ENDOR of Metalloenzymes

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ABSTRACT
This Account examines the role of electron−nuclear double resonance (ENDOR) spectroscopy in furthering our understanding of how metal ions function in biological systems. It briefly describes ENDOR and electron spin−echo envelope modulation (ESEEM) spectroscopies and then illustrates the uses of ENDOR with several case studies from our own research: cytochrome *c* peroxidase compound ES; ribonucleotide reductase intermediate **X**; allylbenzene-inactivated chloroperoxidase; the role of the $[4Fe\text{-}4S]^{+}$ cluster in enzymes of the "radical S-adenosylmethionine" superfamily; dioxygen activation by heme enzymes. Finally, it briefly considers future developments.

Much has happened since an earlier Account[1] described the ways electron−nuclear double resonance (ENDOR) spectroscopy[2] helps us understand how metal ions function in biological systems. Perhaps most noteworthy, and central to this Account, is the expanded application of the technique, with new systems being explored and enzymic intermediates becoming a main focus. In large part, this expansion has been enabled by modern molecular biology, which makes it possible to prepare previously inaccessible proteins and to do so with isotopic labels in both wild-type and site-specifically modified forms. No less important is the increased power of the ENDOR method itself, and of its junior partner electron spin−echo envelope modulation (ESEEM) spectroscopy,[3] as the use and variety of time-domain paramagnetic resonance protocols have increased[3] and microwave frequencies have gotten higher.[4,5] It is thus beneficial to consider the role of these spectroscopic techniques now, at the beginnings of proteomics, when it might become possible to determine the primary sequences and resting-state X-ray crystal structures of all proteins within a chosen organism. This Account briefly recalls the basics of the ENDOR/ESEEM methods and then illustrates the ability of ENDOR to generate all the types of information listed in Table 1, sometimes supplying the "cake", and other times the "frosting".

Background

In studying a metalloenzyme, one wants to know the composition, structure, and bonding at the active site throughout the catalytic cycle. Such information can be obtained through analysis of the electron−nuclear hyperfine and nuclear-electric quadrupole interactions[2] of the metal-ion nuclei themselves, of nuclei that form endogenous and exogenous metal ligands, as well as of enzyme-bound substrates, inhibitors, and products. Sometimes, these interactions are manifest in splittings seen in electron paramagnetic resonance (EPR) spectra. However, for most metalloenzymes no splittings can be resolved for any active-site nucleus, and the chemical information is lost. ENDOR and ESEEM retrieve this information.

Table 1. Spectroscopically Retrievable Information

enzyme mechanism
3D structure
metal-ion: coordination geometry
valence
identification
protein dynamics
electronic and magnetic properties

About the Methods. ENDOR and ESEEM spectroscopies[2,3,6−8] provide NMR spectra of nuclei that interact with the electron spin of a paramagnetic center.

ENDOR monitors the actual NMR transitions induced by an incident radio frequency (rf) field but does not detect the transitions directly. Instead, the rf field is applied in conjunction with a microwave field to a sample in the cavity of an electron paramagnetic resonance (EPR) spectrometer, and the NMR transitions are monitored as a change in the EPR signal intensity as the radio frequency is stepped through a selected range. One thus has "EPR-detected" NMR, hence the term "double resonance". Such NMR spectra display orders-of-magnitude better resolution than the EPR spectrum of the center, and as a result double-resonance methods can be used to characterize hyperfine and quadrupole interactions for systems whose EPR spectra show no hyperfine splittings. Interestingly, this "indirect detection" procedure, which is now well-known in conventional NMR spectroscopy, was introduced with ENDOR spectroscopy.[9]

ENDOR can be performed either in a CW or time-domain (pulsed) mode, which leads to a caveat. The intellectual "undertow" of the far larger NMR community can induce the expectation that time-domain techniques are necessarily better than CW techniques, and that higher microwave frequencies are necessarily better than lower. The former is not correct: for example, CW ENDOR of metalloenzymes almost always has better sensitivity than pulsed because in general a microwave pulse cannot flip all the electron spins in a sample. Conversely, pulsed ENDOR often gives better line shapes/resolution, and the ability to vary pulse sequences and manipulate polarizations/coherences in pulsed ENDOR provides its own benefits.[8,10] For a variety of reasons, not least being difficulties in sample handling, the latter isn't correct either.

ESEEM is an intrinsically time-domain technique that again employs an EPR spectrometer as an NMR detector. However, in this technique the hyperfine and quadrupole

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interactions appear as a time-domain modulation of the amplitude of the electron spin−echo, without the application of an rf field; Fourier transformation then gives the transition frequencies. It can be argued that the "metals-in-biology" community became aware of these advanced paramagnetic resonance techniques through the seminal ESEEM studies of Cu(II) centers by Peisach and Mims,[11] even though they were preceded by applications of ENDOR to both iron−sulfur[12] and heme[13] proteins.

Because the magnetic moment of the electron spin "detector" is $\sim 10^3$ larger than that of nuclei, the EPR-detected NMR methods are orders of magnitude more sensitive than conventional NMR. In addition, ENDOR, and to a lesser degree ESEEM, are inherently broad-banded and can exhibit signals from "nonstandard" nuclei (e.g., ^{2}H, ^{14}N, ^{17}O, ^{33}S, ^{57}Fe, ^{95,97}Mo) as easily, or more so, than "standard" ones (e.g., ^{1}H, ^{13}C, ^{15}N); indeed, it is comparably easy to detect ENDOR signals from essentially every type of nuclear spin covalently linked to an electron spin. Thus, with proper isotopic labeling, it is possible to characterize every atom associated with a paramagnetic metalloenzyme active site.[7] At the same time, ENDOR and ESEEM discriminate against nuclei that are not part of the active site: signals arise only from nuclei that have a hyperfine interaction with the electron-spin system under observation, that is, only from active-site atoms. For example, it is possible to examine ^{57}Fe resonances from one metal cluster without interference from ^{57}Fe in other clusters, and without interference from excess inorganic ^{57}Fe (see below[14]). Even the obvious restriction of paramagnetic resonance methods to paramagnetic centers, be they metal ion or radical, can be circumvented in certain circumstances, as illustrated below.

ENDOR Determination of Active-Site Structure. The derivation of bonding and geometric information through these resonance methods begins with the determination of the hyperfine interaction tensor/matrix,[2] **A**, for hyperfine-coupled nuclei, along with the quadrupole interaction tensor when $I > 2$. This requires a set of measurements taken over a range of orientations of the molecular frame relative to the external field, such as might be collected when a single-crystal is rotated in a field (for a recent metalloenzyme example, see ref 5). However, the samples employed in ENDOR/ESEEM studies of metalloenzymes almost always are frozen solutions; indeed, this is often necessarily so, as with intermediates trapped by rapid freeze-quench (rfq) techniques.[15] A frozen solution contains a random distribution of active-site orientations, and thus it might be thought that the required "orientational" dataset could not be obtained. However, as we have shown[7] and illustrate below, the necessary orientational information can indeed be obtained from a frozen solution through analysis of a "2-D" pattern comprised of "orientation-selective" ENDOR spectra collected at multiple fields (g values) across the EPR envelope of a frozen-solution sample.

A hyperfine matrix thus obtained for a particular nucleus can be decomposed into two components: $\mathbf{A} = \mathbf{A_{loc}} + \mathbf{T}$. The "local" contribution, $\mathbf{A}_{\mathrm{loc}}$, arises from spin on the atom whose nucleus is observed. This term arises from covalent (through-bond) interactions within the paramagnetic catalytic center and generally is announced by the presence of an isotropic (s-orbital) hyperfine coupling. The complete set of nuclei that exhibit an $\mathbf{A_{loc}}$ component thus gives the composition of the covalent network that incorporates the metal ion, which includes but is not limited to the coordination sphere of the metal ion(s). For atoms involved in the covalent network, detailed analysis of $\mathbf{A_{loc}}$ (and the quadrupolar tensor) can yield the valence of the metal ions,[16] covalency parameters for ligands,[17] as well as the coordination geometry of the metal center.

Atoms near to the active site, even if not covalently linked, still exhibit a "nonlocal", dipolar coupling to the electron spin, **T**,[18] whose analysis yields the coordinates of those atoms. As shown below, determination of the positions for several atoms of a bound substrate, product, or inhibitor can provide enough geometric constraints to define its structure, although information derived from ENDOR/ESEEM is most richly interpretable in the context of a related crystal structure.

In principle, **A** can be derived either from ENDOR or from ESEEM and its multidimensional extension, "HYSCORE".[3] ESEEM is technically less demanding because it does not require an applied rf field, and in a minority of cases it is unambiguously the method of choice. Then why do we emphasize ENDOR in our research and in this Account? It is largely a matter of selection rules. The features that are most helpful in analyzing an orientation-selective 2-D dataset are associated with principal values of the hyperfine matrix (and quadrupole tensor), but features associated with hyperfine principal values are intrinsically suppressed in ESEEM.[19] In addition, ESEEM commonly is thought to have a farther detection "horizon" for nuclei in the vicinity of a metal ion, but in fact we find the advantage usually goes to Mims pulsed ENDOR, particularly for nuclei with larger g_n. Overall, however, the two partner techniques have complementary strengths: it needs not be said that the best approach is to use a screwdriver on a screw, a hammer on a nail.

Information Recovered: Case Studies

Not long ago, I attended a meeting in which a speaker showed a list of the types of information needed to characterize a metallobiomolecule, arranged with the "good stuff" toward the top; it is modified here as Table 1. Associated with each type was a technique assigned to "retrieve" it (e.g., X-ray diffraction for 3-D Structure); ENDOR spectroscopy appeared far down the list (slightly below dowsing, as I recall), and ESEEM was not mentioned. This Account will illustrate that, on the contrary, these methods can contribute information of all the types listed in Table 1 and often do so uniquely well; although not part of the Account, this includes information about dynamics,[20] an area where ENDOR/ESEEM spectroscopies might have been thought unsuitable. Most importantly, as is shown, they do so not merely for resting-state metal

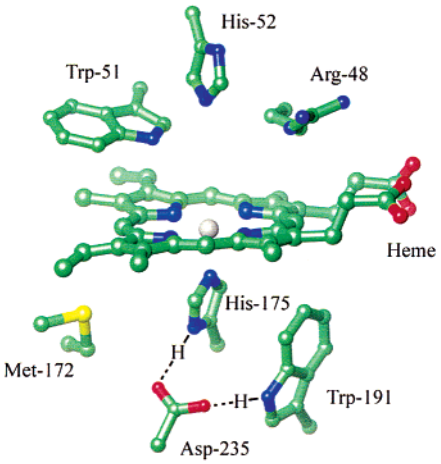


FIGURE 1. Active-site structure of C*c*P, showing the protonated Trp[191] as it occurs in the radical cation of ES.[21]

centers, but for reactive intermediates, which seldom are amenable to X-ray diffraction methods. As a result of all this, in favorable cases these methods can definitively characterize elusive enzymic intermediates and establish enzymic mechanisms, the "highest" goal of Table 1. The application of multinuclear CW and pulsed ENDOR techniques is now illustrated by several, representative, recent examples from our research.

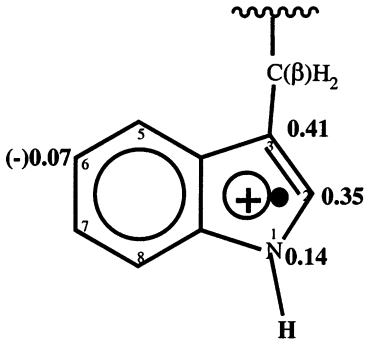
Cytochrome *c* Peroxidase Compound ES.[21] Treatment of cytochrome *c* peroxidase (C*c*P) with H_2O_2 generates a reactive intermediate, called ES, that is oxidized by two equivalents above the ferriheme resting state. One equivalent is stored as the ferry1 (Fe^{IV}=O; $S = 1$) form of the heme. The second exists as a reversibly oxidized amino acid residue (*R*•), whose identity of that radical had been sought for more than a quarter century (see citations in ref 20). ENDOR studies carried out in collaboration with Prof. David Goodin and co-workers provided the answer.[21]

First, a series of ^{1,2}H, ^{13}C, and ^{15}N ENDOR measurements perfomed on enzyme prepared with labeled Trp showed that *R*• is an indole radical of Trp[191], which lies next to the heme, H-bonded to Asp[235] and thence to the proximal His, Figure 1. Detailed analysis of these ENDOR signals then established that *R*• is the π-cation radical formed by one-electron oxidation of the indole moiety, not the neutral radical generated by loss of the N−H proton as well as a π electron. This conclusion was suggested by the presence of a strongly coupled exchangeable N−H proton and was validated by the determination of the indole-ring spin density distribution, Chart 1: the low spin density on N1 and rather large density on C2 distinguishes this form from the neutral radical.[21] Clearly, such a characterization of one electron (hole) and one proton is a spectroscopic, not a crystallographic task, and illustrates the unique abilities of ENDOR spectroscopy.

Ribonucleotide Reductase Intermediate X. This summary proceeds "up" from the bottom of Table 1, first describing the reformulation of a diiron reactive intermediate through the determination of its Fe-ion valencies, followed by the characterization of the metal-ion coordination spheres and the structure of the diiron inorganic core.

Chart 1

C*c*P-ES π-Cation Radical



Ribonucleotide reductases catalyze the conversion of nucleotides to 2-deoxynucleotides in a process that constitutes the rate-limiting step of DNA biosynthesis.[22] The R2 subunit of the *E. coli* enzyme contains a catalytically essential tyrosyl radical located on residue 122 (•Y122), adjacent (5.3 **Å**) to a μ-oxo, carboxylato-bridged diferric cluster. The radical is the "pilot light" that initiates catalysis, while the diiron center is the "match" that lights the pilot light. Oxygen activation during cluster formation generates a diiron intermediate, **X**, which is one-electron oxidized above the diferric resting state and which actually oxidizes Y122 to the radical, Scheme 1.[23] This intermediate is formally equivalent to a hypothetical intermediate in hydroxylations by diiron centers, and so its characterization is not merely important to an understanding of cluster assembly in this enzyme, but perhaps more so for

Scheme 1

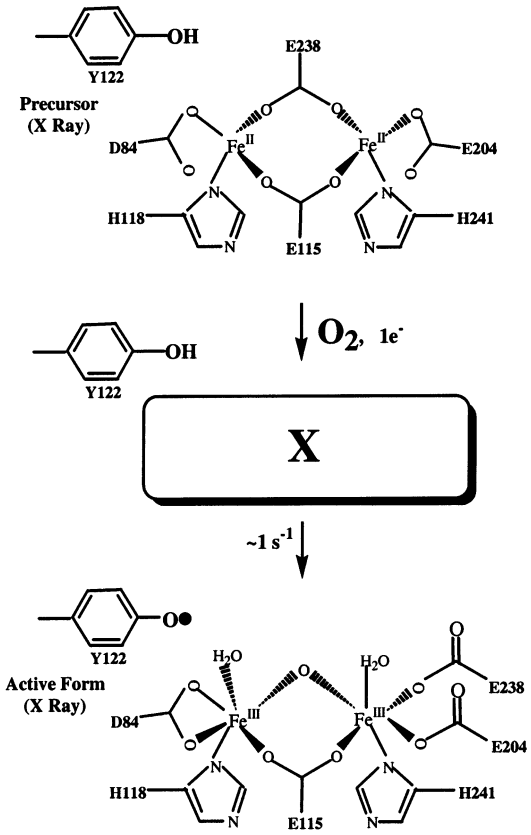
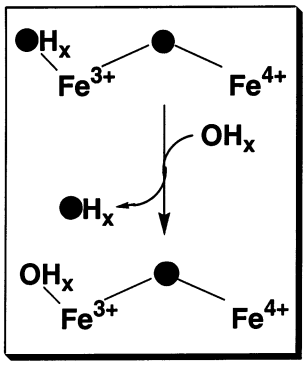
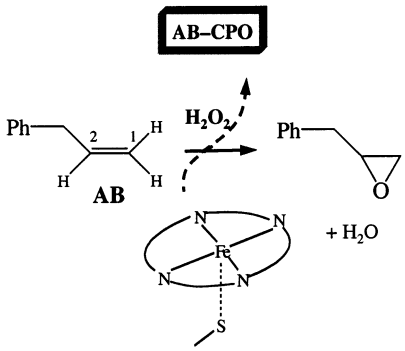


Chart 2



Scheme 2

(*Suicide Inhibition*)



the insight it brings to the general problem of dioxygen activation by diiron centers.

Intermediate **X** was characterized through rapid freeze-quench (rfq) Q-band ENDOR studies in collaboration with Prof. Joanne Stubbe and Dr. Doug Burdi.[14,24,25] Earlier, rfq Mössbauer measurements[26] had suggested that **X** is comprised of two high-spin ferric ions and a $S = {}^1/_2$ ligand radical, mutually spin coupled to give a $S = {}^1/_2$ ground state. However, subsequent rfq ^{57}Fe ENDOR measurements[14] showed that one Fe ion indeed had the isotropic ^{57}Fe hyperfine tensor characteristic of a high-spin ferric ion, but that the tensor of the other Fe is markedly anisotropic, characteristic of a high-spin Fe^{IV} rather than Fe^{III}. This indicated that **X** instead achieves a spin of $S = {}^1/_2$ through antiferromagnetic coupling between $Fe^{III}(S = {}^5/_2)$ and Fe^{IV}-($S = 2$) ions, without the presence of an independent radical. This reformulation of the electronic structure of **X** was made possible by the selectivity of ENDOR: the ^{57}Fe Mossbauer spectrum of **X** had been compromised by an overlapping spectrum from excess ^{57}Fe ion present during the cluster assembly reaction; this species is not EPR-visible in the ENDOR experiment and thus does not contribute to an ^{57}Fe ENDOR experiment.

The reformulation set the stage for ENDOR studies to determine the structure of the diiron core of **X** and the fate of the two O atoms of dioxygen during the cluster assembly/dioxygen activation process. First, CW and pulsed Q-Band ^{1,2}H ENDOR studies of **X** in H_2O and D_2O buffers[24] showed that **X** contains a single terminal aqua ligand (water molecule or 2-fold disordered hydroxyl) bound to Fe^{III}, and neither an aqua ligand bound to Fe^{IV} nor a hydroxyl bridge.

Next, CW and pulsed Q-band ^{17}O ENDOR experiments on **X** prepared with both $H_2{}^{17}O$ and $^{17}O_2$[25] were used to examine the structure of the inorganic core of **X** and to establish the fate of the atoms derived from O_2. These measurements, which included kinetic rfq- ENDOR studies of the formation process, revealed that **X** as formed contains both atoms derived from O_2. These are not bound in an $[Fe_2O_2]$ "diamond core", but rather, one is present as a μ-oxo bridge and the other as the terminal aqua ligand. Analysis of the 2D ENDOR patterns led to the picture of an $[(H_xO)Fe^{III}OFe^{IV}]$ inorganic core, Chart 2 (● again implies origin as O_2), that is further coordinated by one or two additional mono oxo bridges provided by carboxylate oxygens. The dependence of the ^{17}O signals on freeze-quench delay times showed that the terminal oxygen atom rapidly exchanges with solvent; the bridge does not (Chart 2).

Structure of the Alkylated Heme in Allylbenzene-Inactivated Chloroperoxidase.[27] In this case and the following one, ENDOR provided key information about the coordination spheres of two quite different catalytic metal centers, plus three-dimensional structural information about catalytically important states.

The biological role of chloroperoxidase (CPO) is the halogenation of organic substrates, but the enzyme also is found to epoxidize alkenes with high regioselectivity.[28] During the epoxidation of allylbenzene (AB; Scheme 2), CPO eventually is converted to an inactive green species whose low-spin ($S = {}^1/_2$) ferriheme prosthetic group (AB-CPO) is modified by addition of the alkene plus an oxygen atom. Determination of the structure of the adduct was important because it would give insights into the catalytic mechanism, information about the AB binding geometry, and suggestions for mutagenizing the protein so as to improve and/or alter the enantiomeric specificity. In collaboration with Prof. Lowell Hager and Dr. Annette Dexter, ^{15}N, ^{1,2}H, and ^{13}C 35 GHz CW and pulsed ENDOR spectroscopy were therefore used to determine the in situ structure of the alkene-modified heme of CPO.[27]

This effort involved a two-step process. First, covalent connections involving the AB fragment and the heme were established. $^{14/15}$N ENDOR examination of the coordinating pyrrole nitrogens of the AB-CPO ferriheme revealed that three nitrogens of the heme are similar, but the fourth nitrogen is markedly different, indicating that this unique nitrogen is alkylated by AB. It further mapped the **g** tensor coordinate frame, and from this the ultimate structure of the AB fragment, onto the heme molecular framework. ^{13}C ENDOR of AB-CPO prepared with ^{13}C labeled allylbenzene, combined with ^{1}H ENDOR of the protons at the C-2 position, showed that both the C-1 and C-2 carbons of AB are covalently connected to the heme, with C2 closer to Fe, thereby indicating that the heme had been alkylated by formation of a Cl−N bond.

The second step, the 3-D structure of the AB-CPO heme, involved the determination of the coordinates of the two C-1 protons of heme-linked AB; these nuclei exhibit no through-bond coupling and interact with the

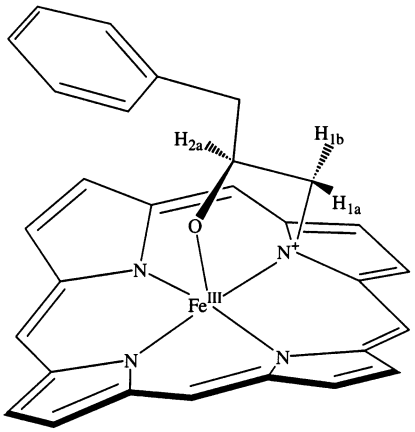


FIGURE 2. ENDOR-derived structure of the alkylated heme, AB-CPO.[27]

Fe^{III} spin only by the nonlocal electron−nuclear dipolar coupling. Analysis of their ^{1}H ENDOR patterns gave the coordinates of the two C-1 protons in the **g**-tensor frame; the results of the $^{14,15}N$ ENDOR measurements then placed these nuclei in the heme framework, and molecular mechanics calculations refined the structure.

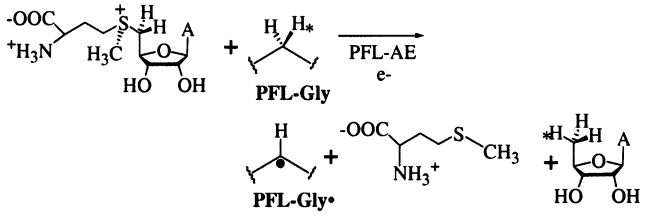
The combined data indicate that inactivation occurs by regioselective oxygen transfer to C2 and attack by C-1 on the pyrrole nitrogen, with the alkoxide oxygen ending up ligated to the heme iron to give the metallacycle shown in Figure 2. This finding formed the basis for a detailed discussion of the mechanism of CPO-catalyzed epoxidation.

Dual Role of the Catalytically Active [4Fe-4S]$^{+}$ Cluster of PFL-AE.[29,30] In recent years, it has been shown that enzymes of the "radical S-adenosylmethionine" superfamily utilize [4Fe−4S] clusters and S-adenosylmethionine (AdoMet) to generate catalytically essential radicals.[31,32] A key mechanistic question regarding this family is the role of the [4Fe−4S] cluster. In particular, the clusters of these enzymes have a "unique" iron site that is not coordinated to the enzyme by a cysteinyl sulfur: does this Fe have a catalytic function, as is true for aconitase?

In collaboration with Prof. Joan Broderick and coworkers, we used EPR and pulsed 35 GHz ENDOR spectroscopy to define the role of the cluster in the pyruvate formate-lyase (PFL) activating enzyme (PFL-AE), which generates a stable glycyl radical on the enzyme PFL, Scheme 3. The experiments disclosed that the cluster plays at least a dual role: the unique Fe anchors the AdoMet cofactor by chelating the amino and carboxyl groups of methionine; electron transfer from the cluster initiates homolytic cleavage of the bond to adenosine.

3-D Structure Information.[29] ^{2}H and ^{13}C pulsed ENDOR spectroscopy was performed on [4Fe−4S]$^{+}$-PFL-AE ($S = 1/2$) with bound AdoMet (denoted [1+/AdoMet]) that had been labeled at the methyl position with either ^{2}H or ^{13}C. The observation of substantial ^{2}H and ^{13}C hyperfine couplings from the labels clearly demonstrated that AdoMet binds adjacent to the 4Fe cluster. The cofactor was shown to bind in the same geometry to both the 1+ and 2+ states of the cluster through cryoreduction of the frozen [4Fe−4S]$^{2+}$/AdoMet complex to form the EPR-active reduced (1+) state which was trapped in the structure of the oxidized (2+) state.

Scheme 3



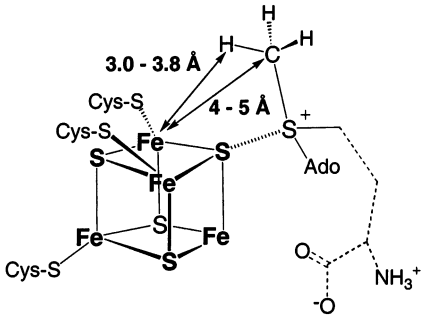


FIGURE 3. Interaction of AdoMet with the [4Fe−4S] cluster of PFL-AE, as deduced from ^{2}H and ^{13}C pulsed Q-band ENDOR spectroscopy.

Modeling of the through-space electron−nuclear dipolar interaction between the cluster electron spin and the methyl-^{13}C and ^{2}H of AdoMet, as derived from orientation-selective, 2-D ENDOR datasets, showed that the shortest distance between an AdoMet methyl proton and an iron of the cluster is ∼3.7(2) Å, with a distance of ∼4.9-(6) Å from the methyl carbon to this iron, Figure 3. Most intriguingly, the analysis disclosed a through-bond (local), isotropic contribution to the ^{13}C interaction, which requires overlap between orbitals on the cluster and on AdoMet. The most plausible origin is a dative interaction of the positively charged sulfur of AdoMet with a negatively charged sulfide of the cluster (Figure 3), rather than with the unique Fe of the cluster. These results led to a proposed reaction mechanism in which inner-sphere electron transfer from the cluster to AdoMet via the sulfide−sulfonium interaction causes cleavage of the sulfonium−adenosyl bond.

Coordination Sphere.[30] The above mechanism offers no role for the unique cluster iron, although it appears that the [4Fe−4S] cluster of every enzyme in the Fe−S/AdoMet family has one. To examine the coordination sphere of the unique Fe, we performed 35 GHz pulsed **ENDOR** spectroscopic studies of [1^{+}/AdoMet] labeled with ^{17}O/^{13}C in the carboxyl group of the methionine fragment, and with ^{15}N in the amino group. **ENDOR** signals observed with all three labels showed that both the carboxylato and amino groups of methionine are coordinated to the unique iron of the [4Fe-4S] cluster in a classical five-membered-ring N/O chelate. This interaction anchors the methionine end of AdoMet, thereby fixing the geometry of the sulfonium linkage for subsequent radical chemistry, as in Figure 4.

Dioxygen Activation by Heme Enzymes: Heme Oxygenase (HO); P450cam; Nitric Oxide Synthase (NOS).[33−35] Dioxygen activation is one of the major functions of

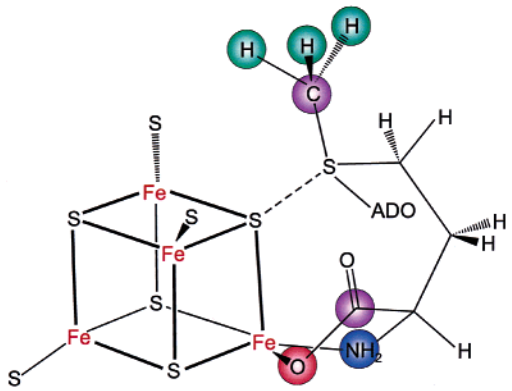


FIGURE 4. Representation of the proposed interactions of AdoMet with the [4Fe−4S] cluster of PFL-AE, as derived from ENDOR spectroscopy of the colored atoms: ^{2}H, ^{13}C, ^{14,15}N, and ^{17}O.[29,30]

biological metal centers. Heme-catalyzed hydroxylations in particular are perhaps the most widely studied bioinorganic reactions: during the 1990s, roughly one paper was published on cytochromes P450 alone, every 5 h of every day (Prof. Martin Newcomb, private communication, 2002). The chemically relevant portion of the enzymatic cycle for the monoxygenation of substrate, RH, begins with one-electron reduction of the oxyferroheme (O_2Fe(P)) enzyme and ends with the hydroxylated product, water, and the ferriheme state:

$$O_2Fe(P) + RH + e^- + 2H^+ \rightarrow ROH + H_2O + Fe^{3+}(P) \quad (1)$$

It had long been thought that heme-catalyzed hydroxylations involved the bracketed intermediates in Scheme 4.[36] The rate-limiting reduction of the dioxygen-bound ferroheme was thought to generate the peroxo-ferriheme, which then was presumed to accept one proton to generate the hydroperoxo-ferriheme, followed by delivery of the second proton and heterolytic O−O cleavage to produce compound I, the reactive, hydroxylating intermediate. However, none of the three intermediates had been characterized before our work, and all three are potentially capable of reacting with a substrate.[37]

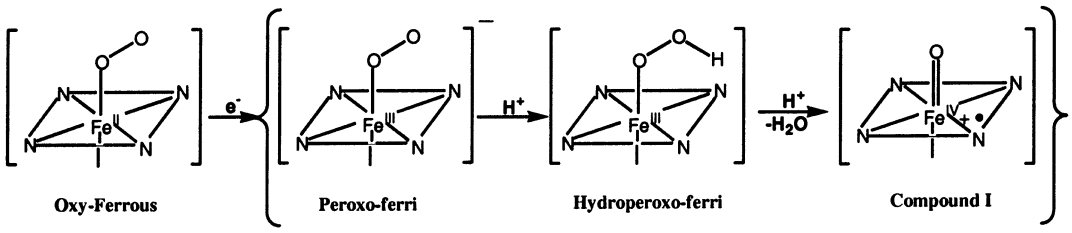
Dr. Roman Davydov in my laboratory therefore initiated a program to characterize the intermediates in dioxyen activation. We employ γ irradiation at low temperature to carry out the first step in Scheme 4,[37] the injection into an oxy-ferrous enzyme of the electron that initiates catalysis. This creates a reduced oxy-heme that is trapped when the temperature of cryoreduction is sufficiently low; stepwise annealing then advances the enzyme through the successive intermediate stages of the catalytic cycle, all the way to product. A combination of EPR and ENDOR measurements provides an ideal means of characterizing the catalytic intermediates thus created.

This approach has been applied to heme oxygenase in collaboration with Prof. Masao Ikeda-Saito and co-workers,[34] P450cam in collaboration with Prof. Steve Sligar and co-workers,[33]and nitric oxide synthase in collaboration with Profs. Bettie Sue Siler Masters, John Dawson, and co-workers.[35] It has allowed us to characterize the peroxo-ferriheme states of P450cam and NOS and the hydroperoxo-ferriheme states of HO and P450cam (Scheme 4). Our data clearly established that compound I indeed is the hydroxylating species in P450cam and tentatively indicate the same for the first step in NO formation, the hydroxylation of L-arginine to form N-hydroxyarginine. However, the same approach instead established that hydroperoxo-ferri-HO is the reactive species that self-hydroxylates the heme, as had been suggested.[38] The purposes of this Account are best served by focusing on the ENDOR measurements that established compound I as the hydroxylating species in P450cam,[33] even though neither we nor anyone else has characterized the Compound I of a P450.

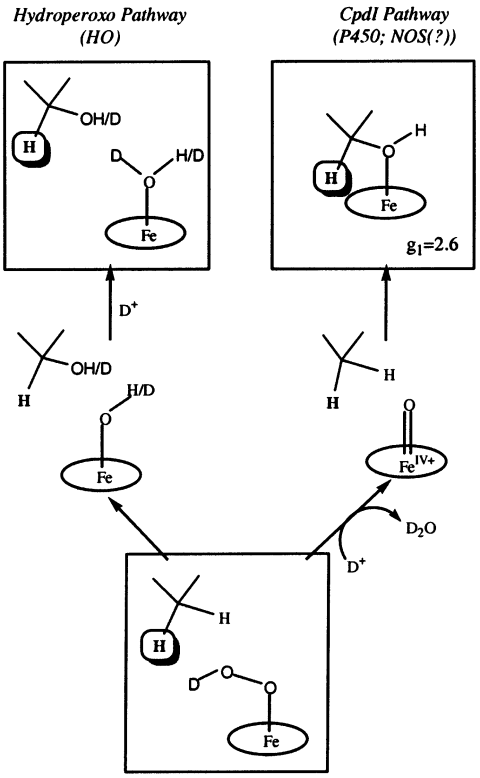
EPR and ENDOR results obtained during cryoreduction and annealing of oxy-P450cam included the following. (i) Cryoreduction generates the end-on, "peroxo" intermediate which converts at cryogenic temperatures to the hydroperoxo-ferriheme species (Scheme 4). (ii) Upon brief annealing at ~200 K, the hydroperoxo-ferriheme state disappears while the 5-exo-hydroxycamphor enzymatic product forms quantitatively and stereospecifically, with no direct spectroscopic evidence for the buildup of a compound I intermediate during this process. Scheme 5 presents the two alternate hydroxylations pathways that begin with the hydroperoxo-ferriheme: left, direct reaction by this intermediate; right, intervening formation of Compound I as the (undetected) reactive species.

The actual pathway was disclosed by ^{1,2}H ENDOR data from the primary product state. The observation of two signals with large hyperfine couplings, one from the nonexchangeable C5 proton remaining on the hydroxycamphor product, the other from the exchangeable hydroxyl, showed that the product state contains hydroxycamphor trapped in a nonequilibrium geometry, with its hydroxyl group bound to the heme ferric ion. Scheme 5 (right) shows that such a primary product arises when compound I is the reactive, hydroxylating species. The alternate mechanism, direct hydroxylation by the remote oxygen atom of a hydroperoxo-ferriheme, instead would leave a water/hydroxide bound to Fe, Scheme 5 (left),

Scheme 4



Scheme 5



contrary to observation. Thus, the cryoreduction/ENDOR measurements show that Compound I is the hydroxylating intermediate in P450cam, despite its being too reactive to accumulate for direct detection,

The Future? One can envisage continued growth in the applications of advanced paramagnetic resonance techniques to bioinorganic problems. New applications to metalloenzymes will continue to emerge, and one notes in particular that the study of metal ions associated with nucleic acids has begun.[39] New technical developments also will continue and will include advances in multifrequency and time-domain approaches.[3,5,40] Although it is our view that a microwave frequency in the vicinity of 35 GHz in general provides an optimum compromise between competing demands of spectral dispersion and sample handling, numbers of biological problems will demand the higher frequencies being developed, while certain spectroscopic constraints sometimes will demand lower. A future third Account might look quite different from this one!

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