How O₂ Binds to Heme
REASONS FOR RAPID BINDING AND SPIN INVERSION*

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We have used density functional methods to calculate fully relaxed potential energy curves of the seven lowest electronic states during the binding of O₂ to a realistic model of ferrous deoxyheme. Beyond a Fe–O distance of \(-2.5 \text{ Å}\), 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 O₂ with heme (deoxyheme is a quintet and O₂ a triplet, whereas oxyheme is a singlet). Thus, despite 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 nonbiological Fe–O²⁻ 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 O₂ may bind to heme, thereby increasing the overall efficiency of oxygen binding. The activation barrier is estimated to be <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 O₂ binding to iron by \(-10^{11}\) compared with the Fe–O²⁻ system. A similar near degeneracy between spin states is observed in a ferrous deoxyheme model with the histidine ligand hydrogen bonded to a carboxylate group, i.e. a model of heme peroxidases, which bind H₂O₂ in this oxidation state.

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 O₂. There is a strong thermodynamic drive of O₂ to oxidize organic matter to H₂O and CO, but because these products (as well as the organic molecules) are singlets (whereas O₂ 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 O₂ 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 O₂. There are many reasons for this choice. First, most transition metals also contain unpaired electrons, allowing reactions with triplet O₂. Second, transition metals are relatively heavy atoms, which increases spin-orbit coupling (SOC),¹ and thereby provide 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 alone to 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 O₂ to hemoglobin, i.e. the binding of O₂ 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 3d orbitals of iron (it is a quintet), and triplet O₂ 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 α and β electrons. As discussed already by Pauling and Coryell (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 cooperativity (5–7). The movement of iron into the heme plane is assumed to trigger a transition from a tense state to a relaxed state after the binding of two oxygen molecules, and this trigger, in the form of the Fe–Nax 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 O₂ adduct (8), whereas state-of-the-art density functional theory (DFT) pro-

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¹ The abbreviations used are: SOC, spin-orbit coupling; DFT, density functional theory.
vides excellent geometries of porphyrins in general (9–13). Among these, the B3LYP density functional predicts very close-lying quintet and triplet states in deoxymyoglobin models, sometimes with a triplet ground state (14). Recently, DFT was used to compute the electronic spectrum of FeII-porphine with 2-methylimidazole as the axial ligand, making the quintet state the lowest in energy, but the triplet state only 12 kJ/mol higher (15), in excellent agreement with the experiment. These circumstances indicate that the treatment of spin states in porphyrins is delicate because of 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 Poli and Harvey (17).

In this work, we have optimized the ground state and several low-lying excited states at many points along the heme–O2 binding curve. Our results indicate the reason for the facilitated binding of O2 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 carbonyl group. Hence, we suggest a new role for the choice of an axial ligand in such systems, viz. to bring spin states close in energy and thereby facilitate spin-forbidden binding of ligands.

**MATERIALS AND METHODS**

In the present work, we studied the reversible binding process

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

**REACTION 1**

with particular emphasis on analyzing possible states along this reaction coordinate. Such a detailed approach seems 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 (B86) (18, 19). Accurate energies were then estimated by single-point calculations using the Becke three-parameter hybrid method with the local spin-density approximation correlation functional of Vosko-Wilk-Nusair and the nonlocal 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 (26–28). However, in our experience, B86 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-ζ basis set of Schäffer et al. (32), augmented with two p, one d, and one f function (DZpfd, exponents: 0.14198 and 0.043402 f) (d); 1.5357 (d); and 1.6200 (/f) with the contraction scheme (14s11p6d4f/8s7p4d1f). Only the pure five d and seven f-type functions were used. Our basis set is balanced and, based on experience (34), is 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 μmol) for the energy and 10−3 atomic units for the maximum norm of the gradient.

**Model System**—All calculations were performed on the FeII-PorImO2 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 geometric 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 FeII-PorImO2 model had C5 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–Fe–N–a torsion angle of 45°. O2 adopts a 4-fold occupancy in the thermally disordered structure at staggered conformations with respect to the equatorial Fe–N–a bonds: two coplanar with imidazole and two orthogonal to it. Hence, this structure indicates that there are two binding modes of O2, one with C5 symmetry and the other unsymmetrical C2. We have optimized the energies of both states, and the symmetric one is more stable than the unsymmetrical one. The geometries, charges, and spin densities of the two states were identical to within the accuracy of the present work, i.e. ±0.01e 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 O2 group in the model shown in a barrier of 1.9 kcal/mol (17).

Likewise, another unsymmetrical conformation arising from a 45° rotation of imidazole (staggered oxygen and eclipsed imidazole with respect to the Fe–N–a bond) was 2 kcal/mol less stable than the C5 conformation. The spin densities and charges were similar to within 0.02e of the C5 state, but the geometry showed differences of up to 0.07 Å from the Fe–O bond. Thus, we can conclude that the structure is the most stable geometry of this system and we have therefore employed C5 symmetry in all the calculations. This strongly facilitates the optimization and characterization of the various excited states. In C5 symmetry, the electronic states are labeled as symmetric A′ or antisymmetric A″, respectively, depending on whether their wave function preserves or changes sign upon reflection in the symmetry plane (xz, yz). The wave function for one and multiplicity (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–Fe–O bonds, the x-axis through two methine bridges (not the a–x nitrogen), and the y-axis through the other two methine bridges (Fig. 1). Thus, the imidazole and O2 molecules lie in the xz plane. The two unpaired π-electrons on oxygen are situated in two degenerate antibonding π orbitals, which transform as a′ and a″ in the reduced C5 symmetry when binding to deoxymyoglobin. In our coordinate system, three of the Fe d-orbitals transform as a, viz. xz, yz, and x′ y′ and would therefore couple to the a′ unpaired π-electron of O2, whereas the other two orbitals, xy and yx, 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 α and β 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 a a′ orbitals, 45 electrons in a a″ orbitals, 74 electrons in b a′ orbitals, and 45 electrons in b a″ orbitals). Some orbitals were found to be very high in energy and were subsequently avoided. For example, the state (74 45 74 45) had 521 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 I. The states are unrestricted Kohn-Sham wave functions with a large degree of spin polarization in most cases.
There are 238 electrons in the model, and they are partitioned into the \( \alpha \) or \( \beta \) orbitals of symmetry \( \alpha' \) or \( \alpha'' \), 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 wave function is antisymmetric (\( A' \)) if the total number of \( \alpha' \) electrons is odd; otherwise it is symmetric (\( A'' \)).

\[
\text{State} \quad a'' - a' - a'' - b' - b'' - E_{\text{rel}}
\]

\[
\begin{array}{cccccc}
\text{State} & a'' & a' & a'' & b' & b'' & E_{\text{rel}} \\
\hline
A'(1) & 74 & 45 & 74 & 45 & 0.0 \\
A'(1) & 75 & 45 & 73 & 44 & 22.0 \\
A'(1) & 74 & 46 & 73 & 45 & 24.1 \\
A''(2)^a & 74 & 46 & 73 & 45 & 19.7 \\
\text{A''(1)} & 75 & 47 & 72 & 44 & 28.5 \\
\text{A''(1)} & 73 & 46 & 74 & 45 & 28.9 \\
\text{A''(1)} & 74 & 46 & 74 & 44 & 24.7 \\
\end{array}
\]

\( ^a \) Antiferromagnetic state obtained from the septet dissociation product.

**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 I (the lowest closed-shell singlet with the same occupation numbers is 5 \( \text{kJ/mol} \) higher in energy). Its geometry is displayed in Table II and Fig. 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 \( \text{Å} \). For the more soft Fe–N \(_{\text{eq}}\) bond, the error is what can be expected with state-of-the-art DFT methods, 0.03 \( \text{Å} \). The optimized structure of this FeIII–O2 complex (Table II) agrees well (within 0.02 \( \text{Å} \)) with the crystal structure of oxymyoglobin at 1.15 \( \text{Å} \) resolution (36). It is notable that both structures show a strongly distorted porphyrin ring with the iron ion –0.3 \( \text{Å} \) 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 \( \text{O}_2 \), there are six unpaired electrons in the total system. The unpaired spin on deoxyhemoglobin and \( \text{O}_2 \) may be either parallel, giving rise to a septet, \( 7A' \), or antiparallel, which gives rise to a triplet state, which turns out to be \( 3A' \). At long (noninteracting) Fe–O distances, these two states are degenerate, as expected. Ideally, both states should give rise to rapid \( \text{O}_2 \) binding (i.e., all active sites of hemoglobin should be able to bind all \( \text{O}_2 \) molecules, independent of their spin states). However, as the Fe–O distance is decreased, the degeneracy is lifted. In the optimal structure, the \( 7A' \) state has a Fe–O bond length of 2.52 \( \text{Å} \), whereas it is 1.89 \( \text{Å} \) for state \( 3A' \) (cf. Table II). The potential energy surface of the \( 7A' \) state is flat around the minimum, and the energy is close to the dissociation limit, which is at 27 \( \text{kJ/mol} \) when calculated from separated species. The two states have very similar energies in their optimum geometries. Interestingly, the B3LYP method gives a quite different behavior of the \( 3A' \) state. The energy of this state increases steadily as the Fe–O bond length is decreased, with an energy of ~50 \( \text{kJ/mol} \) at the BP86 minimum. The B3LYP curve shows a very shallow minimum at Fe–O = 2.39 \( \text{Å} \), with an energy close to the dissociation limit.

The lowest triplet (intermediate-spin) state of deoxyhemoglobin 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 \( \text{kJ/mol} \) more stable (4 \( \text{kJ/mol} \) when optimized at the B3LYP level; hence the dissociation limit of the lowest triplet state is 27 \( \text{kJ/mol} \)). 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 \( 7A'(1) \) and a singlet state, which actually turns out to be the dissociation product of the singlet ground state \( 1A'(1) \).

**Excited States**—In Table I, the relaxed electronic spectrum is presented for the oxyhemoglobin model. It shows that there are six states within 30 \( \text{kJ/mol} \) (25 \( \text{kJ/mol} \) if optimized B3LYP structures are used) of the open-shell singlet ground state of oxy-
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 discussed previously, \(^7\Sigma^+(1), ^5\Delta^+(1),\) and \(^3\Sigma^+(2),\) there is another low-lying antisymmetric triplet state \(^3\Delta^+(1)),\) a symmetric triplet \(^3\Sigma^+(1)),\) and an antisymmetric singlet \(^1\Sigma^+(1)).\)

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 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 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 resulted in a similar ground state and low-lying \(^3\Delta^+\) and \(^1\Sigma^+\) states (at 0.47 and 1.54 eV), but in general, the spectrum had much larger energy separations than we had. 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 III. From these, it can be seen that the two dissociative states, \(^7\Sigma^+(1)\) and \(^5\Delta^+(1),\) are quite close to triplet \(O_2\) and high-or intermediate-spin \(Fe^{III}\), also in their optimum structures. The \(^3\Delta^+(2)\) state is quite well described as intermediate-spin \(Fe^{III}\) (2.98 unpaired electrons) antiferromagnetically coupled to \(O_2\) (1.13e) and the \(^3\Sigma^+(1)\) state is low-spin \(Fe^{III}\) (1.05e) ferromagnetically coupled to \(O_2\) (0.96e). The \(^1\Sigma^+(1)\) state has spin densities similar to the singlet ground state (i.e. low-spin \(Fe^{III}\) antiferromagnetically coupled to \(O_2\) ), whereas the \(^3\Delta^+(1)\) state...
is intermediate between O$_2$ and O$_3$ (1.57 unpaired electrons).

Mulliken charges for the various excited states are compiled in Table IV. Interestingly, whereas the spin densities on iron and O$_2$ vary appreciably for the various states, the charges are much more similar. For example, the variation in the charge of the N$_{ox}$ and N$_{oq}$ atoms is 0.05e and 0.09e, respectively. A somewhat larger variation is seen for the charge on the iron ion, varying between 0.58 and 0.73e. 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$_{ox}$ atoms in Table IV.

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, however, differs significantly within the various states. Therefore, the notion of Fe$^{III}$–O$_2$ and Fe$^{II}$–O$_3$ is only justified in terms of spin density and not in terms of the charge.

Binding of O$_2$—We now turn to the actual association mechanism. How does O$_2$ bind to heme in hemoglobin or myoglobin, facing the restrictions of spatial and spin symmetry? To answer this question, we calculated the fully relaxed geometry and potential energy as a function of the reaction coordinate at the right low-spin state upon binding to triplet O$_2$ (52). In this case, the primary electronic reorganization takes place in iron at an equilibrium between the quintet and triplet states already before 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 O$_2$. To see this, we will consider the Eyring expression of the reaction rate constant $k$ of O$_2$ binding,

$$k = k_BT/h \exp(-\Delta G^*/k_BT)$$  \hspace{1cm} (Eq. 1)

where $h$ and $k_B$ are Planck and Boltzmann constants, and $\Delta G^*$ 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).

$$P_{AB} = 1 - \exp(-2\pi\Delta E_{AB}/hv|S_A - S_B|)$$  \hspace{1cm} (Eq. 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

### Table IV

<table>
<thead>
<tr>
<th>State</th>
<th>Fe</th>
<th>O$_1$</th>
<th>O$_2$</th>
<th>N$_{ox}$</th>
<th>N$_{oq}$</th>
<th>N$_{ox}$</th>
<th>N$_{oq}$</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>
<td></td>
</tr>
<tr>
<td>$^5A'(1)$</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>
<td></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>
<td></td>
</tr>
<tr>
<td>$^3A'(2)$</td>
<td>0.70</td>
<td>-0.10</td>
<td>0.10</td>
<td>-0.36</td>
<td>-0.52</td>
<td>-0.51</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>$^5A'(1)$</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>
<td></td>
</tr>
<tr>
<td>$^3A'(1)$</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>
<td></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>
<td></td>
</tr>
</tbody>
</table>
the states when the perturbation that lifts their degeneracy has been applied, in our case the SOC.

To get a feeling for the various terms in this expression, we will insert reasonable values and compare the results for oxyhemoglobin with a similar but nonbiological process, the dissociation of the diatomic Fe–O system, studied by Danovich and Shaik (56). The term \( h\nu \) 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 nonbiological system, \(-5 \text{ kJ/Å/mol}\).

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 \(-1 \text{ kJ/mol}\) between various spin states in free iron (57), and it is 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 \(-0.8 \text{ kJ/mol}\) (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 \(-300 \text{ kJ/mol/Å}\) for sextet and quartet states (56). With such a large difference in the slopes, Danovich and Shaik (56) 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.

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 1A(1), 3A(2), 5A(1), and 7A(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 Fig. 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 $\frac{75}{25}$ 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 7A(1), for which the slope is 4 kJ/mol/Å or less).

However, even more important for the binding of O₂ to heme is the activation energy of the reaction, $\Delta G^\ddagger$ in Equation 1. From Fig. 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 with 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 O₂), this corresponds to a rate enhancement of ~$10^{10}$.

Thus, we can conclude that the facile binding of O₂ 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, despite 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 O₂ 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 resulted in a closed-shell ground state for oxyhemoglobin, which gives a Fe–O geometry different from the 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 O₂. 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 Fig. 2.

Comparison with Peroxidases—We have seen that the facile binding of O₂ 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 with the peroxidases.

Peroxidases are heme proteins that oxidize various substrates in one-electron reactions, using H₂O₂ 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 H₂O₂, and this reaction is spin-forbidden like the globin binding of O₂, because H₂O₂ is a singlet, whereas peroxymethemoglobin 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 backbone 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 imidazole 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 deoxyhemoglobin (33). 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 (33, 70).

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 a 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 of their spin state (two unpaired α or β 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, despite a modest spin-orbit coupling for iron. In fact, the transition probability, and therefore also the rate constant of O₂ binding, is increased by at least a factor of 15 compared with similar nonbiological iron complexes. Third, the detailed shape of curves enforces 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 O₂ 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 produce 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 found in the globins) results in near degeneracy for the Fe(II) state, whereas imidazole or imidazole, hydrogen-bonded to a carboxylate group (as is found in the peroxidases), instead produces degeneracy for the Fe(III) state (33).

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.

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REFERENCES
