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Bis-Histidine-Coordinated Hemes in Four-Helix Bundles: How the Geometry of the Bundle Controls the Axial Imidazole Plane Orientations in Transmembrane Cytochromes of Mitochondrial Complexes II and III and Related Proteins

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Abstract

Early investigation of the EPR spectra of bis-histidine-coordinated membrane-bound ferriheme proteins led to the description of a spectral signal that had only one resolved feature. These became known as “highly anisotropic low-spin” (HALS) or “large g_{max} ” ferriheme centers. Extensive work with small-molecule model heme complexes showed that this spectroscopic signature occurs in bis-imidazole ferrihemes in which the planes of the imidazole ligands are nearly perpendicular, $\Delta\phi = 57\text{--}90^\circ$. In the last decade protein crystallographic studies have revealed the atomic structures of a number of examples of bis-histidine heme proteins. A frequent characteristic of these large g_{max} ferrihemes in membrane-bound proteins is the occurrence of the heme within a four-helix bundle with a left-handed twist. The histidine ligands occur at the same level on two diametrically opposed helices of the bundle. These ligands have the same side chain conformation and ligate heme iron on the bundle axis, resulting in a quasi-2-fold symmetric structure. The two non-ligand-bearing helices also obey this symmetry, and have a conserved small residue, usually glycine, where the edge of the heme ring makes contact with the helix backbones. In many cases this small residue is preceded by a threonine or serine residue whose side chain hydroxyl oxygen acts as a hydrogen-bond acceptor from the $\text{N}^{\delta 1}$ atom of the heme-ligating histidine. The $\Delta\phi$ angle is thus determined by the common histidine side-chain conformation and the crossing angle of the ligand-bearing helices, in some cases constrained by H-bonds to the Ser/Thr residues on the non-ligand-bearing helices.

A structural motif for transmembrane helical-bundle cytochromes

In this report we describe a tertiary structural motif found in a number of membrane-spanning cytochromes, including mitochondrial respiratory Complexes II and III (Figure 1) and their bacterial homologues, as well as formate dehydrogenase and related dehydrogenases and hydrogenases. The motif consists of a transmembrane antiparallel 4-helix bundle enclosing a heme moiety ligated by the $\text{N}^{\epsilon 2}$ atoms of two histidine residues at the same level on two diametrically opposite helices near one end of the bundle. This is illustrated by close-up views of the heme-binding site of Complex II in Figure 2, A–C. In some cases a heme is found near both ends of the bundle, but this will be considered two separate instances of the motif, sharing the same four helices. In addition to the histidines on the heme-bearing helices, the motif is characterized by glycines in the non-heme-bearing helices at a position where the heme ring

touches the peptide backbone. The presence of these conserved glycines was noticed as early as 1991 for cyt *b* of the *bc₁* complex, and their role in heme binding was deduced [1]. The importance of two of these glycines in the bacterial *bc₁* complex was confirmed by site-directed mutagenesis [2,3]. The residue preceding this glycine is often serine or threonine, and we have noticed that where structures are available, the side-chain of this residue is in rotamer 2¹, putting the O^γ atom in a position to make an H-bond with the N^{δ1} atom (hindered N) of the heme-ligating histidine in the following helix.

The heme-ligating histidines are usually in the third most common conformation (rotamer 3 of the rotamer library¹ distributed with the modeling program “O” [4]) and are related to each other by a two-fold rotation about the axis of the helix bundle. The imidazole planes of the two ligating histidines are, in most cases, within 30° of being perpendicular in those examples where structures are available. As described below, this may be important for the electronic properties of the heme; in fact, we suggest that maintaining this less-stable perpendicular orientation may be one of the constraints leading to the architecture of this motif. When present, the Ser or Thr residue preceding the conserved glycines in the non-histidine-bearing helices may serve to fine-tune or constrain the distribution of the angle by the hydroxyl oxygen acting as H-bond acceptor from the imidazole N^{δ1}-H.

Examples of the motif are found in cytochromes *b* of the mitochondrial and bacterial cytochrome *bc₁* complexes (Complex III; See Figure 1) [8–23], chloroplast or cyanobacterial cytochrome *b₆f* complexes [24–28], mitochondrial and bacterial succinate:ubiquinone oxidoreductases (SQR, Complex II; Figure 1) [29,30], fumarate:menaquinone reductase from *Wolinella succinogenes* [31], and formate dehydrogenase of *E. coli* [32]. Table 1 below lists these examples together with residue numbers of the axial-ligand histidines and average values for some structural parameters discussed below. Other proteins for which structures are unavailable, but which show significant sequence homology with one of these members, include diverse bacterial heme-containing fumarate:menaquinone reductases and succinate dehydrogenases [33,34] and a variety of dehydrogenases and hydrogenases related to formate dehydrogenase [35–37]. Although sequence alignment between the different families is not meaningful due to low sequence homology, structure-based sequence alignments can be made where structures are available, and other sequences sufficiently homologous to one of the structurally aligned proteins can be brought into the alignment by their sequence. Such an alignment illustrating the conserved “notch” Gly residues, frequently preceded by Ser or Thr, in the non-ligating helices is provided as Figure S1 in the Supporting Information.

¹Side-chain conformations are described in terms of the nearest common rotamer, and deviation from that rotamer, using the small set of most common rotamers distributed with the program O [4]. The χ^1 , χ^2 sidechain dihedral angles corresponding to rotamers 2 – 4 of histidine, as well as serine rotamers 1 and 2, are given in the following table. “Freq” is the frequency with which the rotamer occurs in the population originally used to define the rotamers [5].

Residue	Rotamer	Freq.	χ^1	χ^2
His	2	19%	–59	100
	3	16%	–169	80
	4	12%	–169	–90
	5	8%	–59	169
Ser	1	45%	63	-
	2	30%	–62	-

Symmetry of the prototypical 4-helix heme bundle

The alternating up/down orientation of helices going around the antiparallel four-helix bundle imparts a two-fold symmetry about the axis of the bundle, as 90° rotation superimposes helices of opposite orientation while 180° rotation superimposes helices of the same orientation. To a good approximation the heme iron is on the bundle axis, and the heme plane is parallel to, i.e. contains, the bundle axis. Thus 180° rotation about the axis brings the heme plane onto itself. However the pseudo-two-fold axis of the heme moiety is not along the bundle axis, so this rotation does not superimpose individual atoms of the heme. The two histidine axial ligands are in the same conformation (rotamer 3¹) in the prototype, and use the unhindered ring nitrogen N^ε2 to bind the heme iron on the bundle axis, and as a result they are superimposable by two-fold rotation about the bundle axis.

This is illustrated for Complex II in Figure 2A, where the four-helix bundle is superimposed upon itself after rotations to bring helices 1 and 2 onto helices 3 and 4 and vice versa. In Complex II, the bundle is actually formed from two separate homologous chains C and D, each contributing two adjacent helices to the four-helix bundle. This suggests that this motif may have originated as a homodimeric structure, with the monomers coming together in a symmetrical way and binding one heme molecule between them.² In fact there is evidence that over-expression of chain D of *E. coli* SDH leads to a heme-containing homodimer [38]. However, we do not know whether the four-helix heme bundle structures have a common evolutionary origin or represent convergent evolution from separate origins.

While the heme ligand-bearing helices (C2 and D2) superimpose very well, there is significant asymmetry in the other two helices in this particular example, with helix C1 (brown) significantly closer to the heme than helix D1 (green). Reasons for this will be considered in a later section.

Standard orientation of the prototypical arrangement

For comparing the different examples, and later for explaining the angles between imidazole planes, it is convenient to define a standard orientation. This allows us to refer to top and bottom, left and right, front and back, in a non-ambiguous way. In this standard orientation the helix bundle axis is vertical, with the N → C direction of the ligand-bearing helices directed upward. The imidazole rings of the ligating histidines do not lie on a line between ligand-bearing helices 2 and 4, but lean toward the previous, non-ligand-bearing helices so as to be about equidistant from these two adjacent helices: 1 and 2 on one side and 3 and 4 on the other. As shown in Figure 2A, this results in the line between the two imidazole N^ε2 atoms passing through the gaps between helices 1 and 2 on one side and helices 3 and 4 on the other, and the heme plane which is perpendicular to this line passing between helices 2 and 3 on one side and 4 and 1 on the other, in other words helices 1 and 2 are on one side of the heme and 3 and 4 on the other. The helix bundle has a left-handed twist, so opposing helices in the bundle are not parallel but cross at a significant angle to each other. This crossing angle, together with the conformation of the His ligand side chains, determines the angle between the imidazole planes of the two ligands, as discussed further below.

All of the 4-helix bundle examples discussed here have the helices going around the bundle in a clockwise direction (when numbered according to position in primary sequence) when viewed from the cytoplasmic/matrix (N) side of the membrane. All have their N-terminus on the N side (conforming to the “positive inside” rule of von Heijne [39]) so that helices 1 and

²This would be similar to the heme-binding sites of bacterioferritin, which receive one methionine axial ligand from each of two identical monomers.

3 are directed toward the P side while helices 2 and 4 are directed toward the N side. Except for the distal hemes of QFR and FDH, which deviate in other ways as well, all have the heme ligand on helices 2 and 4, directed toward the N side, which thus becomes the “top” in standard orientation. However there is no reason why it could not be the other way around, either by violating the “positive inside” rule or by having an additional helix before the first helix of the bundle.

We still need to define the orientation about the bundle axis in the standard position. For this we take the view along the heme plane normal, i.e. with the heme in the plane of the paper, helices 1 and 2 in front of it and helices 3 and 4 behind it. At this point we are describing a 2-fold symmetric structure, so exchanging 3 and 4 for 1 and 2 makes no difference. Later the asymmetry of the heme will allow us to define the front and back side in order to calculate unique values for the angles, but the biological significance of this is questionable. Either way, we have two helices in front of the heme, slanting up and to the left, with the helix on the left (helix 2) bearing the ligand, and two helices behind the heme, slanting up and to the right, with the one on the right (helix 4) bearing the ligand (Figure 2).

Significance of the angle between the imidazole planes of the axial ligands ($\Delta\phi$)

EPR spectroscopy has been utilized for the past thirty-seven years as a valuable first tool for characterizing the heme and iron-sulfur centers of mitochondrial and bacterial Complexes II–IV of the respiratory chain [40–50]. The EPR spectra of the low-spin bis-histidine-coordinated ferrihemes of mitochondrial Complexes II and III are very distinctive, in that they are single-feature spectra of low-spin Fe(III) hemins having the g-value of that feature close to the maximum possible for this electron configuration, $g = 3.8$: For Complex III [8–23] (Figure 3), the cytochrome bc_1 complex, heme b_L (the low-potential *b*-ferriheme) has a g-value of 3.75–3.78 [40–46], while heme b_H (the high-potential *b*-ferriheme) has a somewhat smaller g-value of 3.41–3.44 for vertebrates [40–43,46], or 3.60 for yeast [44], depending on the oxidation states of other cofactors and the presence or absence of inhibitors. Likewise, for chloroplast cytochrome b_6f [24–28], and for mitochondrial Complex II [29,30] and the related bacterial fumarate reductase [31], similar g-values have been reported for the mitochondrial complex [47], which has only one heme, and the bacterial complex [48], which has two. Other cases where the single-feature EPR signals have been observed include one of the hemes of the cytochromes c_3 [51–57], and one of the hemes of the membrane-associated tetraheme cytochrome *c* of the bacterial photosynthetic reaction centers, [58–60] the only heme that has bis-histidine coordination. It has been shown, using model ferrihemes [61–66], that g-values larger than about $g = 3.2$ are indicative of the axial imidazole ligands being in nearly perpendicular planes, and although that definition has recently been modified to include imidazole plane dihedral angles of about 57–90° [67], the cytochrome b_L centers of ten recent structures of the bc_1 complex have this dihedral angle $\Delta\phi = 84^\circ \pm 5^\circ$ (Table 1), while the cytochrome b_H centers of the same structures have that dihedral angle $\Delta\phi = 58^\circ \pm 13^\circ$, both of which are consistent with the EPR g-values observed [40–46].

As mentioned above, the single-feature EPR signal, which has variously been called a Highly Anisotropic Low-Spin (HALS) [61] or “large $g_{\max}$ ” [63,64] or Type I [68] EPR signal, arises from ferriheme centers that have imidazole ligands in near-perpendicular planes. In this geometry the energy splitting of the three lowest-energy d-orbitals, d_{xy} , d_{xz} and d_{yz} , is as shown in Figure 4, where the two so-called d_π orbitals are quite similar in energy. With five electrons in these three d-orbitals, there is an odd number in the d_π set. This is an unstable situation for the complex because of the Jahn-Teller effect [69], which would, in the case of model hemes having no steric constraints, cause the ligands to assume parallel orientation in order to make one in-plane axis different from the other and thus place one d_π orbital, d_{yz} for example, at

much higher energy than the other; thus a pair of electrons goes into the d_{xz} orbital and the remaining electron into d_{yz} , and the overall energy of the system is lower than if the ligands are in perpendicular planes. In complexes where this can occur, such as those having two non-hindered imidazole ligands bound to the ferriheme iron, this leads to a rhombic EPR spectrum with three observable g -values, whereas in complexes having two hindered imidazole ligands such as 2-methylimidazole, the ligands cannot bind to the iron with bond lengths appropriate for a low-spin complex (≤ 2.0 Å) if the ligands are in parallel planes. In these cases, the bulky ligands are found to bind in perpendicular planes, thus giving rise to a small splitting between the d_{xz} and d_{yz} orbitals. This is a higher-energy state than for the case of axial ligands in parallel planes. In heme proteins, however, nature can choose to place axial ligands (especially histidine imidazoles) in particular orientations by the design of the protein.

Structures are available now for one or more examples of each of the membrane-bound cytochromes of the mitochondrial electron-transfer chain, Complexes II, III and IV, the bacterial reaction centers, the b_6f complex of chloroplasts and cyanobacteria, and Photosystem II. We have calculated $\Delta\phi$ angles from each. As can be seen in Table 1, most of these membrane-bound bis-histidine heme centers have axial ligands in perpendicular planes, with mitochondrial cytochrome a of Complex IV [70] and cytochrome b_{559} of Photosystem II [71–73] being two of the very few exceptions. It is thus of interest to understand how and why nature chooses to have histidine ligands in the high-energy state, with their imidazole planes approximately perpendicular. One clear observation is that the systems with perpendicular ligand planes tend to have a structure similar to the four helix-heme bundle described above and in more detail in the next section, while those examples with the intrinsically more stable parallel ligand planes have a different architecture. However the central heme in the cytochrome c subunit of bacterial reaction centers, which has nearly perpendicular His ligands (with $\Delta\phi$ as large as 73° in the latest structure, PDB file 2JBL), and shows the large $g_{\max}$ signal, is not in a 4-helix bundle. Thus while the 4-helix heme bundle described here seems to be the most common way to arrive at perpendicular imidazole ligands, it is not the only way.

As a consequence of having imidazole planes approximately perpendicular, the orbital energies of Figure 4, calculated from the g -values of the two extremes of ligand orientation, were used to estimate that the midpoint potential for $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ reduction should be more positive than if the heme ligands were coplanar, by at least 50 mV [64]. However, there are other factors that affect the E_m s of the membrane-bound cytochromes in addition to the histidine dihedral angle, one of the most important of which is buried charges near the hemes. Indeed, for cytochrome b of Complex III, near heme b_L is E271(272,295) of the PEWY sequence, mutation of which to Ala shifted the E_m of heme b_L ~ 60 mV positive [74]. And R313 near heme b_H may be responsible for shifting E_m ~ 60 mV more positive than it would otherwise be. The oxidation state of the other heme also affects the E_m by up to 60 mV [75]. Thus it is difficult to deconvolute the separate contributions of each of these factors to the observed E_m s of these complex redox proteins.

Defining dihedral angles over a 360° range

For calculating molecular orbital energies and EPR spectra, it is the degree of orthogonality or coplanarity of the imidazole planes that is important. An angle of 70° results in the same properties as an angle of 110° , both being 20° from orthogonal. However, from a structural point of view the absolute angle is important, as clearly different scaffolding will be required to arrive at an angle of 110° than an angle of 70° . Furthermore, calculating the absolute angle is important for making sense of the distribution of angles observed in a given example. For example, the low-potential heme of cyt b in current crystal structures exhibits $\Delta\phi$ values above and below 90° , with the average quite close to 90° . If an angle between 0 and 90° is calculated, clearly all the angles will be less than or equal to 90° , the mean will be significantly less than

90°, and the distribution will be asymmetric with a peak near 90° dropping to zero immediately beyond. This is simply because those values above 90° have been “folded over” onto the equivalent angles below 90°.

In order to calculate angles over a 360° range we take the upward direction of the bundle axis to be zero, and clockwise rotation when viewed in the standard orientation to be positive. (This is consistent with the right-hand rule if the direction of the rotation axis along the heme normal is taken as going from side 1 to side 2, i.e. away from the viewer.) The orientation of a histidine imidazole plane is taken as a vector along the intersection of the planes of the imidazole and heme rings, in the direction of the imidazole C^{δ2} to C^{ε1} vector, or equivalently the C^γ to N^{δ1} vector. (By taking the intersection, we ignore rocking of the Fe-N bond and only measure rotation about it.) The angle between this vector and the bundle axis is then the orientation of the imidazole, and subtracting the angles of the two imidazoles gives the $\Delta\phi$ value.

Notice that because we take clockwise as positive for both the imidazole in front of the heme and that behind, if the two histidines are in identical conformations related by the two-fold around the bundle axis, their imidazoles will have angles of the same magnitude but opposite signs. Furthermore if we choose to look from the other side of the heme, i.e. interchange helices 1 and 2 with helices 3 and 4, the angle of each imidazole will change sign, however the difference, front angle minus back angle, will be the same in sign and magnitude. In order to have a basis for choice, we calculate the angles looking from the side of the heme such that the pyrrole rings (A, B, C, D by crystallographic conventions, IV, I, II, III by the chemist's) go around in a counterclockwise direction. It is good that this has little consequence for the final result, as determining heme orientation in moderate resolution structures can be non-trivial, and the possibility exists that heme orientation about its 2-fold axis is promiscuous (so-called “heme disorder” often found in NMR spectra of water-soluble heme *b*-containing proteins [76]). This would certainly be the case in our hypothetical homodimer, as the site would have perfect two-fold symmetry in the absence of heme, so the two binding modes would have the same energy.

Up until now we have ignored the orientation of the heme itself within its plane, i.e. rotation of the ring about the iron. This is measured as the angle of the heme N_{II} to N_{IV} direction (NC→NA in atom-names from the PDB heme) to the vertical direction of the bundle axis. While using the heme pseudo-2-fold axis to define the heme orientation would have the advantage of being insensitive to uncertainty about the heme 2-fold, the N_{II} to N_{IV} direction (or the reverse) has traditionally been used to measure the angles of the imidazole planes to the heme [76,77].

The approach we have been using, of measuring all angles relative to the bundle axis, which we call the vertical direction, is obviously only applicable when the heme is in a 4-helix bundle. A more general approach that has been in use for describing general bis-histidyl heme coordination complexes uses ϕ , the angles of the imidazole planes with the N_{II} to N_{IV} direction in the heme plane [77]. Since we are using the N_{II} to N_{IV} direction of the heme to measure its angle, subtracting the angle of the heme and the imidazole gives the ϕ angle for that imidazole. Likewise subtracting the angles of the two imidazoles gives the $\Delta\phi$ value. Details of the calculations are summarized in the Appendix.

Three things to consider when discussing the heme orientation are *i*) the energetic interactions, both steric and orbital, between the heme and the axial ligand histidines, *ii*) the tendency of the heme propionates to face toward the aqueous phase (perhaps a consequence of the mechanism of heme insertion), and *iii*) the orientation of the propionates relative to that of the imidazole N^δ atoms. Close contacts between the porphyrin N atoms and the histidine ring results in steric clashes which are very sensitive to rotation of the plane of the histidine about the N-Fe bond

relative to the heme ring. As defined above, this is given by the angle ϕ which is zero when the imidazole plane intersects the heme plane along the $N_{II} \rightarrow N_{IV}$ ($NC \rightarrow NA$) vector. At even multiples of 90° the imidazole ring clashes with porphyrin N atoms, whereas at 45° and multiples of 90° from there, the ring fits in the notch created by the *meso*-C-H between two pyrrole ring N atoms. As discussed above in the section on the relationship of the dihedral angle to EPR spectroscopic data, orbital electronic interactions favor coplanar imidazole ligands for Fe(III).

A survey of a large number of water-soluble histidyl-bound heme proteins [78] found that orientation of histidine $N^{\delta 1}$ toward the propionates predominates. Because the $C^\gamma \rightarrow N^{\delta 1}$ of histidine is in the same direction as $C^{\delta 2} \rightarrow C^{\epsilon 1}$, which we take as the histidine direction, and one heme propionate is on the N_{IV} , or “A” corner of the porphyrin ring which we take as the heme direction, if the histidine and heme have the same direction ($\phi = 0^\circ$) the $N^{\delta 1}$ is directed toward that heme propionate. The other propionate is on the D ring, which in standard orientation is 90° clockwise, or positive, of the A ring. Thus a histidine angle 90° more positive than the heme angle ($\phi = 90^\circ$) corresponds to $N^{\delta 1}$ directed toward the D propionate.

As described in the next section, the helical bundle arrangement results in imidazole planes with their $C^{\delta 2} \rightarrow C^{\epsilon 1}$ direction approximately 45° from vertical in front and -45° in back. Orientation of the heme at -45° from vertical would thus result in the front imidazole $N^{\delta 1}$ directed toward the D propionate and the back imidazole directed toward the A propionate. This orientation puts both propionates directed upwards with the heme pseudo-2-fold axis on the helix bundle axis. This orientation is not observed, probably because it puts the imidazole rings over the heme nitrogens rather than between them. Rotating the heme 45° alleviates the steric hindrance and puts one or the other propionate directed upwards between the two imidazole $N^{\delta 1}$ directions: heme orientation of 0° puts the A propionate upwards, heme at 90° puts the D propionate upwards. Either of these has the imidazole $N^{\delta 1}$ atoms more toward the heme propionates than away, and so tend to conform to the observations of Zaric and coworkers [78]. The other two sterically favorable orientations have heme at $+90^\circ$ which puts the D propionate downwards, and 180° which puts A downwards. These have the imidazole $N^{\delta 1}$ directed more away from the propionates than toward them, the configuration found to be less common in the study of Zaric et al. [78]. Orientation of the heme in actual proteins will be discussed below in the section on the orientation of the heme within the resulting framework.

Structural parameters of the ideal prototypical four-helix heme bundle

Given that the heme ligand histidines are attached to opposing helices of the bundle and assume a conformation close to rotamer 3¹, the angle between imidazole planes clearly depends on two things: how a helical histidine in rotamer 3 holds the imidazole plane relative to the helix axis, and how the helix is oriented relative to the bundle axis, or more specifically, the crossing angle of the two ligand-bearing helices viewed in projection on the plane of the heme.

Any four-helix-bundled heme conforming to the description provided here can be briefly characterized by the five angles defining the orientations of the heme, the two imidazole planes, and the two ligand-bearing helices relative to the bundle axis, all in projection onto the heme plane. These angles, which explain how the structure determines $\Delta\phi$, can be visually represented in a schematic diagram of the type shown in Figure 2D. The long brown arrows in the front and back indicate the direction of the ligand-bearing helix axes. The short green arrows represent the ligand imidazole plane. The magenta arrow represents the heme $N_{II} \rightarrow N_{IV}$ direction. All vectors are taken in projection on the heme plane, and are shifted to pass through the same central point. Note that in the real projection (Figure 2C), the helices pass over the edges of the tetrapyrrole ring, not the center. Tabulation of these orientation values for available structures (Supporting Information Tables S1 S6, and Figure 7 below) shows the

angle between the imidazole plane and the axis of the helix to which it is attached is relatively constant, and the variation in $\Delta\phi$ is largely accounted for by variation in the helix crossing angle.

Six-coordination of heme iron implies the N-Fe-N bond angle for the two axial ligands is linear and perpendicular to the heme plane. The assumed 2-fold symmetry about a vertical axis passing through the Fe atom in our ideal prototype then implies that the N-Fe-N bond is perpendicular to the 2-fold axis, i.e. horizontal. Now, assuming the N-Fe bonds are in-plane of their respective imidazoles and the Fe-N-C bond angles are symmetric, and considering strong symmetrical steric pressure from the heme N atoms on the C δ^2 and C ϵ^1 atoms of the histidine imidazole, leads us to expect that the imidazole will bond squarely on the heme so the N-Fe-N axis projects along the middle of the imidazole ring through the midpoint between the N δ^1 and C γ atoms on the opposite edge. We refer to this line in the imidazole plane as the ligating axis. Orientation of the imidazole plane can then be defined by the amount of rocking of the ligand axis, which affects linearity of the N-Fe-N bond angle or coplanarity of N-Fe with the imidazole; and rotation about the ligand axis, which affects the ϕ and $\Delta\phi$ angles. The latter can be measured as the orientation of a line perpendicular to the ligand axis, say from C γ to N δ^1 ; which we call the cross-bond axis, of the imidazole plane.

The orientation of the imidazole ring is largely determined by the position of the helix bearing the His ligand relative to the heme. Once the bundle forms (the protein folds) and binds heme, there is little rotational freedom for the imidazole planes. As illustrated in Figure 5, non-coplanarity of the χ^2 dihedral angle of the histidine side chain with the N-Fe bond restricts rotation. As mentioned above the histidine side chain is usually close to rotamer 3, implying the geometry of the bundle and the heme binding site is such as to orient the histidine backbone so that this relatively stable rotamer of the side chain positions the imidazole ideally for heme binding.

Next we take a look at the orientation of the imidazole ring for a histidine in a canonical α -helix, relative to the helix axis. Figure 6, shows the orientation of the imidazole for histidine in rotamer 3 and, for future reference, also in rotamer 2¹. In rotamer 3, the ligand axis of the imidazole plane makes an angle of 59° with the helix axis (N-term to C-terminal direction), i.e. 31° from perpendicular to the helix. Thus if the helices were all vertical, i.e. parallel to the bundle axis, the ligand axis of the imidazoles would be directed upward toward the iron from either side at an angle of 31°, resulting in an N-Fe-N angle of 118° instead of 180°. The cross-bond axis makes an angle of 75° with the helix axis (15° from horizontal), and the $\Delta\phi$ angle for a symmetric bundle of untwisted parallel helices would be 150° (75° for the front imidazole and 75° for the back), or 30° from coplanar.

These angles are modified by the superhelical pitch of the helices twisting about the bundle axis, to obtain a linear N-Fe-N bond angle and more nearly perpendicular imidazoles. Viewed at the level of the histidine ligands, the pitch can be seen as a rotation of each helix about a line perpendicular to the bundle axis and passing through the helix axis. Because the N-Fe-N bond is not along this line (Figure 2A) but on a line passing between helices, a left-handed helical pitch results not only in rotation about the ligand axis (counterclockwise, decreasing ϕ for the front imidazole) but also in rocking the ligand axis of the imidazole down toward horizontal to make a linear N-Fe-N bond. In real structures this may be achieved also partly by distortion of the superhelical pitch, achieved mainly by kinks in the helices, and by deviations of the side chain conformation from rotamer 3. H-bonds from the N δ^1 of the imidazole to the protein may bias the ϕ angle toward one end or the other of its allowable range.

This is not to say that the linear, coplanar N-Fe-N bond and the stability of rotamer 3 force the bundle to assume a left-handed helical pitch. Rather this left-handed coiled-coil is stable due

to interactions between the coiled helices, and it just happens that a histidine in rotamer 3 at the correct helical-wheel position in one of the helices will hold its ligand axis horizontal and at the right position to ligand a Fe on the bundle axis. However, it is easier to visualize this by considering what attitude a helix must take to the bundle axis in order to bring a histidine into this orientation.

In order to tabulate and compare values from the different structures, we measure the angle of the helix axis and imidazole plane to the vertical bundle axis. The angle of the imidazole plane is then seen as the sum of the angle of the helix from vertical and the angle of the imidazole plane from the helix axis. These data can be displayed graphically in a “compass diagram” as shown in Figure 7 for representative structures of each group. These show that in fact the angle between helix axis and the imidazole plane, projected on the heme plane, is quite close to 75° in all cases, and the variation results mainly from the helix crossing angle.

Orientalional parameters calculated from available structures

To avoid confusion by differing orientations (possibly incorrect) of the heme in various proteins, we calculate angles of the helices and histidines relative to the helix bundle axis, as described below in the section on defining dihedral angles over a 360° range. This is expected to emphasize the similarities and make comparisons more meaningful, and still allows calculation of $\Delta\phi$. This section tabulates such data. In the next section we will consider how the heme is oriented about the N-Fe-N bond, and the results for ϕ_1 and ϕ_2 .

Tables S1–S6 in the Supporting Information list, for each of the example families, the average orientation of the five components in order from front to back of the bundle in standard orientation (helix 2, imidazole, heme, imidazole, helix 4) relative to the vertical axis, for each protein. Also listed are some important differences: the helix crossing angle (helix 2 - helix 1), ϕ angles (imidazole vs. heme), and $\Delta\phi$ (imidazole 2 vs. imidazole 1). The latter is expressed first in the range 180° to +180° ($\Delta\phi$), then as an angle 0 – 90° between the two planes ($\Delta\phi^*$). The average values presented in Table 1 were obtained from these data, and from similar tabulations for cytochrome oxidase, photosynthetic reaction center, and cytochrome *b*₅₅₉ (Supporting Information Table S6).

Cytochromes *b*/*b*_L of the *bc*₁/*b*_L complexes contain two hemes in a single 4-helix bundle. Both hemes are ligated by histidines from the second and fourth helices (B and D), residues 82 and 183 for *b*_L and residues 96 and 197 for *b*_H in the yeast sequence. These helices go from the intermembrane space side to the matrix side, so the standard orientation for both has matrix side on top. Heme *b*_H is then near the top, with its propionates directed upwards toward the matrix, with heme *b*_L near the bottom and propionates directed downwards toward the intermembrane space. The ligating residues are separated by 14, two heptad repeats. “Notch” glycines are found in helices A and C, also separated by 14 (residues 33, 47, 117, and 131). The residue corresponding to 46 is a fully-conserved threonine, and structures show its side chain hydroxyl oxygen is an H-bond acceptor from the N^{δ1} NH of His 82. In cytochromes *b*₆ residues corresponding to 116 and 130 are also threonine, and 32 is serine, but these are not conserved in cyt *b*.

Bovine *bc*₁ structures PDB files 1QCR, 1L0L, 1NTM, 1SQQ, 1SQV, and 2FYU have heme *b*_L in the opposite orientation from the majority of structures, including some recent ones from the same laboratory. The chicken and yeast structures all have the majority orientation, while among *b*_L structures 1VF5 has the majority and 1Q90 has the minority orientation. For the analysis we used the majority orientation as in 1PPJ, according to which His182 (183) is ligand 1 and His82(83,84) is ligand 2.

In Supporting Information Table S1 we see $\Delta\phi$ values for heme b_L are mostly between 80 and -95° . Values from the higher resolution structures are mainly in the range -86 and -94° , although surprisingly 2FYU gives -103° . The mean is -87.7° with a standard deviation of 7.6° , and all are within the range predicted to be large $g_{\max}$ by the 57° rule [67] (here we are omitting values from the early structures 1BE3 and 1BGY, which apparently had Thr47 and His83 modeled incorrectly resulting in $\Delta\phi$ angles around -50°). If the values are converted to the spectrally relevant value between 0 and 90° before averaging, which we are calling $\Delta\phi^*$, the mean is 84° with standard deviation 4.7° . Clearly the narrower distribution is due at least in part to folding over the distribution at 90° , and this seems a plausible explanation for the sharp, asymmetric peak observed for the EPR signal of heme b_L (Figure 4). This sort of explanation was proposed by Salerno [45].

The b_H heme is unusual, in that the helices of cyt b are nearly parallel to the bundle axis at the level of heme b_H , making angles of 6 to 9° from vertical. This results in a helix-crossing angle of only 15° which in turn results in a large negative $\Delta\phi$ angle. Instead of being distributed around 90° all are further rotated to between -90 and -180° , and thus there is no folding over of the distribution. The values of $\Delta\phi$ shown in Supporting Information Table S2 show a considerable range within that quadrant, leading to $\Delta\phi^*$ from extremes of 29° (1SQB) to 77° (1BGY). The mean is 58° , just above the threshold for large $g_{\max}$ characteristics. The large variation results not from movement of the helices (the helix crossing angle ranges from 9° to 18°), as much as from variation in the conformation of the histidine side chain. It is likely that a large part of the variation is due to inaccuracy in determining the precise angle of the histidines in the electron density.

Complex II

Complex II, or succinate:ubiquinol oxidoreductase; SQR) is another example of the helix-bundled heme fitting our description, as illustrated in Figure 2. As is observed for the bc_1 complex, the heme of Complex II also has the two histidines on symmetry-related transmembrane helices, in this case of separate polypeptides, and both histidines in rotamer 3, with the helices likewise twisted in such a way as to produce perpendicular imidazole planes.

In this example the bundle consists of two helices from each of two homologous subunits, called “anchor” polypeptides, which are chains C and D in the deposited structures (Yankovskaya et al. [79], Sun et al. [42], and Huang et al. [43]). The heme ligands are His98 in transmembrane helix 2 of chain C and His46 in helix 2 of chain D in the mature chicken sequence, or C101 (C84) and D79 (D71) in the porcine (bacterial) sequence.

The similar membrane topology and slight sequence homology between C and D suggests that the ancestor of these subunits was a homodimer with proper 2-fold symmetry with the heme bound on the 2-fold axis. The dimer was then put together in such a way that the helix bearing the heme ligand in the two monomers would cross at such an angle (and distance) that, if the His were in rotamer 3, the imidazole planes would be perpendicular and the right distance apart to bind heme iron in between the two histidines. The heme would not have its pseudo-2-fold axis along the symmetry axis but would be rotated slightly in its plane so that one or the other of the two ligands has its plane in the favored orientation with small ϕ angle. Then, later in evolution, the gene duplicated and C and D differentiated to stabilize one or the other orientation of the heme.

Of the two chains, chain C is more prototypical, with the usual “notch” glycine in helix 1, preceded by Thr or Ser. In the chicken structure, this is 45TG. The potential H-bond acceptance of Thr45 O γ from the ligand His98 NH is long, 3.33 Å, and similar to that with the backbone O of 42 (3.29 Å).

In helix 1 of the *E. coli* enzyme the corresponding residues are 33SG. His81 N^{δ1}H has potential H-bonds to Ser33 O^γ at 2.81 Å, and 30 O at 3.12 Å. Yang et al. [80] showed that Ser 33 can be mutated to Thr with little effect, but that replacement with Ala or Cys led to significantly reduced activity and poor growth on succinate. Chain D deviates from the prototype in that the “notch” glycine is not conserved in non-ligand-bearing transmembrane helix 1, being replaced by Ala in *E. coli* and Ser in vertebrate mitochondria.

The deviation is less severe in *E. coli* SQR, with the notch glycine replaced by Ala23, preceded by Thr22. The length of a putative H-bond from His71 to Thr22 O^γ is 2.8 Å, and to the carbonyl O of 19 is 3.35 Å. Thr22 O^γ is coplanar with the imidazole ring, while D:19 O is about 20° out of plane. No waters were modeled in the heme cleft. The C^β atom of Ala23 is in van der Waals contact with several atoms of the porphyrin ring.

In the chicken structure the notch glycine is replaced by Ser17, the preceding residue is valine, and the D1 helix is displaced upward compared to the *E. coli* structure. This moves D:13 O out of H-bonding range, at 4.8 Å. Ser17 itself is a candidate for H-bond acceptance at 3.4 Å, and there is a water molecule (unusual in heme clefts) at 2.8 Å. Both are out-of-plane by about 30°, on opposite sides of the imidazole ring.

While Daldal and co-workers found that even a conservative replacement of a notch glycine of the *bc_L* complex with Ala resulted in severe assembly problems [3], presumably due to steric clash of the larger side chain with the heme and imidazole ring, Complex II seems to accommodate the Ser side chain by moving the helix farther from the heme. This can be seen in Figure 2A, in which helix D1 (green) is farther from the center of the bundle and the heme than helix C1 (brown) which has conserved the notch glycine and preceding Thr.

Surprisingly, in yeast Complex II chain C (genbank CAA52088), the conserved glycine is replaced by a bulky leucine residue. This and lack of conservation of the His ligand in chain D (genbank NP_010463) suggests that a four-helix bundle made with these two gene products might not bind heme. In fact, Schilling *et al.* [81] reported isolation of active heme-free Complex II from *S. cerevisiae*. Recent work, however, suggests the enzyme does bind heme [82]. There are actually two genes for each of subunits C and D in the *Saccharomyces* genome, and the alternate chains (genbank EDN64505 and NP_013265) have the conserved notch glycine and ligand histidine. Thus it may be that *Saccharomyces* can express Complex II with or without the heme.

The helix bundle is highly twisted at the level of the heme (note kinks in one helix are obvious in Figure 1), resulting in a large helix-crossing angle of 65°, with a narrow range of 63° to 67° over all the vertebrate and bacterial structures. The large helix-crossing angle makes the $\Delta\phi$ less negative, -78 to -83° for the vertebrate structures. The *E. coli* structure has $\Delta\phi = -101^\circ$ due to a 20° difference in the orientation of the ligand from chain D, which may not be significant. In any case, this is ~10° from being horizontal as are the vertebrate structures, so the agreement in $\Delta\phi^*$ is good, $81 \pm 2.0^\circ$. This is in agreement with the HALS (large $g_{\max}$) EPR spectra observed for the bovine [83], *E. coli* [84] and *Paracoccus* enzymes [85], with $g_{\max}$ values of 3.46, 3.63 and 3.6, respectively. Interestingly, when the extrinsic subunits were removed from the anchor domain, the EPR spectrum became rhombic [83], suggesting the importance of a finely tuned protein structure in maintaining the nearly-perpendicular imidazole ligands.

The orientation of the heme about its pseudo-two-fold axis is different in the models of *E. coli* and porcine Complex II. In our work with chicken complex II, this assignment has been quite difficult. Both vinyl C^β atoms were removed to give a symmetrical structure for refinement, and difference density maps gave no clue as to the correct location of these atoms

at the time we deposited the highest resolution structure 2H88. The deposited structures all have the orientation of the bacterial complex which was used in solving the chicken structure. With further refinement since deposition there is a weak preference for the orientation of the porcine complex. Therefore, in this work we have treated the orientation of the porcine structure 1ZOY as correct, and effectively flipped the hemes in the chicken and *E. coli* structures before calculating the angles. With this choice our convention assigns the ligand from subunit C to be His 1 and that from subunit D to be His 2. The geometric parameters for the heme of Complex II are summarized in Supporting Information Table S3.

Quinol:Fumarate Reductase (QFR)

The only heme-containing QFR whose structure is known is that of the QFR of *Wolinella succinogenes* [31], which is considered to have evolved from the same ancestor as mitochondrial Complex II [86], even though it has two hemes rather than one, and the two anchor subunits, C and D, are fused into a single subunit with five trans-membrane helices. While the subunit designations A, B, C and D presumably arise from their sizes, in fact the order of the genes in the operon is well conserved in eubacterial SQR and QFR, with C always preceding D in the 4-subunit enzymes [86]. Thus it would be expected that the N-terminal two helices of FRD correspond to chain C of SDH and the C-terminal three helices to chain D, and in fact sequence homology is slightly higher that way. However, superimposing Complex II with *W. succ.* QFR, based on the well-conserved extrinsic subunits A and B, aligns chain C of SDH with the *last* three helices of QFR chain C, and the first two helices of chain D of SDH with the *first* two helices of QFR chain C. This implies that if 4-subunit SQR and 3-subunit QFR arose from a common ancestor by a gene fusion or fission event, either the order of C and D in the operon has reversed, or the external subunits changed their binding orientation by 180°.

The menaquinol oxidation site of QFR has not been identified crystallographically, but mutational evidence points to a cavity on the distal (periplasmic) side of the transmembrane region [87]. Potentiometric titration of the hemes reveals two midpoint potentials of -9 and -149 mV [87]. Although there is no experimental evidence assigning the high and low potential hemes to the structurally located hemes in this species, electrostatics calculations using multi-conformational continuum electrostatics, MCCE [88], and assuming an intrinsic E_m (in water) of -220 mV [89] for bis-histidyl heme, resulted in predicted midpoint potentials of -12 to -48 for the proximal heme and -125 to -149 for the distal heme [90]. This makes the assignment of the low potential to the distal heme fairly reliable. The difference is attributed largely to interaction of four strongly basic residues with the propionates of the proximal heme, and only one with the distal heme.

The proximal heme is ligated by H93 and H182 in the second and fourth transmembrane helices. Helix 1 and Helix 3 both have glycine at the notch position for the proximal heme, both preceded by threonine (33TG and 132TG). Glutamines 30 and 129, four residues before the notch glycines, are well conserved (Figure S1 in supporting materials) and H-bond the heme propionates (Gln30 directly, Gln129 via an ordered water). The threonines are H-bond acceptors of the hindered N-H of the ligand histidines, at distances of 3.13 (33T) and 2.78 (132T) in structure 1QLA. The imidazole planes are 55° and -30° from vertical, resulting in a nearly perpendicular -85° for $\Delta\phi$. The region around the proximal heme superimposes well with the two mitochondrial and *E. coli* Complex II proteins.

The superposition of the polypeptide backbones is about equally good when superimposed in the normal gene order (C on N-terminal, D on C terminal domain; 41 atoms with RMSD 0.81 Å) or inverted order (C on C-terminal, D on N-terminal domain; 39 atoms with RMSD 0.75). However the heme rings superimpose better with the normal order.

Most of these calculations were made using the original FRD structure 1GLA from 1999. Applying our definitions to the heme as oriented in that structure, His1 is (C)H93 and His2 is (C)H182, and the heme orientation is the same as in Complex II for the porcine structure. While this work was in progress, further structures were submitted including the 1.78 Å structure 2BS2, and in these structures the heme orientation has been reversed. Structure factors released with this structure strongly support the revised heme orientation. Hence it is likely that the definitions of side 1 and side 2 should be reversed. As mentioned above the orientation of the heme of Complex II about its two-fold axis is even more unclear. But whatever choice is made, superimposing Complex II and FRD approximately superimpose the heme two-fold axes, and thus superimpose the rest of the atoms directly or with the pseudosymmetry mates in the other heme. Also as mentioned above, it would not be surprising if there is significant promiscuity in the orientation of the heme. Energetic preferences for one orientation over the other could only come from interactions of the asymmetric vinyl and methyl groups with the protein in the two orientations.

The distal heme of fumarate reductase is a rather aberrant example, with the ligand histidines in helices 1 (His44) and 3 (His143) rather than 2 and 4, His44 near rotamer 4¹ rather than 3, neither notch glycine conserved, and the helices are splayed out in an open and asymmetrical way. The histidine angles are about -90° and -10° from vertical, giving $\Delta\phi = -80^\circ$.

Note that, since helices 1 and 3 are going in the opposite direction as 2 and 4, the standard position has the distal, periplasmic surface of the membrane up. In order to compare the structure of the distal heme with that of the (standard) proximal one, we need to superimpose them by flipping the distal heme-binding site in the plane of the membrane, so that the heme-bearing helices go in the same direction. This results in the helices going around the bundle in the opposite direction. However, if we ignore connectivity of the helices it is still possible to form a structure identical to that of the proximal heme by superimposing helices 2, 1, 4 and 3 on helices 1, 2, 3, 4. This would correspond to symmetry about a 2-fold axis in the plane of the membrane, and would be complemented by the fact that the heme propionates tend to be directed toward the nearest surface, i.e. in opposite directions. However the distal heme of QFR does not have this kind of symmetry because one of the ligands, His44 is in rotamer 4¹ rather than 3, and the protein around the heme does not superimpose well with the proximal heme binding site or other prototypical examples.

The pathogens *Helicobacter pylori* and *Campylobacter jejuni* have QFRs very similar to that of *W. succinogenes* [33]. More distantly related, but still probably structurally homologous, is the diheme SDH of *Bacillus subtilis* [34]. Sequence alignment with the *Bacillus* protein is not entirely clear. A reasonable alignment (Figure S1 in supporting materials) gives conservation of the four heme ligand histidines and the proximal-heme “TG” in the third transmembrane helix. The notch glycine in helix 1 is only present if one allows for an insert of one residue between it and the distal heme ligand histidine in the same helix. In this case S/T and Q, 1 and 4 residues before the notch glycine, are replaced by Val and His.

EPR data are not available for the hemes of the *Wolinella* QFR. For *Bacillus subtilis* SQR, both hemes have large $g_{\max}$ signals, at 3.68 for the heme that is reducible by succinate ($E^\circ = +65$ mV), and at 3.42 for that which is not reducible by succinate ($E^\circ = -95$ mV) [48]. The proximal heme of *B. subtilis* is believed to be the high potential one and the distal heme the low potential one [91]. It may be a general principle with these transmembrane diheme cytochromes that the heme on the P side has the more negative potential, as this is also the case in the *bc₁* complex. As a result, when the membrane is energized the equilibrium for electron transfer between them would be close to unity. Thus in systems like the *bc₁* complex, FDH, and hydrogenase, where electron transfer from the low potential heme to the high potential heme is responsible for generation of the membrane potential, the redox potential difference

between the two hemes drives forward electron transfer until the electrical potential increases enough to offset the redox potential difference, stalling the reaction. In systems like SQR from *B. subtilis*, where the proton gradient drives electron transfer from succinate to menaquinone, the high potential heme readily accepts electrons from succinate and the low potential heme is a strong enough reductant to reduce menaquinone. Again, the electric potential difference (resulting from the membrane potential) offsets the redox potential difference, allowing the high potential heme to reduce the low potential heme efficiently.

Finally, in FRD from *Wollinella succinogenes*, the forward reaction (from menaquinone to the low potential heme to the high potential heme and on to fumarate) is spontaneous, but not enough so to contribute one proton per electron to the gradient. Experiments show that in fact the reaction is not coupled to proton translocation, and it has been hypothesized that a proton moves through an adjacent channel in parallel with the electron (E-pathway hypothesis [92]) so that the reaction is electroneutral and the scalar protons from quinol oxidation are effectively released on the cytoplasmic side, to be consumed in fumarate reduction with no contribution to ΔpH . The heme-less FRD of *E. coli* and the monoheme Complex II of *E. coli* or mitochondria may then have arisen by movement of the quinol reduction site to the proximal side of the membrane so that the scalar protons could in fact be taken up from or released to the cytoplasmic side, making the reaction electroneutral without the need for the E pathway or the hemes to carry the electrons to/from the distal site. The substrate of Complex II is ubiquinol which is significantly easier to reduce than menaquinol, allowing it to function efficiently as an SQR without the need to couple it to the proton gradient. The geometric parameters for the hemes of QFRs are summarized in Supporting Information Table S4.

Formate dehydrogenase

Formate dehydrogenases (FDHs) catalyze the last step in oxidation of C_1 substrates such as methanol and methylamine to CO_2 . There are at least two families, including soluble pyridine nucleotide-linked enzymes and membrane-bound quinone-linked enzymes. Of the latter, the structure of FDH *n* from *E. coli* has been solved [32]. It consists of two peripheral subunits, one of which has a single transmembranous anchor, and a transmembrane helical domain containing four transmembrane helices and two *b*-type hemes. It is this latter subunit, C, that concerns us here. Superficially the formate dehydrogenase (Structures 1KQF, 1KQG) seems quite similar to *Wollinella* QFR. In both cases the 4-helix bundle is comprised of adjacent transmembrane helices of a single polypeptide (chain C). The proximal heme (ligated by His57 and His155) looks like our canonical prototype, with His ligands in opposite helices (2 and 4) of the bundle and in rotamer 3, while the distal heme is deviant in having one ligand (His169) in rotamer 3 and the other (His18) in rotamer 4 and (even more deviant than in QFR) having ligands in the adjacent helices 1 and 4 rather than opposite helices of the bundle.

However, while the N-terminus of the transmembrane subunit of QFR is on the proximal side, in FDH it is on the distal side, meaning that the orientation of each helix is opposite in the two proteins. Furthermore, the ligands of the proximal heme are in helices directed proximally in QFR and distally in FDH, and the four helices are connected in a clockwise direction when viewed from the proximal end of the bundle in QFR but from the distal end of FDH. Thus the standard orientation defined above in the section on the standard orientation of the prototypical arrangement has the hydrophilic domains of QFR upwards but those of FDH downward.

As it turns out, FDH in fact has its peripheral subunits on the periplasmic side of the membrane, while those of QFR are on the cytoplasmic side, so C subunits of both obey the “N-terminal inside” rule [39]. Thus it is not that the protein flipped in the membrane, but rather one protein acquired catalytic subunits on the cytoplasmic side to couple cytoplasmic succinate/fumarate to membranous quinone, while the other acquired catalytic subunits on the periplasmic side to transfer electrons from periplasmic formate to membranous quinone. Therefore the prototype-

conforming “proximal” hemes of each correspond to the deviant distal hemes of the other, and their similarity (described below) can only be the result of convergent evolution or conservative evolution from an ancestor in which both hemes conformed to the prototype.

Once this is realized, the proximal heme-binding sites superimpose well, not only the His ligands but also the [TS]G dipeptides in the non-ligating helices: 31SG on 33TG of QFR and 130TG on 132TG of QFR. (Note, however, the orientation of the hemes about their normal is the opposite in any case, as the propionates of the proximal heme extend toward the proximal side in both proteins). Using the other two helices allows the findings of Zaric et al. [78] to be obeyed, and flipping one His violates it. Geometric parameters for the hemes of FDH are summarized in Supporting Information Table S5.

The transmembrane subunit of FDH bears significant homology to subunits of several hydrogenases and nitrate reductases, and in fact the four conserved histidines in these proteins had been suggested to be heme ligands long before the FDH structure became available [35–37].

Orientation of the heme within the resulting framework

The orientation of the heme itself within its plane, i.e. rotation of the ring about the iron, is given in the column labeled “heme” of Supporting Information Tables S1–S5 as the angle of the heme N_{II} to N_{IV} direction (NC→NA in atom-names from the PDB heme) to the vertical direction of the bundle axis. As discussed above in the section on the structural parameters of the ideal prototypical four-helix heme bundle, sterically stable orientations given symmetric imidazole angles near $\pm 45^\circ$ are at whole multiples of 90° , i.e. with the tetrapyrrole oriented in “diamond” fashion with the corners up, down, left and right.

Actual orientations close to all four of these are observed, with heme b_L at 93° , heme b_H at -79° , Complex II heme and the proximal heme of QFR at -21° and -30° , respectively, and the proximal heme of FDH at 166° . With the exception of heme b_L these all deviate $10\text{--}30^\circ$ from even multiples of 90° , suggesting that the imidazole angles do not bisect the heme $N \rightarrow N$ angles but orient closer to one pair of nitrogens than the other. In fact in heme b_L the imidazole directions are asymmetric, -57° but $+34^\circ$, so here also the ϕ angles are not equal and opposite, rather one is large and one is small, being nearly complementary angles. Heme b_L and the hemes of Complex II and proximal heme of QFR have the propionates directed upwards toward the N surface which is adjacent to these heme sites, while heme b_H and the proximal heme or FDH have the propionates directed downwards toward the P surface to which they are adjacent. The small ϕ angles are made by His82(83,84) in heme b_L , His 96(97,98) in heme b_H , the His from chain D in Complex II, His182 in FRD proximal, and His57 in FDH proximal heme. These ligands with the small ϕ angle are in each case the ligand we have designated His 2 based on the side of the heme, however given the small number and the uncertainty in heme sidedness, this is not very significant.

Comparison with other bis-imidazole hemes; correlation of EPR spectral features

Another membrane-bound heme protein that has heme ligated by histidines in two transmembrane helices is cytochrome b_{559} of Photosystem II [71,72]. Both histidines - (E)H23 and (F)H24 - are in conformations closest to rotamer 2¹, although (F)H24 deviates significantly. In rotamer 2 the imidazole plane is nearly parallel to the helix axis, pointing downwards, as shown in Figure 6. His23 is nearly vertical (178°), while H24 is 53° off vertical (-127°) resulting in the dihedral angle $\Delta\phi = 54^\circ$ and the EPR spectral type is normal rhombic, with $g = 2.95, 2.26, 1.52$ for the intermediate/low-potential form, $g = 3.10, 2.16, 1.49$ for the high/

intermediate-potential form [71,72]. The heme orientation is off vertical in the opposite direction (+145°) so both ϕ angles are positive (33° and 87°). Note the latter ϕ value close to 90° would result in severe steric interaction with the nitrogens of the heme. However, this is a relatively low resolution structure of a very challenging protein, so this angle may not be very accurate.

For heme *a* of cytochrome *c* oxidase we find the ϕ_1 angle (for H61) is -118.3° (+61.7°) and the ϕ_2 angle (for H378) is 42.4°, yielding $\Delta\phi = 19.3^\circ$ for the mammalian cytochrome oxidase structures 1V45 and 2OCC [70]. The rhombic EPR spectrum *g*-values for heme *a* are $g_z = 3.02$ -2.98, $g_y = 2.25$ -2.23, $g_x = 1.5$ -1.45 [8,10] and are thus well within the range normally associated with “parallel” axial histidine imidazole planes. This is a major departure from what is observed for the *b* hemes of Complexes III and II. The histidines are mounted at approximately the same level in transmembrane helices, as in Complexes II and III. However there is no 4-helix bundle, the helices are separated by ~14.5 Å center to center vs. ~16 Å for the bundled binding sites. While His61 is in a slightly distorted rotamer 3, His378 is in a distorted rotamer 2 conformation, with its imidazole plane nearly parallel to the helix axis (Supporting Information Table S6).

The results of this analysis provide a confirmation of the hypothesis presented previously [29] that the orientation of the heme ligands of these membrane-bound bis-histidine-coordinated cytochromes *b* should not change when ferriheme reduction takes place, since changing rotamers from 3 to 2 (or any other rotamer) to achieve the apparently-favored parallel imidazole plane orientation of low-spin Fe(II) [29,93] would require breaking the histidine imidazole-Fe bond, an extremely high-energy process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

Details of the geometric calculations used in preparing Table 1 and Supporting Information Tables S1 – S6.

Protein structures were obtained from the PDB. EPR spectra are from the literature, as referenced. Calculation of angles and distances were by standard geometric methods using simple programs written for the purpose. All angles are calculated in the projection onto the heme plane, i.e. they are the angles seen when viewing the system along the normal to the heme plane. The plane of a heme is taken as the least-squares best-fit plane to the 25 atoms of the heme porphyrin ring plus the iron.

To calculate angles defining the rotation of the ligating imidazoles and their helices about the heme normal in a 360° range, we need to assign a direction to the helix axis and to the intersection of imidazole with the heme plane.

The axis of an alpha helix at the level of the heme ligand histidines was approximated by using the program LSQMAN [94] to find the rotation-translation operator best superimposing the backbone atoms of three adjacent residues of a canonical α -helix, located at the origin with its axis along the z axis, onto the corresponding atoms of the helical histidine and the preceding and following residues. This operator was then applied to a unit vector along the z axis in order to obtain a unit vector along the axis of the actual helix at the level of the heme-ligand histidine. The projection of this vector on the heme plane was then taken to get the direction of the helix

axis viewed along the heme normal. The 4-helix bundle axis (“vertical” direction) is approximated by taking the average of the axes of the two helices at the level of the His ligands, projected onto the heme plane. One consequence of this is that the angles of the helix axes relative to the bundle axis are always equal and opposite; this does not reflect the symmetry of the bundle but just the way we define the bundle axis.

For calculating the ϕ angles of axial-ligand imidazoles, the intersection of the imidazole plane with the heme plane was found. This measurement thus ignores rocking of the imidazole ring away from the heme normal and only counts rotation about the heme normal. The ligand angles were calculated by finding the best-fit plane to the 5 atoms of the imidazole ring, taking two points on the intersection of this with the heme plane, subtracting them to get a vector along the intersection, and multiplying by -1 if this was not in the same direction as the imidazole $C^{\delta 2} \rightarrow C^{\epsilon 1}$ vector (as indicated by the inner product with that vector). The in-plane rotational orientation of the heme about its normal was calculated as the vector from atom N_{II} to atom N_{IV} (NC to NA using PDB ligand atom nomenclature).

We calculate $\Delta\phi$ as the rotation of ligand 2 relative to ligand 1, where ligand 1 is toward the viewer and ligand 2 is away from the viewer in this orientation. The right hand rule then dictates that clockwise rotation is positive. Since it is conventional to take the direction that moves atom N_{IV} toward atom N_{III} (NA to ND) as positive, we need to choose ligand 1 and ligand 2 such that N_{III} is clockwise of N_{IV} , i.e. the rings of the heme go around counterclockwise, when viewed from the side of ligand 1.

The orientation of the heme about its pseudo-2-fold axis is not always clear from crystallographic analysis, depending only on the positioning of methyl and vinyl groups. In some cases (enumerated in the text) there is disagreement between different structures of the same protein. In these cases we have nonetheless made a single choice for ligand 1 and ligand 2, so that everything but the heme orientation will be consistent [5].

Figure 1.

Overall structure of Complexes II and III, mitochondrial membrane proteins with bis-histidyl hemes suspended between two helices of a 4-helix bundle. The hemes are partially visible, as stick figures with C atoms and bonds colored magenta. In Complex II (right), PDB file 1YQ3, the four-helix bundle is composed of the first two transmembrane helices of the C (brown) and D (green) subunits. Heme b_H is ligated by histidine residues on the second transmembrane helix of each of these chains. The flavoprotein (yellow) and iron-sulfur protein (blue) are extrinsic on the matrix (N) side of the membrane. In Complex III (cytochrome bc_1 complex, physiological dimer shown on the left), for the bovine complex, PDB file 1PPJ, the four-helix bundle consists of the first four transmembrane helices of cytochrome b (colored green), with two hemes suspended between the second and fourth helices (helices αB and αD). Heme b_L is near the P side, and heme b_H is near the N side of the membrane. The C-terminal half of cyt b (brown) includes four more transmembrane helices not involved in heme binding. Cytochrome c_1 (blue) and the iron-sulfur protein (yellow) of the same monomer are also colored, while the rest of the dimer is shown in gray. The Fe_2S_2 cluster is shown in yellow and gold, just at the P-side membrane surface. The figure was made using the Molscript [6] and Raster-3D [7] programs, as were Figures 2, 5, and 6.

Figure 2.

Four-helical-bundle binding site geometry. In A, the psuedo-two-fold symmetry of the motif is demonstrated by showing the C α trace and side-chain of the His ligands in the vicinity of the heme of Complex II before and after rotation about the bundle axis in such a way as to superimpose chain C on D and vice versa. The helices are labeled as transmembrane helices 1 or 2 of chain C or D. The view is along the bundle axis, viewed from the N side which corresponds to the top side in standard orientation: the helices go clockwise around the bundle, and the heme-bearing helices have their C-termini directed toward the viewer. Panels B and C show cartoon renditions of the helices in this area of Complex II, viewed from the “bottom” (P side of the membrane) (B) or sideways from within the membrane, perpendicular

to the heme plane (C). Chain C is brown and D is green. The H-bond from His C:98 to Thr C:45 is shown as a white dotted line. Panel 2D shows the graphical representation described in the text of the five characteristic angles of the helix-bundled heme in avian Complex II. These and the coordinates for panels A–C were derived from the structure PDB 2H88. Panels A B, and C were made using Molscript [6] and Raster3D [7].

Figure 3.

EPR spectra of bovine cytochrome bc_1 , or complex III, recorded at 7 K. A: Cytochromes b_{566} , b_{562} and c_1 (fully oxidized by ferricyanide); B: Cytochrome b_{566} (b_L) and b_{562} (b_H) (ascorbate reduced); C: cytochrome c_1 (A–B); D: isolated cytochrome c_1 . Reprinted from reference [43] with permission from Academic Press.

Figure 4.

The d-orbital splitting pattern observed for low-spin d^5 systems such as ferriheme centers. The splitting between the three lowest-energy and the two highest-energy d-orbitals (Δ_{Oh} for octahedral symmetry) is in reality much larger in all cases than the splittings within the lower- and higher-energy subsets shown in the center and the right, thus confirming that all complexes are indeed low-spin, with a single unpaired electron. For these ferriheme systems, the porphyrin ring is of somewhat lower crystal field than that of the axial imidazole ligands, which leads to a tetragonal distortion of the complex and the energies of the d-orbitals; perpendicularly-oriented planar axial ligands give rise to this tetragonal splitting pattern. Note that the d_{xz}, d_{yz} set (also called the d_{π} set) is degenerate and unsymmetrically filled, making this, as well as the octahedral splitting pattern at the left both Jahn-Teller states [69]. If the planar axial ligands are in parallel planes a rhombic distortion is introduced, and the degeneracy of the d_{xz} and d_{yz} orbitals is removed. The rhombic splitting pattern is thus lower in overall energy than the tetragonal or octahedral splitting patterns.

Figure 5. Histidine side-chain: rotatable dihedrals. Rotation about the N-Fe bond (vertical line) determines the ϕ and $\Delta\phi$ angles. However, noncolinearity of this dihedral axis and the side-chain χ_2 angle limits rotation once the histidine backbone atoms and heme are positioned. In many examples an H-bond from the $N^{\delta 1}$ -H to a serine or threonine residue in the preceding helix of the bundle may further restrict or influence the rotation angle. The dotted line indicates the hydrogen bond to a threonine not visible in this top view because it is below the ribbon of the helix. This is the same structure (1H88) shown from the bottom in Figure 2b, where the threonine is visible. The figure was made using Molscript [6] and Raster-3D [7] programs.

Figure 6.

Histidine side-chain conformations. Three different views of a histidine in an α -helix, in two different rotamers. Each figure shows rotamers 2 and 3, the different figures show different rotation about the helix axis. The helix is oriented vertically with N-terminal end below. Note that rotamer 2 holds the imidazole plane approximately parallel to the helix axis, while in rotamer 3 it is more nearly perpendicular, or horizontal in this orientation. Lines are drawn through the imidazole planes indicating the “ligand axis” and “cross axis” directions as defined in the text. In the left figure the ligand axis of the imidazole plane is directed toward the viewer (and upwards). The cross-axis direction is in the plane of the picture and makes an angle of about 15° with the horizontal. In the right figure the ligand axis is in the plane of the picture,

making an angle of about 31° with horizontal. In order to have the N-Fe-N bond linear and perpendicular to the heme plane, this must be leveled by slanting the helix. The slant results from the superhelical twist of the 4-helix bundle. The figure was made using Molscrip [6] and Raster-3D [7] programs.

Figure 7.

Arrow diagrams illustrating the five characteristic angles for the ten heme groups discussed here. As described more fully in the text, the magenta arrow represents the orientation of the heme N_{II} → N_{IV} direction, the green arrows in front and behind it represent the orientation of the imidazole ligands His1 and His2, and the brown arrows represent the orientation of the helical axes for the two ligand-bearing helices; all with the structure oriented so that the bundle axis is vertical.

Table 1

Angles of ligand imidazole planes relative to heme $N_{II}-N_{IV}$ vector. The ϕ and $\Delta\phi$ values were calculated for available mitochondrial and bacterial bc_1 complexes, individual results listed in the Supporting Information, Tables S1–S6. The angles ϕ_1 and ϕ_2 between the intersection of the ligand imidazole planes and the heme plane are calculated in the range $-180^\circ < \phi < 180^\circ$ as defined in the text, and subtracted to obtain $\Delta\phi$. $\Delta\phi^*$ is then that value expressed as the angle from coplanarity, in the range $0^\circ < \Delta\phi^* < 90^\circ$.

Cytochrome	His1 res#	His2 res#	ϕ_1	ϕ_2	$\Delta\phi$	$\Delta\phi^*$	large ϕ_{max}
Cyt bc_1 heme b_L	182/183	82/84	57.1 ± 5.2	145.7 ± 5.9	-87.7 ± 7.6	83.7 ± 4.8	Y
Cyt b_{6f} heme b_L	187	86	43.6 ± 6.2	163.7 ± 1.1	-120.1 ± 7.2	59.9 ± 7.2	Y
Cyt bc_1 heme b_H	196/197	97/98	-137.9 ± 7.1	-16.2 ± 7.6	-121.8 ± 13.1	58.2 ± 13.1	Y
Cyt b_{6f} heme b_H	202	100	-145.7 ± 3.0	-44.8 ± 0.8	-101.0 ± 3.8	79.1 ± 3.8	Y
Complex II heme b	C98	D46	-64.5 ± 2.6	19.6 ± 7.6	-84.0 ± 7.7	80.9 ± 1.9	Y
<i>E. coli</i> SQR heme b	C84	D71	-64.6	36.5	-101.1	78.9	Y
QFR prox. Heme b	C93	C182	-60.68	24.6	-85.3	85.3	Y
QFR dist. heme b	C143	C44	8.5	-71.7	80.2	80.2	Y
FDH prox. Heme b	C155	C57	102.2	219.7	-117.5	62.5	?
FDH distal heme b	C169	C18	-45.4	$+77.3$	-122.7	57.3	?
PS-II Cyt b_{559}	E23	F24	-34.7	-77.6	42.9	42.9	N
Cyt oxidase cyt a	61	378	118.3	-42.4	160.7	19.3	N
PRC cyt c	309	124	-31.07	30.1	-61.2	61.2	Y