Temperature and pH Dependence of the Proton Magnetic Hyperfine Resonances of Cytochrome c Peroxidase-Cyanide

EVIDENCE FOR HINDERED VINYL GROUP ROTATION AS A MEDIATOR OF THE ENZYME'S ACTIVITY*

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Proton nmr studies of the hyperfine resonances of cytochrome c peroxidase reveal that two pH-dependent processes can be monitored. One of these is the simple pH titration of a resonance which has been assigned to the a-vinyl proton at heme position 4. Combined with this proton's temperature dependence, the pH data indicate that the rotational position of vinyl 4 is changing with a pK which is similar to that which regulates the enzyme's activity. The second process, slow on the nmr time scale, occurs above pH 8. This is beyond what is normally considered to be the optimum pH range for cytochrome c peroxidase's activity and we interpret this to indicate a protein conformational change.

Cytochrome c peroxidase (EC 1.11.1.5; ferricytochrome c oxido-reductase) is a native ferrheme enzyme found in yeast. It is one of the class of heme peroxidases and it functions physiologically to catalyze the hydrogen peroxide oxidation of ferrocyanochrome c (1-3). The proposed mechanism by which cytochrome c peroxidase carries out catalysis involves ligand binding (4). As a consequence, we have undertaken this study of cytochrome c peroxidase-cyanide, a completely low spin six-coordinate form.

Low spin ferric heme proteins have been the object of study by nmr for many years, primarily because their characteristics rapidly spin lattice relaxation time yields relatively narrow resonances (5-9). However, due to a low symmetry (rhombic) perturbation, the spread of hyperfine protein resonances in these proteins is so large that fewer protons are actually resolved outside the diamagnetic region than in the corresponding high spin forms (10). Nevertheless, for practical reasons of signal to noise and resolution, the cyanide-ligated ferric heme proteins have drawn much attention.

Of the heme peroxidases, only horseradish peroxidase (EC 1.11.1.7) has been extensively studied, until recently (11-16). We initiated proton nmr studies of cytochrome c peroxidase which have resulted in characterization of the native protein and the oxidized intermediates (17-19). Detection of heme-centered asymmetry in the cyanide and native forms (20), and unambiguous assignment of many of the hyperfine resonances (21) is difficult in that we take advantage of these assignments to interpret the pH and temperature behavior of the hyperfine spectrum. Combined with crystallographic data (22, 23), we interpret these nmr results to indicate that specific vinyl group rotation is a potential steric and electronic mediator of cytochrome c peroxidase's reactivity.

EXPERIMENTAL PROCEDURES

Cytochrome c peroxidase was isolated and purified as previously described (17, 24, 25). The purity index (ratio of absorbance at 408 and 282 nm) of these enzyme preparations was 1.25 (25). Work has shown that this purity index corresponds to a single species purity of at least 95%. Cytochrome c peroxidase-cyanide was formed by adding a 1.1 mol excess of KCN (Aldrich) directly to the protein solution with careful monitoring of pH to maintain initial pH values between 7.0 and 7.8. Preparation of samples for nmr spectroscopy in "H2O (99.5%, Merck Isotopes) has also been previously described (17, 20).

Reconstitutions of apo-cytochrome c peroxidase were conducted as previously described (20).

Proton nmr spectra were obtained using Nicolet 360 (8.45 Tesla) and 470 (11.03 Tesla) spectrometers at the Purdue University Biochemical Magnetic Resonance Laboratory. Quadrature phase detection, continuous temperature control, and unmodulated decoupler suppression of residual H2O were all employed. Preliminary proton spin lattice relaxation time (T1) measurements on this protein form indicated that the longest hyperfine resonance T1 was on the order of ms.2 As a result, recycle times were varied between 200 and 500 ms and 60°-90° pulses were used, depending upon the exact experiment. All shifts reported in this paper were referenced, initially, to the internal residual water resonance, but are reported relative to external 2.2-dimethyl-2-silapentane-5-sulfonate. Observed shift positions were compensated for the temperature dependence of the residual water resonance. The temperature dependence of the water resonance was determined relative to external 2.2-dimethyl-2-silapentane-5-sulfonate and a standard calibration graph was created. Care was taken during nmr pH titrations to correct the observed resonances for the pH dependence of the H2O reference and to measure the sample pH before and after each data accumulation. An Orion meter equipped with a Beckman combination pH electrode was used for this task and it was calibrated prior to each measurement. The pH adjustments of the nmr samples were carried out with dilute deuterium chloride or deuterium hydroxide as before (17). The pH data such as that gathered for Fig. 5 were plotted in absolute intensity mode, which is a technique whereby subsequent spectra are Y-scaled according to parameters set for an initial spectrum. The pH values are reported as the meter readings in "H2O, as pH. pK values were determined from a nonlinear least squares analysis of these nmr results to indicate that specific vinyl group rotation is a potential steric and electronic mediator of cytochrome c peroxidase's reactivity.

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3 J. E. Erman and D. Dowe, unpublished results.
4 J. D. Satterlee and J. E. Erman, unpublished results.
the raw titration data using the Basic program LSABCP written by Dr. Darow Neves (Purdue University) and provided for our use by Dr. J. T. Jackson (University of California, Davis).

RESULTS AND DISCUSSION

The Spectrum—Fig. 1 shows a cytochrome c peroxidase-cyanide proton spectrum taken in D2O solution and it is labeled with the assigned heme protons. Because this is a ferriheme enzyme, even its low spin, cyanide-ligated state is paramagnetic (S = ½). Under these conditions, 15 resolved proton resonances are shifted away from the diamagnetic protein envelope between 0 and 10 ppm (resonances [1]-[8], [11]-[17]). In low spin heme proteins, these hyperfine or isotropic shifts are found to be caused by two mechanisms: dipolar and contact contributions (Equation 1). Experimentally, it is found that, while the contact contribution dominates low spin heme hyperfine shifts,

\[
\frac{\Delta H}{H} = \frac{\Delta H}{H}_{\text{dip}} + \frac{\Delta H}{H}_{\text{con}}
\]

the dipolar mechanism also contributes through the observed rhombic electronic state (26). This description applies to cytochrome c peroxidase-cyanide which demonstrated a rhombic g tensor (27), so that the dipolar equation must be written as (28):

\[
\frac{\Delta H}{H}_{\text{dip}} = \frac{1}{3N} \left\{ (x_L - 1/2(x_L + x_H)) (3 \cos^2 \theta - 1) \right\}^{-1} - 3/2(x_L - x_H) [\sin^2 \theta \cos 2\Delta \nu^{-1}]
\]

All components of this equation have been previously defined (31). The contact contribution is described by an equation involving the hyperfine coupling constant, \(A_h\), and whose simplest form is (26, 28):

\[
\frac{\Delta H}{H}_{\text{con}} = -A_h g B_{S(S+1)}
\]

Observed resonance positions, \(\frac{\Delta H}{H}_{\text{obs}}\), are related to hyperfine shifts \(\frac{\Delta H}{H}_{\text{hfs}}\) as follows:

\[
\frac{\Delta H}{H}_{\text{obs}} = \frac{\Delta H}{H}_{\text{hfs}} - \frac{\Delta H}{H}_{\text{dip}}
\]

where the last term is the corresponding diamagnetic shift. Changes in either the observed, or hyperfine resonances, for cytochrome c peroxidase-cyanide, as a function of temperature should be approximately linear insofar as the magnetic susceptibility follows Curie behavior. Slight deviations, in the form of line curvature in Curie graphs, may occur due to

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FIG. 1. The proton spectrum of cytochrome c peroxidase-cyanide is shown in the lower part of this figure, together with the assignments made by deuterium labeling (see Footnotes 1 and 2; Ref. 20). The upper portion of this figure shows the heme moiety with the labeling and numbering convention used in the text. Cyanide and the proximal histidine are additional ligands in cytochrome c peroxidase-cyanide. Peak numbers [1]-[17] do not relate to the labeling in the upper figure and are for identification only.

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FIG. 2. Curie graph of cytochrome c peroxidase-cyanide hyperfine resonances. A, downfield shifts; B, upfield shifts. Intercepts from extrapolation to \(T^{-1} = 0\) are indicated in parentheses. Non-Curie behavior is only realized for resonances 5 and 16, which are indicated by asterisks. Note the different shift scales in A and B.
second order Zeeman contributions to the dipolar shift (29). However, as shown in Fig. 2, these deviations are minimal. Only peaks 9 and 10 (Fig. 1), which lie in the diamagnetic region, do not show temperature dependence. Of the resolved resonances, these alone are not influenced by the heme paramagnetism and are probably assignable to aromatic protein protons. The data in Fig. 2 (Curie graph) for the most part exhibit Curie behavior. That is, they mostly show straight lines with intercepts (at $T^{-1} = 0$) at their corresponding diamagnetic positions as predicted by Equations 1-4. Two exceptions, with intercepts outside 0-10 ppm, are resonances [5] and [16].

Resonance [5] has been previously assigned to the hemin 4-position $\alpha$-vinyl proton (Fig. 1). Like all of the assignments in Fig. 1, it was made by reconstituting cytochrome $c$ peroxidase with synthetically deuterated protohemin IX and may be considered to be unambiguous. Its non-Curie behavior, and the fact that its $T^{-1}$ intercept is the same sign as the shift, is characteristic of vinyl group rotational mobility (26, 30, 32). Briefly, as previously demonstrated, because the observed shifts have significant $\pi$-contact contributions, the physical rotational position of the vinyl group mediates its interaction with the heme $\pi$ orbital system. This, in turn, modifies the conjugative and hyperconjugative interactions which govern the hyperfine coupling constant ($A_\delta$, Equation 3) of the vinyl protons (30).

The variable temperature dependence of the cytochrome $c$ peroxidase-cyanide vinyl $\alpha$-H may be understood in the context of Fig. 3 and the crystal structure of cytochrome $c$ peroxidase (22, 23). In Fig. 3, the two extreme rotational positions which the vinyl group may adopt relative to the heme plane and molecular $\pi$ system are shown. Models show that the vinyl group oriented parallel to the heme plane (Fig. 3B) results in severe steric interactions so that the preferred vinyl orientation is more perpendicular (Fig. 3A). The latter minimizes steric repulsions and is the orientation which appears in the crystal structure (22, 23). At low temperature, this is likely the major orientation for the population of vinyl groups. Whereas at higher temperatures, population of more in-plane conformations will increase.

For this analysis, it must be recalled that compared to methyl or methylene hyperfine couplings, the factors that affect $A_\delta$ for vinyl groups are less well documented (32, 33) but must include contributions from both hyperconjugative and conjugative effects. It is likely that the hyperconjugative interaction for the vinyl $\alpha$-H is defined by an equation which depends upon the dihedral angle between the $\pi$ hemin orbital and the $\alpha$-H ($\theta$, Fig. 3) as in the McConnell type relation for methylene groups (30, 32-34).

$$A_{\delta \chi} (\theta) = |B_\delta + B_\alpha \cos \theta|^2 \delta_{\alpha \chi}$$

By making the reasonable assumption of such an angular dependence for $A_{\delta \chi}^{hyper}$, the two limiting orientations indicate that the hyperconjugative mechanism has a minimum value for $3\theta$ (cos $\theta = 90^0$), but a maximum contribution for $3\theta$ (cos $\theta = 0$). Therefore, a purely hyperconjugative contribution to $A_\delta$ predicts the largest contact shift for orientation $3\theta$, increasingly smaller shifts for more co-planar rotamers, with $3\theta$ exhibiting no hyperfine coupling, hence, no shift.

For an appended $\pi$ system, like vinyl groups, the conjugation mechanism which demonstrates rotational position dependence also influences $A_\delta$ (35, 36). Thus, $A_\delta$ and the contact shift are maximum when the nodal planes of the hemin and vinyl $\pi$ systems are parallel (3D), with minimum shifts when they are perpendicular (3C).

With this, the non-Curie behavior of the 4-vinyl, $\alpha$ proton may be understood. At low temperature, the perpendicular orientation is preferred, resulting in a downfield shift originating from the hyperconjugative mechanism. With increasing temperature, rotamers tending toward more parallel orientations are established. Although one expects to observe upfield movement in the $\alpha$-H resonance due to effects from both the decreasing contribution from the hyperconjugative mechanism and the general temperature behavior predicted by Equations 1-4, the observed shift decrease is less than Curie behavior predicts. This is rationalized by the increasing contribution to the observed shift, at higher temperatures, of the conjugation between hemin and vinyl $\pi$ systems. That effect superimposes upon the general upfield shift trend a strong downfield bias. Because the conjugation mechanism produces downfield shifts of greater magnitude (by 1-2 ppm) than hyperconjugation, it more than compensates for the declining hyperconjugative shift contribution and offsets a degree of the normal Curie temperature dependence (30).

Although non-Curie upfield shifts in vinyl $\alpha$-H resonances are generally seen for hemeproteins as the temperature is increased (Fig. 2, Ref. 31, Figs. 4 and 5), actual downfield shifts are observed for hemin model systems (31). This indicates that greater rotational mobility is allowed for hemin vinyl groups outside the protein environment.

We have neglected two things in this analysis. First, the possible contribution of the rotationally modified dipolar shift to the observed non-Curie behavior is minimal for two reasons. Calculation of the rhombic terms (Equation 2) for each of the parallel and perpendicular vinyl orientations shows that they differ by less than 10%, whereas the non-Curie behavior is different from the Curie by substantially greater amounts as

![Fig. 3. Possible extreme vinyl group orientations relative to the heme plane. Qualitative evaluation of the contribution to the contact shift (through $A_\delta$) for the hyperconjugation (A and B) and conjugation (C and D) mechanisms are given. Refer to the text for a complete description.](image)
NMR of Cytochrome c Peroxidase-Cyanide: Temperature and pH

The text) as a function of temperature for the assigned 4-vinyl form) even though it is less than observed above pH 8.0. We line. Below pH 5.5, significant broadening also occurs (low pH state. Reason that may be demonstrated in the next section, the pH-induced changes are by far the largest for the 4-position α-H, even compared to its neighbor on pyrrole II, the 3-position CH₃, indicating that this effect is highly localized. When pH titration data for deuterohemin-reconstituted cytochrome c peroxidase-cyanide are considered, only one of the two pyrrole protons titrates, indicating that this is a localized change. We reason that ΔM is, in fact, dependent upon the vinyl rotational position and is not a consequence of altered iron electronic state.

Second, we have not mentioned the β-vinyl 4-position protons in this analysis. Only one of these (peak [15]) has been assigned and whether it is cis or trans to the α-H has yet to be determined. However, its observed behavior is consistent with that seen in models and monomeric hemoglobins (30, 31) in which little deviation from Curie behavior can be detected.

LaMar et al. (30) have shown that an index of average rotational position is the factor R, defined as the ratio of β proton to a proton hyperfine shift, plotted as a function of temperature (Fig. 4). The data for cytochrome c peroxidase-cyanide shows a small slope approaching 1.0, as do the data for hemin models and the monomeric insect hemoglobins. A more complete comparison with other proteins cannot be made until the relative contributions from dipolar and contact mechanisms to the hyperfine shift are delineated for these proteins.

**pH Dependence**—The pH dependence of observed hyperfine resonances is shown in Figs. 5 and 6. There are three aspects, line broadening, formation of a new protein form above pH 8, and normal titration behavior between pH 4 and 8. In Fig. 5, data plotted in the absolute intensity mode (see "Experimental Procedures") reveal that at both extremes of pH the entire hyperfine spectrum broadens significantly. Above pH 8.0, a new set of resonances develops (high pH form) after the initial spectrum has broadened into the baseline. Below pH 5.5, significant broadening also occurs (low pH form) even though it is less than observed above pH 8.0. We believe that this broadening indicates the limits of the native protein conformation and possibly indicates the transition to a different enzyme form below pH 5.5. Similar pH titrations for aquo-cytochrome c peroxidase (17) also indicate a second conformation exists below pH 5.5 which is in slow exchange with the native conformation on the nmr time scale (17).

The transition between pH 7.5 and 10.45 is a transition between intermediate pH and high pH forms. Consider the hemin methyl resonances. Above pH 8.0, they broaden into a transition state (pH 8.80) and, finally, a new form is established above pH 9.50 with what likely are hemin methyls at 26.4 ppm (peaks [1'] and [2']). Similar processes occur for all of the downfield hyperfine resonances and comparison integrations of the total downfield intensities at pH 10.45 and pH 6.23 indicate that only about 80% of the original intensity remains at pH 10.45. The rest probably is lost through denaturation.

Fig. 6 demonstrates that within the normally accepted limits of cytochrome c peroxidase's stability (pH 4–8) several resonances exhibit pH dependence characterized by smooth titration curves. Both methyl resonances ([1] [2]) show slight pH sensitivity, while the 4-vinyl α-H is dramatically pH-dependent. Resonance [4], probably a propionic acid α-H, displays a lesser effect. These data are summarized in Tables I and II.

Because the variable temperature data have suggested vinyl group oscillatory mobility as a source of hyperfine shift changes, we focus our attention on the vinyl α-H titration. The experimental titration curve is well fit to the Henderson-Hasselbach equation and corresponds to a single ionization with a pK of 5.2 ± 0.2 (Table II). This pK is virtually identical with the one which kinetics studies (37, 38) indicate governs the reactivity of cytochrome c peroxidase. This correspondence allows the conclusion that this observed pK reflects the pH-linked transition between active and inactive enzyme forms. Resonance [4] exhibits a nearly identical pK as does
The pH dependence of cytochrome c peroxidase-cyanide hyperfine resonances. A, downfield resonances; B, upfield resonances. Note the different scales for A and B. pH' indicates the meter reading in deuterium oxide solution.

### Table I

**Data for hyperfine resonances in cytochrome c peroxidase-cyanide**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Assignment</th>
<th>Low pH (4.17)</th>
<th>Intermediate pH (6.84)</th>
<th>High pH (10.45)</th>
<th>(\Delta) (L, I)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Hyperfine</td>
<td>Observed</td>
<td>Hyperfine</td>
<td>Observed</td>
</tr>
<tr>
<td>1</td>
<td>3-CH₃(H)</td>
<td>30.1</td>
<td>26.5</td>
<td>30.7</td>
<td>27.1</td>
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<tr>
<td>2</td>
<td>8-CH₃(H)</td>
<td>28.2</td>
<td>24.6</td>
<td>27.7</td>
<td>24.1</td>
</tr>
<tr>
<td>3</td>
<td>6,7a-H(?)</td>
<td>19.5</td>
<td></td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>4</td>
<td>6,7a-H(?)</td>
<td>19.5</td>
<td></td>
<td>18.3</td>
<td>18.3</td>
</tr>
<tr>
<td>5</td>
<td>4β-H</td>
<td>18.4</td>
<td>9.8</td>
<td>16.0</td>
<td>7.4</td>
</tr>
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<td>6</td>
<td>14.6</td>
<td>14.7</td>
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<tr>
<td>14</td>
<td>4β-H</td>
<td>-2.71</td>
<td>[-9.01]</td>
<td>-2.83</td>
<td>[-8.45]</td>
</tr>
<tr>
<td>15</td>
<td>4β-H</td>
<td>-4.41</td>
<td>-10.7</td>
<td>-3.90</td>
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<tr>
<td>16</td>
<td>-3.78</td>
<td>-4.52</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

- Assignments for heme protons from Footnotes 1 and 2.
- Shifts in ppm at 24 °C, pH 7.2, 0.1 M potassium phosphate. Shifts are reported as either observed or hyperfine.
- For definition, see text or Ref. 26.
- Average rotational factor for vinyl groups (4β shift/4α shift) as defined in the text.
- Magnitude of the shift change between intermediate and low pH forms.

Given the evidence presented here from pH and variable the vinyl \(\beta\)-H, although this latter proton's behavior is complicated by its overlap with neighbor resonances, resulting in larger error in the pK value (Table II). The data in Fig. 6 and Table I demonstrate that the vinyl group is the focus of cytochrome c peroxidase's pH behavior. A similar effect has been found for insect hemoglobin (30) where it was suggested that a pH-dependent vinyl rotational position operates. For cytochrome c peroxidase-cyanide, such a mechanism is consistent with the shift titrations of the vinyl \(\alpha\) and \(\beta\) protons.
TABLE II

<table>
<thead>
<tr>
<th>Resonance number</th>
<th>Assignment</th>
<th>Calculated pK*</th>
<th>Largest error (ppm)</th>
<th>n'</th>
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<tr>
<td>[4]</td>
<td>4-H</td>
<td>5.5 ± 0.2</td>
<td>0.08</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>[5]</td>
<td>4- H</td>
<td>5.2 ± 0.2</td>
<td>0.07</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>[15]</td>
<td>4- H</td>
<td>5.3 ± 0.3</td>
<td>0.10</td>
<td>1.12 ± 0.1</td>
</tr>
</tbody>
</table>

*pK determined from a nonlinear least squares fit to the observed hyperfine shift data.

This error is the largest deviation from the observed hyperfine shift for an individual data point in the theoretically calculated fit to the Henderson-Hasselbach equation.

Hill parameter required to fit the experimental data.

Conclusions

Evidence is presented here which strongly suggests that the 4-position vinyl group of the cytochrome c peroxidase hemin ring displays rotational mobility corresponding to the pK which regulates cytochrome c peroxidase's reactivity. This conclusion is reached from a combination of pH and variable temperature data and offers tempting speculation about how such rotational mobility could contribute to regulating peroxidase function.

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REFERENCES