# Resonance Raman Investigations of Chloroperoxidase, Horseradish Peroxidase, and Cytochrome *c* Using Soret Band Laser Excitation†

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ABSTRACT: Resonance Raman spectra of the heme protein chloroperoxidase in its native and reduced forms and complexed with various small ions are obtained by using laser excitation in the Soret region (350–450 nm). Additionally, Raman spectra of horseradish peroxidase, cytochrome P-450$_{cam}$, and cytochrome *c*, taken with Soret excitation, are presented and discussed. The data support previous findings that indicate a strong analogy between the active site environments of chloroperoxidase and cytochrome P-450$_{cam}$. The Raman spectra of native chloroperoxidase are found to be sensitive to temperature and imply that a high → low spin transition of the heme iron atom takes place as the temperature is lowered. Unusual peak positions are also found for native and reduced chloroperoxidase and indicate a weakening of porphyrin ring bond strengths due to the presence of a strongly electron-donating axial ligand. Enormous selective enhancements of vibrational modes at 1360 and 674 cm$^{-1}$ are also observed in some low-spin ferrous forms of the enzyme. These vibrational frequencies are assigned to primary normal modes of expansion of the prophyrin macrocycle upon electronic excitation.

Chloroperoxidase, a heme glycoprotein isolated from *Caldariomyces fumago*, is a monomer of molecular weight 42 000 containing a single ferriprotoporphyrin IX (Morris & Hager, 1966). Chloroperoxidase catalyzes the peroxidative oxidation of hydrogen donors, such as pyrogallol, guaiacol, and ascorbate, and decomposes hydrogen peroxide in a catalase-type reaction (Thomas et al., 1970). In addition, in the presence of suitable halogen donors ($Cl^-$, $Br^-$, or $I^-$, but not $F^-$) at low pH, chloroperoxidase catalyzes the peroxidative formation of a carbon–halogen bond. Recent physical studies which use optical (Hollenberg & Hager, 1973), EPR (Champion et al., 1973), Mössbauer (Champion et al., 1975), NMR (Krejcarek et al., 1976), MCD (Dawson et al., 1976), and Raman (Champion et al., 1976) spectroscopies have probed many aspects of the active site of chloroperoxidase. The results from a number of studies show strong similarities between chloroperoxidase and P-450 type cytochromes (Champion et al., 1973, 1975, 1978; Gunsalus et al., 1974; Dawson et al., 1976; Hanson et al., 1976), suggesting that the heme environments of these two apparently unrelated proteins are very similar. The present study of the resonance Raman spectra of chloroperoxidase should add substantially to the body of physical and biochemical knowledge of this unique enzyme.

In an earlier paper (Champion et al., 1976), we reported preliminary results for the resonance Raman spectra of native (oxidized) and reduced chloroperoxidase. A principal finding was that several of the characteristic Raman frequencies were anomalous for high-spin heme proteins. In the present paper, we report the results of a more complete study of the Raman spectra of chloroperoxidase. Laser wavelengths in the near-ultraviolet (at 363.8, 406.7, 413.1, and 415.4 nm) in addition to standard visible laser sources are used for selective resonance enhancements. Moreover, background fluorescence observed in the previous work is largely eliminated by better sample preparation. Spectra are measured for both native and reduced chloroperoxidase, for native complexes with cyanide, fluoride, and chloride, and for the reduced complexes with cyanide and carbon monoxide. The effects of changing pH, cooling to 78 K, and denaturation are also investigated.

## Materials and Methods

Samples of native chloroperoxidase were isolated from the mold *Caldariomyces fumago* and purified ($Rz$ = 1.37) as reported elsewhere (Morris & Hager, 1966). A typical Raman sample was buffered in a 50 mM pH 3.6 or 6.3 sodium phosphate buffer to give a concentration of 50–500 μM in heme. The complexes of chloroperoxidase were prepared by careful addition of ethylene glycol or buffered solutions of potassium cyanide, potassium halide, or sodium hydroxide to the buffered enzyme. The reduced enzyme was prepared by adding buffered or solid sodium dithionite (Hardman and Holdman, Manchester, England) to deoxygenated native enzyme under an argon atmosphere (Matheson purity, Matheson, East Rutherford, NJ). Reduced derivatives were prepared by adding deoxygenated buffered solutions of potassium cyanide and sodium hydroxide to the reduced enzyme, also under an argon atmosphere. All chemicals used were reagent grade or better.

The samples were held at 4 °C in a 2 × 10 mm dual path length quartz optical cell with an extra bottom window for the laser beam. Optical absorption spectra were taken with a Cary 14 spectrophotometer before and after each Raman run to verify that the sample was in the proper state and to confirm that no changes had occurred during the run. Figure 1 shows the absorption spectra of several of the samples measured.

Raman spectra were taken with the laser lines indicated at the top of Figure 1. These were chosen to lie near the Soret and visible absorption bands of the heme, to enhance selectively the porphyrin vibrations which couple to these localized electronic transitions. The laser lines used were 363.8 nm (from a Coherent CR-12 argon ion laser), 406.7 and 413.1 nm (from a Spectra Physics 171 krypton ion laser), and 457.9 and 514.5 nm (from a Coherent CR-3 argon ion laser).

The incident laser light was passed through an interference filter or a Claassen filter (Collins et al., 1977) to remove

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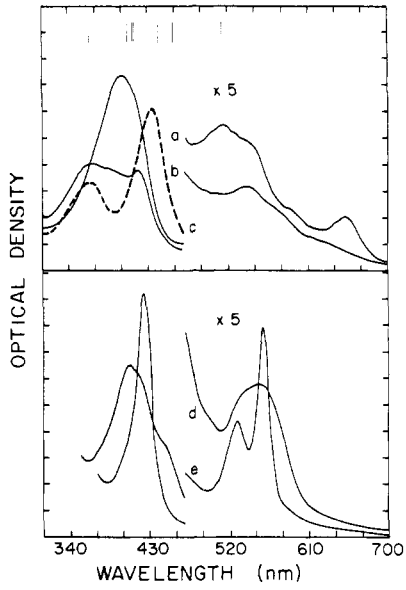


FIGURE 1: Absorption spectra of oxidized and reduced chloroperoxidase, their sodium hydroxide treated forms, and the oxidized cyanide enzyme. (a) Native chloroperoxidase (41 $\mu$M); (b) sodium hydroxide treated enzyme (39 $\mu$M); (c) chloroperoxidase cyanide (37 $\mu$M); (d) reduced chloroperoxidase (45 $\mu$M); (e) reduced sodium hydroxide treated enzyme (37 $\mu$M).

incoherent emission from the laser and then was passed through a polarizing prism and focused inside the sample cell. Laser powers were always less than 25 mW at the 50-$\mu$m diameter focus to avoid sample heating or damage.

Both right-angle and back-scattering geometries were used. In the back-scattering geometry, low frequency modes (<500 $cm^{-1}$) were largely obscured by Raman scattering from the quartz windows. This was avoided in the 90° scattering, but the longer optical path needed in the sample required lower heme concentrations (<$10^{-4}$ M).

The scattered light was collected and analyzed with a Spex 1401 double monochromator using a photon-counting detector with digital data storage. The polarization of the scattered light was analyzed either with a stationary analyzer or with a multiplex scheme in which the scattered intensity polarized parallel and perpendicular to the incident polarization was measured in alternate channels. A quartz "scrambler" was used to ensure uniform sensitivity of the system to both polarizations.

The spectra were averaged and plotted with a small computer. No corrections were made for the instrumental spectral response nor for selective absorption of the scattered light. Neither correction was severe for the conditions used. For instance, even at the peak of the Soret band in 90° scattering, the optical density of the ~100-$\mu$m depth of sample between the laser beam and the exit face of the cell was less than 0.03 for the concentrations used.

## Results

*Native Chloroperoxidase.* Raman spectra of native chloroperoxidase have been examined at several different laser excitation wavelengths, at two different temperatures, and at different pHs.

Figure 2A shows the Raman spectrum of native chloroperoxidase taken with 457.9-nm laser excitation. Figure 2B shows the same spectral region excited with the 406.7-nm laser line. Both polarizations of the scattered intensity are shown, namely, $I_{\parallel}$ and $I_{\perp}$, where the scattered light is polarized parallel and perpendicular, respectively, to the incident laser polarization. Some of the noise in this spectrum has been

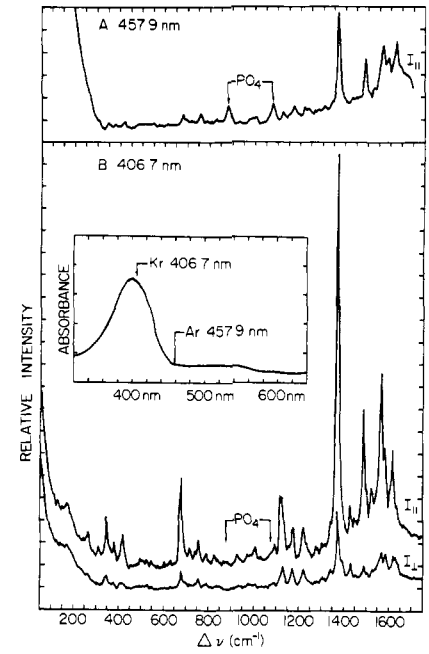


FIGURE 2: Comparison of visibly excited and Soret-excited resonance Raman spectra of native chloroperoxidase. (A) Parallel polarized Raman spectrum taken with 457.9-nm laser excitation. (Unless otherwise indicated a 90° scattering geometry was used.) The two peaks at 877 and 1075 $cm^{-1}$ are due to nonresonant scattering from the phosphate ion in solution. (B) Parallel and perpendicular polarized resonance Raman spectra of chloroperoxidase taken with 406.7-nm laser excitation. Resonantly enhanced spectral features are observed throughout the region from 300 to 1700 $cm^{-1}$. The strength of this enhancement is seen by noting the absence of phosphate Raman bands. Peak intensities in the 406.7-nm spectrum are thus estimated to be $\gtrsim$20 times those in the 457.9-nm spectrum. The insert shows the absorption spectrum of native chloroperoxidase. Arrows indicate the relative positions of the krypton 406.7-nm and argon 457.9-nm lines. Typical experimental conditions for this and all subsequent figures: protein concentrations, 200–600 $\mu$M and 15–50 $\mu$M for 180° and 90° scattering geometries, respectively; laser power, 10–20 mW; effective slit resolution after averaging data in adjacent channels, 6–8 $cm^{-1}$; step size, 0.5–2.0 $cm^{-1}$; counting time, 3–10 s/channel.

suppressed by averaging together signals in adjacent channels. This laser line is very near the peak of the Soret band (as shown in the inset in the figure). The strength of certain features in this spectrum is very much enhanced. Although no quantitative measurements were made, this enhancement can be judged by examining the relative intensity of nonresonant Raman lines from the phosphate buffer. From the relative intensity of these lines, at 877 and 1075 $cm^{-1}$, the peak intensities in the 406.7-nm spectrum are estimated to be at least 20 times those in the 457.9-nm spectrum. The enhancement makes it possible to observe many more Raman lines of chloroperoxidase than were reported before. These lines are listed in Table I. The peak positions were checked in a few cases with neon calibration lines and are accurate to ±2 $cm^{-1}$, except where indicated.

The depolarization ratio ($\rho_l = I_{\perp}/I_{\parallel}$) was measured for each line where possible. For most of the lines, $\rho_l$ does not show dispersion and the line can be characterized as polarized (p), depolarized (dp), or anomalously polarized (ap) depending on whether $\rho_l$ is ~1/8, ~3/4, or larger. These assignments are also listed in Table I. Most of the features which are resonantly enhanced for Soret band excitation are seen to be polarized (p).

Figure 3 shows more examples of the variation of intensity with laser wavelength of the Raman spectra of native chloroperoxidase. The most striking difference is the marked variation in intensity of different Raman bands. Peaks which

Table I: Raman Frequencies of Native Chloroperoxidase for Various Laser Excitation Wavelengths[a]

| laser wavelength (nm) | | | | | | | |
|---|---|---|---|---|---|---|---|
| 363.8 nm | | 406.7 nm | | 457.9 nm | | 514.5 nm | |
| | | 172 | b | | | | |
| | | 264 | w | | | | |
| | | 306 | w | | | | |
| 346 | m | 347 | m, p | 348 | m | | |
| | | 378 | w, p | 381 | w | | |
| | | 408 | sh | | | | |
| 414 | w | 418 | m, p | 418 | m | | |
| 491 | w | | | | | | |
| 676 | m | 677 | s, p | 677 | m, p | | |
| 714 | w | 714 | w, p | 714 | w | | |
| 720 | w | | | | | | |
| 753 | w | 753 | w | 753 | m, dp | | |
| 783, 789 | w | 789 | w, p | 787 | w, dp | | |
| 972 | w | | | 972 | w, p | | |
| 1004 | w | 1007 | w, p | 1004 | m, p | | |
| 1075 | w | | | | | | |
| 1090 | w | 1091 | w, mp | | | | |
| 1122 | m | 1119 | m, p | 1119, 1130 | w, mp | | |
| | | 1130 | m, p | | | | |
| 1169 | m | 1169, 1176 | m, mp | 1168, 1173 | m, dp | | |
| 1218 | m | 1216, 1223 | m, mp | 1215, 1220 | m, p dp | | |
| | | 1303 | vw | 1304 | w, dp | 1301 | w, ap |
| 1340 | w | 1341 | sh, dp | 1340 | w, dp | 1338 | w, ap |
| 1369 | s, p | 1373 | vs, p | 1368 | s, p | 1369 | m, p |
| 1382 | w, p | | | | | | |
| 1396 | sh | 1396 | sh, dp | 1396 | w, dp | 1398 | w, dp |
| 1429 | m | 1430 | w, dp | 1430 | m, dp | 1430 | w, ap |
| 1490 | w, p | 1491 | s, p | 1489 | s, p | 1488, 1498 | vw, mp |
| | | 1504 | sh | 1504 | sh | | |
| 1525 | m | 1525 | w, p | 1529 | w, dp | 1527 | w, dp |
| 1554 | sh | | | 1554 | sh, dp | 1555 | m, dp |
| 1567 | vs, p | 1567 | s, p | 1566 | s, mp | 1564 | m, ap |
| | | 1584 | w, dp | | | | |
| 1591 | s, p | 1591 | sh | 1590 | m, mp | 1590 | w, dp |
| 1619 | sh | 1618 | s, p | 1616 | sh, p | 1617 | sh |
| 1627 | vs, dp | 1628 | sh, dp | 1627 | s, dp | 1627 | m, dp |
| | | 1640 | w, dp | 1640 | sh, dp | 1640 | w, dp |

[a] Frequencies are in units of $cm^{-1}$ and are labeled with a band strength; polarization. w, weak; b, broad; m, medium; s, strong; v, very; sh, shoulder; p, polarized; dp, depolarized; and ap, anomalously polarized as described in the test. In addition, some peaks had depolarization ratios near 1/2. These are labeled mp, medium polarized. The accuracy is $\pm 2$ $cm^{-1}$, except for peaks labeled sh, vw, w, or b in which case the accuracy is $\pm 3$–4 $cm^{-1}$.

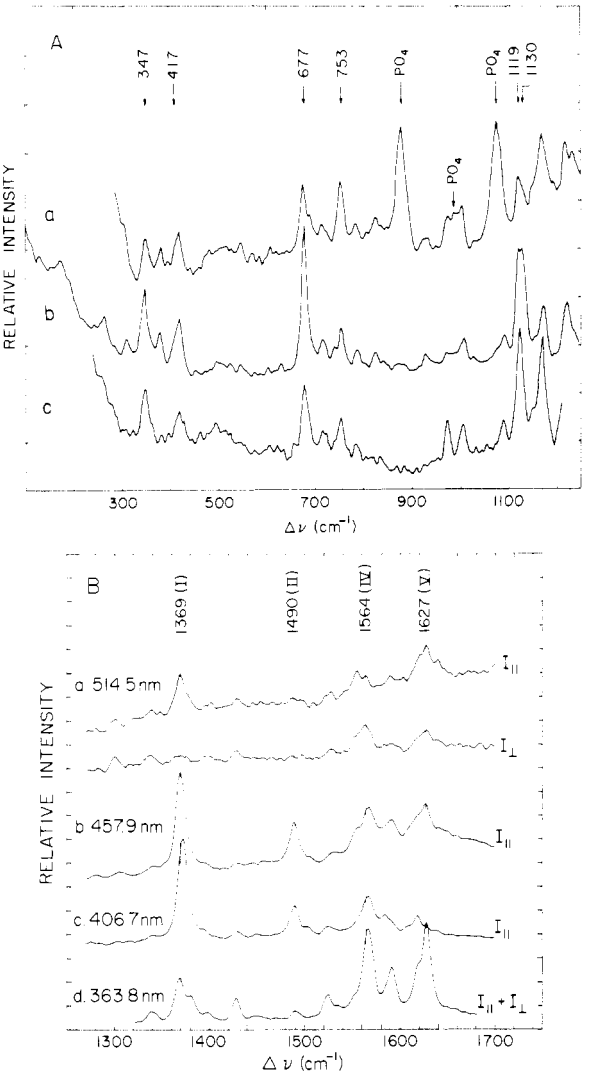


FIGURE 3: Resonance Raman spectra of native chloroperoxidase taken with various laser wavelengths. (A) Low frequency region: (a) [457.9 nm] Relatively strong peaks at 877 and 1075 $cm^{-1}$ are due to the presence of 50 mM sodium phosphate buffer. (b) [406.7 nm] The absence of intense phosphate peaks ($[PO_4]$ = 50 mM) attests to the strength of the observed bands. (c) [363.8 nm] Again the phosphate peaks are very weak. Spectra a and b are parallel polarized, while spectrum c is unpolarized. (B) Marker band region: (a) [514.5 nm] Both parallel and perpendicular polarized spectra are shown. Scattering geometry is 180° (spectra b–d were taken using a 90° scattering geometry). Marker bands I, II, IV, and V are observed at 1369, 1490, 1564, and 1627 $cm^{-1}$. Note that band IV is visible only at 514.5-nm excitation. (b) [457.9 nm] Parallel polarization only is shown. There is a polarized peak at 1567 $cm^{-1}$ which is nearly degenerate with the spin state marker band IV. (c) [406.7 nm] Only marker bands I and II can be detected. Most noticeable is the lack of resonance enhancement of marker band V and the appearance of a relatively strong polarized peak at 1618 $cm^{-1}$. (d) [363.8 nm] The spectrum is unpolarized. Band V is now visible and in fact is the dominant peak in the spectrum.

dominate at one wavelength may be nearly absent at another. In some cases these changes are so pronounced that they lead to apparent "shifts" in the Raman bands.

A prominent example of such a "shift" is observed for the band near 1370 $cm^{-1}$ in Figure 3B. This appears to shift from 1369 $cm^{-1}$ for 457.9- and 363.8-nm excitation to 1373 $cm^{-1}$ for 406.7-nm excitation. On closer examination, however, this band is seen to be a composite band with a new component resonantly enhanced for Soret excitation. Figure 4 presents an enlarged scan of this band made with 1.7-$cm^{-1}$ slit resolution and 0.5-$cm^{-1}$ step size. These and similar scans at other wavelengths provide a basis for decomposing this "band" into its separate components. This example is just one reminder that "bands" in heme spectra may often be composites and that examination at different laser wavelengths is a good way to test for this.

The apparent shift of the peak in Figure 3Bc could result from intense Soret band enhancement of the component at 1373 $cm^{-1}$. This distinct component may be due to a low-spin fraction of chloroperoxidase present in the sample (note the appearance of a low-spin marker band as a shoulder at ca. 1503 $cm^{-1}$ in Figure 3Bc). A similar phenomenon was observed with cytochrome P-450 and was attributed to a minority species of low-spin ferric heme (Champion et al., 1978). Native chloroperoxidase is also known to exist in a high/low spin equilibrium (Champion et al., 1973).

The spectral region shown in Figure 3B for native chloroperoxidase contains the characteristic heme marker bands whose frequencies have been found to be sensitive to the valence and spin state of the iron atom (Spiro & Strekas, 1974) and, possibly, to the identity of the axial ligand (Champion

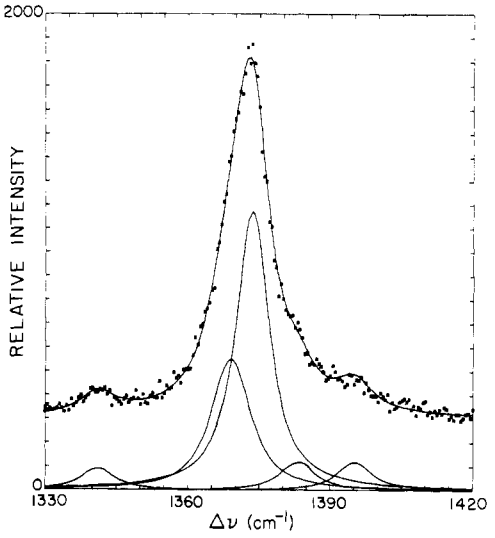


FIGURE 4: High resolution scan of the 1373-$cm^{-1}$ peak of oxidized chloroperoxidase taken with 406.7-nm laser excitation. Squares are the actual data points; the solid line is the sum of the five bands shown below. Peak positions for four of these bands were obtained from Raman spectra taken at other wavelengths. The peak position for the fifth and strongest band (seen only for Soret excitation) was chosen to give the best fit. Relative strengths of bands were also taken as variable. Instrumental conditions: slit width, 1.7 $cm^{-1}$, step size 0.5 $cm^{-1}$ with 10 s counting time per step.

Table II: Marker Band Frequencies of Various Derivatives of Chloroperoxidase[a]

| derivative of chloroperoxidase | marker band | | | |
|---|---|---|---|---|
| | I | II | IV | V |
| native ($Fe^{3+}$) | 1369 ± 1 | 1490 ± 1 | 1564 | 1627 ± 1 |
| cyanide ($Fe^{3+}$) | 1373 ± 1 | 1503 | | 1633 |
| ethylene glycol ($Fe^{3+}$) | 1374 | 1505 | | 1640 |
| NaOH ($Fe^{3+}$) | 1372 | 1490 | | |
| | | 1502 | | |
| reduced ($Fe^{2+}$) | 1348 ± 1 | 1470 ± 1 | | 1612 |
| cyanide ($Fe^{2+}$) | 1360 | 1492 | | |
| NaOH ($Fe^{2+}$) | 1360 | 1492 | | |

[a] Frequencies are in units of $cm^{-1}$ and accurate to within ±2 $cm^{-1}$ except where indicated.

et al., 1976). These bands can be identified most easily in spectra taken with blue or green laser excitation, on the basis of peak location, relative intensity, and depolarization ratio. Four marker bands, labeled I, II, IV, and V in Figure 3B, were identified and are listed in Table II. Band I, the strong polarized band at 1369 $cm^{-1}$, was identified from spectra taken with visible excitation. Band II, a polarized band at 1490 $cm^{-1}$, was sufficiently isolated that there was no ambiguity in assignment. Band IV, the anomalously polarized band discernible at 1564 $cm^{-1}$, was identified using the $I_\perp$ spectrum taken at 514.5 nm. Band V, the depolarized peak at 1627 $cm^{-1}$, was also identified from $I_\perp$ spectra taken at 514.5, 457.9, and 363.8 nm. As noted previously (Champion et al., 1976), these marker bands are located at positions that are atypical for high-spin ferric heme proteins. Of all heme proteins studied so far, only cytochrome P-450 has similar marker band positions (Ozaki et al., 1976; Champion & Gunsalus, 1977; Champion et al., 1978).

The Raman spectra of native chloroperoxidase do not appear to depend on pH. We examined the spectra of samples at pH 3.6 and pH 6.3. No significant differences were detected, except for very minor variations in peak intensity. Consequently, in the remainder of the work, spectra were measured at a convenient pH in this range.

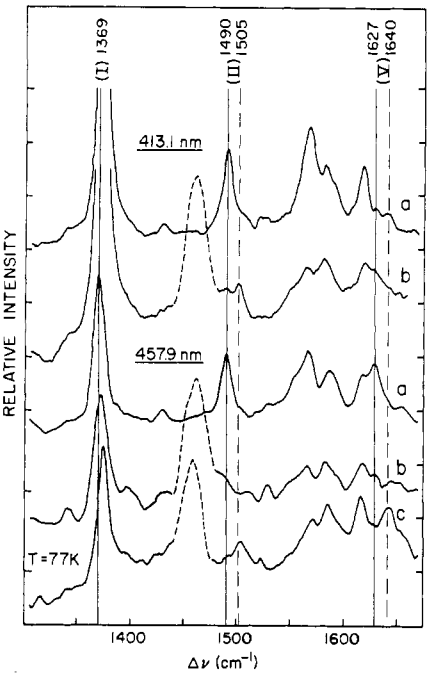


FIGURE 5: Resonance Raman spectra of the low- and high-spin forms of oxidized chloroperoxidase at two laser wavelengths. (a) Spectra of the native enzyme at 277 K. Spectra of the ethylene glycol treated enzyme at (b) 277 K and (c) 77 K. The dashed peaks are nonresonant scattering from the ethylene glycol solvent. A 180° scattering geometry was used for the 457.9-nm spectra.

Spectra were also measured for chloroperoxidase in different spin and oxidation states. In most cases, changes were induced by chemical manipulation as discussed in following sections. However, one change, conversion from high spin to low spin, can be accomplished by lowering the temperature. A 40/60 mixture of 500 $\mu$M native chloroperoxidase and ethylene glycol was prepared and then cooled to 77 K. This procedure is known from both optical absorption and Mössbauer studies to convert nearly all of the chloroperoxidase to the low-spin ferric state (Debey & Balny, unpublished experiments; Champion et al., 1973). Addition of ethylene glycol at room temperature without cooling produces partial conversion. Figure 5 shows examples of Raman spectra taken during this procedure. The solid lines indicate the location of the marker bands initially in the native chloroperoxidase. As conversion to low-spin ferric heme proceeds, these bands decrease in intensity and new bands grow at the positions shown by the dashed lines. These new peak positions are listed in Table II. This observation of shifts in heme protein Raman bands as the temperature is changed has also been observed in various methemoglobin derivatives (K. C. Cho, R. D. Remba, and D. B. Fitchen, unpublished experiments).

*Complexes of Native Chloroperoxidase.* The complexing of small ions to a heme protein often alters the position and width of the Soret absorption band and it may change the spin of the iron atom. These changes affect the Raman spectra of the heme protein complexes in at least two ways. First, different resonance enhancement is expected for a given laser frequency when the resonant electronic transition is changed in energy or width; second, the frequency shifts of the "marker bands" should indicate whether or not spin state changes have occurred.

In Figure 6 we display the Raman spectra of a variety of chloroperoxidase derivatives in which native chloroperoxidase is complexed to various small ions. These spectra are all parallel polarized and taken with 406.7-nm excitation. The upper curve is a spectrum of native chloroperoxidase, allowing

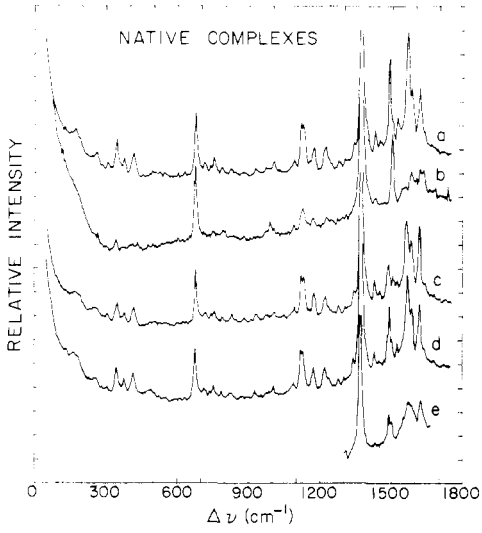


FIGURE 6: Parallel polarized resonance Raman spectra of various oxidized derivatives of chloroperoxidase taken with Soret excitation. (a) Oxidized chloroperoxidase (406.7 nm); (b) chloroperoxidase cyanide (406.7 nm); (c) chloroperoxidase fluoride (406.7 nm); (d) chloroperoxidase chloride (406.7 nm); and (e) sodium hydroxide treated chloroperoxidase (oxidized P-420 form of the enzyme) (413.1 nm).

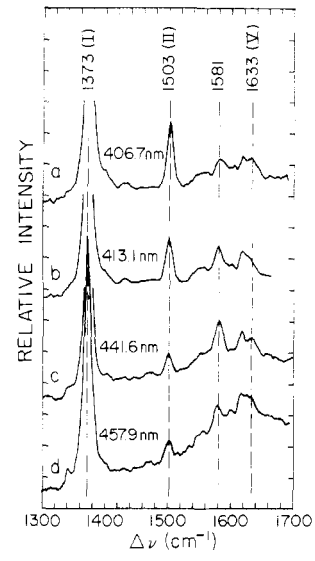


FIGURE 7: Partial resonance Raman spectra of chloroperoxidase cyanide obtained with excitation wavelengths within the Soret absorption region. The spectra were plotted so that band I in each spectrum had the same peak height in the figure. Band I in a–c is truncated for clarity. Laser wavelengths as shown.

direct comparison with the spectra of the cyanide, fluoride, chloride, and sodium hydroxide complexes found below. (The latter is considered to be an inactive "P-420" form of the enzyme.) Peak positions, intensities, and polarizations of the spectra in Figure 6 can be found in Table III.

The 406.7-nm Raman spectra of the cyanide complex and the oxidized enzyme appear very different. For example, the chloroperoxidase cyanide Raman spectrum shows few low frequency modes and the spectral region above 1500 $cm^{-1}$ has no distinctive peaks. This is in direct contrast to the abundant features in the spectrum of oxidized chloroperoxidase and probably reflects a change in the excitation efficiency due to the shift in the Soret absorption band. Figure 1 shows the change in the Soret absorption spectrum when cyanide is complexed to the oxidized enzyme. The cyanide complex shows a "split" Soret peak (characteristic of a hyperporphyrin spectrum; Hanson et al., 1976) with maxima at 363 nm and 430 nm,[1] while the oxidized enzyme shows a single maximum near 400 nm. Hence, the 406.7-nm krypton laser line which is in resonance with the latter is seen to be relatively far from the Soret maxima of the former.

Figure 7 shows Raman spectra of chloroperoxidase cyanide in the marker band region taken using four different wavelengths. Differential enhancement effects are quite apparent. For instance, as the laser wavelength is increased, band II (1503 $cm^{-1}$) decreases monotonically relative to band I (1373 $cm^{-1}$), while the relative intensity of band V (1633 $cm^{-1}$) apparently does not change. The band at 1581 $cm^{-1}$ appears to increase by a factor of $\sim 2$ in the spectra taken with excitation lines close to the 430-nm absorption peak.

The marker bands of chloroperoxidase and its cyanide complex differ characteristically. Band II shifts from 1490 $cm^{-1}$ in the oxidized enzyme to 1502 $cm^{-1}$ for the cyanide complex. Band V shifts from 1627 to 1633 $cm^{-1}$. These shifts indicate a transition from a high-spin iron atom in the case of oxidized chloroperoxidase to a low-spin form in the case of chloroperoxidase cyanide. Other spectral differences are also discernible in the region 1550–1600 $cm^{-1}$.

Chloroperoxidase fluoride and chloroperoxidase chloride have Raman spectra very similar to the Raman spectrum of the oxidized enzyme. The basic change observed in the halide derivative Raman spectra (Figure 6) compared with that of oxidized chloroperoxidase is the decrease in intensity of the peaks at 1490 $cm^{-1}$ (band II) and 1567 $cm^{-1}$ relative to all other Raman peaks. While the reason for these relative intensity changes is not certain, the overall similarity between the chloroperoxidase halide and oxidized enzyme Raman spectra is consistent with the fact that only minor changes in the absorption spectra occur.

Addition of sodium hydroxide to the native enzyme radically alters the absorption spectrum as seen in Figure 1. The Raman spectrum seen in Figure 6 is also very different from the spectrum of the native enzyme. In particular, band II shows two components at 1490 and 1502 $cm^{-1}$, and the region between 1550 and 1600 $cm^{-1}$ has a very broad hump with no distinguishable features.

*Reduced Chloroperoxidase.* The Raman spectra of the reduced form of chloroperoxidase are limited because it is difficult to make a reduced sample that is stable during the time required for Raman measurements (typically 1–4 h). We were able to overcome the problem of reoxidation somewhat by use of careful reduction techniques. In Figure 8, we present the Raman spectra of reduced chloroperoxidase taken with laser excitation wavelengths of 457.9, 413.1, and 406.7 nm. As the excitation frequency is moved from the visible to the ultraviolet, dramatic intensity and polarization variations in the Raman modes of reduced chloroperoxidase are observed. The depolarization ratio of the polarized 1348-$cm^{-1}$ peak increases to a value of $\rho_l \simeq 0.5$ at 413.1-nm excitation, while the two peaks at 1390 and 1425 $cm^{-1}$ have values of $\rho_l$ approaching unity. A cluster of depolarized peaks between 1530 and 1615 $cm^{-1}$ also appears when deep blue wavelengths are used for excitation. In contrast, the presence of a polarized peak at 1471 $cm^{-1}$ shows that the polarization measurements

[1] Actually two cyanide derivatives were prepared which could be distinguished by their optical and Raman spectra. The "437 form" prepared in 50 mM KCN also shows a "split" Soret band with maxima at 437 and 363 nm. However, the Raman spectrum of this complex taken with 413.1-nm laser excitation is nearly identical with that of native chloroperoxidase. This is consistent with the cyanide having a lower affinity for the heme iron than for a chloride binding site which is not at the iron atom (Krejcarek et al., 1976). The "430 form", with absorption maxima at 430 and 363 nm, is prepared in 300 mM KCN.

Table III: Raman Frequencies of Derivatives of Chloroperoxidase[a]

| native | | cyanide | | derivative: ethylene glycol | | derivative: NaOH[c] | | reduced | | reduced cyanide[c] | | reduced NaOH[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 172 | b | | | | | | | 171 | m | | | | |
| 264 | w | 265 | w; b | | | | | | | | | | |
| 308 | w | | | | | | | | | | | | |
| 347 | m; p | 343 | m; p | | | | | 348 | m | 346 | s; p | | |
| 378 | w; p | 372 | | | | | | 378 | w | 383 | w; p | | |
| | | 410 | w | | | | | 405 | w | 412, 422 | m; p | | |
| 418 | m; p | 432 | w | | | | | 415 | m | | | | |
| 677 | s; p | 677 | s; p | 678 | m | | | 676 | m; mp,[c] p | 674 | vs; p | | |
| 714 | w; p | | | | | | | 714 | w; p | 714 | w; p | | |
| 753 | w, m; dp | 753 | w | | | | | 749 | m; dp, mp[c] | 747 | s; dp | | |
| 789 | w; p | 790 | w; p | | | | | 785 | w; dp | 787 | w; p | | |
| 825 | w; p | | | 823 | m | | | 827 | w; p | | | | |
| | | 991 | m; p | | | | | 996, 1003 | w; p | 993, 1003 | m; p | | |
| 1007 | w; p | | | | | | | | | | | | |
| 1091 | w, mp | 1090 | w; p | | | | | 1088 | w; p | | | | |
| 1119 | m; p | 1118, 1133 | m; mp | | | | | 1114 | m; p | 1116 | m; p | | |
| 1130 | m; p | | | | | | | 1133 | w; p | 1130 | m; p | | |
| 1169, 1176 | m; mp | 1171 | w; mp | | | | | 1172, 1178 | m; dp | 1172 | m; mp | | |
| 1216, 1223 | w; mp | | | | | | | 1214 | w; dp | | | | |
| | | 1228 | w; mp | | | | | | | 1225 | m; mp | | |
| | | 1247 | m | | | | | 1238 | w | | | | |
| | | 1276 | | | | | | 1279 | w | | | | |
| | | | | | | | | 1292 | w | | | | |
| 1301[d] | w; ap | | | | | | | 1304 | w; dp | 1310 | w; p | | |
| 1338[d] | w; ap | | | | | | | 1319 | w | | | | |
| 1341 | w; dp | 1342 | w | 1342 | w | | | 1336 | sh; dp | | | | |
| 1369 | vs; p | | | | | | | 1348 | s; mp,[c] p | | | | |
| 1373[c] | vs; p | 1373 | vs; p | 1374 | s; p | 1372 | vs | 1362 | w, dp | 1360 | vvs; p | 1360 | vvs; p |
| 1382 | w; p | | | | | | | | | | | | |
| 1395 | w; dp | | | 1395 | w | | | 1392 | w, s;[c] dp | 1393 | m; dp | | |
| 1430 | w, m; dp ap[d] | 1431 | w; dp | | | | | 1424 | w, s;[c] dp | 1422 | w; dp | | |
| | | | | | | | | | | 1432 | m; dp | | |
| 1490 | w, s; p | 1503 | s; p | 1502 | m; p | 1490 | m | 1471 | w,[d] s; p | 1492 | m; p | 1492 | m; p |
| | | | | | | 1503 | m | | | | | | |
| | | | | | | | | 1534 | sh; dp | | | | |
| 1555 | w, m; dp, | 1556 | w; dp | | | | | 1544 | s; p | 1555 | m; p | 1556 | m; p |
| 1564[d] | m; ap | | | | | | | | | | | | |
| 1567 | s; p, mp | | | 1572 | w | 1575 | b | 1564 | m; dp | | | | |
| 1584 | w; dp | 1581 | m; dp | 1585 | m | | | 1578 | m; dp | 1582 | m; mp | 1582 | m |
| 1618 | w, s;[c] p | 1618 | m; p | 1618 | m; p | 1624 | b | | | 1618 | m; p | 1618 | m; p |
| 1627 | w,[c] s; dp | 1633 | m; dp | 1640 | m; dp | | | 1612 | m, s;[b] dp | | | | |

[a] See Table I. [b] Spectra taken with 363.8-nm laser excitation. [c] Spectra taken with 406.7- or 413.1-nm laser excitation. [d] Spectra taken with 514.5-nm laser excitation.

are reliable. A very similar polarization and intensity pattern has been observed for the reduced form of cytochrome P-450 upon excitation in the deep blue (Champion et al., 1978).

The dashed vertical lines indicate the positions of each of the marker bands in the reduced (high-spin ferrous) state. From Figure 8a, the assignment of bands I (1348 $cm^{-1}$) and II (1470 $cm^{-1}$) is straightforward since each is polarized and fairly well isolated. Depolarized band V, however, is rather difficult to identify. The position of band V at 1612 $cm^{-1}$ indicated in Figure 8 is considered tentative.

The peak positions and polarizations of the spectra in Figure 8 are listed in Table III. As with native chloroperoxidase, the marker bands of the reduced enzyme do not correlate very well with those of other heme proteins having an analogous spin and valence state (high-spin ferrous; Spiro & Burke, 1976). Band I is found at 1348 $cm^{-1}$, 10 $cm^{-1}$ lower than normal. Band II (1471 $cm^{-1}$) is 2 $cm^{-1}$ lower, and band V (1612 $cm^{-1}$) is 5 $cm^{-1}$ higher than the corresponding bands of "normal" high-spin ferrous heme proteins. The only other heme protein having similar marker band frequencies is the reduced form of cytochrome P-450.

The peak at 1362 $cm^{-1}$ in the Soret excited spectra of Figure 8 appears as a shoulder with variable intensity in different runs. The following evidence indicates that it probably represents the presence of a reduced "P-420" form of chloroperoxidase. First, Soret excited spectra of a reduced P-420 form of chloroperoxidase have a very strong polarized band at 1360 $cm^{-1}$ very close to the position of the observed shoulder. Second, a large increase in the intensity of the peak at 1362 $cm^{-1}$ relative to the 1348-$cm^{-1}$ band is seen when the Raman spectrum is taken immediately after a great excess of sodium dithionite (which can cause inactivation and P-420 formation) is added to the sample. Finally, a similar phenomenon has been observed in reduced P-450, where a peak with variable intensity at 1361 $cm^{-1}$ has been attributed to a reduced P-420 species (Champion et al., 1978).

*Complexes of reduced chloroperoxidase*, with NaOH, $CN^-$, and CO, were prepared and Raman spectra were taken. Figure 9a presents Raman spectra of the reduced cyanide complex. The reduced NaOH (reduced P-420 form of the enzyme) complex has virtually the same Raman spectrum; however, this form of the enzyme is unstable. The Raman frequencies are listed in Table III. The laser excitation wavelength of 413.1 nm is very close to the maximum of the Soret band of both of these complexes.

Figure 9a shows some interesting relative enhancement effects. The tremendous relative intensities of the 674- and 1360-$cm^{-1}$ peaks in Figure 9a overpower all other modes in

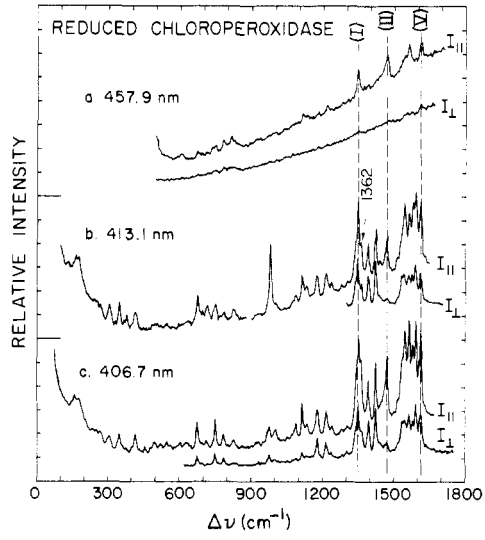


FIGURE 8: Polarized resonance Raman spectra of reduced chloroperoxidase obtained with excitation wavelengths in the Soret absorption region. Dashed lines indicate marker bands I, II, and V. The spectra in b and c are composites with intensities scaled to the 981-$cm^{-1}$ peak of a 0.2 M $SO_4$ internal standard.

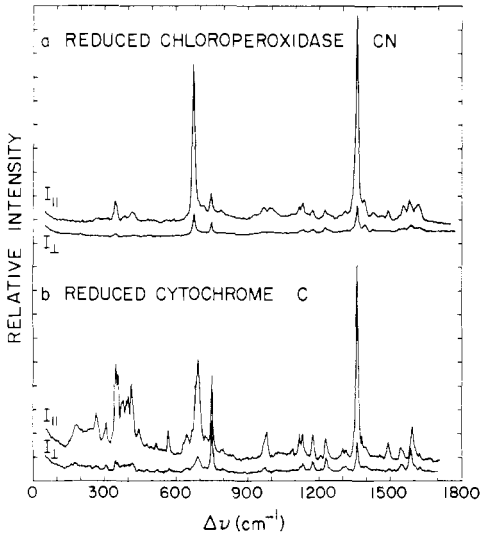


FIGURE 9: Comparison of the polarized resonance Raman spectra of (a) reduced chloroperoxidase cyanide and (b) reduced cytochrome *c*, taken with 413.1-nm laser excitation.

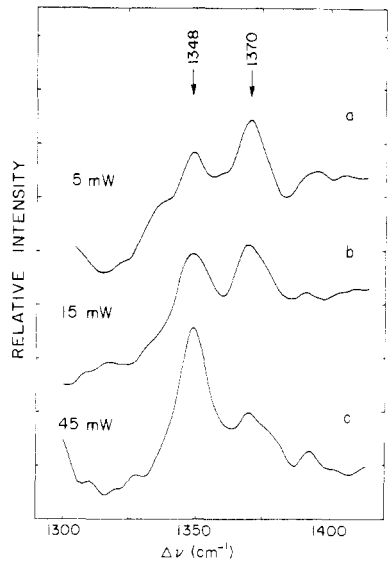


FIGURE 10: Partial resonance Raman spectra of reduced carbonmonoxychloroperoxidase in the region of marker band I. All spectra were taken with 457.9-nm laser excitation on the same sample (concentration = 40 $\mu$M). The laser power was varied from 5 mW (a) to 45 mW (c).

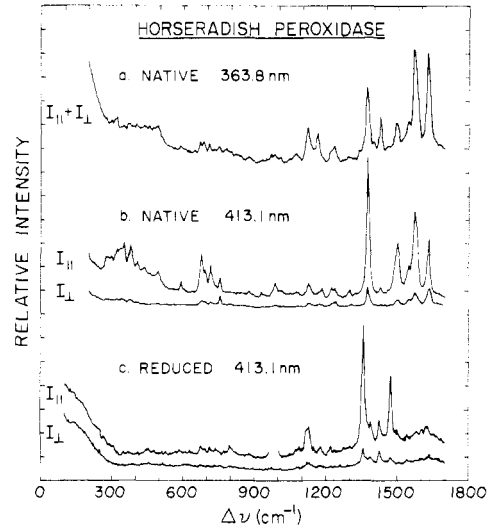


FIGURE 11: Resonance Raman spectra of native and reduced horseradish peroxidase.

the spectrum. The use of a 0.2 M sulfate internal standard, for intensity calibration, indicates that these peaks in the reduced cyanide and reduced P-420 form spectra are approximately ten times stronger than the corresponding bands in native chloroperoxidase when using 413.1-nm excitation. A combination peak (674 $cm^{-1}$ + 1360 $cm^{-1}$ = 2034 $cm^{-1}$) in the reduced chloroperoxidase cyanide spectrum was also observed in this case.

We can assign two marker bands from the Raman spectrum in Figure 9a, one at 1360 $cm^{-1}$ (I) and one at 1492 $cm^{-1}$ (II) (see Table II). The positions of these bands are typical of low-spin ferrous heme complexes (Spiro & Burke, 1976).

The reduced carbon monoxide complex of chloroperoxidase is photolabile as are the analogous complexes of hemoglobin (Haldane & Smith, 1896), myoglobin (Gibson & Ainsworth, 1957), and cytochrome P-450 (Austin et al., 1975). This makes accumulation of the laser-excited Raman spectrum rather difficult because the CO tends to separate from the heme iron under laser illumination. This problem has been overcome, to some degree, by varying the incident laser power, observing concomitant changes in the Raman spectrum, and extrapolating to zero power. Marker band I for the reduced species (at 1348 $cm^{-1}$) is expected to decrease in intensity with decreasing laser power as less CO is photodissociated.

Figure 10 presents the results of such an experiment on the carbonmonoxy complex using 5, 15, and 45 mW of laser power. The shift of the peak (I) from 1348 $cm^{-1}$ characteristic of reduced chloroperoxidase in c to 1370 $cm^{-1}$ characteristic of the CO complex in a is clearly shown.

*Horseradish Peroxidase, Cytochrome P-450, and Ferrocytochrome c.* We have also recorded near-ultraviolet Raman spectra of horseradish peroxidase, cytochrome P-450, and ferrocytochrome *c*, and we present them in this section for comparative purposes. Figure 11 shows spectra of native and reduced horseradish peroxidase taken with 363.8- and 413.1-nm laser excitation. These are the first reported Raman spectra of horseradish peroxidase taken with Soret excitation wavelengths. Above 1300 $cm^{-1}$ six main Raman bands appear in the spectra of the native protein, while below 800 $cm^{-1}$ a rich pattern of vibrational structure develops at 413.1-nm excitation. The relative intensities of these bands are clearly

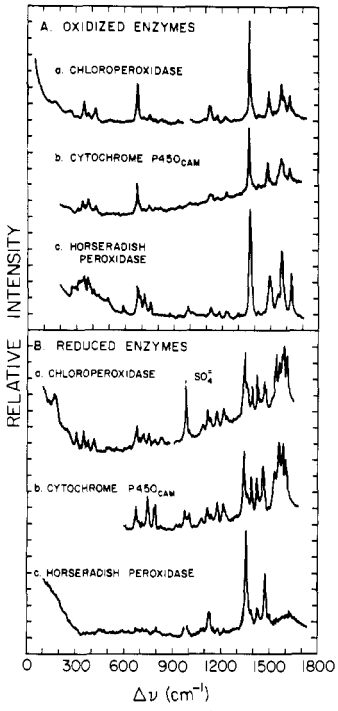


FIGURE 12: Resonance Raman spectra of various heme proteins taken with 413.1-nm laser excitation.

sensitive to the wavelengths used for excitation. The native (oxidized) protein has marker band frequencies at 1373 $cm^{-1}$ (band I), 1499 $cm^{-1}$ (band II), and 1630 $cm^{-1}$ (band V) that are typical of a high-spin ferric heme protein (Spiro & Burke, 1976).

The spectrum of reduced horseradish peroxidase displays very weak structure below 1300 $cm^{-1}$, with the exception of a moderately strong peak at 1129 $cm^{-1}$. In the high frequency region major features occur between 1300 and 1500 $cm^{-1}$; a cluster of weak peaks appears between 1500 and 1700 $cm^{-1}$. The marker bands I and II are typical for a high-spin ferrous heme protein and are easily identified at 1356 and 1470 $cm^{-1}$; however, band V is difficult to identify in Figure 11. A weak peak does appear at 1605 $cm^{-1}$, the position of band V determined from visible excitation (Rakshit & Spiro, 1974), but it is difficult to make the assignment only on the basis of the Soret-excited Raman spectrum. The peak postions and polarizations of both oxidized and reduced horseradish peroxidase are presented in Table IV and are similar to those reported using visible excitation sources.

The Soret excited spectra of native horseradish peroxidase, chloroperoxidase and cytochrome P-450 can be compared directly in Figure 12A. In contrast to horseradish peroxidase, the marker bands of chloroperoxidase and cytochrome P-450 are found at atypical positions. Notable differences in the spectra of the three proteins also occur in the low frequency region at about 750 $cm^{-1}$ and below 500 $cm^{-1}$. In the high-frequency range the spectra of chloroperoxidase and cytochrome P-450 are found to be very similar except for a somewhat better resolution of the two peaks near ca. 1600 $cm^{-1}$ in the chloroperoxidase spectrum. The reduced forms of these enzymes are also compared in Figure 12B, where all three proteins are in the high-spin ferrous state. Both reduced chloroperoxidase and reduced cytochrome P-450 display spectra with polarization and intensity patterns that are significantly different from those of reduced horseradish peroxidase. The overall similarity of the reduced chloroperoxidase and reduced cytochrome P-450 spectra is striking, although there are a few differences that will be discussed in the next section.

Table IV: Comparison of Raman Frequencies of Different Heme Proteins Obtained with 413.1-nm Laser Excitation[a]

| enzyme | | | | | |
|---|---|---|---|---|---|
| oxidized horseradish peroxidase | | reduced horseradish peroxidase | | reduced cytochrome *c* | |
| | | | | 182 | b |
| 277 | w, p | | | 264 | m, p |
| 285 | w, p | | | | |
| 297 | w, p | | | | |
| 319 | w, p | | | 307 | m, p |
| 333 | w, p | | | | |
| 348 | m, p | 350 | w, p | 347 | s, p |
| | | | | 356 | s, p |
| | | 370 | w, p | 373, 380 | m, p |
| 378 | m, p | | | | |
| | | | | 392, 400 | m, p |
| 407 | m, p | | | 413 | s, p |
| | | | | 422 | sh |
| 435 | w, p | 445 | w | 445 | m, p |
| | | 456 | w | | |
| 495 | m, p | | | | |
| | | | | 519 | w, mp |
| | | | | 568 | m, p |
| 592 | m, p | | | | |
| | | | | 646 | b, p |
| | | | | 667 | sh |
| | | | | 681 | sh |
| 677 | s, p | 675 | m, p | 692 | vs, p |
| 693 | sh | | | 700 | sh |
| 716 | m, p | 714 | w, p | 726 | w |
| | | 738 | w, p | | |
| 755 | m, dp | | | 750 | vs, dp |
| | | 797 | m, p | | |
| 877 | w, p | | | | |
| 929 | w, p | | | | |
| 985 | m, p | | | | |
| | | | | 1089 | w, p |
| | | 1120 | m, p | 1118 | m, p |
| 1127 | m, p | 1128 | | 1132 | m, p |
| 1183 | m, p | 1179 | w, p | 1175 | m, mp |
| 1223 | m, p | 1220 | w, p | | |
| 1238 | m, dp | | | 1230 | m, dp |
| 1298 | m, p | | | 1299 | w, mp |
| | | | | 1313 | w, mp |
| 1375 | vs, p | 1356 | vs, p | 1361 | vs, p |
| | | | | 1382 | w, p |
| | | 1387 | sh | 1397 | w, dp |
| 1428 | w, p | 1425 | m, dp | | |
| 1489 | sh | | | | |
| 1500 | s, p | | | 1491 | m, p |
| | | | | 1544 | m, mp |
| 1548 | w, dp | 1541 | w, mp | 1550 | |
| 1574 | s, p | | | | |
| | | 1582 | w | 1585 | m, ap |
| | | | | 1592 | m, p |
| | | 1623 | m, p | | |
| 1631 | s, mp | 1604 | w, dp | 1622 | w, dp |

[a] See Table I.

In Figure 9, we compare the Raman spectra of two low-spin ferrous complexes, obtained using 413.1-nm excitation. The upper spectrum is the reduced cyanide derivative of chloroperoxidase and the lower curve is a spectrum of ferrocytochrome *c*. The spectrum of cytochrome *c* shows a large number of intense low frequency vibrational modes. This structure has not been observed previously because visible excitation does not enhance these vibrations. The ferrocytochrome *c* spectrum differs substantially from the reduced chloroperoxidase cyanide spectrum with regard to both the relative intensities and positions of the peaks. The marker bands of cytochrome *c* appear at 1361 (I), 1491 (II), and 1622

$cm^{-1}$ (V) and agree with those reported using visible excitation. In addition, the anomalously polarized marker band IV is also observed at 1585 $cm^{-1}$ in the cytochrome *c* spectrum. This is the first anomalously polarized peak we have observed with excitation in direct resonance with the Soret band. The peak positions and polarizations of ferrocytochrome *c* are listed along with those of the other reduced proteins in Table IV.

## Discussion

The resonance Raman spectra displayed in the previous sections present a variety of examples of resonance with the Soret band of heme proteins. An analogous Raman study of cytochrome P-$450_{cam}$ (Champion et al., 1978) also utilized Soret excitation sources and made preliminary comparisons between the spectra of P-$450_{cam}$ and other heme proteins. In this discussion, we focus on the chloroperoxidase data and condense the results into three main categories: (1) Soret band resonance; (2) high frequency porphyrin modes; and (3) comparisons between Soret excited Raman spectra of heme proteins.

*Soret Band Resonance.* The interpretation of resonance Raman scattering from porphyrins has been discussed before in several places (Spiro & Strekas, 1974; Friedman & Hochstrasser, 1973, 1976); in particular, a careful treatment of resonance scattering in the Soret band region will be presented very soon by one of us (P. M. Champion and A. C. Albrecht, unpublished experiments) and will not be repeated here. Strong scattering from only the totally symmetric vibrations is expected for Soret band excitation. The modes which are maximally enhanced are those along whose normal coordinates the excited state potential energy well gets displaced the most (with respect to the ground state equilibrium position). The intensity of overtone and combination scattering is also quite sensitive to the magnitude of these displacements.

The selective enhancement of only totally symmetric modes can be seen quite clearly in Figures 2 and 3. These spectra of native chloroperoxidase, taken with different incident laser frequencies, show how the 753- and 1627-$cm^{-1}$ depolarized bands are not enhanced with 406.7-nm excitation. In spectra 3Ab and 3Bc, the depolarized bands at 753 and 1627 $cm^{-1}$ (band V) appear minute compared with the strongly enhanced polarized modes at 677, 1373, 1490, 1567, and 1618 $cm^{-1}$.

Careful measurements of depolarization ratios for "polarized" bands of porphyrins reveal that $\rho_l$ is seldom exactly 1/8, but is more often larger, about 1/6. (For chloroperoxidase, only the 1490-$cm^{-1}$ band has $\rho_l \simeq 1/8$). This discrepancy in $\rho_l$ is not understood, but may be related to differences in effective local symmetry seen by different vibrational modes.

The selective enhancement of the 674- and 1360-$cm^{-1}$ modes in the reduced cyanide complex (Figure 9) provides another example of the resonance scattering process. These modes are polarized and have $A_{1g}$ symmetry (in $D_{4h}$). The fact that other $A_{1g}$ modes in the reduced chloroperoxidase cyanide spectrum are not so strongly affected by the Soret resonance is quite interesting. The most probable explanation is that the excited state associated with the Soret transition has an expanded porphyrin macrocycle that is stretched primarily along the normal coordinates corresponding to the 674- and 1360-$cm^{-1}$ modes.

Normal mode calculations (Stein et al., 1975; Sunder & Bernstein, 1976) indicate that this intense mode (oxidation state marker band I) is primarily due to breathing of the outer porphyrin ring. However, recent experiments (Kitagawa et al., 1977, 1978) using $N^{15}$ enriched metallooctaethylporphyrins and a new calculation (Abe et al., 1978) indicate that band I is basically a breathing mode of the pyrrole rings with an appreciable contribution from the $C_\alpha N$ symmetric stretching vibration, associated with the in-phase displacement of the four pyrrole nitrogens toward the metal ion. This change in equilibrium position of the iron nitrogen system, accompanying the porphyrin macrocyle expansion in the excited state, may give rise to relatively strong enhancement of low frequency iron–nitrogen vibrations observed in Soret excited spectra of chloroperoxidase, P-$450_{cam}$, reduced chloroperoxidase cyanide, ferrocytochrome *c* (Figure 9), and horseradish peroxidase (Figure 11).

Since the half-width of the Soret band in native chloroperoxidase is nearly 2000 $cm^{-1}$, it may hide other types of electronic transitions. For example, charge-transfer bands could be present within the Soret band envelope. The enhancement pattern of both high and low frequency Raman modes that we have observed within the Soret band may give clues to the underlying structure of this transition. Figures 3 and 7 show some examples of this phenomenon for native chloroperoxidase and chloroperoxidase cyanide. The selective variation of Raman intensities using excitation within the Soret band may indicate the coupling of modes to a variety of electronic states within the Soret band or to states located at even higher energies. The formation of a hyperporphyrin spectrum (split Soret band) upon cyanide binding to chloroperoxidase is further indication of the complex nature of the Soret transition and probably reflects mercaptide–sulfur ligation in this compound (Hanson et al., 1976). The cyanide complex of cytochrome P-$450_{cam}$ also has a hyperporphyrin type Soret band (Gunsalus et al., 1974).

*High Frequency Raman-Active Vibrations of the Heme.* Characteristic peaks in the Raman spectra of heme proteins shift frequency upon change of spin or oxidation state of the iron atom. This attribute has been used by several groups to define Raman marker band schemes that reflect the electronic configuration of the heme complex. As the variety of heme complexes studied with Raman spectroscopy increases, these classification schemes must be modified to take into account chemical and structural effects on the heme vibrational modes other than the spin and valence of the central iron atom.

In particular, the marker bands (I) of chloroperoxidase and cytochrome P-450 are found at positions that are anomalously low when compared with other heme proteins having analogous spin and valence states. The shift of chloroperoxidase band I from 1369 $cm^{-1}$ in the oxidized state to 1348 $cm^{-1}$ in the reduced state is similar to the shift observed for horseradish peroxidase (1375 to 1358 $cm^{-1}$), although the absolute positions of the bands in chloroperoxidase (and also cytochrome P-450) are lower in energy. As pointed out in an earlier note (Champion et al., 1976), the anomalous band I positions of chloroperoxidase and cytochrome P-450 are probably due to the presence of a strongly electron-donating axial ligand increasing the population of the $\pi^*$ antibonding orbitals and weakening the porphyrin bond strengths. The mercaptide sulfur of cysteine is a likely candidate for this role. Thus it appears that marker band (I) is not only sensitive to the formal oxidation state of the iron atom but also to the electronic properties of the axial ligand.

The binding of carbon monoxide to reduced chloroperoxidase causes a shift of band I back near the oxidized position (1369 $cm^{-1}$). This indicates that electron density is removed from the antibonding orbitals and shuttled (presumably) onto the bound CO molecule since the iron must remain in the $Fe^{2+}$ state as required by Mössbauer data (Champion et al., 1973). A similar phenomenon has been observed in cytochrome

P-450$_{cam}$. This transfer of excess electron density from porphyrin $\pi^*$ orbitals to the bound ligand could relate to the binding and activation of oxygen by these enzymes.

The marker bands (II and V) observed with Soret excitation can be used as indicators of spin state. Chloroperoxidase is an especially good example to investigate because it undergoes a spin transition as a function of temperature. The native enzyme, at room temperature, contains a small fraction of low spin heme that becomes the dominant species at low temperatures. In Figures 2 and 6, the small peak near 1503 cm$^{-1}$ that appears as a shoulder to the high-spin marker band II located at 1490 cm$^{-1}$ is evidence of this small low-spin fraction. As ethylene glycol is added and the temperature is lowered, a spin transition takes place (Figure 5); at 77 K chloroperoxidase is completely converted into a low-spin ferric form. This spin transition is in agreement with observations using Mössbauer and electron paramagnetic resonance (Champion et al., 1973) and optical spectroscopies (Debey & Balny, unpublished results).

The complexation of chloroperoxidase to small molecules such as cyanide, fluoride, chloride, or hydroxide also appears to affect the spin-state equilibrium. The strongly binding cyanide ligand achieves a complete conversion to low spin as evidenced by the strong peaks at 1503 cm$^{-1}$ (band II) and 1633 cm$^{-1}$ (band V) in Figures 6 and 7. Addition of the halide ligands does not drastically alter the spectral features of the native enzyme[2] (Figure 6a), although a slight shift in the relative intensities of the 1490- and 1502-cm$^{-1}$ bands can be discerned (Figures 6c and 6d). The P-420 form of the enzyme, made from treatment with NaOH, shows a distinct decrease in the high-spin/low-spin ratio as determined from the relative intensities of these bands.

*Comparison of Soret-Excited Heme Protein Spectra.* One of the most striking qualitative similarities in the Raman spectra of the heme proteins studied is found in Figure 12B. The reduced forms of chloroperoxidase and cytochrome P-450$_{cam}$ display intensity patterns and polarization properties that are quite distinct from the reduced forms of horseradish peroxidase or cytochrome *c*. The correlation between the spectra of reduced chloroperoxidase and cytochrome P-450 is not perfect, however. For example, tentative assignments of the marker band V positions in these two compounds differ by ca. 10 cm$^{-1}$. This difference may be due to the difficulty of resolving band V using Soret excitation. No visibly excited Raman spectra of reduced P-450 have been obtained, and the Soret-excited spectra contain a variety of depolarized or nearly depolarized modes in the 1500–1700-cm$^{-1}$ region.

Mössbauer studies of the reduced forms of these enzymes at low temperatures also reveal unique similarities between chloroperoxidase and cytochrome P-450 (Champion et al., 1975). The central iron atom of both proteins appears to reside in a very low symmetry ligand field environment. If this is a reflection of lowered porphyrin symmetry, it may account for the relatively large values of the depolarization ratio, $\rho_l$, found for some of the Raman vibrational modes ($\rho_l$ = 0.5 for the polarized 1348-cm$^{-1}$ mode). The size of the depolarization ratio by itself would indicate that the heme symmetry had been reduced by the protein environment to $D_{2h}$ or lower. However, $\rho_l$ also shows dispersion as a function of the exciting laser frequency. This is reminiscent of an effect observed previously in reduced cytochrome *c* (Collins et al., 1973). In that case, the dispersion was adequately described as due to a perturbation from $D_{4h}$ symmetry resulting in the mixing of the $D_{4h}$ basis states (namely, $A_{1g}$, $B_{1g}$, $A_{2g}$, $B_{2g}$).

Comparison of spectra of the oxidized proteins in Figure 12A shows interesting similarities between P-450 and chloroperoxidase, although some minor differences are apparent, most notably at ca. 1600 cm$^{-1}$ and in the low frequency region. The marker band frequencies, however, are distinctly different from those of horseradish peroxidase and, in conjunction with other physicochemical evidence (Champion et al., 1973; Gunsalus et al., 1974; Champion et al., 1975; Hanson et al., 1976; Dawson et al., 1976), suggest that these two proteins have similar active site structure and share a common electron-donating axial ligand (the mercaptide sulfur of cysteine).

Comparison of Soret excited Raman spectra of heme proteins in the low-spin ferrous state shows dramatic differences between ferrocytochrome *c* and reduced chloroperoxidase cyanide (Figure 9). This is probably a reflection of the differing axial ligands and heme group coordination in the two complexes. The unusually intense low frequency modes and the complex structure found in the cytochrome *c* spectrum suggest that a wealth of information may be extracted from this region of the Raman spectrum by using tunable ultraviolet laser excitation (P. M. Champion and A. C. Albrecht, unpublished experiments).

Acknowledgments

The authors are very grateful to Professor A. Lewis for allowing the use of his Raman apparatus for part of this work. We thank Spectra Physics, Inc., for the generous loan of a Spectra Physics Model 171 krypton ion laser. We wish to thank Cynthia Wolberger very much for taking the ferrocytochrome *c* Raman spectrum. The use of Material Science Center facilities at Cornell University is also acknowledged.

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# Proton Coupling in the Cytochrome P-450 Spin and Redox Equilibria†

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ABSTRACT: Ferric bacterial cytochrome P-450 is known to exist as a mixture of high-spin ($S = 5/2$) and low-spin ($S = 1/2$) configurations of the heme iron d-shell electrons. This equilibrium between the two forms of P-450 has been shown to control both substrate affinity and the observed redox potential. Room temperature binding of a solvent-exchangeable proton to high-spin, substrate-free P-450 occurs with p$K$ = 6.5, while camphor association shifts this equilibrium to a p$K$ of 5.8. No $H^+$ ligation to the low-spin forms of P-450 is observed. Proton coupling is also observed in the reduction of camphor-bound, high-spin ferric P-450. Analysis of these structures by a four-state, free-energy coupling model yields p$K$s of 5.8 and 8.0 for $H^+$ binding respectively to $Fe^{3+}$ and $Fe^{2+}$ P-450 and inherent electrode potentials of −106 and −238 mV for the oxidation/reduction of protonated and unprotonated protein. Analysis of the free-energy interaction diagram yields the standard state potential $E^{\circ\prime} = -173$ mV at pH 7 for camphor-bound material. Proton modulation of the putidaredoxin–cytochrome P-450 electron transfer in a stabilized dienzyme complex is quantitated by stopped-flow spectrophotometry. Protonation of the multiprotein complex is seen to occur on cytochrome reduction–redoxin oxidation with p$K$ = 5.8, indicating that, at pH 7, there is little equilibration with solvent protons. The possibility of concerted $H^+/e^-$ transfer during P-450-catalyzed, mixed function oxidation is discussed.

Cytochrome P-450 is the terminal mixed function oxidase of a short flavoprotein and iron–sulfur protein electron transport chain linking pyridine nucleotide oxidation, with $O_2$ reduction and concomitant substrate oxygenation through the production of water and hydroxylated substrate. Enzymatic hydroxylations are found ubiquitously in living species as a means of xenobiotic and steroid metabolism and exhibit varying degrees of substrate specificity. The most commonly studied hydroxylases are the hepatic microsomal P-450 responsible for general detoxification and hydrocarbon solubilizations (Orrenius & Ernster, 1974), the adrendal mitochondrial enzymes active in the conversion of cholesterol to the various steroid hormones (Hamberg et al., 1974), and the camphor 5-*exo*-hydroxylase from the soil bacterium *Pseudomonas putida* (Gunsalus et al., 1975). This last system, discovered and investigated in detail by the Gunsalus laboratory (Hedegaard & Gunsalus, 1965), offers the outstanding advantages of high purity and yield necessary for precise, quantitative biochemistry, and will be the enzyme used in the investigations reported herein.

The two central areas of P-450 mechanism which have been under active investigation in our laboratories center on the chemistry of $O_2$ bond cleavage/substrate oxygenation and the physical biochemistry of the redox transfer events leading from flavoprotein dehydrogenation, through the iron–sulfur protein, to the cytochrome. This area provides a molecular description of the regulation and control of redox equilibria in cytochrome P-450 through substrate ligation (Sligar & Gunsalus, 1976) and the interconversion of the $d^5$-ferric heme iron from a low-spin ($S = 1/2$) to high-spin ($S = 5/2$) configuration (Sligar, 1976). This communication adds to the interacting equilibria the binding of protons to the cytochrome to illustrate an ionic control of spin and substrate binding, thus suggesting a possible concerted electron/proton transfer event within the

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*Received August 17, 1978; revised manuscript received March 2, 1979.* Supported by grants from the National Institutes of Health (GM 24976, GM 18051) and the Biomedical Research Support (RR-07015).

0006-2960/79/0418-2290$01.00/0 