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COMMENTARY

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When an amide is more like histidine than imidazole: the role of axial ligands in heme catalysis

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Abstract Of the many subtle protein-cofactor interactions which facilitate oxidative catalysis by heme enzymes, the role of the axial ligand has for some time appeared to be fairly well understood. Recent studies from several laboratories, however, have provided good reason to reemphasize the importance of secondary interactions between the axial ligand and protein, as the results suggest that simple ligand identity is neither necessary nor sufficient for function. It has been widely proposed that the strong hydrogen bond between a proximal carboxylate and the histidine ligand of peroxidases assists O–O bond heterolysis and stabilizes the Fe(IV)=O center that is produced. Recent replacements of the axial ligand in a number of heme proteins have produced a few surprises, suggesting that the subtle interactions between the ligand and protein may in some cases be more important than the actual identity of the ligand.

Key words Axial ligands · Peroxidases · Heme enzymes · Imidazole · Electron transfer

Of the many subtle protein-cofactor interactions which facilitate oxidative catalysis by heme enzymes, the role of the axial ligand has for some time appeared to be fairly well understood. Recent studies from several laboratories, however, have provided good reason to reemphasize the importance of secondary interactions between the axial ligand and protein, as the results suggest that simple ligand identity is neither necessary nor sufficient for function. At the least, it is clear that structural and chemical descriptions of these experiments have yet to be formulated with enough clarity to make complete sense in terms of established models.

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The traditionally proposed roles played by the axial ligand are well illustrated by the peroxidases versus the P_{450} type mono-oxygenases. As described in the commentary by Poulos [1], O–O bond heterolysis may be facilitated by residues positioned on both proximal and distal heme faces. It has been widely proposed that the strong hydrogen bond between a proximal carboxylate and the histidine ligand of peroxidases assists O–O bond heterolysis and stabilizes the $Fe^{+4} = O$ center that is produced [2, 3]. Mutagenesis studies have shown that this hydrogen bond indeed affects the Fe-His bond strength [4, 5], redox potential, and iron electronic properties [6, 7] in the direction expected. For enzymes such as P_{450}, the "push-pull" hypothesis [8] persists in which the more nucleophilic cysteine ligand is required to promote O–O bond heterolysis due to the fact that substrate access to the distal heme face precludes the positioning of distal catalytic groups. Secondary conserved hydrogen bonding interactions and the electrostatic environment near the cysteine of these enzymes may also be important in tuning the properties of the sulfur ligand for optimum function [1].

Recent replacements of the axial ligand in a number of heme proteins have produced a few surprises, suggesting that the subtle interactions between the ligand and protein may in some cases be more important than the actual identity of the ligand. While replacement of the cysteine ligand in cytochrome P_{450} has been difficult to achieve, the successful introduction of a cysteine [9, 10] or tyrosine [11] proximal ligand into myoglobin (Mb) has been described. For the cysteine variant, the spectroscopic features of the ferric state suggest axial sulfur ligation, but the ligand appears to be lost upon reduction, and the simple introduction of cysteine has not, on its own, produced a protein with P_{450}–like reactivities. A different approach to ligand replacement has followed from observations that the deletion of protein side chains can leave structural cavities that can be complemented by exogenously provided compounds [12–15]. This method has been recently applied to the axial ligand of a number of heme proteins including Mb

[16], cytochrome *c* peroxidase (CCP) [17], and heme oxygenase (HO) [18]. Deletion of the proximal histidine of Mb and its replacement by imidazole gave a protein which was comparable to the native enzyme in spectroscopic properties, ligand binding, and structure, although a small rotation of the ligand about the Fe-imidazole bond was noted [16]. That its properties are similar to the native protein in spite of this small distortion may reflect the fact that secondary interactions between the histidine and protein are weak, and perhaps not as critical to protein function as they are for peroxidases and mono-oxygenases. In a similar vein, replacement of the presumed axial histidine of HO with alanine resulted in an inactive protein, which could be reactivated upon imidazole binding [18]. Resonance Raman data have indicated that the histidine in these enzymes is also not strongly hydrogen bonded [19]. So, as with Mb, the properties of the ligand may not be significantly modulated by whatever differences exist between the geometry of the native histidine and the exogenous imidazole. When this approach was applied to CCP, imidazole binding to the "histidine cavity" was also observed, producing spectroscopic properties which were similar to those of the native enzyme [17]. However, in this case, the imidazole complex did not significantly restore cytochrome *c* (cyt *c*) oxidation activity. In perhaps the most intriguing experiments to date, the Poulos group has observed that replacement of the histidine ligand of CCP with glutamine resulted in a fully active enzyme [20], while a glutamate ligand produced a hyperactive variant [21, 22]. Thus, the peroxidases appear on one hand to be exquisitely sensitive to perturbations of their native ligand, and on the other able to accept completely different ligands with minor functional consequences.

These results have raised some rather focused questions about the proximal ligand in peroxidase function. As noted by Poulos [1], the difference in function observed between the Gln/Glu and imidazole variants could be in the efficiency of reducing Compound I by substrate, or in the initial reaction of the enzyme with peroxide to give Compound I. The first possibility could result from differences in the energetics of the ferryl intermediate, and, while this is as yet unknown, recent advancements in the direct electrochemistry of these states may soon provide some answers [23]. It is noted that the Fe^{+3}/Fe^{+2} midpoint potential of the Gln variant is increased by 75 mV over wild-type [21], while that for the imidazole complex is only ~20 mV higher (Goodin, unpublished results). If this information can be extrapolated to the $Fe^{+3}/Fe^{+4} = O$ states, the Gln may produce a more reactive ferryl, yet this provides no electrochemical understanding of the inactive imidazole complex. As previously noted [21], the reduction potential of the $Fe^{+4} = O$ cannot be increased very much above its wild-type value of +740 mV [23]. In fact, as the overall reaction for oxidation of cyt *c* by the ferryl state is very over-driven anyway ($\Delta G << 0$), it is difficult to rationalize the hyperactive mutants in terms of driving force alone. Intramolecular electron transfer has been shown to be much faster than the overall enzyme turnover [24], so that the rate-limiting step is likely to be attainment of the most efficient electron transfer complex. The other possibility raised by Poulos [1] is that the initial rate of Compound I formation is compromised in the imidazole complex. The Gln and Glu variants react readily with peroxide to form the ferryl intermediate, demonstrating either that the proximal "push" is not critical for O–O bond heterolysis in the peroxidases, or that other ligands can serve this role. This complements well the observation that the distal histidine is critical for this reaction [25]. However, while the imidazole complex reacts with H_2O_2 to give a compound I-like state, we find that this reaction is slow and is either incomplete or the ferryl state is unstable (Goodin, unpublished results). In addition, recent results show that substituted imidazoles, particularly 4-methylimidazole, significantly affect this reaction with H_2O_2. Thus, while it remains to be shown whether this is the sole cause of inactivity, it appears that alteration of the proximal ligand can but does not always impair O–O bond heterolysis.

It is also possible that the normal mode of electron transfer has been altered in these CCP axial ligand variants. Several studies have indicated that electron transfer from cyt *c* proceeds via the Trp191 cation radical for one or both reduction steps [26], even though this pathway may not be required for other substrates or in other peroxidases lacking a tryptophan at this position [29]. In this view, the imidazole complex may have destroyed the efficiency of the specific pathway used for cyt *c* by subtly altering the coupling of the Trp radical with cyt *c* and/or the CCP ferryl heme. In fact, while it was noted that the imidazole complex was inefficient at oxidizing cyt *c* [17], this variant is capable of oxidizing aniline with rates similar to those caused by the native enzyme (Goodin, unpublished results). In addition, the radical EPR signal observed in the oxidized imidazole complex is very different in lineshape from that in the wild-type enzyme [17], and this lineshape is known to be the result of a distributed exchange coupling to the ferryl heme [27]. As shown in Fig. 1, the proximal aspartate hydrogen bonds to both the axial ligand and the Trp radical site, so that small variations in the geometry of the imidazole could conceivably modulate the coupling between heme and radical. In the case of the Gln/Glu axial ligand variants, an important question is whether electron transfer is proceeding through the normal pathway or whether new ones have opened up. Several other seemingly unrelated hyperactive CCP mutants have been characterized [28], one of which, A147Y, is not near the heme active site residues or the proposed electron transfer pathway [29]. In addition, the H175Q/W191F double mutant was shown to rescue partial activity from the inactive W191F variant [30], and electron transfer in this mutant at least cannot proceed through the missing Trp radical. Thus, it may be possible that introduction of the Gln ligand by itself im-

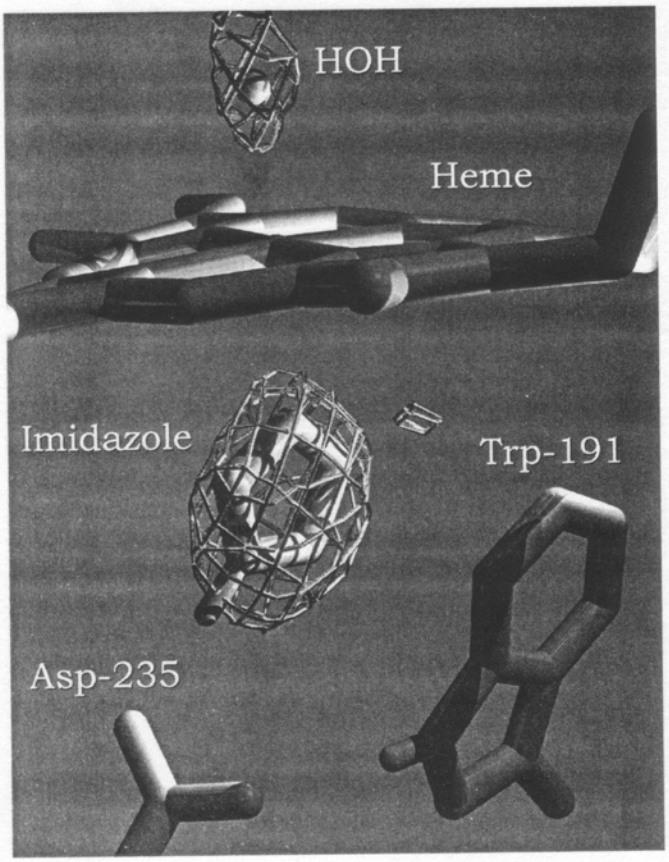

Fig. 1 A crystal structure of the H175G mutant of cytochrome *c* peroxidase containing a proximally bound imidazole. Electron density contours are shown at 5 σ for the F_o–F_c omit map in which both proximal imidazole and distal water were removed from the model before refinement. Also shown is the hydrogen bonding network linking the heme, proximal imidazole, apartate-235 and the tryptophan-191 radical site

pairs efficient electron transfer through the Trp radical. Indeed, the Trp radical signal observed for the oxidized Gln/Glu mutants was also reported to be unusual [30]. In this case, a novel short circuit pathway would be needed to explain the activity of the Gln mutant. Given such a new pathway, the hyperactive glutamate mutant may also provide such a short circuit without completely destroying the normal one.

Important questions also remain to be answered concerning the inactive imidazole complex, primarily: In what ways is imidazole as a ligand different from the native histidine? One possibility is that electronic differences between imidazole and the histidine side chain, by virtue of the 4-methylene substitutent, are functionally significant. For example, the pK_a for methylated imidazoles is elevated by 0.5–1 pH unit with respect to unsubstituted imidazole, and this would make it more difficult for Asp-235 to impart imidazolate character to the native histidine relative to imidazole. Assuming that this is the role of the aspartate, a fully deprotonated histidine should be a stronger nucleophile than Im^-. Alternatively, the loss of a covalent tether to the protein may be the important parameter, and this could be felt in a number of ways. While the covalent link between the protein and heme may provide efficient electron transfer pathways, the linkage onto the proximal α-helix is not one that has been proposed or considered likely. It is also possible that electron transfer is coupled in some way to a conformational trigger or to the vibrational modes of the protein, and the untethered imidazole is thus disconnected from these mechanisms. However, our currently favored hypothesis is that the imidazole may be "frustrated" by competing interactions between the iron and its aspartate hydrogen bond partner, and may require tight structural tethering to force a crucial balance of these interactions. An untethered imidazole may be free to "choose" one of these at the expense of the other, and, unlike the cases for Mb and HO, this could have a modulating effect on its properties as a ligand and/or as a hydrogen bond partner. If one or both of these is important to function, for example in providing the proper "push" for O–O bond heterolysis or mediating a coupling mechanism between the ferryl and Trp radical, the effect could be devastating for function. An attractive example of how this coupling could be mediated by the axial ligand through delocalization of electrons from a porphyrin π radical onto the axial ligand is discussed in the commentary by Weiss et al. [31]. Thus, while a number of new puzzling questions have been raised about the role of axial ligands in heme enzymes, indeed more than appear to have been answered, these questions will help to stimulate new research and discussion of this aspect of catalysis.

References

1. Poulos TL (1996) JBIC 1:356–359
2. Valentine JS, Sheridan RP, Allen LC, Kahn PC (1979) Proc Natl Acad Sci USA 76:1009–1013
3. Finzel BC, Poulos TL, Kraut J (1984) J Biol Chem 259:13027–13036
4. Smulevich G, Mauro JM, Fishel LA, English AM, Kraut J, Spiro TG (1988) Biochemistry 27:5477–5485
5. Spiro TG, Smulevich G, Su C (1990) Biochemistry 29:4497–4508
6. Goodin DB, McRee DE (1993) Biochemistry 32:3313–3324
7. Ferrer JC, Turano P, Banci L, Bertini I, Morris IK, Smith KM, Smith M, Mauk AG (1994) Biochemistry 33:7819–7829
8. Dawson JH (1988) Science 240:433–439
9. Adachi S, Nagano S, Ishimori K, Watanabe Y, Morishima I, Egawa T, Kitagawa T, Makino R (1993) Biochemistry 32:241–252
10. Hildebrand DP, Ferrer JC, Tang H-L, Smith M, Mauk AG (1995) Biochemistry 34:11598–11605
11. Hildebrand DP, Burk DL, Maurus R, Ferrer JC, Brayer GD, Mauk AG (1995) Biochemistry 34:1997–2005
12. Toney MD, Kirsch JF (1989) Science 243:1485–1488
13. Denblaauwen T, Vandekamp M, Canters GW (1991) J Am Chem Soc 113:5050–5052
14. Eriksson AE, Baase WA, Wozniak JA, Matthews BW (1992) Nature 355:371–373
15. Fitzgerald MM, Churchill MJ, McRee DE, Goodin DB (1994) Biochemistry 33:3807–3818
16. Barrick D (1994) Biochemistry 33:6546–6554

17. McRee DE, Jensen GM, Fitzgerald MM, Siegel HA, Goodin DB (1994) Proc Natl Acad Sci USA 91:12847–12851
18. Wilks A, Sun J, Loehr TM, Ortiz de Montellano PR (1995) J Am Chem Soc 117:2925–2926
19. Sun J, Loehr TM, Wilks A, Ortiz de Montellano PR (1994) Biochemistry 33:13734–13740
20. Sundaramoorthy M, Choudhury K, Edwards SL, Poulos TL (1991) J Am Chem Soc 113:7755–7757
21. Choudhury, Sundaramoorthy M, Hickman A, Yonetani T, Woehl E, Dunn M F, Poulos TL (1994) J Biol Chem 269:20239–20249
22. Smulevich G, Neri F, Willemsen O, Choudhury K, Marzocchi MP, Poulos TL (1995) Biochemistry 34:13485–13490
23. Mondal MS, Fuller HA, Armstrong FA (1996) J Am Chem Soc 118:263–264
24. Hahm S, Miller MA, Geren L, Kraut J, Durham B, Millett F (1994) Biochemistry 33:1473–1480
25. Erman JE, Vitello LB, Miller MA, Shaw A, Brown KA, Kraut J (1993) Biochemistry 32:9798–9806
26. Miller MA, Vitello L, Erman JE (1995) Biochemistry 34:12048–12058
27. Houseman ALP, Doan PE, Goodin DB, Hoffman BM (1993) Biochemistry 32:4430–4443
28. Roe JA, Goodin DB (1993) J Biol Chem 268:20037–20045
29. Wilcox SK, Jensen GM, Fitzgerald MM, McRee DE, Goodin DB (1996) Biochemistry 35:4858–4866
30. Choudhury K, Sundaramoorthy M, Mauro JM, Poulos TL (1992) J Biol Chem 267:25656–25659
31. Weiss R, Mandon D, Wolter T, Trautwein AX, Müther M, Bill E, Gold A, Jayaraj K, Terner J (1996) IBIC 1:377–383