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Resonance Raman Spectroscopy of Soybean Peroxidase

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Resonance Raman spectra (600–1700 cm^{-1}) for the heme enzyme soybean peroxidase (R_Z = 2.5) were obtained using Soret band excitation at 406.7 nm. The vibrational frequencies and depolarization data indicate a strong similarity between the active sites of soybean and horseradish peroxidase. This similarity suggests that the active site in the resting form of soybean peroxidase contains a ferric iron, is a high-spin 5-coordinate heme binding His as a fifth axial ligand. © 1997 Academic Press

Peroxidases represent an important class of heme-containing enzymes that catalyze the oxidation of a wide range of organic substrates using hydrogen peroxide. (1, 2) Recently, a peroxidase, soybean peroxidase (SBP), isolated from the seed hulls of soybeans has attracted a great deal of attention. (3) The 37kD protein is acidic (pI 4.1) and appears to contain one predominant isozyme. (3) The enzyme possesses a wide substate specificity and has been shown to catalyze the oxidation of nonphenolic compounds such as veratryl alcohol (3,4-dimethoxybenzyl alcohol; $E_{1/2}$ = 1.5 V) to veratraldehyde in the presence of hydrogen peroxide and 2 mM $CaCl_2$ at pH 2.4. (4) Reaction kinetics for this reaction at low peroxide concentrations (< 1mM) follow a ping-pong bi-bi mechanism. (4) SBP is a very potent oxidant with an apparent oxidation potential of 1.42 V. (4) Soybean peroxidase shows unusual thermal stability exhibiting a melting temperature of 90.5°C at pH 8.0 in the presence of 1 mM $CaCl_2$. (5) Clearly, soybean peroxidase shows particular promise as a biocatalyst. Thus, it was surprising to us to discover that no spectroscopic structural information has been reported for this enzyme to date.

Resonance Raman spectroscopy has become an invaluable means of characterizing the active site structure of heme proteins. (6) Spectral frequencies in the 900–1700 cm^{-1} range yield information on the iron oxidation state, spin state, and heme core size. Resonance Raman spectra have been reported for a variety of peroxidases, peroxidase-complexes, and peroxidase intermediates. (7-20) In view of the demonstrated promise of soybean peroxidase as an emergent biocatalyst, the lack of available structure information on SBP reported to date, and the continued interest in the physicochemical characterization of peroxidases, we wish to report the first fruits of our vibrational study of SBP.

MATERIALS AND METHODS

Soybean peroxidase (R_Z 2.5) was a gift from Enzymol, International (Ohio). SBP samples were prepared for vibrational study by adding 0.1 M sodium phosphate buffer, pH 6.0, to soybean peroxidase powder to produce a final concentration of 0.68 mM. Optical spectra were obtained at room temperature on a Hewlett-Packard 8452A diode array UV-vis spectrometer using 1.0 cm pathlength rectangular supracil cuvettes (Hellma).

Raman spectra were run at room temperature on a home-built instrument: The system is based on a Coherent INNOVA 302 Krypton ion laser (6 W all lines) with UV-option, spatial filter assembly, a SPEX triplemate spectrometer (0.6 m, f/6.3), a Vivitar 58-mm 85-205 zoom camera lens, and a liquid nitrogen-cooled Princeton Instruments CCD detector. A pentium personal computer controls the data acquisition. An uncoated broadband depolarizer (Optics for Research) was used in all measurements. Polarization measurements were made with a polaroid analyzer placed in the beam after the sample before the entrance slit. Samples contained in 3-mm i.d. quartz tubes were typically excited with 120 mW of 406.7 nm light. The resolution of the spectrometer is ca. 3 cm^{-1} at the excitation wavelength used here. Fenchone was used as an external calibrant for Raman frequencies. (21) Carbon tetrachloride was used to calibrate the polarization measurements. All observed intensities are relative and not true intensities. Reported intensities have not been corrected for the spectral sensitivity of the instrument. All frequency and polarization data represent the average of at least three replicate analyses.

RESULTS AND DISCUSSION

Soybean peroxidase exhibited good thermostability and photostability upon exposure to 406.7 nm laser light. We found that the SBP sample could be maintained in the beam for up to 30 min without detectable changes in either the observed vibrational frequencies or in observed intensities. Figure 1 shows the quality of data we obtained for soybean peroxidase with 406.7 nm excitation. Excitation at this wavelength, slightly

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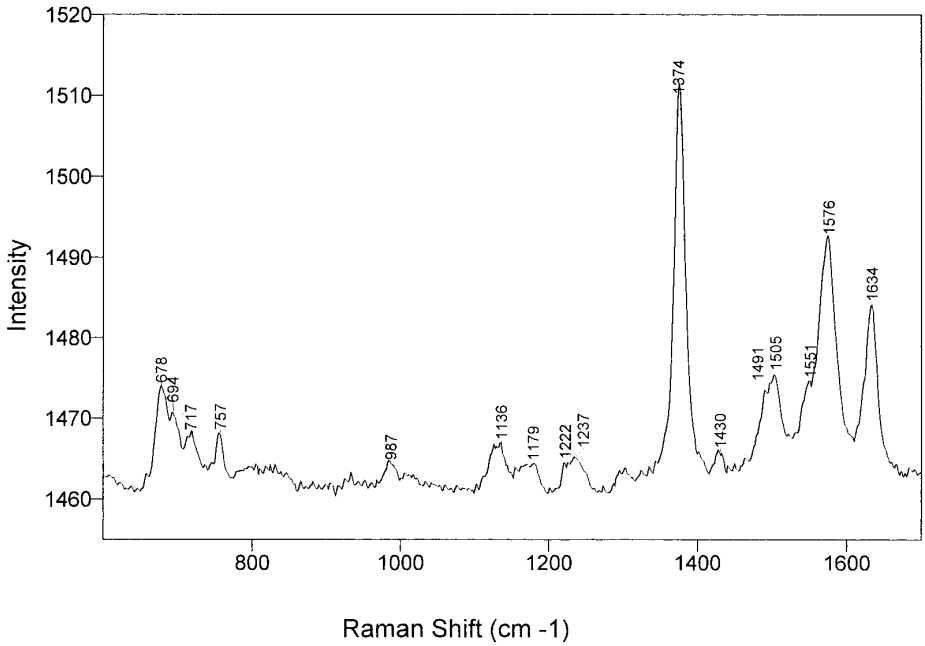

FIG. 1. Resonance Raman spectrum for 0.68 mM SBP in 0.1 M sodium phosphate buffer, pH 6.0. Spectral conditions: wavelength, 406.7 nm; power at sample, ≈120 mW at sample; integration time, 2 s/scan. The spectra are composites of 60 scans.

to the red of the Soret band which peaks at 402 nm, was expected to result in A term scattering. Sixteen characteristic vibrational features can be observed in the spectral range between 600 and 1700 cm^{-1}. As expected the in-plane C-C and C-N stretching vibrations of the porphyrin represent the dominant features of the vibrational spectrum of SBP with Soret band excitation. The symmetry of the vibrational modes in the resonance Raman spectrum were determined by measuring the depolarization ratio, ρ, in order to assign the vibrational modes. Figure 2 shows typical spectra for SBP in parallel and perpendicular polarization. Fre-

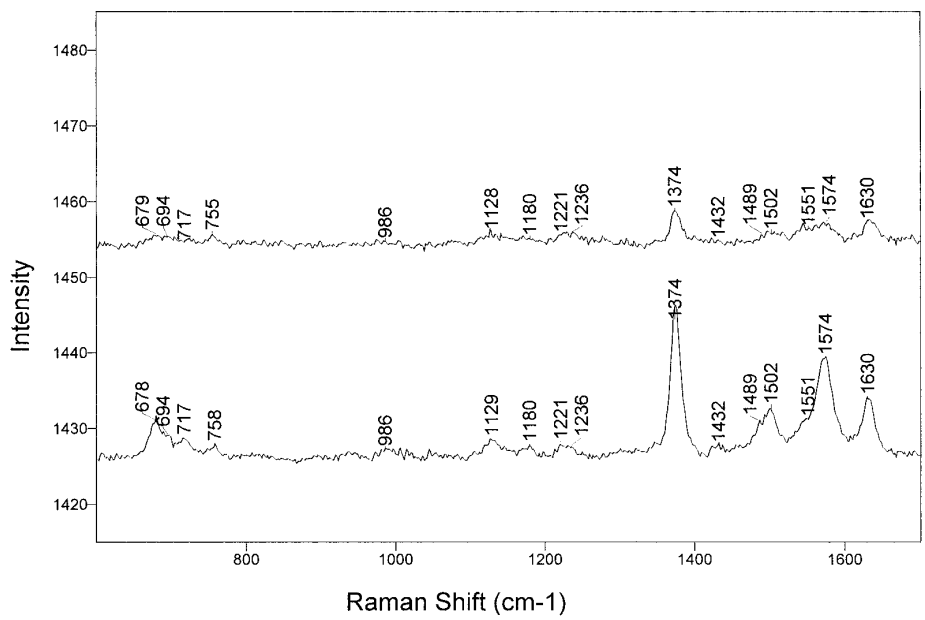

FIG. 2. Perpendicular (top) and parallel (bottom) polarized resonance Raman spectra for 0.68 mM SBP in 0.1 M sodium phosphate buffer, pH 6.0. Spectral conditions: wavelength, 406.7 nm; power at sample, ≈120 mW at sample; integration time, 2 s/scan. The spectra are composites of 60 scans.

TABLE 1

Vibrational Frequencies, Polarization Data, and Vibrational Assignments for SBP

Mode	Symmetry	SBP average relative intensity (sd)	SBP (cm^{-1})	SBP ρ (sd)	SBP polarization assignment	HRP-C[a] (cm^{-1})	HRP polarization assignment[a]	HRP[b] (cm^{-1})	HRP polarization assignment[b]
ν_{10}		0.43 (0.01)	1633	0.6 (0.1)	p	1630	p	1631	dp
ν_{19}	A_{2g}	0.60 (0.02)	1574	0.33 (0.1)	p	1574	ip	1575	p
ν_{11}	B_{1g}	0.24 (0.01)	1550	0.65 (0.1)	dp	1553	dp	1550	dp
ν_3	A_{1g}	0.25 (0.02)	1492	0.4 (0.2)	p	1499	p	1500	p
δ (=CH_2)		0.08 (0.02)	1428	0.78 (0.1)	dp	1427	p	1430	dp
ν_4	A_{1g}	1.00 (0.01)	1376	0.24 (0.1)	p			1372	p

[a] Smulevich, G., Paoli, M., Burke, J. F., Sanders, S. A., Thornely, R. N. F., Smith, A. T. 1994, *Biochemistry* **33,** 7399–7407.
[b] Palaniappan, V., Terner, J. 1989, *J. Biol. Chem.* **264,** 16046–16053.

quency and polarization data and the resulting vibrational assignments are summarized in Table 1.

The frequencies and depolarization data for the marker bands in the 1000–1700 cm^{-1} spectral range are similar to those reported recently for horseradish peroxidase isozyme C (HRP-C). For example, the frequency of ν_4, the so-called oxidation state marker band, is consistent with Fe^{3+}. The frequencies of ν_{10}, ν_{11}, and ν_{19}, the core size marker bands, are consistent with a high-spin, 5-coordinate heme system. On the basis of these data, we conclude that SBP has a 5-coordinate, high spin, ferric active site with axial His ligation that is similar to that of HRP-C.

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