asp and $\beta^2$102Asn. The overall quaternary structure of deoxy hemoglobin Yakima is similar to that of normal deoxy hemoglobin. Analysis of the difference electron density map of deoxy hemoglobin Yakima also suggests a partial rotation of the αβ dimers toward the liganded quaternary conformation.

Overall, it appears that deoxy hemoglobin Yakima exhibits some structural features intermediate between deoxy and liganded hemoglobin, while retaining an essentially deoxy quaternary structure.[40] It loses the hydrogen bonds involving β99Asp characteristic of the deoxy form while at the same time gaining an interaction ($\alpha^1$94Asp–$\beta^2$102Asn) characteristic of oxy hemoglobin. The limited dimer rotation toward the liganded conformation, together with the absence of difference density near either $\beta^2$97His or $\alpha^1$41Thr and $\alpha^1$44Pro,[40] imply that β97His remains in the pocket on the αC helix characteristic of the deoxy quaternary form. The deoxy form of hemoglobin Ypsilanti also crystallizes in a form isomorphous with crystals of normal deoxy human hemoglobin (F.R. Smith, unpublished). These crystals also diffract to high resolution and will permit examination of effects of the β99Asp-Tyr mutation on the deoxy quaternary structure.

In contrast, liganded hemoglobin Ypsilanti has a totally new quaternary structure, Y, involving rotation of the αβ dimer beyond the limits observed for the direct pathway from deoxy to oxy quaternary forms. Nevertheless, the $\alpha^1\beta^2$ contact retains hydrogen bonding interactions characteristic of normal liganded hemoglobin ($\alpha^1$94Asp – $\beta^2$102Asn) and gains a new set ($\beta^2$99Tyr with $\alpha^1$97Asn and $\alpha^1$38Thr) reminiscent of those associated with β99Asp in normal deoxy hemoglobin. Moreover, CO-YPS exhibits only a limited set of those electrostatic interactions previously identified in deoxy and oxy hemoglobins, having lost the majority of the salt bridges characteristic of the canonical structures. Of particular interest is the fact that in the Y-state, the β97His is fully disengaged from packing interactions along the αC helix which are generally believed to enforce two-state behavior in ligand binding. An obvious implication of the composite nature of the $\alpha^1\beta^2$ interface and the disengaged switch region in CO-YPS is that substitution of β99Tyr for Asp may stabilize a quaternary structure, Y, that occurs transiently along a discontinuous pathway from normal deoxy to oxy hemoglobin.

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