

Molecular description of dioxygen bonding in hemoglobin*

(porphyrin/myoglobin/cooperativity/generalized valence bond/configuration interaction)

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ABSTRACT From *ab initio* quality calculations on model systems, we conclude that in unliganded Fe-porphyrin the Fe lies in the plane for both the high-spin (*q*) and intermediate-spin (*t*) states. Thus, the high-spin d^6 Fe is not too big to fit into the porphyrin plane (as often suggested). We find the *q* state lower for a porphyrin hole radius >1.94 Å and the *t* state lower for smaller sizes. For the five-coordinate complex including an axial nitrogenous ligand [a model for myoglobin (Mb) and hemoglobin (Hb)], we find the ground state to be *q* with the Fe 0.3 Å out of the plane (recent x-ray data on deoxy Mb suggests about 0.4 Å). The origin of this out-of-plane displacement is the nonbonded repulsions between the axial ligand and porphyrin nitrogen orbitals. Pushing the Fe of the five-coordinate complex into the plane does not lead to a stable low-spin state (as usually suggested), the *q* and *t* states being the low-lying states.

Bonding the O_2 to form the six-coordinate complex stabilizes the *t* form of the Mb model, leading to a singlet state of MbO_2 with Fe in the plane. (It has often been suggested that the Fe of MbO_2 and HbO_2 is low-spin Fe^{2+} ; however, we find this not to be the case.) The bonding in the MbO_2 model confirms the ozone model of the bonding, leading to a structure consistent with the Pauling model (our calculated $FeOO$ bond angle is 119°). The total charge transfer to the O_2 is 0.10 electron, in disagreement with the Weiss model. Molecular orbital calculations (Hartree-Fock) incorrectly lead to a septet ground state ($S = 3$) for the MbO_2 model.

The implications for the cooperative O_2 binding in hemoglobin and protein modifications of the energetics of the active site are considered. Use of our calculated force constants for displacement of Fe perpendicular to the heme plane suggests that the movement of the Fe upon a change in the quaternary structure from the *T* to the *R* form is only about 0.04 Å toward the heme plane.

We will discuss the bonding of oxygen to hemoglobin (Hb) or myoglobin (Mb) using the results of theoretical calculations of the electronic structure of model systems representing Fe-porphyrin, Fe-porphyrin plus an additional nitrogenous axial ligand (deoxy Mb), and O_2 bound to the latter five-coordinate complex (MbO_2). In all cases the Fe is found to be high- or intermediate-spin ($S = 2$ or 1) with six electrons in the *d* orbitals. In no case (not even with the six-coordinate complexes) do we find a low-lying state in which the Fe is in a low-spin state (t_{2g}^6 , $S = 0$). We find that the properties of these model complexes are consistent with the observed properties of the active sites of Mb and Hb and that these calculations give additional insight into the protein-modified behavior of the active site of Hb. In this paper we outline the qualitative description that emerges; detailed results will appear elsewhere.

Model calculations

As a model for the theoretical study of the bonding of O_2 to Mb and Hb, we have used four NH_2 groups‡ in a plane (D_{4h} sym-

Abbreviations: Hb, hemoglobin; Mb, myoglobin; *q*, high-spin; *t*, intermediate spin; *s*, low-spin.

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‡ The geometry used was $R_{NH} = 1.014$ Å and $\angle HNH = 120^\circ$. The four NH_2 groups were always taken with C_{4v} symmetry.

metry) to represent the four-coordinate porphyrin ligand and an NH_3 group to represent the axial imidazole ligand. *Ab initio* quality calculations including electron correlation (many-body) effects [generalized valence bond (26) plus configuration interaction] were carried out for a number of different molecular geometries of the four-, five-, and six-coordinate complexes§. The four-coordinate calculations were carried out with both minimal basis set and valence double zeta basis; the other calculations used only the former¶. An effective potential was used to replace the 18 core electrons of Fe (1-3). All of our model systems have overall charge neutrality, as is appropriate for the systems being modeled.

By representing the porphyrin ligand as four NH_2 groups, we lose the geometric stabilization resulting from the bridging methene groups. We have made no attempt to correct our calculated results for this effect in this paper, and therefore our unconstrained model system will lead to optimized geometries in which the hole radius is artificially smaller than the corresponding porphyrin system. This effect appears to result in calculated porphyrin N-Fe distances that are systematically underestimated by about 0.03 Å.

Four-coordinate model complexes

First we consider the four-coordinate complex with the *x* and *y* axes along the Fe-N bonds. The two important states are high-spin Fe (denoted as *q* for quintet, $S = 2$)

$$q: (d_{zz})^2(d_{xy})^1(d_{yz})^1(d_{xz})^1(d_{x^2-y^2})^1 \quad [1]$$

and intermediate-spin Fe (denoted as *t* for triplet, $S = 1$)

$$t: (d_{zz})^2(d_{xy})^2(d_{yz})^1(d_{xz})^1. \quad [2]$$

A third state, corresponding to low-spin Fe (denoted as *s* for singlet, $S = 0$)

$$s: (d_{zz})^2(d_{xy})^2(d_{yz})^2,$$

is also considered because it has often been thought to be important for the five- or six-coordinate cases. Other states with different configurations of *d* electrons were calculated, but the above states were the lowest lying states of their respective spin

§ There is one correlated pair involving the "porphyrin N" π system in all calculations; in cases having an empty $d_{x^2-y^2}$ orbital, one porphyrin N σ pair is correlated; in the oxygenated complex, the FeO and OO σ bonds are correlated as is the FeOO π bond (see ref. 19). An extensive configuration interaction was carried out over the doubly occupied valence orbitals, the open-shell orbitals, and the correlated orbitals (17 total orbitals), allowing up to 3056 spin eigenfunctions (4456 determinants).

¶ The basis used for Fe was a modification of the Gaussian basis of Wachter [(1970) *J. Chem. Phys.* **52**, 1033-1035] using (5d4s4p) contracted to [1d1s1p] for minimum basis set calculations and [2d3s2p] for valence double zeta calculations. The Pople STO-4G basis was used for O, N, and H in the minimum basis set calculations [(1969) *J. Chem. Phys.* **51**, 2657-2664] and the Pople STO 4-31G basis was used for N in the valence double zeta calculations [(1971) *J. Chem. Phys.* **54**, 724-728].

for all the complexes we considered. Optimizing the Fe–N bond length of the four-coordinate complex, we find the optimal bond length of the q state to be 1.95 Å, which is 0.14 Å longer than the optimal bond length, 1.81 Å, found for the t state. This difference is due to the q state containing an electron in the antibonding $d_{x^2-y^2}$ orbital; however, our calculated optimal Fe–N bond distance for high-spin Fe of 1.95 Å is far shorter than the value (approximately 2.2 Å) previously estimated (4, 5). The reason for this discrepancy is that previous estimates were based on Fe and Co complexes consisting of *saturated* nitrogen ligands (possessing a coordinate bond to the Fe), whereas the ligands of our complex (and porphyrin) should be viewed as partially *unsaturated* (two of the four Fe–N complexing bonds have significant covalent character).

Of course the constraints in the porphyrins do not easily allow radii less than 1.9 Å [free base porphyrins have hole radii of about 2.05 Å (6)]; however, Baldwin and Huff (7) have made an analogous planar four-coordinate complex which has a much shorter unconstrained radius, and they find an “anomalously” short Fe–N bond of 1.84 Å (8); this is in good agreement with our value of 1.81 Å for the t state.

We find that the t state is lower than the q state for hole radii ≤ 1.94 Å. The calculations clearly suggest that the Baldwin-Huff compound has a triplet ground state [in agreement with the measured experimental magnetic moment of $2.8 \mu_B$ at 28° (8)]. Fe-porphyrin systems with Fe–N distances close to 1.94 Å should have a small difference in energy between the q and t states [e.g., Fe tetraphenylporphyrin has Fe–N = 1.972 Å and a magnetic moment of $4.4 \mu_B$ at 25° (9)]. We find that the s state is 2.9 eV higher than the q state (for a hole radius of 2.01 Å); consequently, geometry searches were not carried out for this state.

An important result from these calculations is that, even for porphyrin hole radii as small as 1.85 Å, the optimal position of the Fe is *in the plane* for the q state (and for the t state). That is, even for hole radii as small as 1.85 Å, the attraction of the Fe atom for the macrocycle (favoring the planar geometry) is larger than the antibonding interactions of the $d_{x^2-y^2}$ orbital (favoring nonplanarity). This is contrary to the commonly accepted belief (13) that high-spin Fe is too large to fit into the porphyrin pocket and hence, that in order to form high-spin complexes, the Fe must move out of the porphyrin plane. However, the presence of the electron in the $d_{x^2-y^2}$ antibonding orbital *does* lead to a smaller force constant for out-of-plane motion in the q state as compared with the t state. This difference in force constants is important in analyzing the cooperativity of Hb, as discussed below.

Five-coordinate model complexes

It has long been known that five-coordinate Fe-porphyrin complexes are generally high-spin (14, 15) with the Fe significantly out of the porphyrin plane (5, 16, 17). The usual explanation is that high-spin Fe is too big to fit in the plane of the

porphyrin. The above calculations contradict this argument since high-spin Fe *does* lie in the porphyrin plane for the unliganded Fe-porphyrin complex for the range of accessible porphyrin hole radii.

To model deoxy Hb we used NH₃ for the fifth ligand and optimized the Fe-to-plane distance and also the Fe–NH₃ distance. The results are: (i) the ground state is q with the Fe 0.25 Å out of the plane, (ii) the state t is 20 kcal higher and only 0.13 Å out of the plane, and (iii) for Fe 0.25 Å out of the plane, the Fe–NH₃ distance is 2.1 Å.

The five-coordinate Fe porphyrin systems on which crystal structure information is available all possess a domed porphyrin ring—e.g., in Fe tetraphenylporphyrin (2-methylimidazole) the porphyrin N plane is displaced 0.13 Å from the mean porphyrin plane and the pyrrole groups rotate by about 9° (18). We considered such doming in our model and find that the optimal Fe position is 0.28 Å from the N plane. In comparing with experiment, we will ignore any corrections resulting from replacing the NH₃ axial ligand with imidazole.

Comparisons with experiment are difficult because no published crystal structure analysis has led to a reliable value for the distance of the Fe from the porphyrin on an analogous system. Radonovich and Hoard (18) examined Fe 2-methylimidazole and found that the Fe is 0.42 Å out of the N plane and 0.55 Å out of the mean porphyrin plane. The methyl group leads to a short contact with the porphyrin and hence may lead to an increase over the value for unsubstituted imidazole. Recent high-resolution (2 Å) x-ray diffraction studies on Mb (T. Takano, personal communication) lead to Fe 0.55 Å from the mean porphyrin plane with the Fe about 0.42 Å from the porphyrin N plane, suggesting that the protein causes at most only a slight increase in the Fe-porphyrin separation.

Since the q state has the same electronic configuration for four- and five-coordinate complexes and since the high-spin Fe is *in the plane* for the four-coordinate, a natural question is, “Why does the Fe move so far out of the plane for five-coordinate complexes?” We find that the answer is *nonbonded interactions* between the bond pairs of the fifth ligand and the N orbitals of the porphyrin.

To test this idea we repeated the calculations of the five-coordinate complex but with the Fe atom deleted. Moving the axial ligand from infinite separation to a position 2.1 Å from the porphyrin–N plane (appropriate for a five-coordinate complex with Fe in the plane) leads to an increase of 15 kcal in the energy of the system. The origin of this energy increase is just the nonbonded repulsions (arising from the Pauli principle) between the NH₃ and porphyrin electron pairs. As a result, Fe moves out of the plane until there is a balance between the five attractive Fe–N interactions and the repulsive nonbonded interactions. These calculations are a strong indication that the size of high-spin Fe is not the only major factor in determining the Fe out-of-plane distance for five-coordinate complexes.

These nonbonded interactions are sensitive to porphyrin ring size. Decreasing the porphyrin hole size from 2.01 Å to 1.85 Å leads to a doubling in the calculated distance of the Fe from the N plane. However, the calculated force constant increases by only 13%.

For the five-coordinate complex (q state, Fe 0.3 Å out of plane) we find an optimal porphyrin hole radius 0.02 Å longer than that found for the four-coordinate q state.

O₂ bonded to the five-coordinate complex

Next we consider the bonding of dioxygen to the five-coordinate complex. It has generally been assumed that pushing the Fe into the porphyrin plane (as required for the six-coordinate

|| There seem to be difficulties in interpreting the magnetic susceptibility and Mössbauer experiment on Fe tetraphenylporphyrin, Fe phthalocyanine, and the Baldwin-Huff compounds. For example, the magnetic susceptibility of Fe phthalocyanine can be fit for a triplet ground state ($^3B_{2g}$), but only with a very large zero-field splitting parameter ($D \approx 64 \text{ cm}^{-1}$) (10, 11). Also, considering only the d orbitals of Fe, a $^3B_{2g}$ state of Fe would yield an electric field gradient whose major component has a negative sign, in disagreement with Mössbauer experiments (12). Our calculations indicate that the $^3B_{2g}$ state lies 0.3 eV higher than the t state. In light of these difficulties and considering the similar energies calculated for the t and q states, there is no conclusive experimental evidence of the ground configuration of the Fe in such systems.

oxygenated complex) stabilizes the s state of Fe, leading hence to the observed diamagnetic character. We find that the s state plays no role, lying about 2 eV above the ground state.

The current calculations including all six ligands confirm our "ozone model" for the bonding of dioxygen to Hb (19). Thus, we find that (i) a covalent sigma bond is formed between the d_{z^2} orbital of Fe and an oxygen p orbital and (ii) the d_{yz} orbital is paired with the three-electron pi system of the O_2 ligand. The result is that bonding O_2 to the t state of Mb leads to an overall diamagnetic singlet state ($S = 0$) for MbO₂.

$$t: [(d_{z^2})^1, (O_2\sigma)^1] \\ [(d_{yz})^1, (O_2\pi)^1] \\ (d_{xy})^2 \\ (d_{xz})^2 \quad [3]$$

in which orbitals in brackets correspond to bond pairs. Similarly, bonding O_2 to the q state leads to an overall paramagnetic triplet state ($S = 1$) for MbO₂.

$$q: [(d_{z^2})^1, (O_2\sigma)^1] \\ [(d_{yz})^1, (O_2\pi)^1] \\ (d_{xy})^1 \\ (d_{xz})^2 \\ (d_{x^2-y^2})^1. \quad [4]$$

With either state, in order for O_2 to form a bond with Fe, the Fe must move back into the plane of the porphyrin to decrease the repulsive nonbonded interactions between O_2 and the porphyrin nitrogens.

The question now is: "Which state, q or t , should be lower for MbO₂?" We find that, even with the Fe in the plane of the five-coordinate complex, the q state is lower than the t state (for porphyrin radii >1.95 Å); hence, one might expect that the paramagnetic q state (Eq. 4) of MbO₂ would be the ground state. However, our calculations lead to the diamagnetic t state as the ground state of the oxy complex (with the q state 8 kcal higher).

This result may seem mysterious. Both the q and t states can make similar bonds to O_2 and the q state is lower than t for the deoxy complex; yet the t state is lower for the oxy complex. The reason behind this interchange in ordering of q and t lies in the exchange interactions. The q state is lower than the t for the deoxy complex because of the extra stabilization due to the exchange interactions of the four unpaired orbitals of the q state (six exchange terms) as compared with the t state (three exchange terms arising from the corresponding four electrons). Pairing two orbitals in each state with the O_2 to form the bonds leaves two unpaired orbitals for q (decreasing the number of exchange terms by three) and no unpaired orbitals for t (decreasing the number of exchange terms by only one). Thus, the relative stabilization of q with respect to t is reduced by two exchange terms upon bonding the O_2 . Since each exchange term is about 1 eV, this effect is enough to interchange the ordering of q and t between deoxy Mb and MbO₂.

We calculate that 0.10 electron is transferred to the O_2 [in agreement with our earlier calculations (19)], contradicting the Weiss model (20) of the bonding. We calculate an optimal FeOO angle of 119°, in reasonable agreement with the value

of 136° for model MbO₂ systems (27) and 120° for model CoO₂ systems (28). Indeed, our overall wavefunction and geometry are quite consistent with the early model of Pauling (21). The optimal ring size for the six-coordinate complex is 0.03 Å larger than that found for the four-coordinate complex. Again, this lengthening of the hole radius is apparently due to nonbonded repulsions between the porphyrin N and the axial ligands.

We also carried out Hartree-Fock or molecular orbital calculations on the six-coordinate complex. We found that this leads to a ground septet state ($S = 3$) with additional quintet ($S = 2$) and triplet ($S = 1$) states lying below the closed-shell singlet state. Thus, these calculations are not consistent with a diamagnetic ground state of MbO₂. Consequently, it is essential that theoretical studies of this system include electron correlation (many-body) effects as in these generalized valence bond-configuration interaction studies. Recently, *ab initio* Hartree-Fock calculations (22, 23) have been reported for an Fe-porphyrin complex using an NH₃ ligand and one O_2 ligand. They report a lower energy for the triplet paramagnetic state than for the singlet diamagnetic state (in agreement with our finding) but did not report energies for the quintet and septet states.

Cooperative oxygen binding

We will now consider the implications of the above molecular model in understanding the nature of heme-heme interaction leading to cooperative oxygen binding. That is, how might the protein modify the function of the heme binding site?

It is generally believed that the difference in affinity for O_2 is dependent mainly on the quaternary state of Hb. Hoard (13) suggested that the large displacement of the Fe to the porphyrin plane accompanying the high-spin to low-spin transformation associated with bonding of O_2 could require stereochemical alterations near the heme that would trigger the cooperative oxygenation in Hb. Perutz *et al.* (17, 24) provided a detailed model of these stereochemical alterations, showing how they lead to both cooperativity and the Bohr effect. However, there is still much to learn concerning the microscopic details of the difference in O_2 bonding for the two quaternary forms.

We can separate the different energy (enthalpy) contributions of O_2 binding in Hb as follows.

1. Starting with Hb in its optimal geometry for the q state (Fe out of the plane) and moving the Fe-imidazole group to the point where Fe is in the heme plane will change the energy of the system by

$$\Delta E_q = -E_q (\text{Fe at equilibrium}) + E_q (\text{Fe in the plane}).$$

2. With Fe in the plane, the energy separation of the q and t states of Hb is

$$\Delta E_{qt} = E_t (\text{Fe in the plane}) - E_q (\text{Fe in the plane}).$$

3. Given Hb in the t state with the Fe in the plane, the bond energy for bonding O_2 is

$$D_t = E_t (\text{Hb} + O_2, \text{Fe in plane}) - E_t (\text{HbO}_2, \text{Fe in plane}).$$

The total energy for binding O_2 to a particular heme in deoxy Hb becomes

$$\bar{D} = D_t - \Delta E_{qt} - \Delta E_q. \quad [5]$$

The difference in O_2 affinity for the T and R quaternary forms of Hb (T being the tense or low-affinity form stable for Hb and R being the relaxed or high-affinity form stable for fully oxygenated HbO₂) must arise from one or more of the energy terms in Eq. 5 being dependent upon the quaternary form. For example, (i) steric effects of side chains on the distal side of the

** This analysis has been simplified a bit; in the configuration interaction wavefunction a part of these intra-atomic exchange terms is retained at the sacrifice of a slightly worse pairing of the bonding orbitals.

heme could modify D_t between the T and R forms, (ii) rotation of the vinyl groups or propionic side chains into or out of the porphyrin plane could modify the conjugation of the porphyrin and thereby affect ΔE_{qt} or ΔE_q , (iii) movement of groups such as valine $E11$ or histidine $E7$ into the porphyrin could modify the conjugation and thereby ΔE_{qt} or ΔE_q , (iv) distortions in the protein modifying the effective size of the porphyrin hole would change ΔE_{qt} and ΔE_q , and (v) hindered motion of the proximal imidazole could change ΔE_q .

Nuclear magnetic resonance evidence by Shulman *et al.* (25) argues against significant changes in the porphyrin conjugation and thereby against (ii) and (iii) and probably (iv) above, indicating that the difference in O_2 affinity between the R and T forms is probably due to ΔE_q being different between the two forms. If this is the case, our calculated potential energy surfaces for movement of this Fe-imidazole group in the deoxy complex allows us to estimate the difference in the Fe displacement between the unliganded T and R forms [the analysis is similar to that of Hopfield (29)]. Earlier results from x-ray crystallographic studies indicated a very large difference in Fe position, perhaps as large as 0.3–0.4 Å, between the two forms. Our calculations indicate a much smaller change, about 0.05 Å Fe movement, as a result of the change in quaternary structure.

From our calculations we find the optimal Fe out-of-plane distance for the five-coordinate q state to be 0.28 Å with $\Delta E_q = 7.8$ kcal††. We calculate a force constant for moving the Fe-imidazole unit toward the porphyrin plane of $k = 200$ kcal/Å:

$$\begin{aligned} E_q &= 7.8 \text{ kcal} \\ x &= 0.28 \text{ Å} \\ k &= 200 \text{ kcal/Å.} \end{aligned}$$

The crystal structure data (T. Takano, personal communication) on Mb lead to $x = 0.42$ Å, whereas we calculate $x = 0.28$ Å for the free complex. Such a difference could be real; however, considering the uncertainties in both experimental and theoretical values, we will assume that the forces on the Fe for Mb and for the R form of Hb are much the same as in the free complex:

$$\begin{aligned} x_R &= 0.42 \text{ Å} \\ k_R &= 200 \text{ kcal/Å.} \end{aligned} \quad [6]$$

Assuming the cooperative effect to be due to ΔE_q then requires that

$$\Delta E_q^T = \Delta E_q^R + 3.6 \text{ kcal.}$$

We assume that this increase in ΔE_q is due to the increased rigidity of the tertiary structure in the T form of the protein (e.g., due to the extra salt bridges). Pulling the Fe into the plane must act against this additional protein force, F_p , increasing ΔE_q . This force will act upon the unliganded Fe to distort it to a new position, x^T . Assuming F_p is constant, x_T and x_R are related by

$$3.6 \text{ kcal} = \frac{1}{2} k(x_T^2 - x_R^2)$$

leading to

$$x_T - x_R = 0.041 \text{ Å.}$$

†† Note that this barrier is for the case in which the porphyrin is not allowed to distort. The adiabatic barrier should be smaller.

Thus, the force of the protein on the Fe-imidazole group merely shifts the energy surface for pulling Fe-imidazole into the plane by an amount such that ΔE_q is increased by 3.6 kcal. This small displacement corresponds to $F_p = 8.2$ kcal/Å.

The most critical assumption in the above analysis is that the Fe-imidazole group is constrained by the protein in the T form and is essentially free of protein forces in the R form. The presence of additional protein forces in the R form will increase the displacement of the Fe between the two forms. Experimental data consistent with such a small change are now emerging. Recent crystal structure studies (T. Takano, personal communication) of deoxy Hb and deoxy Mb suggest a displacement $x_T - x_R = 0.05$ Å.

Summary

On the basis of model calculations we conclude the following.

1. In unliganded Fe-porphyrin, the ground state is q or t , depending upon the radius of the porphyrin hole.

2. The optimal position of the four-coordinate Fe is in the plane, even for the q state.

3. Addition of an axial ligand leads to an optimal Fe position out of the plane due to a balance between attractive interactions of the Fe to the axial and porphyrin ligands and repulsive nonbonded repulsions of the axial and porphyrin nitrogens. For five-coordinate complexes these effects can serve to stabilize the q state, even for porphyrin radii for which the t state is lower at the Fe in-plane geometry.

4. It was previously assumed that pushing five-coordinate Fe into the plane stabilizes the s state of Fe. We find that, even with Fe in the plane, both high-spin Fe and intermediate-spin Fe are significantly better than low-spin. In addition, for porphyrin hole radii typical of MbO₂ the q state is the lowest (even with the Fe in the plane).

5. Bonding an O₂ to the five-coordinate complex pairs two of the Fe orbitals and moves the Fe back into the plane, leading to an overall bond much as in ozone and a geometry close to that suggested by Pauling. This stabilizes the t state of the Fe, leading correctly to a diamagnetic ground state of MbO₂. The Fe of FeO₂ is not in the s state as is usually assumed. The charge transfer from Fe to O₂ (0.10 electron) is in disagreement with the Weiss model.

6. Electron correlation effects are of great importance in the oxygenated complex; Hartree-Fock (or molecular orbital) calculations lead to a septet (paramagnetic) ground state with quintet and triplet states also lying below the diamagnetic closed-shell singlet state.

7. Considering this model allows us to partition the O₂ affinity into three logically distinct terms that can be affected differently by the change from the T to R quaternary forms. Using our calculated force constant of the free five-coordinate complex, we predict that in the T quaternary form of Hb the Fe is displaced only about 0.04 Å farther from the N plane than in the R form.

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