Transient Raman Study of Horseradish Peroxidase

EVIDENCE FOR A LACK OF EXTENSIVE HEME POCKET RELAXATION SUBSEQUENT TO CARBON MONOXIDE PHOTOLYSIS*

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Time-resolved resonance Raman spectroscopy is a valuable tool for the study of the dynamics of heme-protein interactions. In particular, the photolysis of a ligand by short laser pulses allows for the examination of the dynamic evolution of heme-protein interactions subsequent to ligand dissociation. To date, such studies have been confined largely to hemoglobins and myoglobins. Here we present the results of the first transient Raman study of a peroxidase. Resonance Raman spectra of horseradish peroxidase were obtained within 10 ns of ligand (CO) photolysis at a variety of pH values. We find that there is only minimal relaxation of the heme pocket of horseradish peroxidase in response to ligand photolysis. This relaxation is pH-dependent and most probably involves the heme vinyl substituents. Such behavior is in sharp contrast to the transient behavior of most hemoglobins and beef heart cytochrome oxidase.

Recent advances in pulsed laser and transient detector technology have opened the way for the application of nanosecond and picosecond time-resolved Raman spectroscopy to complex biological systems. In particular, heme-containing proteins are ideal candidates for such studies since many possess active sites that readily bind photolabile ligands and have been extensively characterized in their steady-state configurations by vibrational and magnetic techniques. The photolysis of a photolabile ligand by a short laser pulse initiates a series of events that can include a diabatic heme pocket relaxation and geminate ligand rebinding and electron transfer depending upon the specific protein system. The inherent structural specificity of resonance Raman scattering allows the dynamic evolution of heme-protein interactions to be probed on a molecular level at time scales ranging from picoseconds to milliseconds. To date, transient Raman investigations of this type have been confined to hemoglobin and myoglobin (1-9) and, quite recently, mammalian cytochrome oxidase (10, 11). Here we present the preliminary results of our effort to apply these techniques to new classes of heme proteins. The resonance Raman spectra of ferrous horseradish peroxidase within 10 ns subsequent to carbon monoxide photolysis have been obtained at a variety of pH values. We find that there are only minimal responses of the local heme environment to ligand photolysis indicating that either the relaxation of the local protein environment about the heme proceeds in less than 10 ns or there is no extensive reordering of heme-protein interactions engendered by ligand binding. This is in marked contrast to the behavior of most hemoglobins (1-8) and cytochrome oxidase (10) and is similar to the lack of response exhibited by myoglobin (9).

MATERIALS AND METHODS

Horseradish peroxidase (isoenzyme c) was purchased from Sigma. Subsequent purification of the enzyme via gel filtration produced no effects upon the spectra obtained and, thus, most spectra were gathered from samples that were not further purified. The exact solution conditions for each sample are specified in the figure captions. Ferrous anaerobic samples were made by equilibrating solutions under an anaerobic nitrogen atmosphere and adding a very slight excess of sodium dithionite (Baker-purified grade). Carbonmonoxymy samples were obtained by flushing the ferrous samples with CO gas that had been passed through a bubbler containing 1 M KOH. Raman spectra were obtained from samples in a homemade anaerobic cuvette with instrumentation described elsewhere.† A single pulse train of 440-nm radiation (10-ns pulse width) from a Molectron DL 14/UV 24 nitrogen pumped dye laser was used for both photolysis of CO and generation of the transient resonance Raman spectra. A backscattering geometry was employed. Both the optical absorbance and pH of the samples were checked before and after the Raman experiments.

RESULTS AND DISCUSSION

The application of transient Raman techniques to a heme protein system first requires the demonstration that the higher incident powers and repetition rate of the pulsed laser system produce no artifacts in the steady-state protein spectra. Additionally, the protein must rebind the photolyzed ligand and relax to its steady-state liganded configuration before the next photolytic pulse interrogates the sample. Nonlinear effects or long-lived (2×10³ ns) electronic states may also be evident in the spectra generated with pulsed lasers. Figs. 1 and 2 demonstrate that these potential complications are absent in our investigation of horseradish peroxidase. The spectra of steady-state ferrous horseradish peroxidase that we obtain with our pulsed laser system are in complete agreement with those obtained by Kitagawa (12, 13) and others (14, 15) via cw laser illumination. In particular, we have reproduced exactly the pH-dependent shift known to occur in the ironhistidine stretching vibration assigned via isotopic substitution to the ~245 cm⁻¹ mode in ferrous horseradish peroxidase (12). Transient absorption studies (18) have determined CO-horseradish peroxidase binding rate constants of ~5×10⁹ and ~1×10⁴ M⁻¹ s⁻¹ for the acidic and alkaline forms of horseradish peroxidase, respectively. Thus, at the CO concent.

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Fig. 1. Low frequency resonance Raman spectra of equilibrium ferrous horseradish peroxidase (isoenzyme c) and the transient species within 10 ns of carbon monoxide photolysis from ferrous horseradish peroxidase. Spectra were obtained with 10-ns pulses at 440 nm and a 12-14-Hz repetition rate. An 8-10 cm$^{-1}$ spectral bandpass and backscattering geometry were employed. Samples were 50-100 μM in horseradish peroxidase and were buffered with 0.2 M MES$^2$ (for pH 5.5 samples) or 0.2 M Tris (for pH 8.0 samples). These traces are the unsmoothed sums of 5-7 scans and represent: a, horseradish peroxidase photolytic transient at pH 5.5; b, horseradish peroxidase equilibrium species at pH 5.5; c, horseradish peroxidase photolytic transient at pH 8.0; and d, horseradish peroxidase equilibrium species at pH 8.0.

Fig. 2. High frequency resonance Raman spectra of ferrous horseradish peroxidase equilibrium and transient species. Conditions were the same as for Fig. 1 except only 3 scans were summed for each spectrum: a, horseradish peroxidase photolytic transient at pH 5.5; b, horseradish peroxidase equilibrium species at pH 5.5; c, horseradish peroxidase photolytic transient at pH 8.0, and d, horseradish peroxidase equilibrium species at pH 8.0.

$^2$ The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
TABLE I

Comparison of the transient heme species generated within 10 ns of carbon monoxide photolysis from various proteins.

<table>
<thead>
<tr>
<th></th>
<th>Hemo-globin*</th>
<th>Myo-globin*</th>
<th>Mammalian cytochrome oxidase*</th>
<th>Horseradish peroxidase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-His mode</td>
<td>+8 to +15†</td>
<td>0</td>
<td>+6 to +10†</td>
<td>0</td>
</tr>
<tr>
<td>$v_1$ (π* electron density)</td>
<td>-2 to -4†</td>
<td>0</td>
<td>0 to +1</td>
<td>0</td>
</tr>
<tr>
<td>Geminate recombination in ≤10 ns</td>
<td>Yes†</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Low frequency shifts (other than Fe-His)</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>High frequency shifts</td>
<td>Yes</td>
<td>No</td>
<td>Yes†</td>
<td></td>
</tr>
<tr>
<td>Vinyl group mode</td>
<td>+1 to +3</td>
<td>?</td>
<td>?</td>
<td>+2.0†</td>
</tr>
</tbody>
</table>

* From Refs. 1–7 and Footnote 2.  † From Refs. 5 and 9.  ‡ From Ref. 10.  § This work.  ‡§ Dependent upon pH.  ‡§§ Dependent upon protein quaternary structure.

spectra) and a 0.5 to 0.01 mJ/pulse range in laser energy (for the steady-state spectra). This makes us confident that CO recombination occurs completely between laser pulses and that we have no power-dependent artifacts in our spectra.

Spectra of horseradish peroxidase within 10 ns of CO photolysis were obtained in both the low (200–375 cm⁻¹) and high (1300–1700 cm⁻¹) frequency regions and are displayed in Figs. 1 and 2. Since steady-state species of horseradish peroxidase display some pH-dependent spectral changes, transient spectra from samples at pH values that spanned the apparent pKₐ of the equilibrium species (~7.0) were gathered. It is evident from these data that only minor (though quite reproducible) differences exist between the equilibrium and photolytic transient species of horseradish peroxidase. At either pH extreme, there is no change in the position of the Fe-histidine mode frequency (to ±1 cm⁻¹) after CO photolysis. This is in marked contrast to the 8–15 cm⁻¹ shift to higher frequency in this mode upon ligand photolysis from hemoglobins (1–8) and mammalian cytochrome oxidase (10). At both pH values there appears to be a general response of the heme upon photolysis leading to changes in the relative intensity of the Fe-histidine mode and a shifting and decreased intensity of the ~314 cm⁻¹ mode which has been assigned as a bending motion of the heme vinyl substituent (21). Modes in the 300–400 cm⁻¹ region also respond on a nanosecond time scale to ligand photolysis in hemoglobins (1–8) and cytochrome oxidase (10).

Examination of the high frequency spectra further demonstrates that despite the fact that hemoglobin and horseradish peroxidase contain the same heme active site, their dynamic responses to ligand binding are radically different. Although $v_4$ (porphyrin mode designation follows Ref. 19) displays systematic shifts to lower frequency (indicating increased porphyrin π* electron density) in hemoglobin photolytic transients relative to the equilibrium species, no such shifts in this mode are evident in horseradish peroxidase transients. On the other hand, systematic shifts at the limit of detectability (with our spectral resolution) apparently occur in several high frequency modes. A shift in $v_2$ (~1470 cm⁻¹) to lower frequency is observed in the high pH horseradish peroxidase transient species that is not observed in hemoglobins. A general shift to lower frequency is observed in the $v_2$ region of the spectrum (1550–1590 cm⁻¹) in the photolyzed transients. Several different modes contribute to scattering in this region (16) and thus we cannot unambiguously assign the position of $v_2$. Since $v_2$ and $v_3$ have been shown to be sensitive to porphyrin core size, a slightly expanded heme core size may be indicated for the transient species at this pH.

Finally, a small but reproducible shift to higher frequency (2 cm⁻¹) in the mode assigned as the peripheral vinyl group frequency (17) is observed in the high pH transient spectra. Transient differences in the local vinyl environment are also corroborated by the fact that the only low frequency mode to exhibit significant transient differences presumably also involves vinyl substituent motion. NMR studies of ferric cytochrome c peroxidase indicate that one of the vinyl groups at the heme displays a pH-dependent change in its local mobility which may be mechanistically significant (20). A similar situation in horseradish peroxidase may be indicated by our data. Preliminary studies indicate a small shift to higher frequency of the vinyl group frequency in transient hemoglobin.

Collectively, the small shifts in the modes above 1400 cm⁻¹ suggest that the heme pocket of horseradish peroxidase appears to be more responsive to ligand binding at high pH, than at low pH. The pH dependence of equilibrium ferrous horseradish peroxidase has been ascribed to protonation of the distal histidine residue (12). Our data suggest that the dynamic properties of the heme pocket are also modulated to a small degree by ionization of this residue.

The lack of a discernible shoulder at a frequency corresponding to the liganded value of $v_4$ (~1373 cm⁻¹) at either pH suggests that little or no geminate rebinding of CO occurs on a 10-ns time scale irrespective of the degree of protonation of the distal histidine. This is, again, different from the behavior of hemoglobin which shows definite CO recombination within 10 ns under similar conditions. It is, however, not unexpected in view of the measured on rates for CO binding to ferrous horseradish peroxidase (18).

In conclusion, horseradish peroxidase and hemoglobins, despite the generic similarities of their heme active sites, display completely different behavior in the molecular dynamics of carbon monoxide binding. This is not terribly surprising in view of their widely divergent physiological functions. The differences in transient behavior among hemoglobin, myoglobin, mammalian cytochrome oxidase, and horseradish peroxidase are summarized in Table I. These data demonstrate the diversity of dynamic responses of heme active sites to ligand binding among these proteins and indicate the utility of transient Raman spectroscopy for the investigation of the differences in molecular dynamics at protein active sites. This is particularly evident in the comparison of hemoglobin and myoglobin which display the widest difference in their transient behavior despite the close similarities of their equilibrium spectra. Indeed, the differences between the dynamics of heme pocket relaxation can be used to rationalize the differences in ligand binding kinetics between R-state hemoglobins and myoglobin (9). Obviously, much more work must be done to further quantify the behavior of the less well-studied proteins (oxidase and horseradish peroxidase) and to extend these studies to other ligand binding heme proteins (P-450, for example). These studies are currently underway in our laboratory.

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REFERENCES