Oxygen-carrying proteins: three solutions to a common problem

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Introduction

Both vertebrates and invertebrates derive most of their energy by ‘combustion’ of organic compounds, and thus require O₂. Given the surface-to-volume ratio of most higher organisms, simple diffusion of O₂ across body surfaces at ambient partial pressures would result in internal O₂ concentrations that are insufficient to sustain life. Furthermore, in response to various stresses, certain tissues or organs require a rapid infusion of O₂, for which passive diffusion would be insufficient. The existence of O₂-carrying proteins and their presence in large concentrations (approaching 20 mM for haemoglobin in red blood cells [1]) in higher organisms is thus rationalized. These proteins can be defined as those capable of undergoing the reversible reaction shown in eqn. (1) to some measurable degree at ambient temperatures and partial pressures of O₂:

\[
\text{Protein + O}_2 \rightleftharpoons \text{Protein - O}_2
\]

Three types of O₂-carrying protein are known: haemoglobin (Hb)/myoglobin (Mb), haemerythrin (Hr)/myohaemerythrin (myoHr) and haemocyanin (Hcy). Mammalian species contain Hb/Mb as the only known O₂-carrying proteins. The Hr/myoHr family has so far been found only in marine invertebrate species, and Hcy occurs in arthropods and molluscs. This Chapter attempts to summarize the current state of knowledge as well as some recent advances in
our understanding of chemical and biochemical aspects of O$_2$-carrying proteins, and focuses on the active sites of these proteins. Since all of these proteins contain either iron or copper at their active sites, some relevant chemical properties of O$_2$ and transition-metal ions are first summarized.

**Reactivity of molecular oxygen with transition-metal ions**

Thermodynamically, molecular oxygen has the capacity to be quite reactive, but kinetically it is quite sluggish in its reactions with most organic molecules. These two seemingly contradictory properties are rooted in the energies of the highest-occupied molecular orbitals (HOMOs) and lowest-unoccupied molecular orbital (LUMO) of the dioxygen molecule, which are shown in Figure 1. Since most organic molecules have paired electrons, i.e. singlet ground states, they are spin-forbidden from reacting with the triplet ground state of O$_2$. Complexation of dioxygen with transition-metal ions having unpaired electrons provides a relatively low-energy pathway for overcoming the spin restrictions to reactivity and, thus, the kinetic inertness of O$_2$. This low-energy pathway derives from overlap of metal-ion d-orbitals with the HOMOs and LUMO on O$_2$ upon complexation, and this overlap also provides a facile pathway for exchange of electrons between the metal ions and dioxygen, as shown schematically in Figure 2. Given the propensity of O$_2$ to accept electrons under ambient conditions and the multiple, readily accessible, positive oxidation states of many transition-metal ions, complexation usually results in net transfer of electron density from metal ion to dioxygen. As

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<tr>
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<th>(A)</th>
<th>(B)</th>
<th>(C)</th>
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<tbody>
<tr>
<td>O$_2$ oxidation state:</td>
<td>O$_2$</td>
<td>O$_2^-$</td>
<td>O$_2^{2-}$</td>
</tr>
<tr>
<td>O–O bond order:</td>
<td>2</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>LUMO:</td>
<td>(\sigma^*_{2p})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOMOs:</td>
<td>(\pi^<em>_{2p}(x), \pi^</em>_{2p}(y))</td>
<td>(1)</td>
<td>(1)</td>
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</tbody>
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![Figure 1. HOMOs and LUMO of the dioxygen molecule and its one- and two-electron reduced forms](image-url)

(A) Dioxygen; (B) superoxide; (C) peroxide. Shapes of the HOMOs and LUMO are shown to the right of the \(\pi^*\) and \(\sigma^*\) energy levels, respectively, with the O–O bond axis (defined as the z-axis) oriented horizontally. Only one of the two identically shaped HOMOs is shown. The other HOMO would have its lobes oriented perpendicular to the plane of the page.
shown in Figures 1 and 2, this electron transfer occurs into effectively anti-bonding orbitals on O₂. This transfer of electron density manifests itself in lower O–O stretching frequencies and longer O–O distances (due to weaker O–O bonds) in metal–dioxygen adducts compared with molecular oxygen (see Table 1). However, in order for the O₂ binding to be reversible, the extent of electron transfer from metal ion to dioxygen must be delicately balanced, i.e. enough to form a stable complex, but not so much that the O–O bond is cleaved irreversibly.

Table 1. Metal-ion complexation weakens the O–O bond [2]

<table>
<thead>
<tr>
<th>Species</th>
<th>νO–O (cm⁻¹)*</th>
<th>O–O Bond length (Å)</th>
</tr>
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<tbody>
<tr>
<td>Dioxygen</td>
<td>1555</td>
<td>1.21</td>
</tr>
<tr>
<td>Metal-superoxo</td>
<td>1100–1150</td>
<td>1.24–1.31</td>
</tr>
<tr>
<td>Metal-peroxo</td>
<td>800–900</td>
<td>1.35–1.5</td>
</tr>
</tbody>
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*O–O Stretching frequency in wavenumbers.

The active sites of oxygen-carrying proteins

Studies of synthetic metal–dioxygen interactions (reviewed extensively in [3]) have shown that oxygen-carrying proteins modulate the delicate balance referred to above in at least three general ways: the choice of metal ion, the type of complex (i.e. type and number of ligands to the metal ion, coordination geometry etc.), and the environment of the complex. The natural
selection of iron (in Hb/Mb and Hr/myoHr) and copper (in Hcy) as the metal ions in oxygen-carrying proteins could not necessarily have been predicted from studies of synthetic complexes. Although synthetic iron and copper complexes that reversibly bind O₂ are known, synthetic cobalt complexes that reversibly bind O₂ have also been known for many years, but have no known biological counterpart. One rationale for Nature’s selection of iron and copper over other transition metals for reversible O₂ binding is that the large concentrations of O₂-binding proteins necessary for life in higher organisms can be attained only by using metals with relatively high bio-availabilities, such as copper and particularly iron [4].

Figure 3. The active site structures of O₂-carrying proteins
Active sites are shown schematically for (A) sperm whale Mb, (B) Themiste dyscritum (‘peanut’ worm) Hr, and (C) Limulus polyphemus (horseshoe crab) Hcy. Deoxy forms are shown on the left, and oxy forms on the right. Metal ligands are represented as atom-undifferentiated line drawings, and are identified in the text. Blue spheres represent either iron or copper ions, and grey spheres represent bound dioxygen atoms. Black spheres in (b) represent oxo or hydroxo ions. The drawings are based on X-ray crystal structure co-ordinates deposited in the Brookhaven Protein Data Bank (Mb, 1MBD, 1MBO [5,6]; Hr, 1HMD, 1HMO [7]; Hcy, ILLA, IOXY [8,9]).
Fairly high-resolution X-ray crystal structures are now available for all three types of O₂-carrying protein in both their oxy and deoxy forms. The deoxy and oxy active-site structures are shown in Figure 3, and the backbone

Figure 4. The backbone structures of O₂-carrying protein subunits

(a) Sperm whale Mb, (b) T. dyscritum Hr, and (c) L. polyphemus Hcy. Deoxy forms are shown. Spheres represent either iron (Mb, Hr) or copper (Hcy) ions. The drawings are based on X-ray crystal structure co-ordinates (see Figure 3 legend for references) and are scaled to the relative sizes of the subunits.

Fairly high-resolution X-ray crystal structures are now available for all three types of O₂-carrying protein in both their oxy and deoxy forms. The deoxy and oxy active-site structures are shown in Figure 3, and the backbone
structures of the protein subunits are shown in Figure 4. Even a cursory inspection of these structures leads to an obvious conclusion: the three types of oxygen-carrying protein bear no detectable structural similarities to each other, at either subunit-fold or active-site levels. This conclusion is borne out upon detailed inspection as well. Thus although helical regions of the polypeptides surround the active sites in all three types of oxygen-carrying protein, and all of the active sites contain at least one histidine ligand, there is no detectable amino acid sequence homology among them. Clearly, Nature has found three distinct solutions to the problem of binding O₂ reversibly.

**Hb and Mb**

Hb and Mb incorporate the macrocyclic tetrapyrrrole, protoporphyrin IX, which binds a single iron ion via the four pyrrole nitrogen atoms located near its centre. The resulting chelate, called haem, is the prosthetic group that reversibly binds O₂ to the distal side of the haem, as shown in Figure 3. A histidine ligand from the protein (referred to as the ‘proximal’ histidine) forms a fifth Fe–N bond, which is approximately perpendicular to the haem plane. Hb is tetrameric (α₂β₂), and each subunit is structurally and functionally very similar to the monomeric Mb. Hb circulates through the body within red blood cells, whereas Mb occurs within muscle tissues. The literature on Hb and Mb is amongst the most voluminous on any protein, and even a relatively limited survey would be impossible in this short essay. Instead, the current status of two intensively studied, but still incompletely resolved, issues about O₂ binding to Hb and Mb will be discussed: (i) the electronic distribution within the Fe–O₂ unit, and (ii) discrimination between O₂ and CO binding.

**The electronic distribution within the Fe–O₂ unit**

Pauling and Coryell’s classic magnetic susceptibility measurements, published in 1936, showed that, whereas deoxyHb is paramagnetic, oxyHb is diamagnetic, consistent with spin pairing of all electrons [10]. This result initiated a controversy about both the electronic distribution and the geometry of the Fe–O₂ unit in Hb. The fascinating history of this controversy, including unsubstantiated challenges to Pauling and Coryell’s results, has recently been summarized by Momenteau and Reed [11], and also by Bytheway and Hall [12]. The question of geometry has been settled largely by X-ray crystallography, which clearly shows that O₂ binds to the haem iron in a bent end-on manner in oxyHb, oxyMb and synthetic haem–O₂ adducts. This geometry is illustrated for oxyMb in Figure 3. The reported Fe–O–O angles are within the range of 115–160° [6], which, given the experimental uncertainty, are consistent with the molecular orbital description for end-on O₂ σ-type bonding in these complexes (Figure 2a).

The paramagnetism of deoxyHb can be rationalized readily and quantitatively in terms of the four unpaired d-electrons expected for high-spin Fe(II)
d_{x^2-y^2}, d_{z^2} & \_ & 1 & _ & _ & _ & _ & _  \\
\_ & d_{xy}, d_{xz}, d_{yz} & \_ & \_ & 1 & \_ & \_ & \_  \\
hs \text{Fe(II)} & Is \text{Fe(II)} & Is \text{Fe(III)}

**Scheme 1.** d-Electron configuration for high-spin (hs) Fe(II), low-spin (ls) Fe(II) and low-spin Fe(III)

(Scheme 1), and a wealth of spectroscopic evidence supports this description. The structural results also show that upon conversion of the deoxy to the oxy forms the iron atom moves closer to the porphyrin plane (see Figure 3). This structural change implies a decrease in the radius of the iron ion upon conversion of the deoxy to the oxy forms, and has been interpreted as indicating a change from high- to low-spin state of the iron, and/or oxidation of Fe(II) in the deoxy forms to a higher formal oxidation state [Fe(III)] in the oxy forms. If the iron remains Fe(II) in the oxy forms, the high-to-low-spin conversion would result in its six d-electrons becoming completely spin paired [ls Fe(II) in Scheme 1]. The pair of electrons in the filled $\pi^5$ orbital of an effectively spin-singlet $O_2$ would then have to be donated to the $d^{2}$ orbital on iron, as shown in Figure 2(a) (\(\sigma\)-type), in order to explain the observed diamagnetism. Back donation of d-electrons on iron into the other $\pi^5$ orbital of $O_2$, as shown in Figure 2(a) (\(\pi\)-type), could also occur, leading to $\text{Fe(II)}-O_2^{2-} \leftrightarrow \text{Fe(IV)}-O_2^{2-}$ resonance structures.

Alternatively, if, upon $O_2$ binding, one electron was transferred from Fe(II) to the co-ordinated $O_2$, the resulting low-spin Fe(III) would have one unpaired d-electron [ls Fe(III) in Scheme 1]. This d-electron could be spin-paired with the remaining unpaired electron on the co-ordinated $O_2^{2-}$ via the orbital overlaps shown in Figure 2(a) (\(\pi\)-type), thereby achieving diamagnetism. The reported O–O distances in oxyHb and oxyMb range from 1.2–1.3 Å [6], which, when compared with the distances listed in Table 1, are consistent with the superoxo formalism. However, the experimental uncertainties on the O–O distances in the proteins are too large to rule out alternative bonding descriptions. More direct evidence that the superoxo formalism accurately describes the oxidation state of co-ordinated $O_2$ comes from vibrational spectroscopy, which gives an O–O stretch near 1100 cm$^{-1}$ for both oxyHb and oxyMb [11]. As can be seen from Table 1, this frequency is in the range expected for metal–superoxo complexes, and is well separated from that for either molecular $O_2$ or $O_2^{2-}$. Thus considerations of basic bonding and reactivity, as well as experimental results, support the notion that some electron transfer occurs from haem–Fe(II) in deoxyHb and deoxyMb to $O_2$ upon formation of the oxy adducts. Many bioinorganic chemists regard low-spin Fe(III)–$O_2^{2-}$ as the most accurate and useful representation of the electronic distribution in
oxyHb and oxyMb, and there seems to be little interest in revisiting this issue. Nevertheless, as for all formal oxidation-state representations, the electronic distribution and charges represent approximations, not literal truth.

**Discrimination between O\textsubscript{2} and CO binding**

Synthetic penta-co-ordinate haems in solution favour binding of CO over O\textsubscript{2} by a factor of approximately 100000, whereas Hb and Mb bind CO only about 100-fold more strongly than O\textsubscript{2} [1]. Even the latter lower ratio is not sufficient to prevent the well-known artificial poisoning caused by CO binding to Hb and Mb. However, this toxicity would be even more acute were it not for the discrimination against CO imposed by the surrounding protein. This discrimination is particularly important because it minimizes interference with O\textsubscript{2} binding from CO generated by biological processes such as haem degradation. How is the CO/O\textsubscript{2} affinity ratio reduced in Hb and Mb relative to those of synthetic haems? Until recently, the ‘textbook’ explanation was that residues lining the O\textsubscript{2}-binding pocket in these proteins, particularly the conserved ‘distal’ histidine (not shown in Figure 3), sterically hindered attainment of the preferred linear Fe–C–O geometry observed in the synthetic haem–CO complexes. On the other hand, as discussed above, the Fe–O–O unit prefers to be bent, so that this steric restriction should be less of a hindrance to O\textsubscript{2} binding. Indeed, X-ray crystal structures of CO adducts of Hb and Mb seem to show a bent, i.e. energetically unfavourable, Fe–C–O unit (Fe–C–O angle of 20–40° measuring the C–O bond axis relative to an axis perpendicular to the average plane of the haem atoms).

However, more recent results are at odds with the distal histidine/steric hindrance explanation for inhibition of CO binding. Both vibrational spectroscopy [13] and polarized infrared spectroscopy [14,15] indicate that the Fe–C–O unit is very close to linear, contrary to the X-ray crystallographic results. Furthermore, site-directed mutagenesis of the distal histidine to residues having either larger or smaller side chains does not have the effects on CO affinity expected for the steric-hindrance explanation [1]. For example, replacement of the distal histidine in Mb with a leucine residue resulted in a 30-fold increase in CO affinity. The effect of the distal leucine replacement on CO affinity could be both hydrophobic and steric in nature, i.e. the isopropylmethyl side chain of leucine is both smaller and less polar than the side chain of histidine. If steric restrictions of the distal side chain dominate CO affinity, then replacement of the leucine with progressively smaller aliphatic side chains, namely those of valine (isopropyl), alanine (methyl) and glycine (hydrogen), should produce larger increases in CO affinity compared with the wild-type than does the leucine replacement. In fact, the opposite occurs; the valine, alanine and glycine mutants show smaller increases in CO affinity than does the leucine mutant [1]. These results strongly suggest that steric restrictions imposed by the distal side chain do not control CO affinity in Mb. Since CO/O\textsubscript{2} discrimination also needs to be addressed, it is important to note that
the distal histidine-to-leucine mutant of Mb showed a 100-fold decrease in O₂ affinity, making the discrimination between CO and O₂ similar to that of synthetic penta-co-ordinate haems.

The following revised explanations for the CO/O₂ discrimination in Hb and Mb are emerging. Pocket polarity rather than steric hindrance is a key factor. This polarity influences ligand binding in two ways. First, the wild-type Mb and Hb crystal structures show a water molecule occupying the O₂-binding pocket in the deoxy forms, but this water is not co-ordinated to the haem iron. The CO adducts show no such ‘pocket water’; it is apparently displaced upon CO binding. Thus ligand binding is inhibited by polar pocket residues, which provide a favourable environment for the pocket water compared with the relatively non-polar co-ordinated CO. The relatively polar Fe⁸⁻–O–O⁸⁻ unit, on the other hand, is stabilized by hydrogen bonding to the polar distal histidine, to such an extent that the pocket water is more easily displaced. This explanation is consistent with the relative changes in CO and O₂ affinities of the distal histidine-to-leucine Mb mutant discussed above. Results of time-resolved infrared polarization spectroscopy following photolytic cleavage of the Fe–CO bond suggest another factor that may discriminate against CO binding [15]. CO can be induced to dissociate from the haem by short (ps time-scale) pulses of green polarized laser light. The orientation of the dissociated CO with respect to the haem can then be probed by ultra-short (fs time-scale) polarized pulses of infrared light having energy corresponding to the stretching frequency of the C–O bond. These experiments show that shortly (within a ps) after photolytic dissociation, the C–O bond axis lies parallel to the haem plane in a ‘docking site’ near the binding pocket, and the CO remains there for a few hundred nanoseconds without rebinding to iron. The protein may thus provide an energetic barrier to the preferred perpendicular orientation required for CO binding to the haem iron, but this barrier does not involve steric hindrance by the distal histidine. However, this issue remains controversial; not all scientists in the field are ready to abandon the distal histidine/steric hindrance explanation.

**Hr and myoHr**

Of the three types of O₂-carrying protein, the molecular details of O₂ binding to the active site of Hr were, chronologically, the next to be clarified. Hr is most often found as an octamer of essentially identical O₂-binding subunits, and is thought to serve primarily as an O₂-storage reservoir in the marine invertebrates in which it occurs [7]. MyoHr fulfils a function more closely related to that of Mb and is confined to muscle tissues of the same marine invertebrates. The structure of the myoHr subunit and active site are both very similar to those of Hr (cf. Figures 3 and 4). Therefore, the following discussion applies, with very few exceptions, equally well to both Hr and myoHr. The two iron atoms at the active site are bound directly to protein side chains: five
histidines, one aspartate and one glutamate, the latter two carboxylates of which bridge the two irons. An accumulation of spectroscopic evidence [16] had established fairly conclusively, prior to the structural results, that the oxidation state changes shown in eqn. (2) accurately describe O₂ binding in Hr and myoHr (where μ indicates a bridging OH⁻ or O²⁻):

\[
[Fe^{II}_1(\mu-\text{OH})Fe^{II}_2]+O_2 \rightleftharpoons [Fe^{III}_1(\mu-O)Fe^{III}_2O_2H^+]^{\text{oxy}}
\]

Thus upon binding, O₂ is formally reduced to the peroxide level by the two Fe(II) in deoxyHr. Perhaps the most direct evidence for the formal oxidation state of the bound O₂ in oxyHr comes once again from vibrational spectroscopy, which shows an O–O stretch at 844 cm⁻¹ [16]. This frequency clearly indicates a peroxide (O₂²⁻) oxidation state, as can be seen by comparison with the values in Table 1. Thus eqn. (2) describes an elegant oxidative addition/proton-transfer reaction, which does not directly involve any amino acid residues. Stenkamp et al. [17] first proposed the active-site structures and mechanism shown in Figure 5 in 1985, and subsequent work has confirmed many of their proposals.

The O₂-binding pocket surrounding Fe2 is hydrophobic, with no nearby water, proton donors or nucleophiles (other than the iron ligands) in either the

![Figure 5. Structural mechanism for O₂ binding to the di-iron site of Hr and myoHr](image)

Formal oxidation-state changes of Fe1, Fe2 and O₂ accompany the structural changes, as described in the text. O₂ enters the binding pocket and co-ordinates in a bent end-on fashion to Fe2. This binding initiates oxidation of Fe2, with concomitant shortening of the Fe2–(μ–O) bond (because increasing the metal oxidation state creates a better Lewis acid, thereby attracting nucleophilic ligands more strongly). The increased competition for the electrons on the bridging oxygen causes lengthening and weakening of the O–H bond, and the proton is also attracted to the incipient negative charge developing on the terminal oxygen atom of end-on co-ordinated O₂. The loss of the proton from the bridging oxygen would, in turn, favour shortening of the Fe1–(μ–O) bond, thereby facilitating oxidation of Fe1, even though Fe1 does not directly interact with O₂.
deoxy or oxy forms [7]. Furthermore, the steric constraints of the O₂-binding pocket, together with the five ligands to Fe₂ (Figures 3 and 5) in the deoxy form, favour a bent, end-on co-ordination of O₂ to Fe₂, but the six ligands to Fe₁ and other protein steric constraints greatly inhibit inner-sphere access of O₂ to Fe₁. Based on comparisons to synthetic di-iron complexes [18], uninhibited interaction of O₂ with both Fe₁ and Fe₂ and/or a more polar O₂ pocket would probably make the di-iron site in Hr much more prone to the autoxidation reaction shown in eqn. (3). Autoxidation of Hr does in fact occur, but on the time scale of a day or two at room temperature and ambient partial pressures of O₂. This time scale is several orders of magnitude slower than for the reversible O₂ binding and release reactions represented by eqn. (2).

\[
[\text{Fe}^{III}({\mu-O})\text{Fe}^{III_2}O_2H^-]+H^+ \rightarrow [\text{Fe}^{III}({\mu-O})\text{Fe}^{III}]^+\text{H}_2\text{O}_2 \quad (3)
\]

The intermediate depicted between oxy and deoxy in Figure 5 has never been observed, even when using rapid kinetics methods. Thus whether Fe₁ is oxidized prior to, concomitant with, or following, transfer of the proton from the oxo bridge to the bound O₂ is not known. Reasonably good evidence exists for formulation of the nitric oxide adduct of deoxyHr as [Fe₁⁺(μ-ΟH)Fe₂⁺NO⁻] [19], suggesting by analogy that [Fe₁⁺(μ-ΟH)Fe₂⁺O₂⁻] is a reasonable formulation for the intermediate, as depicted in Figure 5. However, the failure to detect this (or any other) intermediate implies that the rate-determining step leading to the oxy form occurs either prior to or during formation of this intermediate (with the subsequent conversion to oxy being much faster), and that some combination of reverse proton transfer, electron transfer and cleavage of the Fe–O₂ bond together constitute the rate-determining process for O₂ release. Whereas this rationale is consistent with the available kinetic data [20,21], the elementary steps governing the rates of O₂ binding and release in Hr and myoHr remain to be delineated. Residues lining the O₂-binding pocket, several of which are conserved in all known Hrs and myoHrs, are likely to influence or control some of these steps, and their role is just beginning to be examined [22].

Hcy

Hcys are all large, multidomain, multisubunit proteins and, partly due to their large sizes, the structural and electronic aspects of O₂ binding were the most recent to be clarified among the O₂-carrying proteins. The unusual side-on bridging geometry of O₂ shown in Figure 3 was finally determined by X-ray crystallography in 1994 [8,9]. However, this geometry was accurately predicted from studies of the beautiful model dicopper(II)-peroxo complex synthesized and structurally characterized by Kitajima et al. [23,24]. The diamagnetism and O-O stretching frequency of this synthetic complex also accurately modelled those of oxyHcy and, together with the structure, greatly
clarified what had been difficult-to-explain properties of the oxy protein. The 750-cm\(^{-1}\) O–O stretching frequency of oxyHcy is unusually low, even for metal–peroxo complexes (see Table 1). Based on the structure of Kitajima’s complex, Solomon and co-workers explained this unusually low frequency as due to a π-acceptor interaction of the σ\(^*\) orbital on dioxygen (see Figure 1) with d-orbitals on both coppers [25]. This interaction results in transfer of electron density from the two Cu(II) ions to an effectively anti-bonding orbital on O\(_2\)\(^{2-}\) (i.e. the σ\(^*\)), which is normally unoccupied and non-interacting in other metal–peroxo complexes. In terms of weakening the O–O bond strength, this π-acceptor interaction more than compensates for σ donation from the effectively π\(^*\) orbital on O\(_2\)\(^{2-}\) to d-orbitals on Cu(II), which occurs for the side-on geometry, as illustrated in Figure 2(b). The long-accepted formal oxidation-state changes embodied in eqn. (4) for the oxygenation reaction of Hcy are, thus, rationalized.

\[
[Cu(I), Cu(I)] + O_2 \Leftrightarrow [Cu(II)(\mu-O_2^{2-})Cu(II)]
\]

Complexes containing a single Cu(II) have one unpaired d-electron; therefore, the observed room-temperature diamagnetism of oxyHcy must be due to spin-pairing of the two originally unpaired d-electrons, one from each Cu(II). Since the CuA–CuB distance of 3.6 Å [8,9] in oxyHcy is considered to be too long for direct overlap of d-orbitals on the two Cu(II), the spin pairing is presumably mediated via relatively weak interactions with paired electrons in orbitals of the bridging peroxide in a phenomenon referred to as superexchange [25]. Whatever the most accurate bonding description may be, it is clear that a (nearly) planar, side-on-O\(_2\)-bridged \([Cu(II)(\mu-O_2^{2-})Cu(II)]\) unit results in unusually extensive orbital overlaps and bonding interactions between metal and dioxygen; nevertheless, this unit can retain reversibility.

X-ray crystal structures of deoxyHcys from two arthropod species both show a dicopper site that appears to be well poised for incorporation of an exogenous bridging ligand (see Figure 3). CuA and CuB are each 3-co-ordinate with no atoms from protein side chains or solvent water visible between the two coppers. In the one case where X-ray crystal structures of the same Hcy (from horseshoe crab) in both forms are available [8,9] (see Figure 3), the CuA–CuB distance decreases by about 1 Å (from 4.6 to 3.6 Å) upon transformation of the deoxy to the oxy form. On the other hand, the side chains of the six histidine ligands move very little upon transformation between oxy and deoxy forms, and the remainder of the tertiary and quaternary structures of the two forms are also very similar to each other. This similarity belies the well-documented co-operativity in O\(_2\) binding exhibited by all Hcys [9]. Co-operativity, in which binding of O\(_2\) to one subunit increases the O\(_2\) affinity of other subunits in the oligomer, is seen to some extent for all multisubunit O\(_2\)-carrying proteins, and even a cursory review of this phenomenon would require a separate chapter. A structural mechanism for co-operativity in arthropod Hcys is proposed in [1]. X-ray crystal structures of molluscan Hcys
are eagerly awaited, because they could help define a mechanism of co-operativity and also could confirm the presence of an unusual cysteine–histidine thioether bridge to one of the histidine ligands of the dicopper site [26].

**Perspectives**

How and why did Nature evolve three different solutions to the problem of reversible dioxygen binding? What are the evolutionary antecedents to the three types of O₂-carrying protein? Although fascinating to ponder, these questions may remain forever unanswerable, unless a much more complete picture of evolutionary biology becomes available. A more useful pursuit and realistically attainable goal is probably an understanding of the catalytic chemistry occurring in enzymes with active sites similar to those found in O₂-carrying proteins. Rather than reversibly binding O₂, these enzymes use O₂ as one substrate to oxidize a second substrate, e.g. hydrocarbons, and the enzymic reactions are invariably accompanied by O–O bond cleavage. Parallel examples are known of these so-called ‘O₂-activating’ enzymes with active sites closely resembling those in each type of O₂-carrying protein (Table 2) [27–30]. These parallel examples suggest that the three types of metal site shown in Figure 3 are particularly well-suited to dealing with the chemistry of dioxygen. Subtle alterations in the environment of the metal ion–dioxygen complex within the O₂-activating enzymes must encourage the thermodynamic propensity of O₂ towards its further reduction and, simultaneously, channel this propensity towards biochemically useful oxidations. The existence of O₂-carrying proteins and analogous O₂-activating enzymes apparently represents Nature’s tiptoeing along the edges of the energetic barrier separating reversible O₂ binding from O–O bond cleavage without crossing it. Detailed structure–function comparisons of O₂-binding protein/O₂-activating enzyme pairs may ultimately provide a clearer understanding of the factors required to avoid crossing this barrier while maintaining functionality. For example, it is clear, at least in the cases of the haem and non-haem di-iron active sites, that changes in the type and/or number of metal ligands supplied by the protein constitute one such factor [27,29]. These protein/enzyme pairs are also one reason why the electronic distributions within the metal–O₂ units in O₂-carrying proteins merit detailed

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**Table 2. O₂-carrying protein/O₂-activating enzyme analogues**

Excellent reviews of the O₂-activating enzymes can be found in [27–29].

<table>
<thead>
<tr>
<th>Active site</th>
<th>O₂-Carrier</th>
<th>O₂-Activator</th>
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</thead>
<tbody>
<tr>
<td>Haem</td>
<td>Hb</td>
<td>Peroxidases, cytochrome P450</td>
</tr>
<tr>
<td>Di-iron</td>
<td>Hr</td>
<td>Ribonucleotide reductase, methane mono-oxygenase</td>
</tr>
<tr>
<td>Dicopper</td>
<td>Hcy</td>
<td>Tyrosinase, ascorbate oxidase</td>
</tr>
</tbody>
</table>
and accurate descriptions. Only then can we understand how and why this distribution may differ in the $O_2$-activating-enzyme counterparts.

Finally, perhaps the most intriguing question of all: are there other types of $O_2$-carrying protein with active sites different from those shown in Figure 3 awaiting discovery?

**Summary**

- *Nature has used transition-metal ions with unpaired d-electrons to overcome the kinetic inertness of $O_2$ and to control its thermodynamic tendency towards reduction.*
- *High-resolution X-ray crystal structures of $O_2$-carrying proteins show that Nature has devised three distinct solutions to the problem of reversible $O_2$ binding. The three types can be classified according to their active sites: Hb (haem iron); Hr (non-haem di-iron); and Hcy (dicopper).*
- *The reversible $O_2$ binding to the three types of active site are formally oxidative additions: $Fe(II) to Fe(III)-O_2$ for Hb; $[Fe(II),Fe(II)] to [Fe(III),Fe(III)O_2]$ for Hr; and $[Cu(I),Cu(I)] to [Cu(II)(\mu-O_2Cu(II)] for Hcy. In all cases the O–O bond is weakened, but not cleaved, upon binding.*
- *The ‘textbook’ explanation for discrimination against CO and $O_2$ binding to Hb has been revised: steric constraints to the preferred linear Fe–C–O geometry imposed by the ‘distal’ histidine are no longer thought to play a major role. Instead, recent experimental evidence indicates that the polarity of the binding pocket favours the polar Fe–O–O unit over the relatively non-polar Fe–C–O unit, and that a C–O-binding pocket near the haem also inhibits the preferred linear Fe–C–O geometry.*
- *Reversible $O_2$ binding to the di-iron site of Hr involves an internal proton transfer as well as electron transfer to $O_2$, but the elementary steps governing the rates of $O_2$ binding and release, especially the effects of the surrounding protein, remain to be delineated.*
- *An unusual side-on-bonded $O_2$ that bridges the two copper ions explains both the unusually low O–O stretching frequency and the diamagnetism of oxyHcy.*
- *$O_2$-activating-enzyme counterparts exist for each of the three known types of $O_2$-carrying protein. Detailed comparisons of these protein/enzyme pairs are likely to clarify the factors that tune the delicate balance between reversible $O_2$ binding and controlled O–O bond cleavage.*
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References

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