Allosteric transition intermediates modelled by crosslinked haemoglobins

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The structural end-points of haemoglobin's transition from its low-oxygen-affinity (T) to high-oxygen-affinity (R) state, have been well established by X-ray crystallography[1–7], but short-lived intermediates have proved less amenable to X-ray studies. Here we use chemical crosslinking to fix these intermediates for structural characterization. We describe the X-ray structures of three haemoglobins, $\alpha_2\beta^1S^{82}\beta$, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$, which were crosslinked between the amino groups of residues βVal1 and βLys82 by 3,3′-stilbenedicarboxylic acid (S) or trimesic acid (Tm) while in the deoxy state, and saturated with carbon monoxide before crystallization. $\alpha_2\beta^1S^{82}\beta$, which has almost normal oxygen affinity, is completely in the R-state conformation; however, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$, both of which have low oxygen affinity, have been prevented from completing their transition into the R state and display many features of a transitional intermediate. These haemoglobins therefore represent a snapshot of the nascent R state.

The crosslinked haemoglobins were prepared by reacting human deoxyhaemoglobin with the diacyl bis(methylphosphate) derivatives of 3,3′-stilbenedicarboxylic acid (S)[8] or trimesic acid (Tm)[9] and were crystallized in the carbonmonoxy (CO) form by the method of Perutz[10]. $\alpha_2\beta^1S^{82}\beta$, which has a slightly higher O_2 affinity than haemoglobin A(HbA) and high cooperativity (Table 1), crystallizes isomorphously with respect to CO-HbA[1,7]. The structure has been refined[11] to a crystallographic *R*-factor of 14.6% at 2.4 Å resolution (Table 1). Both $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ show markedly reduced O_2 affinity compared with HbA, high cooperativity, and crystallize in space group *C*2 (Table 1). The structure of $\alpha_2\beta^1Tm^{82}\beta$ was solved by molecular replacement[12] and found to contain a tetramer per asymmetric unit. Refinement converged to an *R*-factor of 15.0% at 1.8 Å

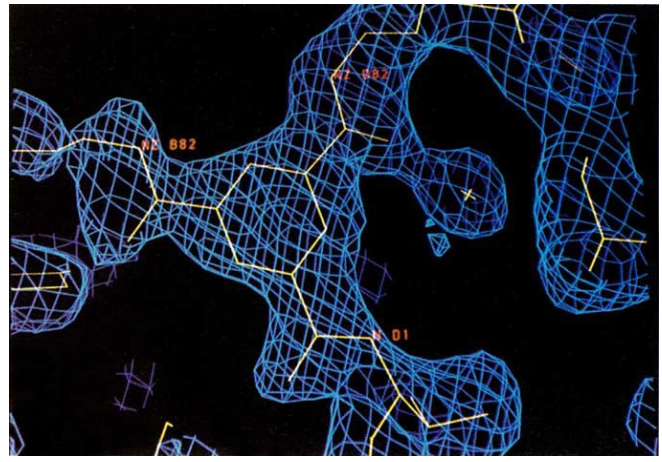


FIG. 1 $2F_o - F_c$ electron density map of the triply linked $\alpha_2\beta^{1,82}Tm^{82}\beta$ haemoglobin contoured at 1.0σ. The atoms are represented as yellow sticks and the electron density map is indicated in blue[25]. The cross represents the location of a water molecule that is hydrogen-bonded to the carbonyl group of the crosslinker. Shown is the trimesoyl crosslinker bonded to the ε-NH_2 of β_1Lys82 (Nζ D82), the α-NH_2 of β_1 Val1 (N D1) and the ε-NH_2 of β_2 Lys82 (Nζ B82), providing the first example of a rationally designed triply linked protein.

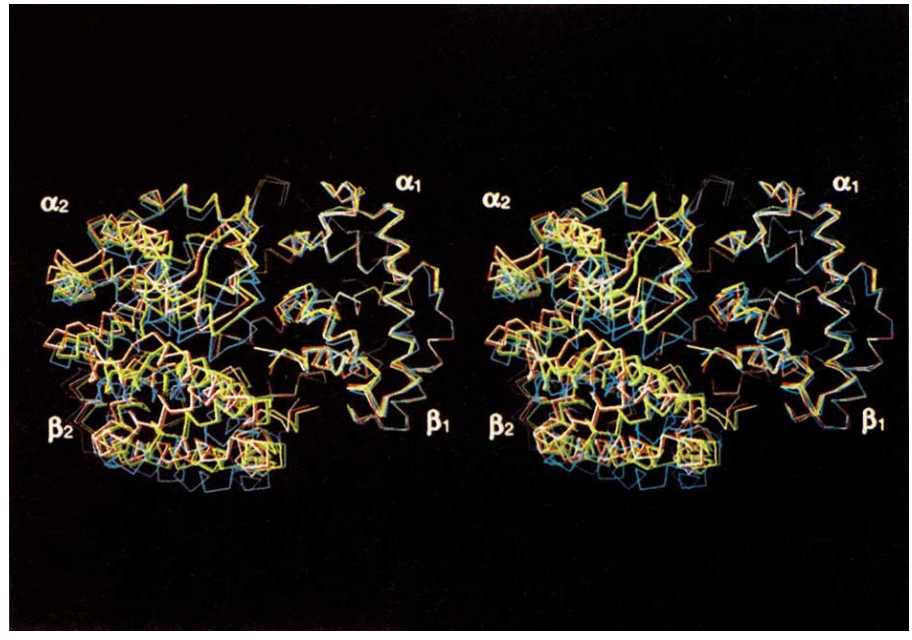
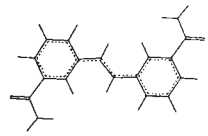
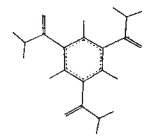
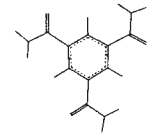


FIG. 2 Stereo view of the overlay of the $\alpha_1\beta_1$ interface of deoxyhaemoglobin (blue Cα trace), $\alpha_2\beta^1Tm^{82}\beta$ (yellow Cα trace), $\alpha_2\beta^{1,82}Tm^{82}\beta$ (green Cα trace) and $\alpha_2\beta^1S^{82}\beta$ (white Cα trace) onto CO-HbA (red Cα trace) using the method of Baldwin and Chothia[1]. This figure dramatically illustrates the quaternary changes that occur in the T-to-R transition. In this overlay method, the regions in which no structural changes occur in going from T to R are used as a reference frame and include residues α30–α36 (B helix); α102–α113 (G helix); α117–α127 (H helix); β30–β36 (B helix); β51–β55 (D helix); β107–β132 (G and H helices). After overlaying this region of the $\alpha_1\beta_1$ dimers, a rotation of 13.6° is required to bring the $\alpha_2\beta_2$ interfaces of the deoxy- and CO-HbA into coincidence. The TmHbs appear to be intermediates in this transition as they have rotated only 7.4° and 8.5° about an axis of rotation nearly coincident to that used by native CO-HbA. Their translation components, approximately half (0.44) of that of native Hb, are also intermediate. No additional rotation or translation is required for the $\alpha_2\beta^1S^{82}\beta$ haemoglobin, consistent with its complete R-state conformation. Coordinates used in all analyses were taken from refs 7 (CO-HbA) and 2 (deoxyhaemoglobin).

TABLE 1 Summary of selected biochemical and crystallographic data

Crosslinked haemoglobin	$\alpha_2\beta^1S^{82}\beta$	$\alpha_2\beta^1Tm^{82}\beta$	$\alpha_2\beta^{1,82}Tm^{82}\beta$
Crosslinker			
P_{50} (mmHg)	3.4[8]	17.1[8]	18.1[9]
Hill coefficient	2.6[8]	2.7[8]	2.6[9]
Space group	$P4_12_12$	$C2$	$C2$
Cell dimensions (Å)	$a=b=54.13$ $c=196.23$	$a=104.43$, $b=72.16$ $c=88.03$ $\beta=108.25°$	$a=104.43$, $b=72.16$ $c=88.03$ $\beta=108.25°$
$\alpha\beta$ dimer per ASU	1	2	2
Data collection			
Resolution (Å)	2.4	1.8	1.8
Number of reflections	9,807	48,608	42,152
R_{Sym} (%)	3.6	6.3	3.0
Number of atoms	2,300	4,576	4,577
Number of solvent molecules	110	393	429
Refinement			
R-factor (%)	14.6	15.0	13.5
Deviations from ideality (r.m.s.)			
Bond distances (Å)	0.019	0.010	0.017
Bond angles (deg)	2.90	2.34	3.01

$\alpha_2\beta^1S^{82}\beta$, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ were crosslinked in their deoxy forms as described[8,9] by reacting human deoxyhaemoglobin with the diacyl bis(methylphosphate) derivatives of 3,3′-stilbenecarboxylic acid (S) or trimesic acid (Tm). $\alpha_2\beta^1S^{82}\beta$ and $\alpha_2\beta^1Tm^{82}\beta$ are crosslinked through the α-NH_2 of β_1 Val1 to the ε-NH_2 of β_2Lys82, where beta subscripts 1 and 2 indicate different β-subunits only. $\alpha_2\beta^{1,82}Tm^{82}\beta$ is crosslinked through the α-NH_2 of β_1Val1 and the ε-NH_2 of β_1Lys82 to the ε-amino group of β_2Lys82. The crosslinkers are depicted in their amide forms. The negative charge of the methyl phosphate leaving groups serves to target the crosslinkers to the cationic 2,3-bis-phosphoglycerate (BPG)-binding pocket, where βLys82 and βVal1 are located. Comparison of the P_{50} values and Hill coefficients of the crosslinked haemoglobins with those of native HbA ($P_{50}=5.0$ mmHg, Hill coefficient = 3.0) shows that $\alpha_2\beta^1S^{82}\beta$ binds oxygen with an increased affinity, whereas $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ show reduced oxygen affinities; however, all remain cooperative[8,9]. Crystals of the carbonmonoxy form of $\alpha_2\beta^1S^{82}\beta$, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ were grown at room temperature by the batch method of Perutz[10] in 2.45 M, 2.35 M and 2.35 M sodium–potassium phosphate solutions, respectively, following carbon monoxide saturation. The $\alpha_2\beta^1S^{82}\beta$ crystals are isomorphous with native carbonmonoxy haemoglobin whereas the Tm crosslinked haemoglobins, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$, are not. Data for the $\alpha_2\beta^1S^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ haemoglobins were collected at room temperature with an Area Detector Systems Corporation (ADSC) area detector using a Rigaku RU200-H rotating anode generator for the X-ray source (40 kV, 150 mA) and the data were processed with the software provided by ADSC. Data for the $\alpha_2\beta^1Tm^{82}\beta$ were collected with an R-Axis II imaging plate at Molecular Structure Corporation (The Woodlands, Texas) and processed with software provided by MSC. The native carbonmonoxy haemoglobin structure minus water molecules was used as the starting model for the $\alpha_2\beta^1S^{82}\beta$ structure and the crosslinker was located in a difference Fourier map and fitted using FRODO[25]. The model was refined using TNT[11]. The $\alpha_2\beta^1Tm^{82}\beta$ haemoglobin was solved by Molecular Replacement using the MERLOT[12] software package. The native carbonmonoxy $\alpha\beta$ dimer[7], minus water, was used as the starting model. Two large peaks in both the rotation and translation functions located the molecule and confirmed the presence of a tetramer in the asymmetric unit (ASU). Refinement[11] and difference Fourier maps located the trimesoyl crosslinker, which was fitted using FRODO[25]. Subsequent cycles of refinement followed until convergence. This structure, minus the crosslinker and waters, was used as the starting model for the $\alpha_2\beta^{1,82}Tm^{82}\beta$ model. The crosslinker was located in a difference Fourier map and the structure was refined to convergence. Coordinates are being deposited in the Brookhaven Protein Data Bank and are available from R.G.B. Full details of the structures will be reported elsewhere (M.A.S. *et al.*, manuscript in preparation). $R_{Sym}=\sum|I_o-\langle I\rangle|/I_o$, where I_o is the observed intensity, and $\langle I\rangle$ is the average intensity from multiple observations of symmetry related reflections. R-factor $=\sum||F_o|-|F_c||/\sum|F_o|$.

resolution (Table 1). This crosslinked haemoglobin served as the starting model, minus crosslinker, for refinement of the triply linked $\alpha_2\beta^{1,82}Tm^{82}\beta$, which converged to a final R-factor of 13.5% at 1.8 Å resolution (Table 1). The final $2F_o-F_c$ electron density map corresponding to the crosslinker region of $\alpha_2\beta^{1,82}Tm^{82}\beta$ is shown in Fig. 1. To our knowledge, the structure of $\alpha_2\beta^{1,82}Tm^{82}\beta$ provides the first example of a rationally designed, triply linked protein[9].

Upon binding carbon monoxide, the $\alpha_1\beta_1$ dimer of HbA undergoes a 13.6° rigid body rotation with respect to the $\alpha_2\beta_2$ dimer[1]. This rotation is also observed for $\alpha_2\beta^1S^{82}\beta$ (Fig. 2). However, the $\alpha\beta$ dimers of $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ (TmHbs) are restricted by the trimesoyl group and rotate only 7.4° and 8.5°, respectively, suggesting that they are trapped in a transitional conformation (Fig. 2). The tertiary and quaternary differences between the crosslinked haemoglobins and deoxy- and CO-HbA were analysed by a series of α-carbon coordinate-difference plots (Fig. 3). These plots confirm the identical conformations of $\alpha_2\beta^1S^{82}\beta$ and CO-HbA, and demonstrate that simply crosslinking the α-NH_2 of βVal1 of one $\alpha\beta$ dimer to the ε-NH_2 of βLys82 of the other $\alpha\beta$ dimer does not induce large structural perturbations. Most important, these plots reveal that, unlike the structures of other proposed transitional intermediates such as R2-HbA[13], YpsilantiHb[14] and CN-metHb[15], those of the TmHbs lie directly on the T-to-R conformational pathway.

The most significant structural differences between the TmHbs and CO-HbA and deoxy-HbA are found in elements involved

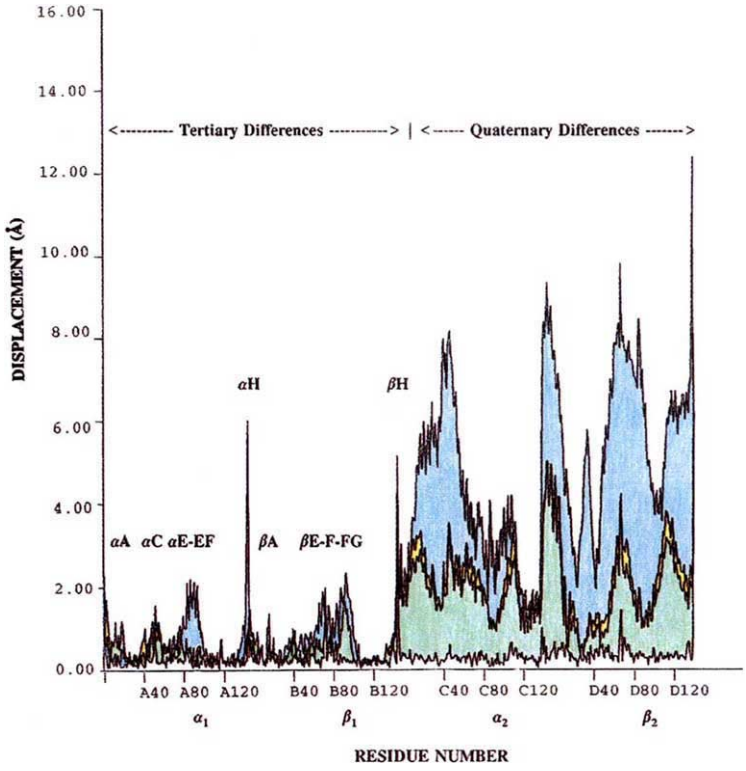
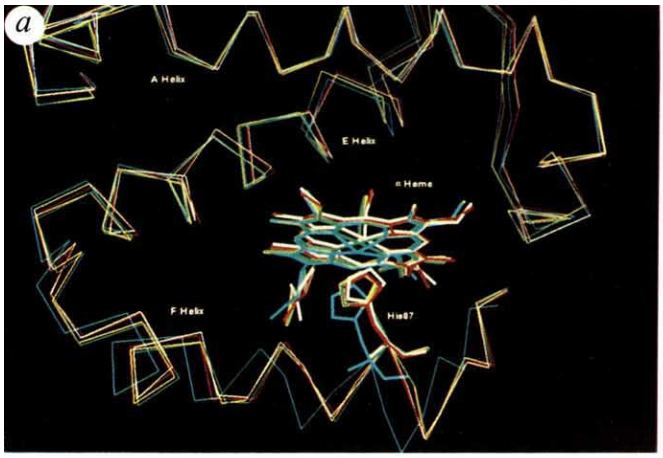
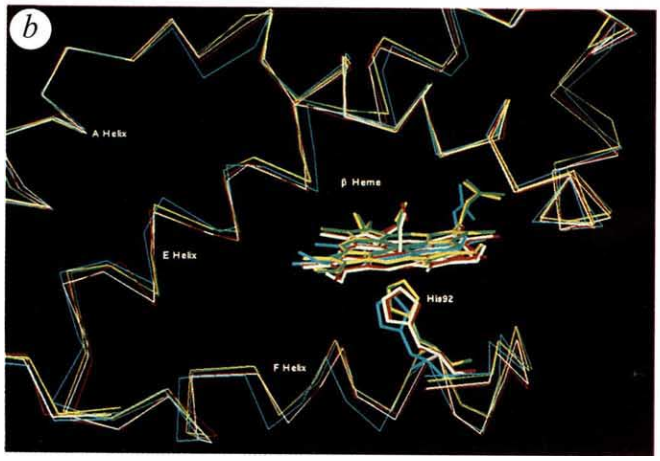
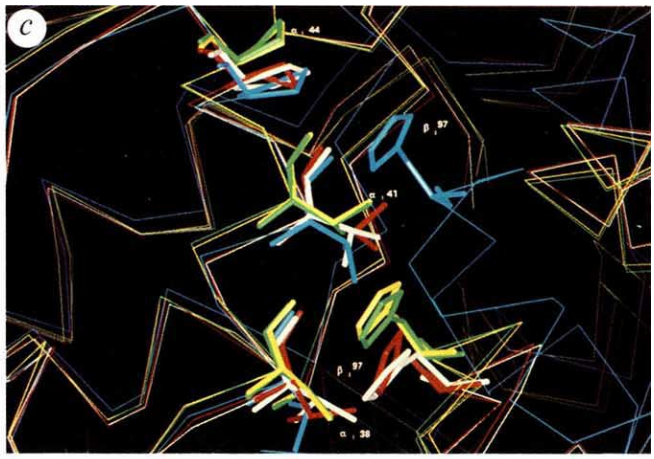
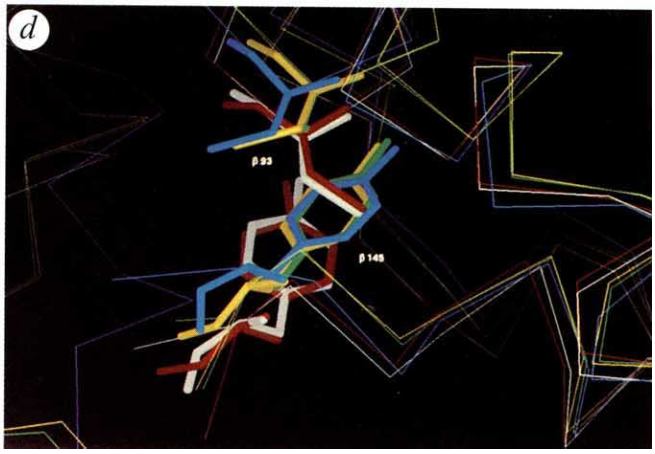


FIG. 3 Coordinate-difference plot (CDP) showing the differences between corresponding Cα positions of deoxyhaemoglobin (background, shaded in blue), $\alpha_2\beta^1Tm^{82}\beta$ (near background, shaded in yellow), $\alpha_2\beta^{1,82}Tm^{82}\beta$ (foreground, shaded in green) and $\alpha_2\beta^1S^{82}\beta$ (white) after their $\alpha_1\beta_1$ interfaces are overlaid on CO-HbA as in Fig. 2. The ordinate indicates the displacement in Å for the corresponding Cα atoms and the abscissa indicates the residue number where the A and B chains correspond to the α_1 and β_1 chains, respectively, and the C and D chains, to the α_2 and β_2 chains, respectively. The region designated 'tertiary changes' corresponds to the $\alpha_1\beta_1$ region which was overlaid and refers to structural alterations between tertiary elements, for example, helical displacements. The structural elements that undergo the most significant displacements are labelled on the plot. The region labelled 'quaternary changes' corresponds to the $\alpha_2\beta_2$ region, which was not overlaid, and corresponds to quaternary differences between deoxyhaemoglobin, $\alpha_2\beta^1Tm^{82}\beta$, $\alpha_2\beta^{1,82}Tm^{82}\beta$ and $\alpha_2\beta^1S^{82}\beta$ and CO-HbA. The quaternary differences between the TmHbs and CO-HbA are made obvious by these plots and suggest that the TmHbs assume an intermediate quaternary conformation. A similar overlay of the $\alpha_2\beta_2$ interfaces of deoxyhaemoglobin, $\alpha_2\beta^1Tm^{82}\beta$, $\alpha_2\beta^{1,82}Tm^{82}\beta$ and $\alpha_2\beta^1S^{82}\beta$ onto that of CO-HbA produces a nearly identical CDP.









directly in the T-to-R transition, namely, the A, C, E and H helices and EF turns of the α-subunits and the A, E, F and H helices and FG turns of the β-subunits (Fig. 3). Inspection of these structural elements reveals that relaxation to the R conformation is incomplete or has not started, and that their locations result in unfavourable steric pressure or strain, which is the probable cause of the reduced oxygen affinities of both subunits of the TmHbs. Specifically, their α-subunit E helices are essentially in the T-state position (Fig. 4*a*), whereas the E helices of the β-subunits are located between the T and R positions (Fig. 4*b*). Ultraviolet Raman studies have shown that movement of the E helices of haemoglobin is critical to relieve the strain imposed by ligand binding[16]. Also intermediate are the locations of the F helices of the β-subunits, which have moved only 0.7 Å in $\alpha_2\beta^1Tm^{82}\beta$ and 0.6 Å in $\alpha_2\beta^{1,82}Tm^{82}\beta$ from the T-state position, as compared with a 1.4 Å shift in CO-HbA (Fig. 4*b*). Their fully ligated haems have also not reached the R-state location, but there is no haem doming. To maintain proper coordination to the haem Fe^{2+}, the proximal histidine, βHis92 (F8), which retains its T-state χ^1 torsion angle, is significantly displaced towards the haem, implying a parabolic trajectory of F8 in the T-to-R transition (Fig. 4*b*). These results suggest that the movements of the β-subunit E and F helices and haems are coordinated.

In contrast to the β-subunit F helices, the F helices of the α-subunits have reached the R-state location in the TmHbs (Fig. 4*a*). The α-subunit haems, which are also fully ligated and planar, are in the T-state location, however, implying that they move independently of their F helices. The conformational transition from the T-state E helices to the R-state F helices is accommodated by the EF turns, which are structurally intermediate (Fig. 4*a*). The α-subunit proximal histidine, αHis87 (F8), is also positioned between its T- and R-state locations to coordinate the haem Fe^{2+} properly. Thus, the T-to-R state transitional pathways of the α and β haems are different. This finding is in accord with the structures of the T-state liganded haemoglobins, T(α-oxy, β-deoxy)Hb and T(α-met, β-met)Hb[17,18] and probably reflects the different packing and chemical environments of the α (tight) and β (looser) haems.

Other notable features of these intermediates are found in the switch region and the β-subunit carboxy termini of the TmHbs. Inspection of the switch reveals that, although located in its R-state position between α_1Thr38 and α_1Thr41, β_2His97 is displaced significantly towards its T-state location (Fig. 4*c*). At the C termini, all critical T-state salt bridges, including that between βHis146 and βAsp94, are disrupted[7], yet the side chains of their neighbouring residues, βTyr145 and the reactive βCys93 (refs 19,20), maintain their T-state locations, which suggests that their movements occur late in the T-to-R transition (Fig. 4*d*).

In conclusion, the carbonmonoxy structures of $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ are probable snapshots of haemoglobin's nascent R state and can explain their lowered oxygen affinities. Using these crosslinked haemoglobins, this idea could also be investigated using dynamic techniques such as ultraviolet Raman[16,21,22] and ultrafast near-infrared[23,24] spectroscopy. Our results, combined with the finding of an inverse correlation between the O_2 affinity and crosslinker bridging distance in a series of $\beta^1X^{82}\beta$ crosslinked haemoglobins[8], suggests that the structures of additional haemoglobin intermediates, either more or less R-like, can be trapped by using crosslinkers of different lengths, an idea that might be extended to other multistate proteins. □

◀ FIG. 4 Snapshots of the T-to-R transition in haemoglobin. All views are the result of the $\alpha_1\beta_1$ overlay described in Fig. 2 legend. As in Fig. 1, deoxyhaemoglobin is shown in blue, $\alpha_2\beta^1Tm^{82}\beta$ in yellow, $\alpha_2\beta^{1,82}Tm^{82}\beta$ in green, $\alpha_2\beta^1S^{82}\beta$ in white and CO-HbA in red. *a*, The α_1 haem environments showing the proximal histidines, αHis87 (F8) and the haem groups as solid rendered bonds and the A, E and F helices as Cα traces. The T-state positions of the E helices and haems of the TmHbs are evident, as are the R-state positions of their F helices. The A helices of the TmHbs are also closer to the T-state position, a finding that is consistent with recent UV–Raman studies[16]. The α haems are fully ligated and planar. The T-state positions of the E helices and the α haems and the R-state positions of the F helices result in steric pressure which requires F(8) to move to a position between its normal T and R state locations so that the Fe^{2+} atom of the α haem maintains proper octahedral geometry. *b*, The β_1 haem environments showing the proximal histidines, βHis92 (F8) and the haem groups as solid-rendered bonds, and the A, E and F helices as Cα traces. The intermediate locations of the A, E and F helices of the TmHbs are evident, as are the significant displacements of their proximal histidines towards the haem. Despite the strain resulting from the intermediate nature of the E and F helices of the TMHbs, the β haems are fully ligated and planar. The shifts of the β F helices were quantified (see text) by averaging the Cα displacements (Å) of each F helix residue of deoxyhaemoglobin, $\alpha_2\beta^1Tm^{82}\beta$, $\alpha_2\beta^{1,82}Tm^{82}\beta$ and $\alpha_2\beta^1S^{82}\beta$ relative to the corresponding residues in CO-HbA. *c*, The switch region of haemoglobin, which is composed of residues 97–102 from the β_2 FG corner and G helix and residues 38–44 from the C helix and CD corner of the α_1 subunit, demonstrating the significant displacements of the key switch residue β_2His97 of $\alpha_2\beta^1Tm^{82}\beta$ (yellow) and $\alpha_2\beta^{1,82}Tm^{82}\beta$ (green) towards the T-state position. The distances from the β_2His97 Cα atoms of $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ to the corresponding β_2His97 Cα atom in CO-HbA (red) are 1.26 and 1.47 Å, respectively, compared to a distance of 7.12 Å for deoxyhaemoglobin (blue). Despite these displacements, the β_2His97 of the TmHbs are clearly in the R conformation, as evidenced by their locations between α_1Thr38 and α_1Thr41. In deoxyhaemoglobin, His 97 sits between α_1Thr41 and α_1Pro44. *d*, The pocket formed by the β-subunit F and H helices showing the T-state position of the side chains of βCys93 and βTyr145 of $\alpha_2\beta^1Tm^{82}\beta$ (yellow) and $\alpha_1\beta^{1,82}Tm^{82}\beta$ (green). The side chains of βCys93 and βTyr145 of the $\alpha_2\beta^1S^{82}\beta$ haemoglobin (white), however, are clearly in the R-state location. Deoxyhaemoglobin and CO-HbA are shown in blue and red, respectively.

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