The T-to-R transformation in hemoglobin: A reevaluation

(allosteric transitions)

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ABSTRACT The relationship between the T, R, and R2 quaternary forms of hemoglobin is examined by computational experiments. Contrary to previous suggestions, we propose that the R quaternary form may lie on the pathway from T to R2. This proposal is consistent with four independent observations. (i) Difference distance maps are used to identify those parts of the molecule that undergo conformational change upon oxygenation. The simplest interpretation of these maps brackets R between T and R2. (ii) Linear interpolation from T to R2 passes through R. (iii) The well-known “switch” region (so called because, upon transition between the T and R quaternary forms, a residue from the $\beta 2$ subunit toggles between two stable positions within the $\alpha 1$ subunit) progresses from T through R to R2, successively. (iv) A hitherto-undocumented feature, diagnostic of the R structure, is noted within the α subunit: upon transformation from T to R, the β -turns at the amino termini of the E and F helices flip from one turn type to another. Upon transformation from R to R2, the latter turn—a strained conformation—flips back again.

The quaternary structure change accompanying cooperative ligand binding by hemoglobin has been analyzed extensively by Perutz (1) and Baldwin and Chothia (2). Both analyses assume the existence of two stable quaternary structures corresponding to the fully oxygenated (R) and completely deoxygenated (T) forms. Recently, two different groups crystallized hemoglobin in a third quaternary structure, termed Y (3, 4) or R2 (5). Unlike previous oxy structures, which were crystallized under high-salt conditions [e.g., Shaanan (6) used 2.5 M sodium/potassium phosphate, pH 6.7], this third quaternary form was crystallized under physiological salt conditions [0.18 M Cl[−], pH 7.4 (4) or 100 mM sodium cacodylate/75 mM Cl[−], pH 5 (5)]. Janin and Wodak (7) analyzed the Y-form structure and concluded that it is a modified form of the R structure that does not lie on the pathway from T to R. In contrast, our analysis of the crystal structures of the three different forms suggests that R2/Y may be the authentic structure of fully oxygenated tetrameric hemoglobin and that R lies on the pathway from T to R2.

Our computational experiments raise questions about the physiological relevance of the classical R structure, and they underscore the admonition (4) that hemoglobin structure–function relationships need to be reexamined in the context of the T–R–R2 framework.

METHODS

Protein structures used in the analysis, designated by their Protein Data Bank (8) identifiers, are 2HHB, the deoxy T structure (9); 1HHO, the oxy R structure (6); and 1BBB, the oxy R2 structure (5). The solvent-accessible surface (10) is the area swept out by the center of a water-sized sphere (i.e., 1.4 Å) that systematically probes the surface of a molecule. Solvent-accessible surface areas were calculated (11) with

atomic radii of tetrahedral carbon = 2 Å, trigonal carbon = 1.7 Å, nitrogen = 1.7 Å, oxygen = 1.5 Å, and sulfur = 2 Å. Contacts between residues were then evaluated from buried surface area. Linear interpolation from one structure to another was performed by superimposing the two structures (12) and then incrementally changing the coordinates of the initial structure to those of the final structure in discrete, uniform steps.

The difference between two similar structures, such as deoxy- and oxyhemoglobin, is typically assessed by superimposing one upon the other (12). Optimal superimposition is achieved by minimizing the root-mean-square (rms) difference between the two structures, where the rms difference = $\{\sum_i[(\Delta x^2 + \Delta y^2 + \Delta z^2)/N]\}^{1/2}$, and $(\Delta x^2 + \Delta y^2 + \Delta z^2)$ is the squared distance between corresponding points in the two structures, the sum being performed over all N atoms. For similar structures, most of the terms in this sum are contributed by atoms from isomorphous regions; thus, small but possibly important differences can easily be submerged.

An alternative way to assess conformational change is to calculate a difference distance map (13, 14). Such maps can be a sensitive probe of structural differences between similar molecules (W. L. Nichols, G.D.R., L. F. Ten Eyck, and B. H. Zimm, unpublished work). In this procedure, corresponding distances between every two atoms are measured in both the oxy and deoxy structures, and their differences are taken. That is, for all i and j , the quantity $\Delta\delta_{ij}$ is reckoned, where $\Delta\delta_{ij} = \delta_{ij}^{\text{oxy}} - \delta_{ij}^{\text{deoxy}}$. Here, δ_{ij}^{oxy} is the distance between atoms i and j in oxyhemoglobin, $\delta_{ij}^{\text{deoxy}}$ the distance between atoms i and j in deoxyhemoglobin, and $\Delta\delta_{ij}$ the difference between the two.‡ Clearly, $\Delta\delta_{ij}$ does not depend on the center of mass.

In practice, difference distance maps were constructed in two steps. First, the distance δ_{ij} was measured between every pair of α -carbons in each of the two structures being compared. Then, the difference in the distances between corresponding atoms in the two structures was evaluated to construct elements of the difference distance matrix, $\Delta\delta_{ij}$. Elements of $\Delta\delta_{ij}$ less than the threshold value of 0.5 Å were set to 0.

RESULTS

Difference distance maps of the $\alpha 1\beta 2$ interface upon transformation from T to R, R to R2, and T to R2 are shown in Fig.

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‡We adhere to the convention of referring to the quantity $\Delta\delta$ as the “difference of distances” or simply the “difference distance.” As pointed out by a referee, this convention seems to depart from familiar English usage where, for example, differences between religions are referred to as religious differences, not difference religions. However, for clarity we note that were there a religion whose tenets consisted solely of the differences between two other religions—say Buddhism and Judaism—then it would be both formally and colloquially correct to refer to this hypothetical faith as the difference religion, just as one would refer to the Buddhist religion. The term “difference distance” is used in this sense.

1. In these maps, + represents the case where two α -carbons are closer in the final structure than in the initial structure, and - represents the converse case. Blank spaces correspond to regions where the magnitude of change does not exceed a threshold value of 0.5 Å.

The difference distance maps probe conformational change in all parts of the hemoglobin molecule in an unbiased way. From the maps (Fig. 1), it is apparent that the path between T and R2 passes through R since, to a close approximation, this transformation is the sum of the T $\rightarrow$ R and R $\rightarrow$ R2 transformations. In particular, the difference distance maps of T $\rightarrow$ R2, T $\rightarrow$ R, and R $\rightarrow$ R2 are all parallel transformations, especially in regions of intersubunit contact. Conversely, in the detailed pathway T $\rightarrow$ R2 $\rightarrow$ R, almost all T $\rightarrow$ R2 displacements oppose their corresponding R2 $\rightarrow$ R displacements (Fig. 1). Thus, if R2 were between T and R (instead of R between T and R2), then those interatomic distances that contract in the T $\rightarrow$ R transition must first expand, while those interatomic distances that expand in the T $\rightarrow$ R transition must first contract—a highly unlikely sequence of events.

Solvent-accessible surface areas of both monomers and dimers are listed in Tables 1–3. These data show that, with the exception of the $\alpha 1\beta 2$ interface, both the individual subunits and the interfaces between them bury additional surface area upon transformation from either T to R or T to R2. This fact can be interpreted as indicating that, upon ligation, interaction between the dimers is reduced, while interactions within both

Table 1. Solvent-accessible surface areas (Å²) for T, R, and R2 structures

State	$\alpha 1$	$\beta 1$	$\alpha 1\beta 1$	$\alpha 1\beta 2$	$\alpha 1\alpha 2$	$\beta 1\beta 2$
T	7859	8186	14,324	14,613	15,089	16,372
R	7717	8014	13,875	14,678	14,593	15,607
R2	7777	7904	13,840	14,731	14,685	15,493

Table 2. Change in solvent-accessible surface areas (Å²) for transformation between T, R, and R2 structures

Transformation	$\alpha 1$	$\beta 1$	$\alpha 1\beta 1$	$\alpha 1\beta 2$	$\alpha 1\alpha 2$	$\beta 1\beta 2$
T $\rightarrow$ R	142	172	449	-65	496	765
T $\rightarrow$ R2	82	282	484	-118	404	879
R $\rightarrow$ R2	-60	110	35	-53	-92	114

Table 3. Surface area (Å²) buried at interfaces in T, R, and R2 structures

State	$\alpha 1\beta 1$	$\alpha 1\beta 2$	$\alpha 1\alpha 2$	$\beta 1\beta 2$
T	1721	1432	629	0
R	1856	1053	841	421
R2	1841	950	869	315

Solvent-accessible surface areas were calculated according to Lee and Richards (10).

monomers and dimers are enhanced. Individual contacts at all interfaces in each of the three forms are listed in Tables 4–7.

Comparisons of the backbone dihedral angles ϕ and ψ for each residue in both subunits of hemoglobin for all three crystal structures are plotted in Fig. 2. Two pronounced changes are seen in the α subunit upon transformation from T to R. One of these involves His⁵⁰ and Gly⁵¹, wherein the amide linkage between these residues flips, changing the local conformation from a type II β -turn to a type I β -turn (15, 16) (Table 8); the other involves a similar flip of the amide linkage between Ala⁷¹ and His⁷², changing the local conformation from a distorted type I β -turn to a type II β -turn (Fig. 2C). These turn flips are situated at the amino termini of the E and F helices, respectively.

These turn flips appear to be diagnostic of the R structure: they are observed in carbonmonoxyhemoglobin (17) and all other R-form structures in the data base (8). The turn flips are not discernible upon examination of α -carbon traces, which do not change appreciably on transformation from T to R.

Importantly, only residues His⁵⁰ and Gly⁵¹ exhibit a turn flip upon transformation from T to R2; the Ala⁷¹-His⁷² flip, observed in the T $\rightarrow$ R transformation, is no longer in evidence. This latter flip (type I $\rightarrow$ type II) at the amino terminus of the F helix is expected to involve an energy penalty because the $i + 2$ position in a type II turn has a positive ϕ angle, a "disallowed" conformation for a histidine residue. In contrast, a corresponding penalty is not paid by the His⁵⁰-Gly⁵¹ turn (type II $\rightarrow$ type I) at the amino terminus of the E helix since, in this case, the $i + 2$ residue has an allowed conformation for all residues. Changes in the β subunit are all much smaller than these two conspicuous turn flips and are not further discussed.

$\alpha 1\beta 1$ Interface. Data in Table 6 show clearly that the $\alpha 1\beta 1$ interface is tighter in the R and R2 forms than in the T form. Within error, the amount of surface area buried at this interface is the same in both R and R2. This observation is corroborated by the difference distance maps; there are substantially fewer points in the R $\rightarrow$ R2 transition, especially for residues within the dimer interface. The difference distance maps for both the T $\rightarrow$ R and T $\rightarrow$ R2 transitions are also very similar, indicating that all displacements are in the same direction.

$\alpha 1\beta 2$ Interface. In contrast to the $\alpha 1\beta 1$ interface, the $\alpha 1\beta 2$ interface loosens upon transition from T $\rightarrow$ R or T $\rightarrow$ R2, with

FIG. 1. Difference distance maps of the $\alpha 1\beta 2$ subunit in the transformation from T to R via R2 (Left) and T to R2 via R (Right). As seen, T $\rightarrow$ R2 and R2 $\rightarrow$ R transformations are in opposite directions (like an image and its photographic negative), while T $\rightarrow$ R and R $\rightarrow$ R2 transformations are in the same direction (like two positive images). In these maps, regions annotated with a + (darker regions) correspond to residues that move closer together upon transformation, while regions annotated with a - (lighter regions) indicate the converse.

Table 4. Contacts at the $\alpha 1\beta 1$ interface

$\alpha 1$	$\beta 1$	T	R	R2
GLU27	PRO124	55.260	62.170	60.280
GLU30	PRO124	82.370	94.900	93.240
ARG31	PHE122	91.290	97.140	91.270
ARG31	THR123	79.750	81.920	80.530
ARG31	PRO124	142.220	151.930	141.180
ARG31	GLN127	120.940	130.060	118.710
LEU34	PRO124	88.740	94.360	92.040
LEU34	PRO125	75.610	91.140	82.360
LEU34	ALA128	78.020	89.100	79.850
SER35	PRO124	0.000	34.300	0.000
SER35	GLN127	79.470	82.810	81.310
SER35	ALA128	92.090	95.770	96.340
SER35	GLN131	82.190	85.070	85.560
PHE36	GLN127	0.000	0.000	33.200
PHE36	GLU131	77.440	88.080	88.560
LYS99	GLU101	0.000	44.370	64.900
HIS103	ASN108	108.310	116.470	116.010
HIS103	VAL109	0.000	29.270	0.000
HIS103	VAL111	70.230	67.920	71.700
HIS103	CYS112	65.470	69.160	70.050
HIS103	GLN127	55.280	50.790	62.350
HIS103	GLN131	71.220	68.690	71.280
CYS104	GLN127	60.770	58.800	66.200
LEU106	CYS112	71.480	59.230	68.190
VAL107	VAL111	78.390	85.360	77.800
VAL107	CYS112	87.430	95.560	86.650
VAL107	ALA115	79.990	87.610	85.950
VAL107	PHE122	57.340	56.850	63.310
VAL107	GLN127	88.420	86.860	89.550
ALA110	CYS112	65.610	55.350	63.620
ALA110	ALA115	85.810	75.050	85.620
ALA110	HIS116	105.910	105.370	104.180
ALA111	ALA115	87.980	88.230	90.020
ALA111	HIS116	54.180	54.440	49.650
ALA111	GLY119	89.150	86.260	87.050
ALA111	LYS120	52.670	69.030	42.430
ALA111	PHE122	39.430	38.520	36.680
HIS112	LYS120	0.000	99.280	33.600
LEU113	HIS116	42.380	0.000	0.000
PRO114	HIS116	99.380	115.060	115.080
ALA115	HIS116	34.860	29.360	34.190
PHE117	ARG30	105.100	101.190	104.580
PHE117	CYS112	66.050	56.830	64.940
PHE117	HIS116	110.220	102.920	91.710
THR118	ARG30	84.050	72.320	80.800
PRO119	GLU26	0.000	44.360	33.740
PRO119	ARG30	131.610	129.520	125.590
PRO119	VAL33	85.710	85.510	85.180
PRO119	VAL34	43.000	44.040	39.530
PRO119	PRO51	49.430	46.720	56.940
PRO119	MET55	87.340	96.890	97.470
ALA120	VAL33	49.220	51.140	50.090
ALA120	PRO51	66.630	57.060	67.470
HIS122	ARG30	110.930	120.830	114.160
HIS122	VAL34	105.620	103.920	107.580
HIS122	VAL109	69.960	56.330	60.720
HIS122	CYS112	73.490	68.420	68.590
ALA123	VAL33	67.930	70.140	72.720
ALA123	VAL34	102.060	96.000	105.100
ASP126	VAL34	68.530	72.790	74.460
ASP126	TYR35	73.070	64.880	77.320

Table 5. Contacts at the $\alpha 1\beta 2$ interface

$\alpha 1$	$\beta 2$	T	R	R2
PRO37	HIS97	0.000	38.290	0.000
PRO37	TYR145	45.140	0.000	0.000
PRO37	HIS146	82.000	0.000	0.000
THR38	HIS97	0.000	102.480	75.830
THR38	VAL98	0.000	43.350	0.000
THR38	ASP99	62.610	79.630	0.000
THR38	PRO100	88.590	0.000	0.000
THR38	TYR145	71.570	60.120	0.000
LYS40	HIS146	91.620	0.000	0.000
THR41	ARG40	0.000	101.080	82.080
THR41	CYS93	45.180	0.000	0.000
THR41	HIS97	107.430	69.180	36.750
THR41	VAL98	74.230	0.000	0.000
THR41	ASP99	83.850	0.000	0.000
THR41	PRO100	45.560	0.000	0.000
THR41	TYR145	62.090	0.000	0.000
TYR42	ARG40	102.450	122.580	135.390
TYR42	ASP99	94.570	0.000	0.000
PRO44	ARG40	0.000	35.080	0.000
PRO44	HIS97	95.830	0.000	0.000
LEU91	ARG40	74.340	56.820	53.320
ARG92	PRO36	38.960	97.290	60.820
ARG92	TRP37	122.500	120.980	102.090
ARG92	GLN39	82.250	113.280	104.600
ARG92	ARG40	162.250	109.610	144.600
ARG92	GLU43	64.600	0.000	84.440
VAL93	TRP37	52.940	69.930	69.760
VAL93	ARG40	53.350	0.000	0.000
ASP94	TRP37	93.370	114.820	111.410
ASP94	ASP99	57.370	52.600	62.080
GLU101	ASN102	82.810	0.000	0.000
ASP94	ASN102	43.460	66.050	67.020
ASP94	LEU105	52.910	0.000	0.000
PRO95	TRP37	88.170	112.790	116.410
VAL96	ASP99	40.280	87.710	92.660
VAL96	GLU101	94.490	54.300	0.000
ASN97	ASP99	68.150	35.970	0.000
LEU100	ASP99	0.000	31.090	0.000
LYS139	PRO36	0.000	0.000	86.930
TYR140	PRO36	51.590	100.920	0.000
TYR140	TRP37	129.470	136.480	0.000
ARG141	VAL34	93.120	0.000	0.000
ARG141	TYR35	99.160	0.000	0.000
ARG141	PRO36	87.550	0.000	0.000
ARG141	TRP37	89.110	0.000	0.000

Table 7. Contacts at the $\beta 1\beta 2$ interface

$\beta 1$	$\beta 2$	T	R	R2
VAL1	HIS146	0.000	49.420	0.000
LYS82	HIS143	0.000	35.220	0.000
LYS82	HIS146	0.000	0.000	75.500
ASN139	TYR145	0.000	0.000	41.520
ASN139	HIS146	0.000	64.210	82.390
HIS143	LYS82	0.000	35.220	0.000
TYR145	ASN139	0.000	0.000	46.260
HIS146	VAL1	0.000	49.420	0.000
ASN139	HIS146	0.000	64.210	85.030
HIS146	HIS146	0.000	0.000	111.640

Table 6. Contacts at the $\alpha 1\alpha 2$ interface

$\alpha 1$	$\alpha 2$	T	R	R2
VAL1	PRO77	0.000	0.000	80.820
VAL1	THR134	0.000	0.000	78.780
VAL1	VAL135	0.000	0.000	71.930
VAL1	SER138	52.860	90.440	94.320
VAL1	LYS139	0.000	83.590	36.420
VAL1	TYR140	0.000	33.350	121.950
VAL1	ARG141	0.000	129.840	0.000
LEU2	TYR140	0.000	0.000	78.040
LEU2	ARG141	0.000	71.900	0.000
SER3	LYS139	0.000	0.000	49.420
SER3	TYR140	0.000	0.000	101.380
SER3	ARG141	0.000	0.000	60.280
PRO4	TYR140	0.000	0.000	54.900
PRO4	ARG141	0.000	0.000	47.460
PRO77	VAL1	0.000	0.000	68.780
LYS99	LYS99	0.000	37.050	0.000
ALA123	ARG141	44.990	0.000	0.000
ASP126	ARG141	103.950	0.000	0.000
LYS127	SER138	0.000	0.000	57.670
LYS127	LYS139	0.000	51.920	87.850
LYS127	TYR140	0.000	93.850	52.450
LYS127	ARG141	174.840	127.360	0.000
ALA130	ARG141	48.660	61.800	0.000
SER131	ARG141	0.000	33.540	0.000
THR134	VAL1	0.000	0.000	76.720
THR134	THR134	0.000	32.550	0.000
THR134	ARG141	0.000	54.170	0.000
VAL135	VAL1	0.000	0.000	68.040
SER138	VAL1	0.000	90.440	89.250
SER138	LYS127	0.000	0.000	59.640
LYS139	VAL1	0.000	83.590	38.240
LYS139	SER3	0.000	0.000	44.550
LYS139	LYS127	0.000	51.920	94.290
TYR140	VAL1	0.000	33.350	118.420
TYR140	LEU2	0.000	0.000	73.960
TYR140	SER3	0.000	0.000	95.130
TYR140	PRO4	0.000	0.000	52.370
TYR140	LYS127	0.000	93.850	46.200
ARG141	VAL1	33.210	129.840	0.000
ARG141	LEU2	0.000	71.900	0.000
ARG141	SER3	0.000	0.000	50.290
ARG141	PRO4	0.000	0.000	45.670
ARG141	ALA123	44.600	0.000	0.000
ARG141	ASP126	104.460	0.000	0.000
ARG141	LYS127	175.870	127.360	0.000
ARG141	ALA130	46.310	61.800	0.000
ARG141	SER131	0.000	33.540	0.000
ARG141	THR134	0.000	54.170	0.000
ARG141	ARG141	0.000	43.280	0.000

Columns 1 and 2 give residue names and numbers in the indicated chain and columns 3–5 show the surface area ($\text{\AA}^2$) buried between the pair of residues.

R2 looser than R (Tables 5 and 6). As analyzed above, the difference distance maps suggest that the R quaternary form lies along the $T \rightarrow R2$ pathway. Such an inference can be tested by a simple calculation. If R is on the pathway between T and R2, then linear interpolation from the coordinates of the T structure to those of the R2 structure should pass through the R structure. Indeed, a structure is encountered at $\approx 60\%$ along this hypothetical pathway that strongly resembles R; the rms deviation (12) between the actual R structure and the hypothetical intermediate structure is 1.04 for all α -carbons. In fact, the measured fit of this hypothetical structure to R is much better than that of either T or R2 to R (Table 9). A superposition of the hypothetical structure on the R structure is shown in Fig. 3.

In their seminal analysis, Baldwin and Chothia (2) noted the presence of the flexible joint and switch regions in the hemoglobin tetramer. Specifically, the switch region involves residues 38–44 of the $\alpha 1$ subunit (C and CD corner) together with residues 97–102 of the $\beta 2$ subunit (FG corner and G helix). In the quaternary T structure, His⁹⁷ of the $\beta 2$ chain is positioned between Thr⁴¹ and Pro⁴⁴ of the $\alpha 1$ chain (Table 5). Upon transition to the quaternary R structure, His⁹⁷ of $\beta 2$ is displaced by one turn of the C helix to a position between residues 38 and 41 of the $\alpha 1$ chain.

Our own analysis of the switch region—which benefits from the recent availability of the Y (3, 4) and R2 (5) structures—prompts us to reinterpret this switching mechanism. In the T structure, His⁹⁷ of $\beta 2$ buries 0, 107, and 96 $\text{\AA}^2$ with Thr³⁸, Thr⁴¹, and Pro⁴⁴ of $\alpha 1$, respectively—a total of 203 $\text{\AA}^2$. In the R structure, the corresponding areas are 102, 69, and 0 $\text{\AA}^2$, respectively—a total of 171 $\text{\AA}^2$. Upon transformation to the R2 structure, the corresponding areas are 76, 37, and 0 $\text{\AA}^2$, respectively—a total of 113 $\text{\AA}^2$. Thus, there is a progressive decrease in the interaction among residues in the switch region upon transformation from T to R and R to R2, as assayed by buried surface. Additionally, the transition from T to R and R2 is accompanied by loss of hydrogen-bonding interactions between Tyr⁴² and Asn⁹⁷ of $\alpha 1$ with Asp⁹⁹ of $\beta 2$, and the generation of a new hydrogen bond between Asp⁹⁴ of $\alpha 1$ and Asn¹⁰² of $\beta 2$.

Upon transformation from T $\rightarrow$ R, a molecular animation (data not shown) of the switch region shows that, with the two components oriented parallel to the y axis, one component is displaced along the positive y and negative x axes, while the other is displaced along the negative y and positive x axes (Fig. 4). This overall trajectory is extended further upon transformation from R to R2, consistent with the hypothesis that R lies on the pathway between T and R2.

FIG. 2. (A) Difference ϕ (continuous lines) and difference ψ (dashed lines) plots of the α subunit for the T $\rightarrow$ R (Top), R $\rightarrow$ R2 (Middle), and T $\rightarrow$ R2 (Bottom) transformations. (B) Difference ϕ (continuous lines) and difference ψ (dashed lines) plots of the β subunit for the T $\rightarrow$ R (Top), R $\rightarrow$ R2 (Middle), and T $\rightarrow$ R2 (Bottom) transformations. (C) Stereoscopic views of the turn flips accompanying the T (solid) $\rightarrow$ R (dotted) transition in the α subunit of hemoglobin. (Upper) Superposition of the turn involving His⁵⁰ and Gly⁵¹. (Lower) Turn involving Ala⁷¹ and His⁷².

As originally conceived (2), the switch mechanism posited that Thr⁴¹ of $\alpha 1$ would function as a major steric barrier to His⁹⁷ of $\beta 2$ in the transformation from its T position to its R

Table 8. ϕ and ψ values (degrees) for residues 50, 51, 71, and 72 of a subunit in T, R, and R2 quaternary forms

Residue	T		R		R2	
His ⁵⁰	-57.95,	127.11	-43.64,	-64.65	-37.34,	-74.85
Gly ⁵¹	86.29,	7.11	-88.80,	19.20	-88.45,	18.59
Ala ⁷¹	-62.66,	-32.98	-97.60,	89.02	-68.68,	-25.97
His ⁷²	-122.08,	45.79	119.82,	17.08	-120.82,	36.51

Each column contains the ϕ and ψ values for the residue separated by commas. For a definition of turn types, see ref. 13. In the T state the residue pair His⁵⁰-Gly⁵¹ is in a type II turn; in both R and R2 it is in a distorted type I turn. The residue pair Ala⁷¹-His⁷² is in a distorted type I turn in both the T and R2 states and in a type II turn in the R state.

position. It is noteworthy that side-chain torsion angles of residues in the switch region change very little upon transition from T to R2, although Thr³⁸ and Thr⁴¹ do undergo side-chain rotations upon transition from T $\rightarrow$ R and R $\rightarrow$ R2.

Table 9. rms deviations for the superposition of T, R, and R2 forms

Forms	All	$\alpha 1\beta 1$	$\alpha 1\beta 2$
T, R	2.26	0.82	1.72
R, R2	1.65	0.45	1.35
T, R2	3.33	0.92	2.74
TR2*, R	1.04	0.44	0.71

Superpositions were performed for the α -carbons only. The superposition for all residues and for residues in the $\alpha 1\beta 1$ and $\alpha 1\beta 2$ dimers is listed.

*Hypothetical intermediate structure on the path from T to R2.

FIG. 3. Least-squares superposition of the R quaternary form (black) with the hypothetical intermediate (gray) recovered from a linear trajectory between T and R2. Superpositions were performed (13) using atoms in the $\alpha 1\beta 1$ interface, similar to the BGH frame of Baldwin and Chothia (2).

It follows that the rearrangement is driven predominantly by alterations in the backbone. As seen in Table 4, the α subunit becomes more compact in the T $\rightarrow$ R transition, with backbone atoms in the switch region being drawn toward the center of the subunit and concomitant displacement of side chains. Linear interpolation of the switch region from T to R shows that no steric barriers would be encountered in this transition, because the subunit motions are in mutually opposite directions.

$\alpha 1\alpha 2$ and $\beta 1\beta 2$ Interfaces. Both $\alpha 1\alpha 2$ and $\beta 1\beta 2$ interfaces bury more surface area upon transformation from T to R and T to R2. The $\alpha 1\alpha 2$ interface is more buried in R2 than in R, whereas the surface area buried at the $\beta 1\beta 2$ interface is greater in R than in R2. However, in both regions the coordinates of the residues in contact are not well resolved, and the calculated surface areas are probably inaccurate.

DISCUSSION

All the computational experiments presented above suggest strongly that the R quaternary form lies on the path from T to R2: (i) the analysis of difference distance maps, (ii) the progression of diagnostic turn flips, (iii) the reinterpretation of events in the switch region, and (iv) the fact that an R-like intermediate can be found by linear interpolation from T to R2. While none of the evidence is conclusive, taken in sum the preceding lines of inquiry constitute a persuasive argument for the need to reexamine hemoglobin structure-function relationships in the context provided by the T-R-R2 framework.

Thermodynamic measurements of partially ligated states of hemoglobin by Ackers *et al.* (18) have revealed the existence of three distinct energy levels on the pathway from the deoxy form to the fully ligated molecule. It was suggested (3, 5) that the R2 or Y structure may correspond to the intermediate energy level observed in these thermodynamic studies (18). However, it should be noted that a simple two-state model could account for the existence of three cooperative energy levels (19).

Arguably, the R structure is a "geometric intermediate" between T and R2, but is it a stable thermodynamic species? Although our computational experiments cannot resolve this question, we suspect that the existence of the R structure may be a consequence of the fact that high-salt conditions serve to impede the fully ligated structure from progressing all the way to R2. Moreover, from our analysis, the switch region does not appear to function as a steric barrier sepa-

FIG. 4. Stereoview of α -carbon trace of the switch region, with T darkest, R lighter, and R2 lightest. The β subunit (Lower) is observed to move away from the α subunit (Upper), with no evident steric barrier. The traces were generated by a least-squares fitting of the BGH frame (2) of T, R, and R2.

rating T from R, as previously believed. In the absence of the switch, there remain no evident steric barriers between T and R2. Hence, we tentatively conclude that, under physiological conditions, only two distinct populations are visited by the hemoglobin tetramer—T and R2.

Note Added in Proof. A study by Korn and Rose (20) that appeared after submission of our manuscript uses backbone torsion angles to identify conformational changes in proteins. The turn flips at the amino termini of the E and F helices are among the examples chosen by Korn and Rose to illustrate their method, and their figures 3A and 3B are comparable to our Fig. 2A.

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