How $O_2$ binds to heme:

Reasons for rapid binding and spin inversion

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Running title: $O_2$ binding to heme
We have used density functional methods to calculate fully relaxed potential energy curves of the seven lowest electronic states during the binding of \( \text{O}_2 \) to a realistic model of ferrous deoxyheme. Beyond a Fe-O distance of \( \sim 2.5 \) Å, we find a broad crossing region with five electronic states within 15 kJ/mol. The almost parallel surfaces strongly facilitate spin inversion, which is necessary in the reaction of \( \text{O}_2 \) with heme (deoxyheme is a quintet, \( \text{O}_2 \) a triplet, whereas oxyheme is a singlet). Thus, in spite of a small spin-orbit coupling in heme, the transition probability approaches unity; using reasonable parameters, we estimate a transition probability of 0.06-1, which is at least 15 times larger than for the non-biological Fe-O\(^+\) system. Spin crossing is anticipated between the singlet ground state of bound oxyheme, the triplet and septet dissociation states, and a quintet intermediate state. The fact that the quintet state is close in energy to the dissociation couple is of biological importance, because it explains how both spin states of \( \text{O}_2 \) may bind to heme, thereby increasing the overall efficiency of oxygen binding. The activation barrier is estimated to be less than 15 kJ/mol based on our results and Mössbauer experiments. Our results indicate that both the activation energy and the spin-transition probability are tuned by the porphyrin as well as by the choice of the proximal heme ligand, which is a histidine in the globins: Together, they may accelerate \( \text{O}_2 \) binding to iron by \( \sim 10^{11} \) compared to the Fe-O\(^+\) system. A similar near-degeneracy between spin states is observed in a ferric deoxyheme model with the histidine ligand hydrogen bonded to a carboxylate group, i.e. a model of heme peroxidases, which bind \( \text{H}_2\text{O}_2 \) in this oxidation state.
Introduction

All electrons have a spin, which is an intrinsic quantum chemical property that can take only two possible values, normally called \( \alpha \) and \( \beta \) (or spin up and down). Almost all normal organic molecules contain an even number of electrons and also an equal number of \( \alpha \) and \( \beta \) electrons. They are then said to have paired spin or to be singlets. Molecular oxygen (\( O_2 \)) is a famous exception to this rule: In its ground state, it has two more electrons of one spin state than the other. Thus, it is said to have two unpaired electrons or to be a triplet. The singlet state of \( O_2 \), with all electron spins paired, is \(~90 \text{ kJ/mol}\) higher in energy than the triplet ground state (1).

A chemical reaction can normally not change the spin state of an electron. Therefore, reactions between singlet and triplet states are formally spin-forbidden, which means that they are slow. This is the reason why organic matter may exist in an atmosphere containing much \( O_2 \): there is a strong thermodynamic drive of \( O_2 \) to oxidize organic matter to \( H_2O \) and \( CO \), but because these products, as well as the organic molecules are singlets, whereas \( O_2 \) is a triplet, this reaction is spin-forbidden and therefore very slow at ambient temperatures. On the other hand, this is a problem when living organisms want to employ \( O_2 \) in their metabolism – the reactions are still spin-forbidden and slow.

Nature has handled this problem by using transition metals to carry, activate, and reduce \( O_2 \). There are many reasons for this choice. First, most transition metals also contain unpaired electrons, making reactions with triplet \( O_2 \) allowed. Second, transition metals are relatively heavy atoms, which increases spin-orbit coupling (SOC) and thereby a quantum-mechanical mechanism to change the spin state of an electron, called spin inversion. However, the SOC of the first-row transition metals is too small to alone allow for spin transitions. Third, transition metals often have several excited states with unpaired electrons close in energy to the ground state. This can also be used to enhance the probability of spin inversion.

One of the most simple biological reactions involving molecular oxygen is the binding of \( O_2 \) to hemoglobin, i.e. the binding of \( O_2 \) to the Fe(II) ion in a heme group. This reaction is formally spin-forbidden, because the reactant deoxyheme contains four unpaired electrons in the 3\( d \) orbitals of iron (it is a quintet) and triplet \( O_2 \) has two unpaired electrons. Thus, depending on the relative direction of these two sets of unpaired electrons, the adduct would be expected to have two (4 – 2) or six (4 + 2) unpaired electrons (i.e. a triplet or a septet state). However, experimentally, the
product complex is a singlet state with an equal number of $\alpha$ and $\beta$ electrons. As discussed already by Pauling (2, 3), this problem makes the hemoglobin reactions troublesome to understand (4), and it is not clear how nature has coped with the spin-forbidden nature of this reaction. The importance of spin inversion is also reflected in the Perutz model of hemoglobin co-operativity (5-7): The movement of iron into the heme plane is assumed to trigger a transition from a tense $T$ state to a relaxed $R$ state after binding of two oxygen molecules, and this trigger, in the form of the Fe-$N_{\alpha}$ pull, depends on the spin state of heme.

Theoretical methods have been successfully applied to many problems in heme chemistry. Already the simple Hartree-Fock formalism correctly predicts the bent form of the $O_2$ adduct (8), whereas state-of-the-art density functional theory (DFT) provides excellent geometries of porphyrins in general (9-13). Among these, the B3LYP density functional predicts very close-lying quintet and triplet states in deoxyheme models, sometimes with a triplet ground state (14). Recently, DFT was used to compute the electronic spectrum of Fe$^{II}$porphine with 2-methylimidazole as the axial ligand, giving the quintet state lowest but the triplet state only 12 kJ/mol higher in energy (15), in excellent agreement with experiment. These circumstances indicate that the treatment of spin states in porphyrins is delicate, owing to the closeness in energy of various spin states. The fact that spin inversion occurs in globins during oxygen binding means that all low-lying states, independent of their number of unpaired electrons, need to be considered in a proper study of the reaction. Spin-dependent mechanisms relevant for the present work have been studied in particular by Franzen (16) and Harvey (17).

In this work, we have optimized the ground state and several low-lying excited states at many points along the heme-$O_2$ binding curve. Our results indicate that the reason for the facilitated binding of $O_2$ to heme is a broad crossing region of the relevant spin states, which provides significant transition probabilities. We show that porphyrin is an ideal iron ligand for the spin-transition problem, because it tunes the spin states to be close in energy, giving parallel binding curves, small activation energies, and large transition probabilities. This finding explains why the porphyrin ring is designed to bring spin states close in energy and why spin inversion and reversible binding is possible in heme proteins. We also provide evidence that similar arguments apply to other heme proteins, e.g. the heme peroxidases, where near-degeneracy, in this case in the ferric state, is caused by strengthening the ligand field
of the proximal histidine by a hydrogen bond to a carboxylate group. Hence, we suggest a new role for the choice of axial ligand in such systems, viz. to bring spin states close in energy and thereby facilitate spin-forbidden binding of ligands.

Methods
In the present work, we study the reversible binding process

\[
\text{Fe}^{II}(\text{heme}) + \text{O}_2 \leftrightarrow \text{Fe}^{II}(\text{heme})(\text{O}_2)
\]

with particular emphasis on analyzing possible states along this reaction coordinate. Such a detailed approach seems to be necessary to study the nature of the reversible process.

Computational details
All geometry optimizations were performed with the Becke 1988 exchange functional together with the Perdew 1986 correlation functional (BP86) (18, 19). Accurate energies were then estimated by single-point calculations with the Becke three-parameter hybrid method with the local spin-density approximation correlation functional of Vosko-Wilk-Nusair and the non-local Lee-Yang-Parr correlation functional (B3LYP) (20-25). B3LYP is probably the most accurate of the generally available exchange-correlation functionals for calculating relative energies and frequencies (26-29). However, in our experience, BP86 provides slightly better geometries for metal complexes than B3LYP and at an appreciably lower cost (30).

The calculations were carried out with the Turbomole software, version 5.6 (31). The basis sets used for geometry optimization were 6-31G(d) for all atoms except iron, which was described by the double-\(\zeta\) basis set of Schäfer \textit{et al.} (32), augmented with two \(p\), one \(d\), and one \(f\) functions (DZpdf) (33) with the contraction scheme (14s11p6d1f) / [8s7p4d1f]. Only the pure five \(d\) and seven \(f\)-type functions were used. Our basis set is balanced and based on experience (34), flexible enough to account for the electronic structure and polarization effects encountered in heme systems. We applied the default (m3) grid size of Turbomole, and all optimizations were carried out in redundant internal coordinates. Unrestricted calculations were performed for all open-shell systems. We made use of default convergence criteria,
which imply self-consistency down to $10^{-6}$ Hartree (2.6 J/mol) for the energy and $10^{-3}$ a.u. for the maximum norm of the gradient.

**Model system**

All calculations were performed on the Fe$^{II}$PorImO$_2$ model, where Por is porphine (heme without side chains) and Im is imidazole, a model of the proximal histidine. We calculated the structure and energetics of Fe-O bond breaking for the seven lowest states by systematically increasing the Fe-O bond distance and optimizing the structure with a fixed Fe-O distance. From the fully optimized potential energy surfaces, we obtain the crossing points of the various spin states involved in the binding mechanism. Strictly speaking, proper crossing points would require identical structures of the states (true transition states). Methods to obtain such structures have been developed, but the geometry effect is usually small and does not significantly affect the location of the crossing point along the reaction coordinate (17, 35).

We found that the most stable state of the Fe$^{II}$PorImO$_2$ model had $C_s$ symmetry. This is consistent with the most accurate crystal structure of oxymyoglobin (1.0 Å resolution) (36), in which the imidazole ligand has a staggered conformation, with a C-N$_{ax}$-Fe-N$_{eq}$ torsion angle of 45°. O$_2$ adopts a four-fold occupancy in the thermally disordered structure at staggered conformations with respect to the equatorial Fe-N$_{eq}$ bonds: two coplanar with imidazole and two orthogonal to it. Hence, this structure indicates that there are two binding modes of O$_2$, one with $C_s$ symmetry and the other unsymmetrical $C_1$. We have optimized the structure of both states, and it turned out that the symmetric one was 0.6 kJ/mol more stable than the unsymmetrical one. The geometries, charges, and spin densities of the two states were identical to within the accuracy presented in this work, i.e. ±0.01 e and 0.001 Å. In addition, the calculation shows that π-bonding and trans electronic effects, including back-bonding to iron d-orbitals, are absent. This implies that imidazole is an innocent ligand. In fact, it has been shown that the rotation of the O$_2$ group in a model similar to ours has a barrier of less than 8 kJ/mol (37).

Likewise, another unsymmetrical conformation arising from a 45° rotation of imidazole (staggered oxygen and eclipsed imidazole with respect to the Fe-N$_{eq}$ bonds) was 2 kJ/mol less stable than the $C_s$ conformation. The spin densities and charges were similar to within 0.02 e of the $C_s$ state, but the geometry showed
differences of up to 0.07 Å in the Fe-O bond. Thus, we can conclude that the symmetric structure is the most stable geometry of this system and we have therefore employed $C_s$ symmetry in all the calculations. This strongly facilitates the optimization and characterization of the various excited states. In $C_s$ symmetry, the electronic states are labeled as symmetric A' or antisymmetric A'', respectively, depending on whether their wavefunction preserves or changes sign upon reflection in the symmetry plane ($xz$, cf. Figure 1). The states that we shall discuss will follow this nomenclature with a superscript in front indicating the multiplicity (the number of unpaired electrons plus one). States with the same symmetry and multiplicity are numbered (in brackets) after their optimum energy. For example, the ground state is a symmetric singlet, $^1A'(1)$.

Throughout this work, we used a coordinate system with Fe in the origin, the $z$-axis along the N$_{tm}$-Fe-O bonds, the $x$-axis through two methine bridge atoms (not the nitrogens), and the $y$-axis through the two other methine bridges (Figure 1). Thus, the imidazole and O$_2$ molecules lie in the $xz$ plane. The two unpaired $\pi$-electrons on oxygen are situated in two degenerate antibonding $\pi$ orbitals, which transform as a' and a'' in the reduced $C_s$ symmetry when binding to deoxyhemoglobin. In our coordinate system, three of the Fe $d$-orbitals transform as a', viz. $xz$, $z^2$, and $x^2-y^2$, and would therefore couple to the a' unpaired $\pi$-electron of O$_2$, whereas the other two orbitals, $xy$ and $yz$, would interact with the a'' electrons instead.

**Selection of states**

We have searched for low-energy states that could contribute to the process of oxygen binding. The states were obtained from a systematic permutation of the occupations of 6 electrons in an “active space” consisting of molecular orbitals 73-75 a' and 45-47 a''. There are four classes of orbitals: symmetric and antisymmetric $\alpha$ and $\beta$ orbitals. Some restrictions were introduced to minimize the search, based on the ground state. We have only examined those configurations that distinguish themselves by one occupied orbital per class from the ground state configuration (74 45 74 45, i.e. 74 electrons in $\alpha$ a' orbitals, 45 electrons in $\alpha$ a'' orbitals, 74 electrons in $\beta$ a' orbitals, and 45 electrons in $\beta$ a'' orbitals). Some orbitals were found to be very high in energy and were subsequently avoided. For example, the state (75 44 74 45) had 212 kJ/mol higher energy than the ground state. Hence, we avoided the (45→75)
excitation. Such selections reduced our number of states to 20, and the seven lowest are presented in this work. The electronic configurations and optimized energies of these states are shown in Table 1. The states are unrestricted Kohn-Sham wavefunctions with a large degree of spin polarization in most cases.

Results and Discussion

The ground state of the adduct

The lowest energy was obtained for the (74 45 74 45) open-shell singlet $^1A'(1)$ state in Table 1 (the lowest closed-shell singlet with the same occupation numbers is 5 kJ/mol higher in energy). Its geometry is displayed in Table 2 and Figure 1. It can be seen that it closely resembles the X-ray structure of oxymyoglobin (36): The Fe-O bond lengths differ by only 0.001 Å. For the more soft Fe-N$_a$ bond, the error is what can be expected with state-of-the-art DFT methods, 0.03 Å, whereas for the average equatorial Fe-N$_e$ bonds, the difference is only 0.006 Å. This gives us confidence that this is the correct ground state and that the description of the Fe-O bond is essentially correct.

The ground state is an open-shell singlet, in accordance with the experimental observation that the O$_2$ adduct is EPR-silent (38). However, the spin is unevenly distributed in the complex, with a surplus of $\alpha$ spin on O$_2$ (0.75 electrons) and a surplus of $\beta$ spin on iron (-0.79 e), as is quantified in Table 3 and illustrated in Figure 1, bottom. Literature is rich on discussions about the nature of the Fe-O bond (38, 39). In particular, it has been argued whether the electronic structure of oxyheme is better described as singlet oxygen, bound to low-spin Fe$^{III}$ (2) or as a superoxide radical antiferromagnetically coupled to low-spin Fe$^{III}$ (40). Some consensus has arisen on the point that the Fe$^{III}$-O$_2$ form agrees better with experiments, e.g. the O-O frequency of 1100 cm$^{-1}$, which is close to what is expected for O$_2$ (3), some aspects of the chemical reactivity (41), and changes in the electric field gradient studied with Mössbauer spectroscopy (42).

Our results are closest to the Fe$^{III}$-O$_2$ description, in accordance with earlier DFT calculations (14) (the Fe$^{II}$-O$_2$ form would be a closed-shell singlet). However, the spin densities are far from ±1, which clearly shows that the electronic structure cannot be fully be described by a single configuration (such as Fe$^{III}$-O$_2$ or
Fe$^{II}$-O$_2$), but rather as a mixture of both these and possibly also other configurations. Thus, our spin densities could be interpreted as a mixture of 75-80% Fe$^{III}$-O$_2$ and 20-25% Fe$^{II}$-O$_2$. This is good accordance with the experimental observation that a “quantum mixture” of ~2/3 ferric and ~1/3 ferrous states gives the best agreement with Mössbauer spectra (43). Thus, oxyheme is inherently multiconfigurational, with an electronic structure that is somewhat analogous to that found in ozone (44). Early CASSCF studies (on a simplified heme model with ammonia instead of imidazole) gave a mainly closed-shell 1$A'$ ground state (45), as did a symmetry-adapted cluster configuration interaction (SAC-CI) study (46), with the lowest open-shell singlet 150 kJ/mol higher in energy. However, the present results gives a better description of the ground state in terms of geometry.

*The dissociated states*
Isolated deoxyheme is experimentally a high-spin quintet (38). The optimized structure of this complex (Table 2) agrees well (within 0.02 Å) with the crystal structure of deoxymyoglobin at 1.15 Å resolution (36). It is notable that both structures show a strongly distorted porphyrin with the iron ion ~0.3 Å out of the ring plane, illustrating that high-spin iron is too large to fit into the ring cavity.

When this complex is associated with triplet O$_2$, there are six unpaired electrons in the total system. The unpaired spin on deoxyheme and O$_2$ may be either parallel, giving rise to a septet, 7$A''$(1), or antiparallel, which gives rise to a triplet state, which turns out to be 3$A''$(2). At long (non-interacting) Fe-O distances, these two states are degenerate, as expected. Ideally, both states should give rise to rapid O$_2$ binding (i.e. all active sites of hemoglobin should be able to bind all O$_2$ molecules, independent on their spin states).

However, as the Fe-O distance is decreased, the degeneracy is lifted. In the optimal structure, the 7$A''$(1) state has a Fe-O bond length of 2.52 Å, whereas it is 1.89 Å for state 3$A''$(2) (cf. Table 2). The potential energy surface of the 7$A''$(1) state is flat around the minimum and the energy is close to the dissociation limit, which is at 27 kJ/mol, when calculated from separated species. The two states have very similar energies in their optimum geometries. Interestingly, the B3LYP gives a quite different behavior of the 3$A''$(2) state: The energy of this state increases steadily as the Fe-O bond length is decreased, with an energy of ~50 kJ/mol at the BP86 minimum. The
B3LYP curve shows a very shallow minimum at Fe-O = 2.39 Å, with an energy close to the dissociation limit.

The lowest triplet (intermediate-spin) state of deoxyheme is close in energy to the lowest quintet state. In fact, in the present calculations (as well as in most previous DFT calculations (11, 34)), it is actually 3 kJ/mol more stable (4 kJ/mol when optimized at the B3LYP level; hence the dissociation limit of the lowest triplet state is 24 kJ/mol). Thus the states are degenerate to within the uncertainty of the method. If this triplet state is associated with triplet \( \text{O}_2 \), we once again obtain two states, depending on the relative orientation of the two sets of unpaired spin, a quintet state \( ^5\text{A}'(1) \), and a singlet state, which actually turns out to be the dissociation product of the singlet ground state \(^1\text{A}'(1)\).

**Excited states**

In Table 1, the relaxed electronic spectrum is presented for the oxyheme model. It shows that there are six states within 30 kJ/mol (25 kJ/mol if optimized B3LYP structures are used) of the open-shell singlet ground state of oxyheme. Scalar-relativistic corrections have only a minor effect on these energies (less than 5 kJ/mol, usually in favor of the low-spin states). Besides the three dissociation states, \(^7\text{A}''(1)\), \(^5\text{A}'(1)\), and \(^3\text{A}''(2)\) discussed previously, there is another low-lying anti-symmetric triplet state, \(^3\text{A}''(1)\), a symmetric triplet, \(^3\text{A}'(1)\), and an antisymmetric singlet, \(^1\text{A}''(1)\).

Thus, these states are nearly degenerate within the uncertainty of current methods (~10 kJ/mol), which makes it hard to assign the spectrum in detail. However, the result is in qualitative agreement with the fact that all three spin states have been found from Mössbauer spectroscopy within 10 kJ/mol in ferrous myoglobin and hemoglobin (corresponding to an excitation at 12 000 nm) (47-49), and the observation of a low-lying triplet state in thermal equilibrium with the singlet ground state of oxyheme at temperatures between 25 K and 250 K (50).

The vertical electronic excitation spectrum was recently calculated (46) with a model identical to ours using the symmetry-adapted cluster configuration interaction (SAC-CI) method on the experimental geometry. This approach gave a similar ground state and low-lying \(^3\text{A}''\) and \(^1\text{A}''\) states (at 0.47 and 1.54 eV), but in general the spectrum had much larger energy separations than we have. The reason for this is probably that they used one geometry (from experiments) to compute all states, whereas we have optimized the geometry of all states. If the excited-state
geometries are optimized, the states will come substantially closer in energy.

The spin densities of the seven low-lying states are shown in Table 3. From these, it can be seen that the two dissociative states, \( ^7A"(1) \) and \(^5A'(1) \), are quite close to triplet \( \text{O}_2 \) and high- or intermediate-spin Fe\(^{III} \) also in their optimum structures. The \( ^3A"(2) \) state is quite well described as intermediate-spin Fe\(^{III} \) (2.98 unpaired electrons) antiferromagnetically coupled to \( \text{O}_2^- \) (1.13 \( e \)) and the \( ^3A'(1) \) state is low-spin Fe\(^{III} \) (1.05 \( e \)) ferromagnetically coupled to \( \text{O}_2^- \) (0.96 \( e \)). The \(^1A"(1) \) state has spin densities similar to the singlet ground state (i.e., low-spin Fe\(^{III} \) antiferromagnetically coupled to \( \text{O}_2^- \)), whereas the \( ^3A"(1) \) state is intermediate between \( \text{O}_2 \) and \( \text{O}_2^- \) (1.57 unpaired electrons).

Mulliken charges for the various excited states are compiled in Table 4. Interestingly, whereas the spin densities on iron and \( \text{O}_2 \) vary appreciably for the various states, the charges are much more similar. For example, the variation in the charge of the \( N_{ax} \) and \( N_{eq} \) atoms is 0.05 \( e \) and 0.09 \( e \), respectively. A somewhat larger variation is seen for the charge on the iron ion, varying between 0.58 and 0.73 \( e \). However, this variation is fully consistent with the spin densities, giving a lower charge for low-spin states, which are better shielded from the nuclei, as rationalized by Slater's rule (51). For the same reason, the high-spin states tend to have more charge in the porphyrin ring as measured at the \( N_{eq} \) atoms in Table 4.

Thus, the total electron density (charge) is quite rigid in the states. This applies for the polarity of the Fe-O bond as well. The spin density, on the other hand, differs significantly within the various states. Therefore, the notion of Fe\(^{III} \)-\( \text{O}_2^- \) and Fe\(^{II} \)-\( \text{O}_2 \) is only justified in terms of spin density, and not in terms of the charge.

**Binding of \( \text{O}_2 \)**

We now turn to the actual association mechanism. How does \( \text{O}_2 \) bind to heme in hemoglobin or myoglobin, facing the restrictions of spatial and spin symmetry? To answer this question, we have calculated the fully relaxed geometry and energy at various fixed Fe-O bond distances, to obtain the binding curves of the seven lowest states shown in Figure 2. The most prominent feature is that there are five potential curves, viz. those for the \(^1A'(1)\), \(^3A"(2)\), \(^5A'(1)\), \(^7A"(1)\), and \(^1A"(1)\) states, which from a Fe-O distance of 2.5 Å and outwards are near-degenerate and virtually parallel, within the accuracy of the present method. Solvent effects, calculated by the COSMO model in water-like solvent (\( \varepsilon = 80 \)), tend to favor the separated species by \(~10 \text{ kJ/mol} \),
thereby making the spectrum even more dense at long Fe-O distances.

The closeness of these states is of course caused by the near-degeneracy of the triplet and quintet state of deoxyheme. As can be seen from the variation of the spin densities with the Fe-O distance in Figure 3, the \(^1\!A'(1), \, ^5\!A'(1),\) and \(^1\!A''(1)\) states dissociate as triplet \(\text{O}_2\) and intermediate-spin deoxyheme, whereas the \(^3\!A''(2)\) and \(^7\!A''(1)\) states dissociate as triplet \(\text{O}_2\) and high-spin deoxyheme. The curves show a smooth transition from the bound to the dissociated state. It is reasonable that all low-lying states end up in triplet \(\text{O}_2\), because the lowest singlet state of dioxygen is more than 90 kJ/mol less stable than the triplet state (1).

Apparently, there must be at least one spin crossing during the binding of \(\text{O}_2\) to deoxyheme. Thus, we could start with the triplet dissociative state \(^3\!A''(2)\) (which, together with the septet \(^7\!A''(1)\) state is the experimental ground state of the deoxyheme-\(\text{O}_2\) system before binding) and then reach the ground state of oxyheme, \(^1\!A'(1)\), by the flip of one spin. However, it would be more effective if all heme sites could bind any \(\text{O}_2\) molecule, independent of its spin state, i.e. if the septet state also could reach the ground state oxyheme. Such a binding would involve at least four spin states, e.g. in the sequence \(^7\!A''(1) \rightarrow ^5\!A'(1) \rightarrow ^3\!A''(2) \rightarrow ^1\!A'(1)\). According to our results, such a pathway is also possible, because all these four spin surfaces are parallel and nearly degenerate. In fact, such an association mechanism would have essentially the same energetics as the triplet association (owing to the near-degeneracy at large Fe-O distances) but different dynamics, because the crossing probabilities may differ.

Alternatively, the binding of \(\text{O}_2\) could take place also directly to the triplet state of deoxyheme, which would lead directly to the right low-spin state upon binding to triplet \(\text{O}_2\) (52). In this case, the primary electronic reorganization takes place at iron in an equilibrium between the quintet and triplet states already before \(\text{O}_2\) approaches heme (53). Of course, such a mechanism would also be facilitated by the near-degeneracy of the spin states of deoxyheme.

This topology with five nearly degenerate states at long and intermediate Fe-O distances explains the rapid and reversible binding of \(\text{O}_2\). To see this, we will consider the Eyring expression of the reaction rate constant \(k\) of \(\text{O}_2\) binding:

\[
k = \kappa k_B T / h \exp(-\Delta G^\ddagger / (k_B T)) \tag{1}
\]
where \( h \) and \( k_B \) are Planck's and Boltzmann's constants, and \( \Delta G^a \) is the activation energy. For a simple one-step reaction involving two states (A and B), the transmission coefficient \( \kappa \) can be approximated by the probability \( P_{AB} \) in the Landau-Zener equation (54, 55):

\[
P_{AB} = 1 - \exp\left[-\frac{(2\pi \Delta E_{AB})^2}{hv|S_A - S_B|}\right] \tag{2}
\]

Here, \( v \) is the crossing velocity (the classical velocity of the particle moving along the potential energy surface) and \( |S_A - S_B| \) is the absolute value of the difference in slope of the potential energy as a function of the reaction coordinate at the crossing point. In addition, \( 2\Delta E_{AB} \) is the difference in energy of the states when the perturbation that lifts their degeneracy has been applied, in our case the spin-orbit coupling (SOC).

To get a feeling of the various terms in this expression, we will insert reasonable values and compare the results for oxyheme with a similar but non-biological process, the dissociation of the diatomic Fe-O\(^+\) system, studied by Shaik et al. (56) The term \( hv \) describes the normal mode of Fe-O dissociation, which can be obtained from temperature and the reduced mass. It will be similar in the biological and non-biological system, ~5 kJÅ/mol.

The SOC constant for iron is intermediate between what you find for first-row elements and for heavy metals, because it grows approximately as \( Z^4 \). It is ~1 kJ/mol between various spin states in free iron (57) and it is in general smaller in metal complexes than in the free ions (58). For ferrous deoxyheme in hemoglobin and myoglobin, it has been estimated from Mössbauer spectroscopy to be ~0.8 kJ/mol (47).

Finally, the gradient differences \( |S_A - S_B| \) will strongly depend on the system under study and the location of the crossing point. For the FeO\(^+\) system, the crossing point was between two surfaces with very different slopes, one negative and the other positive. The calculated \( |S_A - S_B| \) was ~300 kJ/mol/Å for sextet and quartet states (56). With such a large difference in the slopes, Shaik et al. obtain a transmission probability of only 0.004 for the crossing between the high- and intermediate-spin states, with a SOC of 0.61 kJ/mol (56).

However, our results indicate that the behavior of oxyheme is quite different. At Fe-O distances longer than 3 Å, the potential energy curves of the relevant \(^1\!A'(1), \quad ^3\!A''(2), \quad ^5\!A'(1), \quad \text{and} \quad ^7\!A''(1)\) states are nearly degenerate, making up a
crossing “region” of flat and almost parallel potential energy surfaces. We do not know the exact locations of the crossing points between the various spin states, because the energy differences are so small and the triplet and quintet states of deoxyheme are almost degenerate. However, it is known from experiments that the triplet-quintet splitting in deoxyheme is 10 kJ/mol, with the quintet lower in energy. This gives us an experimental bound to the crossing points in Figure 2. Translating the septet and triplet curves (corresponding to quintet deoxyheme) down to such a dissociation energy shows that all crossing points must be at Fe-O distances above 2.5 Å. This means that all curves have a slope of less than ±25 kJ/mol/Å. Moreover, the maximum value of $|S_A - S_B|$ for the curve crossings of interest is less than 20 kJ/mol/Å (all curves have a negative slope except that of $^1A'(1)$, for which the slope is 4 kJ/mol/Å or less). With the other terms similar to the FeO$^+$ case, this increases the transmission probability to 0.06-1, an enhancement by a factor of at least 15. Thus, we see that the special topology of the heme-O$_2$ system with many parallel and nearly degenerate spin surfaces leads to a large increase in the transmission probability and therefore also in the rate of O$_2$ binding by a corresponding factor (the SOC of heme will probably be slightly smaller than that of FeO$^+$, because the average effective nuclear charge that the $d$ electrons see will be smaller in the larger complex, but this effect is minimal).

However, even more important for the binding of O$_2$ to heme is the activation energy of the reaction, $\Delta G^\#$ in Eqn (1). From Figure 2, it can be seen that for the possible crossing points discussed above (i.e. for Fe-O distances longer than 2.5 Å), all curves for the relevant four spin states are less than 15 kJ/mol above the energy of the dissociated states (allowing for a downshift of the heptet and triplet curves to the experimentally observed quintet-triplet splitting (47-49)). This means that the activation enthalpy should be lower than this. For the FeO$^+$ system, the analogous reaction from the high-spin state has an activation enthalpy of binding of 75 kJ/mol. (56) Thus, the design of deoxyheme gives a barrier decrease of ~60 kJ/mol compared to the simplest Fe-O binding complex imaginable. Provided that the entropy of binding is similar in both reactions (it is most likely dominated by the removal of six degrees of freedom from free O$_2$), this corresponds to a rate enhancement of $\sim 10^{10}$.

Thus, we can conclude that the facile binding of O$_2$ to hemo- and myoglobin arises primarily as an effect of the topology of the binding curves for the
four relevant spin states. This topology, with nearly degenerate and parallel curves, is caused by the near-degeneracy (within 10 kJ/mol) of the triplet and quintet states of deoxyheme. Therefore, the design by nature of iron porphines having close-lying spin states of a particular symmetry and energy is a means to tune binding of small ligands and overcome the activation barriers of these spin-forbidden reactions, in spite of the moderate SOC of first-row transition metals. The resulting barrier height makes up most of the rate enhancement due to the exponential dependence on the rate, whereas one or two orders of magnitude may come from the increase in the transmission coefficient.

The different relative rates for the rebinding of NO, CO, and O\textsubscript{2} to heme have recently been studied by DFT (16). That study also used a Landau-Zener formalism to explain the importance of spin states for the rates of ligand binding. Unfortunately, it was based on curves obtained with a fixed geometry except for the Fe-O bond. Moreover, it gave a closed-shell ground state for oxyheme, which gives a Fe-O geometry different from experiment. Relaxation effects are very large for these systems, in particular for the Fe-imidazole and iron out-of-plane distances. This was also observed when three different values of the distance of the iron ion out of the porphyrin plane were tested, giving rise to changes by up to 100 kJ/mol in the energies and a reordering of the spin states. Therefore, none of the curves has any clear significance for the binding of O\textsubscript{2}. An accurate description of spin surfaces and the topology at crossing points can only be obtained with fully relaxed potential energy surfaces, such as those presented in Figure 2.

**Comparison with peroxidases**

We have seen that the facile binding of O\textsubscript{2} to heme in the globins is essentially an effect of the near-degeneracy of quintet and triplet states of deoxyheme. It is then natural to ask whether other heme proteins have solved the problem in a similar way and whether proteins are designed to facilitate the binding of ligands. We will show that this is probably the case by a comparison to the peroxidases.

Peroxidases are heme proteins that oxidize various substrates in one-electron reactions, using H\textsubscript{2}O\textsubscript{2} as the oxidant. The resting state of these proteins is high-spin *ferric* heme (a sextet), in contrast to the ferrous high-spin state in the globins. It is this state that binds H\textsubscript{2}O\textsubscript{2} and this reaction is spin-forbidden like the globin binding of O\textsubscript{2}, because H\textsubscript{2}O\textsubscript{2} is a singlet, whereas peroxyheme is a doublet (57).
Interestingly, many experiments indicate that the sextet and quartet states are very close in energy for the ferric resting state of peroxidases. In fact, the ground state of some peroxidases seems actually to be a quantum chemical (by SOC) mixture of these two states (59-68). This indicates that the same mechanism as we have suggested for O₂ binding to globins also applies for the binding of H₂O₂ to the peroxidases, i.e. that the spin surfaces are nearly degenerate and parallel, caused by a near-degeneracy of the dissociated states.

This suggestion is further strengthened by the fact that peroxidases and globins have a slightly different axial bonding of the heme group. In the globins, the axial histidine ligand makes only weak hydrogen bonds to the back-bone of the surrounding protein. However, in all known heme peroxidases, the axial histidine ligand instead forms a strong hydrogen bond to the carboxylate side-chain of a conserved aspartate residue (69). It has been suggested that such a hydrogen bond may change the properties of the axial ligand (impose some imidazolate character onto it) (70).

Interestingly, we have recently shown that such a hydrogen bond to a carboxylate group changes the relative energies of the spin states of ferrous and ferric deoxyheme (71): Without the hydrogen bond, the high- and intermediate-spin states are nearly degenerate in the ferrous state, but not in the ferric state. With the hydrogen bond, the opposite is true; the two states are degenerate to within 3 kJ/mol in the ferric state. Thus, it seems that evolution has selected an axial ligand that favors spin-degeneracy and thereby a facile binding of a proper ligand in the opposite axial site in both peroxidases and the globins. This would provide a new explanation for the selection of the axial ligand in heme proteins, a subject of much debate (70.71).

**Conclusions**

We have provided evidence that the spin-forbidden reversible binding of oxygen to globins is strongly facilitated by the shape of the potential energy curves of the various spin states during O₂ binding: We have found that the four relevant low-lying spin states, with zero, two, four or six unpaired electrons, form nearly parallel surfaces with almost the same energy along the O₂-binding coordinate beyond at Fe-O distance of 2.5 Å. Such a topology has three important biological consequences for O₂ binding. First, it ensures that the protein may bind all O₂ molecules, independent on
their spin state (two unpaired \( \alpha \) or \( \beta \) electrons). Second, the relative slope of the crossing spin surfaces is small in the crossing region. This leads to a large probability for the necessary spin crossing, in spite of a modest spin-orbit coupling for iron. In fact, the transition probability, and therefore also the rate constant of \( \text{O}_2 \) binding, is increased by at least a factor of 15, compared to similar non-biological iron complexes. Third, the detailed shape of curves ensures that the energy barrier (activation enthalpy) for the curve crossing is small (<15 kJ/mol). This has a large effect on the rate acceleration of \( \text{O}_2 \) binding. Altogether, these parallel and nearly degenerate energy surfaces may accelerate oxygen binding by 11 orders of magnitude.

The unusual topology of the binding surfaces is caused by the near-degeneracy of the two lowest spin states of ferrous deoxyheme. Such near-degeneracy is a basic feature of many porphyrins (7) and is supported by Mössbauer spectroscopy, which finds all three spin states within 10 kJ/mol in ferrous hemo- and myoglobin (47-49). In fact, we have earlier shown that it is an intrinsic property of porphyrin to give a small splitting between the various spins states of iron, because the central cavity of the ring is too large for low-spin iron (11). However, we have also seen that the axial ligand has an important influence of the spin-splitting energies: Imidazole with only weak hydrogen bonds (as is found in the globins) gives near-degeneracy for the Fe(II) state, whereas imidazolate or imidazole, hydrogen bonded to a carboxylate group (as is found in the peroxidases), gives degeneracy instead for the Fe(III) state (71). Thus, we have obtained an important explanation for the selection of the axial ligand and the structural design of heme proteins, with the aim of enhancing the binding of substrates to the proteins.

Acknowledgments

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References

33. Exponents: 0.141308 and 0.043402 (p); 0.1357 (d); and 1.6200 (f)
Sons, New York, 1944


Table 1. Relative energies (kJ/mol) and occupation numbers of the seven lowest states of oxyheme in $C_s$ symmetry. There are 238 electrons in the model and they are partitioned into the $\alpha$ or $\beta$ orbitals of symmetry $a'$ or $a''$ as is specified in this table. A surplus of zero, two, four, or six $\alpha$ electrons gives a singlet, triplet, quintet, or a septet, respectively. The total wavefunction is antisymmetric ($A''$) if the total number of $a''$ electrons is odd; otherwise it is symmetric ($A'$).

<table>
<thead>
<tr>
<th>State</th>
<th>$a'$ - $\alpha$</th>
<th>$a''$ - $\alpha$</th>
<th>$a'$ - $\beta$</th>
<th>$a''$ - $\beta$</th>
<th>$E_{rel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1A'(1)$</td>
<td>74</td>
<td>45</td>
<td>74</td>
<td>45</td>
<td>0.0</td>
</tr>
<tr>
<td>$^5A'(1)$</td>
<td>75</td>
<td>46</td>
<td>73</td>
<td>44</td>
<td>22.0</td>
</tr>
<tr>
<td>$^3A''(1)$</td>
<td>74</td>
<td>46</td>
<td>73</td>
<td>45</td>
<td>24.1</td>
</tr>
<tr>
<td>$^3A''(2)^a$</td>
<td>74</td>
<td>46</td>
<td>73</td>
<td>45</td>
<td>19.7</td>
</tr>
<tr>
<td>$^7A''(1)$</td>
<td>75</td>
<td>47</td>
<td>72</td>
<td>44</td>
<td>28.5</td>
</tr>
<tr>
<td>$^1A''(1)$</td>
<td>73</td>
<td>46</td>
<td>74</td>
<td>45</td>
<td>28.9</td>
</tr>
<tr>
<td>$^3A'(1)$</td>
<td>74</td>
<td>46</td>
<td>74</td>
<td>44</td>
<td>24.7</td>
</tr>
</tbody>
</table>

$^a$ Antiferromagnetic state obtained from the septet dissociation product.
Table 2. Optimum bond distances (Å) of the seven lowest states of oxyhemoglobin, obtained with the BP86 functional.

<table>
<thead>
<tr>
<th>State</th>
<th>Fe-O</th>
<th>Fe-N&lt;sub&gt;ax&lt;/sub&gt;</th>
<th>Fe-N&lt;sub&gt;eq1&lt;/sub&gt;</th>
<th>Fe-N&lt;sub&gt;eq2&lt;/sub&gt;</th>
<th>Fe oop</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹A'(1)</td>
<td>1.807</td>
<td>2.096</td>
<td>2.024</td>
<td>2.001</td>
<td>0.034</td>
</tr>
<tr>
<td>⁵A'(1)</td>
<td>2.679</td>
<td>2.253</td>
<td>2.012</td>
<td>1.998</td>
<td>0.087</td>
</tr>
<tr>
<td>³A''(1)</td>
<td>1.952</td>
<td>2.100</td>
<td>2.011</td>
<td>2.005</td>
<td>0.008</td>
</tr>
<tr>
<td>³A''(2)</td>
<td>1.892</td>
<td>2.133</td>
<td>2.079</td>
<td>2.065</td>
<td>0.018</td>
</tr>
<tr>
<td>⁷A''(1)</td>
<td>2.519</td>
<td>2.200</td>
<td>2.086</td>
<td>2.069</td>
<td>0.144</td>
</tr>
<tr>
<td>¹A''(1)</td>
<td>1.878</td>
<td>2.071</td>
<td>2.012</td>
<td>2.008</td>
<td>0.012</td>
</tr>
<tr>
<td>³A'(1)</td>
<td>2.080</td>
<td>2.135</td>
<td>2.075</td>
<td>2.075</td>
<td>0.044</td>
</tr>
<tr>
<td>oxy&lt;sup&gt;a&lt;/sup&gt; exp</td>
<td>1.806</td>
<td>2.064</td>
<td>2.006&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.006&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.023</td>
</tr>
<tr>
<td>deoxy&lt;sup&gt;c&lt;/sup&gt; exp</td>
<td>---</td>
<td>2.141</td>
<td>2.074&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.074&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.364</td>
</tr>
<tr>
<td>⁷A''(1)&lt;sup&gt;d&lt;/sup&gt; diss</td>
<td>4.300</td>
<td>2.158</td>
<td>2.082</td>
<td>2.079</td>
<td>0.257</td>
</tr>
<tr>
<td>deoxy&lt;sup&gt;c&lt;/sup&gt; calc</td>
<td>---</td>
<td>2.153</td>
<td>2.083</td>
<td>2.079</td>
<td>0.266</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Experimental structure of oxymyoglobin

<sup>b</sup>: Average of the four Fe-N<sub>eq</sub> distances.

<sup>c</sup>: Experimental structure of deoxymyoglobin (36).

<sup>d</sup>: Geometry at largest Fe-O distance, 4.3 Å.
Table 3. Spin densities on various atoms of the seven lowest states of oxyheme, obtained with the BP86 functional.

<table>
<thead>
<tr>
<th>State</th>
<th>Fe</th>
<th>O₁</th>
<th>O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹A'(1)</td>
<td>-0.79</td>
<td>0.26</td>
<td>0.49</td>
</tr>
<tr>
<td>⁵A'(1)</td>
<td>2.25</td>
<td>0.90</td>
<td>0.92</td>
</tr>
<tr>
<td>³A''(1)</td>
<td>3.35</td>
<td>-0.73</td>
<td>-0.84</td>
</tr>
<tr>
<td>³A''(2)</td>
<td>2.98</td>
<td>-0.68</td>
<td>-0.45</td>
</tr>
<tr>
<td>⁷A''(1)</td>
<td>3.89</td>
<td>0.87</td>
<td>0.91</td>
</tr>
<tr>
<td>¹A''(1)</td>
<td>-0.77</td>
<td>0.35</td>
<td>0.41</td>
</tr>
<tr>
<td>³A'(1)</td>
<td>1.05</td>
<td>0.38</td>
<td>0.58</td>
</tr>
</tbody>
</table>
Table 4. Mulliken charges on various atoms of the seven lowest states, obtained with the BP86 functional.

<table>
<thead>
<tr>
<th>State</th>
<th>Fe</th>
<th>O₁</th>
<th>O₂</th>
<th>Nₐx</th>
<th>Nₑq₁</th>
<th>Nₑq₂</th>
<th>Im</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹A'(1)</td>
<td>0.58</td>
<td>-0.17</td>
<td>-0.14</td>
<td>-0.35</td>
<td>-0.48</td>
<td>-0.48</td>
<td>0.22</td>
</tr>
<tr>
<td>³A'(1)</td>
<td>0.59</td>
<td>-0.05</td>
<td>-0.06</td>
<td>-0.37</td>
<td>-0.53</td>
<td>-0.51</td>
<td>0.18</td>
</tr>
<tr>
<td>³A''(1)</td>
<td>0.72</td>
<td>-0.11</td>
<td>-0.07</td>
<td>-0.38</td>
<td>-0.53</td>
<td>-0.53</td>
<td>0.20</td>
</tr>
<tr>
<td>³A''(2)</td>
<td>0.72</td>
<td>-0.10</td>
<td>0.16</td>
<td>-0.36</td>
<td>-0.52</td>
<td>-0.52</td>
<td>0.22</td>
</tr>
<tr>
<td>⁷A''(1)</td>
<td>0.73</td>
<td>-0.06</td>
<td>-0.07</td>
<td>-0.39</td>
<td>-0.56</td>
<td>-0.54</td>
<td>0.18</td>
</tr>
<tr>
<td>¹A''(1)</td>
<td>0.58</td>
<td>-0.18</td>
<td>-0.15</td>
<td>-0.35</td>
<td>-0.49</td>
<td>-0.47</td>
<td>0.22</td>
</tr>
<tr>
<td>³A'(1)</td>
<td>0.58</td>
<td>-0.18</td>
<td>-0.14</td>
<td>-0.36</td>
<td>-0.48</td>
<td>-0.48</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Figure 1. Optimized structure (top) and spin density (bottom) of the oxyheme model, in the $^1A'(1)$ ground state. All wavefunctions that change sign when reflected in the $xz$ plane are antisymmetric, labeled $A''$, whereas those that keep their sign are symmetric, labeled $A'$. 
Figure 2. Energy surfaces (kJ/mol) of the various spin states during the binding of O$_2$ to heme.
Figure 3. Spin densities of Fe, O1, and O2 as a function of Fe-O1 distance, obtained with the BP86 functional. From top to bottom: $^1A'(1)$, $^3A''(2)$, $^1A''(1)$, $^7A''(1)$, and $^5A'(1)$. 

\begin{center}
\includegraphics[width=0.7\textwidth]{figure3}
\end{center}
Figure 3. Continued.

$7\text{A}''(1)$

$5\text{A}'(1)$
How O₂ binds to heme: Reasons for rapid binding and spin inversion
Kasper P. Jensen and Ulf Ryde

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