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Structure of Hemoglobin M Boston, a Variant with a Five-Coordinated Ferric Heme

(x-ray analysis/molecular pathology/inherited blood diseases)

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ABSTRACT **X-ray analysis of the natural valency hybrid $\alpha_2^{+M\ Boston}\beta_2^{deoxy}$ shows that the ferric iron atoms in the abnormal α subunits are bonded to the phenolate side chains of the tyrosines that have replaced the distal histidines; the iron atoms are displaced to the distal side of the porphyrin ring and are not bonded to the proximal histidines. The resulting changes in tertiary structure of the α subunits stabilize the hemoglobin tetramer in the quaternary deoxy structure, which lowers the oxygen affinity of the normal β subunits and causes cyanosis. The strength of the bond from the ferric iron to the phenolate oxygen appears to be the main factor responsible for the many abnormal properties of hemoglobin M Boston.**

Hemoglobin (Hb) M Boston is a variant in which the distal histidines in the heme pockets of the α chains are replaced by tyrosines [His E7(58) → Tyr] (1–3). So far it has been found only in heterozygotes, in whom it causes methemoglobinemia and cyanosis. The former is due to the low redox potential of the hemes in the abnormal α chains, which inhibits their reduction by methemoglobin reductase, and the latter to the low oxygen affinity of the hemes in the normal β chains.

We have investigated Hb M Boston by x-ray analysis. A difference electron density map of deoxyHbM Boston ($\alpha_2^{+B}\beta_2$) minus A shows that the ferric iron atoms in the α chains are coordinated to four nitrogens of the porphyrin and the phenolate of the tyrosine. The bond to the proximal histidine (F8) is absent and the iron lies on the distal side of the porphyrin ring. The rupture of the iron histidine bond causes the helix F to coil up a little, like a spring released from a tension that had kept it slightly uncoiled. The resulting changes in tertiary structure of the α chain have a stabilizing effect on the quaternary deoxy or T structure of the hemoglobin tetramer, which lowers the oxygen affinity of the normal β subunits.

EXPERIMENTAL METHODS AND RESULTS

The Hb M Boston used in these studies was identified in a 38-year-old caucasian male referred to one of us by Dr. Morris Asch. The Hb M was isolated from Hb A by column chromatography at 4° with a weakly acid cation exchange resin (Bio-Rex 70) and a 0.15 M, pH 6.42, sodium phosphate buffer. Under these conditions Hb A elutes readily from the column but Hb M Boston remains attached. Hb M Boston was eluted from the column with the same buffer to which 0.5 M NaCl had been added. The mutant so purified, had the spectral properties found by Gerald *et al.* in the originally described case of Hb M Boston (1). Structural studies demonstrated that the substitution was indeed E7 (58) His → Tyr; peptide maps of the α^M chains, done with the technique described elsewhere (24), showed cathodal displacement of peptides 7 and 7–8. The amino-acid composition of these abnormal tryptic peptides demonstrated the absence of one histidine and the addition of one tyrosine residue.

Structure of $\alpha_2^{+B}\beta_2$. Addition of ferrous citrate to oxyHb Boston reduces the heme irons in the normal β, but not in the abnormal α subunits. We were therefore able to crystallize the hybrid $\alpha_2^{+B}\beta_2$ as described for deoxyHb A and found the crystals to be isomorphous with those of A (4). We measured the intensities of about 14,000 reflections within the limiting sphere of 3.5 $Å^{-1}$ and calculated a difference Fourier synthesis using ($|F_{Boston}| - |F_A|$) as coefficients, together with the phase angles of deoxyHb A determined by Muirhead and Greer (5). Fig 1 shows a superposition of several sections through the electron density map of deoxyHb A, comprising the heme iron and the distal histidine of the α subunits. In deoxyHb A the iron atom is displaced by 0.75 Å from the plane of the porphyrin ring towards the proximal histidine (6). The difference map shows a large negative peak (*b*) superimposed on, and slightly to the right of, the iron peak; this is flanked on the left by an even larger positive peak (*a*), indicating a displacement of the iron atom from right to left.

The difference Fourier synthesis is on an absolute scale, so that the displacement of the iron atom can be calculated following the method of Booth (7). To start with, the magnitudes of the difference peaks were doubled in order to compensate for our having neglected the difference in phase between the reflections from Hbs A and M Boston (8–10). The components of the displacements of the iron along the crystallographic X, Y, and Z* axes were then derived from the expressions

$$\Delta X = \frac{-\text{slope of } \Delta\rho_x}{\text{curvature of } \rho_x} = -\frac{\partial \Delta\rho_x}{\partial x} \Big/ \frac{\partial^2 \rho_x}{\partial x^2}$$

and similarly for ΔY and ΔZ^* (ρ_x is the electron density along a line parallel to x). The components of the slope were obtained from the three projected gradients of the difference map at the iron positions, and the components of the curvature from three projections of the iron peak in an electron density map of deoxyHb A calculated with the same *hkl* terms as the difference map. The results gave the shift of the iron atom as 0.99 Å normal to the plane of the porphyrin from the proximal towards the distal side, which means that the iron atom in Hb

Abbreviation: Hb, hemoglobin.

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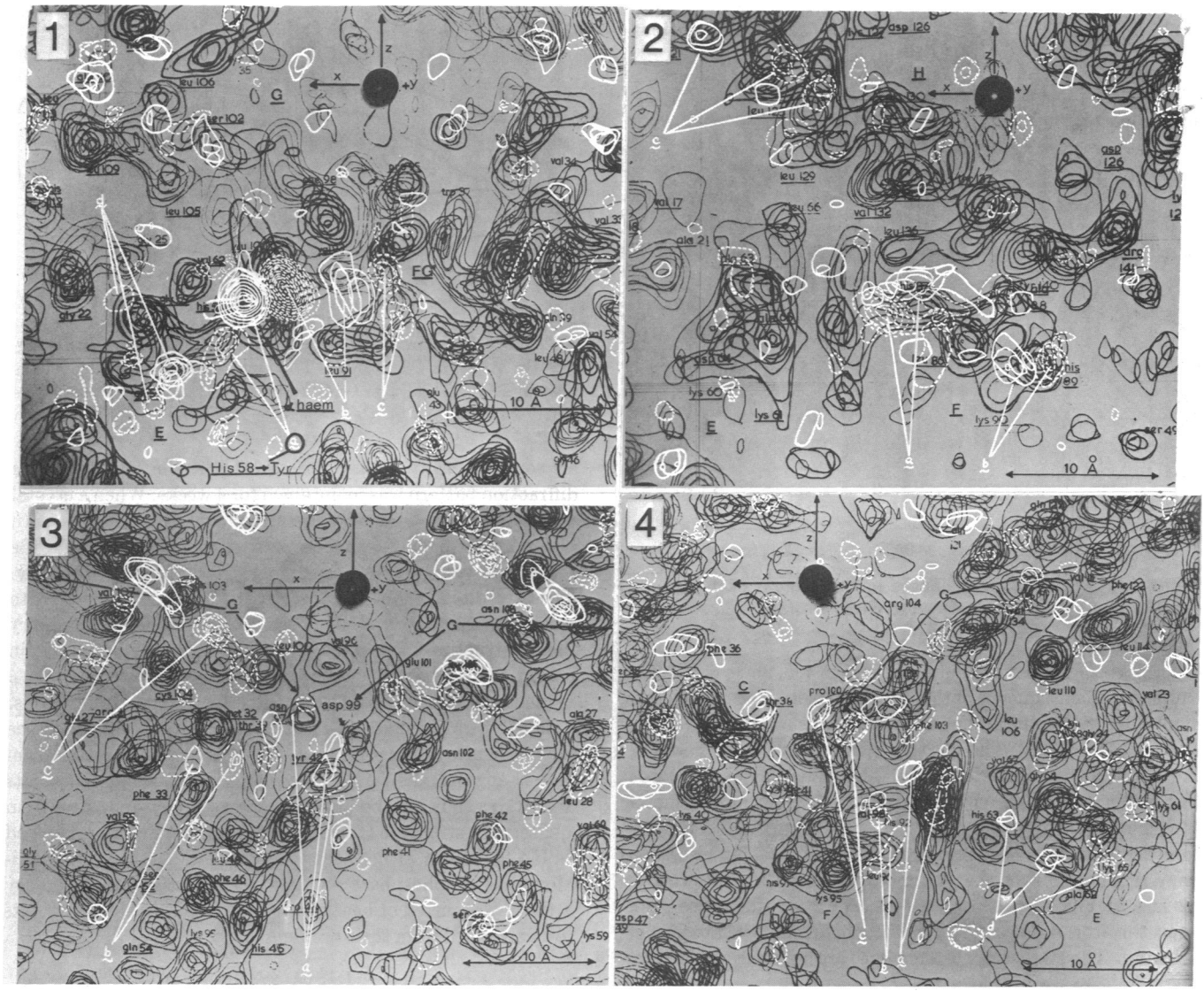

FIGS. 1–4. Sections of the difference electron density map (*white contours*) of $\alpha_2{}^{+B}\beta_2 - \alpha_2\beta_2$ (*A*) superimposed on Muirhead and Greer's electron density map of human deoxyHb at 3.5 Å (*black contours*) (5). *Solid white contours* indicate positive, *broken white contours* negative, difference density. Difference contours are drawn at intervals of ±0.015 e/Å^3 with the zero and ± first levels omitted. Residues in the α chains are underlined, those in the β chains are not. The *black circles* mark the molecular dyad running through the internal cavity. The surface of the molecule is near the lower edges of the sections. Sections are at intervals of 1 Å normal to *y*. *Fig. 1.* Sections y = 4–9 comprising the heme iron and lower half of the heme in the α subunits; on the left of the heme is helix E (*d*) with the distal histidine (*a*); on its right is the FG segment of the α chain (*c*) and further right the C helix of the β chain. Helix G runs from the upper left-hand edge to just below the molecular dyad. The large negative peak caused by the iron displacement and part of the movement of His F8α are pointed out by (*b*). *Fig. 2.* Sections y = 10–14, comprising the upper half of the heme in the α subunits, with helix E on its left and helix F with the proximal histidine (*a*) on its right. Helix H extends from the top left-hand corner diagonally down. *Fig. 3.* Sections y = +1 to −3 showing, on the left, parts of the helix C, CD segment, and helix G of the α chain, and on the right the corresponding parts of the β chain. *Fig. 4.* Sections y = −4 to −10 comprising the upper part of the heme in the β subunits, with helix F on its left, helix E on its right, and helix G just above it. Further to the left is part of helix C of the α chain.

M Boston is displaced by 0.24 Å towards the distal side. Integration over the negative peak in the difference map gives a total of 25.4 electrons, compared to 24 for a ferrous iron. Integration over the positive peak gives 38.1 electrons, which is probably made up of the sum of the 23 electrons contributed by the ferric ion and of the 15 electrons contributed by the unresolved oxygen and adjacent carbon of the phenolate ion. The position of the phenolate ring is indicated by an extension of the iron peak into a narrower one to its left. There is also a negative peak (*a*) superimposed on histidine E7, marking its removal.

Fig. 2 shows sections through the proximal histidine and the upper part of the porphyrin. The histidine peak is flanked by a negative difference peak below and a positive one above (*a* in Fig. 2), indicating that the side chain moves deeper into the heme pocket after the bond to the iron atom has been broken. Peaks *b* and *c* in Fig. 1 and *a* and *b* in Fig. 2 show that this movement of the histidine side chain generates a clockwise rotation of helix F. The rotation pushes the FG, C, and CD segments of the α chain towards the center of the molecule (peaks *a* and *b* in Fig. 3), taking the FG segment of the β chain with them, and causing small shifts in the G helix and

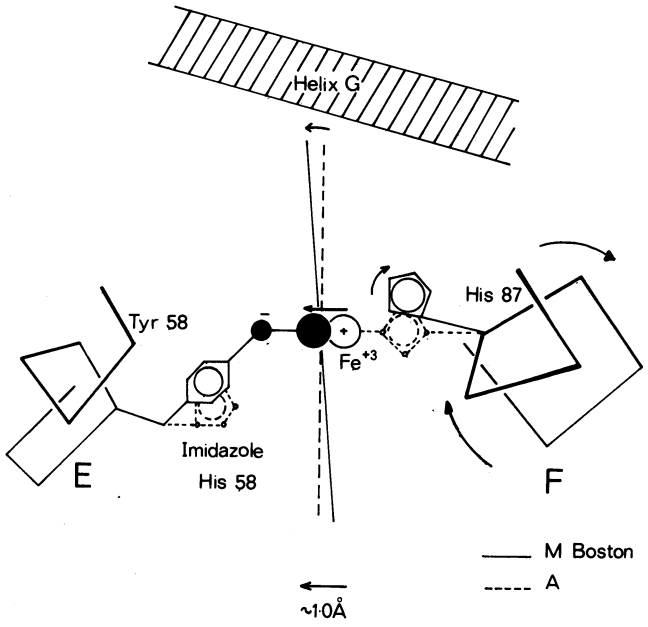

FIG. 5. Stereochemical changes in the heme pocket of the α subunit on going from deoxyHb A to Hb M Boston.

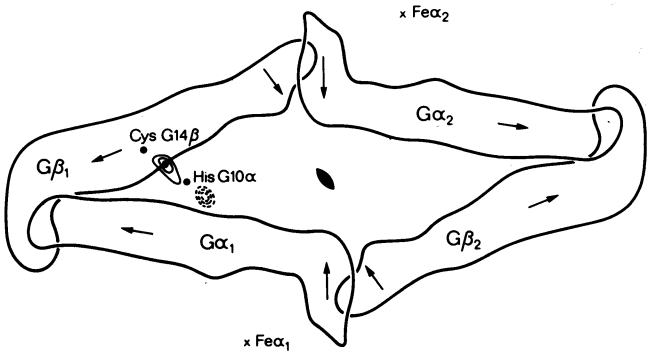

FIG. 6. Movements of helices Gα and β on going from deoxyHb A to Hb M Boston. The unexplained difference peaks near His G10α and Cys G14β (*c* in Fig. 3) are indicated on the left.

other parts of the α and β subunits (peaks *a*, *b*, and *c* in Fig. 4). One curious consequence of this rearrangement is the formation of a bridge of density linking the side chains of His G10(103)α and Cys G14(112)β. The negative and positive peaks flanking the histidine side chain look as though it had moved towards the cysteine (peak *c* in Fig. 3), yet the atomic model shows such a movement to be stereochemically impossible. Therefore, the difference peaks must represent something else, perhaps a counterion or a water molecule. Another noteworthy feature of Fig. 4 is the absence of a positive difference peak near the iron atom of the β heme, confirming the absence of a ligand. The stereochemical changes are summarized in Figs. 5 and 6.

Structure of $\alpha_2^{+B}\beta_2^{O_2}$ or $\alpha_2^{+B}\beta_2^{+H_2O}$. OxyHb M Boston was crystallized from concentrated phosphate buffer, but by the time the crystals had grown, the hemes in the normal β chains had become oxidized to met. 5° Screenless precession pictures about [001] showed that the crystals belong to the point group $\underline{4}2$. 17° Precession photographs about [100] and [110] indicate that the $00l$ reflections are absent for $l \neq 4n$ and $h00$ for $h \neq 2n$, which would make the space group either $P4_12_12$ or $P4_32_12$; however, the $h00$ reflections were really too weak to be sure that all odd orders were absent, so that the space groups $P4_12\ 2$ or $P4_32\ 2$ cannot be excluded. The unit cell dimensions are $a = 55.4$ Å, $c = 378$ Å, with $n = 8$. The tetragonal form of oxy or metHb A belongs to the space group $P4_12_12$ and has the unit cell dimensions $a = 53.7$ Å, $c = 193.5$ Å (11). Comparison of the two sets of dimensions shows that the lattices of metHbs M Boston and A are similar, except for a doubling of the *c* axis in the former.

Reaction of Crystals of $\alpha_2^{+B}\beta_2$ *with Oxygen or Carbon Monoxide.* When crystals of deoxyHb A are exposed to air, their high-angle diffraction pattern usually disappears within a few hours, or a day or two at the most. This is due to the disruption of the crystal lattice by the change in quaternary structure of the tetramer. When a crystal of $\alpha_2^{+B}\beta_2$ was exposed to air some intensity changes occurred, but the quality of the diffraction pattern was maintained for 4 weeks. When a crystal was exposed to CO, on the other hand, the diffraction pattern beyond 6-Å spacing disappeared within a few hours and the reflections below 6 Å showed marked changes in intensity. These results suggest that the lattice forces are sufficient to maintain $\alpha_2^{+B}\beta_2^{+H_2O}$ but not $\alpha_2^{+B}\beta_2^{CO}$ in the quaternary deoxy structure, presumably because it requires less strain energy to maintain the quaternary deoxy structure when the β hemes are high-spin ferric than when they are low-spin ferrous.

In Hb M Milwaukee [Val E11(67)β → Glu] reaction of the ferrous hemes in the normal α subunits with CO caused a spectral change of the ferric hemes in the abnormal β subunits which was shown to be linked to the accompanying change in quaternary structure (12). No comparable effect was observed in Hb M Boston, either because under our experimental conditions (0.1 M phosphate of pH 6.5) no change in quaternary structure occurred or because the iron atoms in the α subunits are uncoupled from their proximal histidines.

DISCUSSION

In the β chains of Hb M Milwaukee the bond from the iron to the proximal histidine (F8) is maintained and the carboxylate of Glu E11 provides the sixth ligand (12). Why is the bond to His F8α broken in Hb M Boston? The reason may be a simple steric one. If a tyrosine is substituted for His E7α in the atomic model of human deoxyHb, its phenolic oxygen comes to lie on a straight line from N_ϵ of His F8 through the center of, and roughly perpendicular to the plane of, the porphyrin, but the O–N distance is 6.5 Å. If the iron were to be 6-coordinated that distance would have to contract to not more than 4 Å, which could be achieved only by severely distorting the globin. Faced with the choice between forming a ferrous complex with the imidazole or a ferric one with the phenolate, the iron binds to the phenolate, for which its affinity is apparently much greater (25). In Hb M Milwaukee no such difficulty arises because the distance between N_ϵ of His F8β and the carboxylate oxygen of Glu E11β is only 4.5 Å. The displacement of the iron in Hb M Boston by 0.24 Å towards the distal side of the porphyrin, estimated from the slope of the difference density, is smaller than the displacement of 0.455 Å found in the comparable 5-coordinated high-spin compound methoxy-Fe^{3+}-mesoporphyrin IX-dimethylester (13). This result suggests that we may have underestimated the displacement of the iron atom.

TABLE 1. *Tentative interpretation of abnormal properties of hemoglobin M Boston*

Ref.	Property	Interpretation
	Clinical picture	
1	Carriers show cyanosis but no hemolytic anemia.	Cyanosis is due to the bias of the allosteric equilibrium towards the T structure, which lowers the oxygen affinity of the normal β subunits. Absence of hemolysis and heat stability are due to the strong bond between the ferric iron and the phenolate which makes the abnormal met α subunits more stable than normal aquometHb.
22	Rate of denaturation of metHb M Boston in 0.1 M phosphate of pH 6.8 at 64° is $^1/_4$ of that of metHb A.	
	α Hemes	
1	Absorption bands at 490 and 600 nm.	Characteristic for high-spin ferric hemoglobins, e.g., fluoro or high-spin form of hydroxy metHb.
20	ESR band at $g = 6.0$ normally present in aquometHb A is split into two bands at $g = 5.71$ and 6.30. Weak band at $g = 2.0$ is also present.	Splitting is due to asymmetry in plane of porphyrin produced by asymmetric position of phenolate.
21	No change in optical or ESR spectra of abnormal α subunits on reaction of normal β subunits with ligands	Absence of a change in quaternary structure and uncoupling of Fe–N_ϵ (His F8) bond inhibit interaction between hemes in β and α subunits.
22	Equilibrium constant for reaction with CN is 1000 times larger than for Hb A.	A sixth ligand can combine with the iron either by leaving the iron oxygen bond intact and pushing His F8 further out of the way, or by reforming the Fe–N_ϵ (His F8) bond and pushing the tyrosine side chain out of the way. Either mechanism would be opposed by steric and chemical factors.
1	Ferric form is reduced much more slowly by $Na_2S_2O_4$; ferrous CO form oxidized 50 times more rapidly by ferricyanide; ferrous oxy form autoxidized 4.5 times more rapidly than Hb A.	Reduction is opposed and oxidation favored because the affinity of ferric iron for phenolate is higher than the affinity of ferrous iron for imidazole (25).
	β Hemes	
18	In 0.2 M phosphate of pH 7.0, $p(O_2)_{50}$ is 4 times larger than that of Hb A. No Bohr effect. $n = 1.2$.	Bias of allosteric equilibrium towards T structure lowers oxygen affinity of normal β subunits and inhibits interaction between them. Absence of Bohr effect suggests that the salt bridges between His 146β, Lys 40α, and Asp 94β remain intact in $\alpha_2{}^{+B}\beta_2{}^{O_2}$.
2	Absorption maxima of β bands in $\alpha_2{}^{+B}\beta_2{}^{O_2}$ and	These may represent spectral differences that arise when the β
23	$\alpha_2{}^{+B}\beta_2{}^{CO}$ appear to be blue-shifted by several nm compared to their positions in Hb A (541 nm in Hb O_2 A and 540 nm in Hb CO A). ESR spectrum of azide complex differs in g values from that of azide metHb A.	subunits form part of either the quaternary R structure as in Hb A or the T structure in Hb M Boston.
21	Normal reactivity towards CN^-, N_3^-, and F^- of $\alpha_2{}^{+B}\beta_2{}^{+H_2O}$.	Affinities of aquometHb A in the T state for N_3^- and CN^- are practically equal to those in the R state (26).

ESR, electron spin resonance.

Another interesting result is the rotation of helix F on release from the iron–nitrogen bond, because it indicates a tension in the bond, pulling the iron upwards and to the right, away from the plane of the porphyrin (Fig. 5). The existence of such a tension in the T state had been inferred from optical and magnetic studies (14, 15), but this is the first direct evidence for it on an electron density map. Note that the tension tends to pull the iron in a direction that lies at an oblique angle rather than being perpendicular to the plane of the porphyrin. Unfortunately we cannot estimate the degree of rotation of helix F for lack of resolution. All the same, we can now begin to understand why the tertiary structure of the abnormal α chains biases the allosteric equilibrium of Hb M Boston towards the quaternary T state. Several crystallographic studies have shown that in Hb A changes in spin state of the heme iron are linked to a rotation of helix F, especially the turn of π helix which links His F8 to the FG corner (refs. 16 and 17 and E. G. Heidner, unpublished results). Looking from the direction of the EF corner, as in Fig. 5, helix F turns clockwise when the spin rises, i.e., when the iron–nitrogen bonds lengthen. This rotation sets in train stereochemical changes at the subunit contacts, thereby changing the allosteric equilibrium between the two alternative quaternary structures (17). In Hb M Boston helix F is turned in the direction of higher spin, i.e., iron–nitrogen distances longer even than in deoxyHb A. This rotation is transmitted to the $\alpha_1\beta_2$ contact and stabilizes it firmly in the deoxy conformation.

The unusually high stability of the quaternary T structure is corroborated by the preservation of the x-ray diffraction pattern on exposure of deoxyHb M Boston crystals to air. It is also consistent with the low oxygen affinity [$\log p(O_2)_{50} = 1.6$], low cooperativity ($n = 1.2$), and absence of a Bohr effect observed in the reaction of the β subunits with oxygen in 0.2 M phosphate buffer (18). If this is correct, then the cyanosis observed in patients heterozygous for Hb M Boston would be caused by failure of the tetramer to undergo the transition to

the quarternary R state on reaction with oxygen in the erythrocyte. On the other hand, the disappearance of the x-ray diffraction pattern on exposure of crystals of deoxyHb M Boston to CO, and the crystallization of $\alpha_2^{+B}\beta_2^{+H_2O}$ in a lattice closely related to that of metHb A indicate that the tetramer is not *locked* in the quaternary T structure. The T → R transition may not take place *in vivo* or in dilute phosphate buffer, but it does seem to occur when the salt bridges that stabilize the T structure are weakened by very concentrated buffers. Hb M Boston exhibits several other unusual properties; they are summarized in Table 1, together with tentative interpretations in the light of its structure.

Finally, we want to consider the relationship of Hb M Boston to other abnormal hemoglobins of the M type. Structurally, its nearest relative is Hb M Saskatoon [His E7(63)β → Tyr] in which the distal histidines of the β chains are replaced by tyrosines. It has a similar absorption spectrum, with high-spin bands at 490 and 600 nm, indicative of a similar stereochemistry at the heme, but its oxygen affinity and Bohr effect are high (19), which suggests that reaction of its normal α subunits with oxygen switches its quaternary structure from T to R as it does in Hb M Milwaukee. We can offer no explanation as yet for this difference in behavior. Functionally, the nearest relative to Hb M Boston is Hb M Iwate [His F8(87)α → Tyr] in which the proximal histidines of the α subunits are replaced by tyrosines. Like M Boston, it remains in the T state on reaction of the normal β subunits with oxygen, but as its x-ray analysis could not be carried beyond 5.5 Å resolution, the stereochemical reasons for the stability of its T structure are not yet understood.

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