DETERMINATION OF OXIDATION AND SPIN STATES OF HEME IRON. RESONANCE RAMAN SPECTROSCOPY OF CYTOCHROME c, MICROPEROXIDASE, AND HORSERADISH PEROXIDASE.

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SUMMARY: Resonance Raman spectra of cytochrome c in solution are compared with those of lyophilized cytochrome c, a heme undecapeptide from cytochrome c, and horseradish peroxidase. Spectral lines sensitive to iron oxidation state and spin state are identified. The spectra are not noticeably influenced by the protein environment of the heme chromophore.

INTRODUCTION: Resonance Raman spectroscopy is proving to be useful in determining the nature of metal-ligand bonding in iron-containing proteins. In an application of this technique to the nonheme-iron protein, rubredoxin, it was found that the iron atom in the oxidized form was tetrahedrally coordinated by sulfurs in both solution and solid states (1,2). Studies of heme-iron proteins indicate that certain Raman bands are sensitive to the valence and spin state of the iron (3). We wish to corroborate and extend these heme-protein correlations with various preparations of cytochrome c, cytochrome c analogs, and horseradish peroxidase.

MATERIALS AND METHODS: Cytochrome c (Type III), microperoxidase, and horseradish peroxidase (Type VI) were obtained from Sigma. Phosphate buffer (0.1 M) of pH 7.3 was used throughout. Reductions and oxidations were performed with sodium dithionite and potassium ferricyanide, respectively.

Oxidation states were verified by visible absorption spectroscopy. The spin state of cytochrome c in acid (pH 1) was verified by electron spin resonance using a polyethylene sample tube. Solid samples of oxidized and reduced cytochrome c were prepared by lyophilization of the protein after passage through a Sephadex column to remove oxidant or reductant.

Raman spectra were recorded using a Jarrell-Ash 25-300 spectrophotometer with the 5145 Å line of an argon ion laser (Coherent Radiation). Laser power was attenuated by a 10 Å spike filter and N. D. filters in order to obtain less than 100 mw at the sample. Liquid samples were placed in 1.6 mm glass capillaries and mounted perpendicular to the incident beam. The direction of polarization of the incident laser light was fixed by a halfwave plate. Spectra were generally recorded at 20 cm^{-1}/min with a 2 sec time constant, a 7 cm^{-1} slit and a sensitivity of 5 x 10^3 photon counts/sec. Tabulated frequencies are accurate to ± 3 cm^{-1}.

RESULTS AND DISCUSSION: Resonance Raman spectra of oxidized and reduced cytochrome c in solution have been reported (4, 5, 6). Yamamoto et al. (3) have correlated the spectral band at 1584 cm^{-1} with the existence of low-spin iron in both oxidation states and the position of a spectral band at ~1375 cm^{-1} or ~1360 cm^{-1} with oxidized and reduced cytochrome c, respectively.

We have noted that several other Raman lines are also sensitive to changes in the oxidation state of iron in cytochrome c. Table 1 shows that reduction of cytochrome c causes frequency shifts from 1640 to 1622 cm^{-1}, from 1566 to 1551 cm^{-1}, and from 1409 to 1401 cm^{-1}. These bands in the 1650 to 1400 cm^{-1} region are more sensitive to Ar^+ laser excitation in the α or β band than the excitation in the Soret band used by Yamamoto et al. (3). The general shift towards lower frequency upon reduction of Fe^{3+} to Fe^{2+}

Table 1: Raman Spectral Band Patterns in Hemoproteins

Frequency, cm^{-1}

	1640/1622/1610	1589/1575	1566/1551[a]	1494/1476	1409/1401	1375/1367/1359
Low-spin Fe(III)						
Ferricyt c solution	+	+	+		+	+
Ferricyt c solid	+	+	+		+	+
Ferrimicroperoxidase	+	+	+		+	+
High-spin Fe(III)						
Ferricyt c, pH 1	+	+	+		+	+
Low-spin Fe(II)						
Ferrocyt c solution	+	+	+	+	+	+
Ferrocyt c solid	+	+	+	+	+	+
Ferromicroperoxidase	+	+	+	+	+	+
Ferro hr peroxidase-CN[b]		+	+	+	+	+
High-spin Fe(II)						
Ferro hr peroxidase-H_2O[b]	+	+	+	+		+

[a] observed at 1538 cm^{-1} in ferromicroperoxidase and ferro hr proxidase-CN complex

[b] hr peroxidase = horseradish peroxidase

also appears to apply to the shift from 1500 to 1490 cm^{-1} which is clearer in the He-Cd laser excited Raman spectrum (4) than in our own.

The vibrational bands in the 1650 to 1400 cm^{-1} region of Raman and infrared spectra of metalloporphyrins are assigned to skeletal vibrations of conjugated bonds in the macrocycle (7), and they change in intensity and frequency upon metal ion substitution (7, 8). Thus, it is reasonable that the Raman bands in this region of the cytochrome c spectrum are sensitive to electronic changes in the iron-porphyrin moiety.

Chemical modifications of heme proteins resulting in the conversion of iron from a low- to a high-spin state have been correlated with a 1584 to 1566 cm^{-1} frequency shift, which is ascribed to the movement of the iron atom out of the heme plane upon increasing its spin state (3, 9). We find that several other bands are dependent on spin state in addition to oxidation state. Reduced horseradish peroxidase (cyano complex) shows a spectral band pattern (Table 1) identical to that of reduced cytochrome c and, presumably, typical of low-spin ferrous hemoproteins. In the absence of strong-field axial ligands, however, reduced horseradish peroxidase (aquo complex) has high spin iron and there is a consequent shift of the 1622 cm^{-1} band to 1610 cm^{-1} , the 1494 band to 1476 cm^{-1}, and the 1367 band to 1359 cm^{-1}.

Cytochrome c in the solid state has a Raman band pattern identical to that of the protein in solution (Table 1) with small differences in spectral intensities (Figure 1) being due to polarization scrambling from the solid. Raman spectra of the solid show no evidence for high spin iron, although a $g = 6$ signal has been detected in the low temperature electron spin resonance spectrum of lyophilized ferricytochrome c (10). Treatment of ferricytochrome c with acid produces a substantial amount of high-spin iron (11) which can be detected by an increase in the Raman intensities at 1622 cm^{-1} and 1575 cm^{-1}.

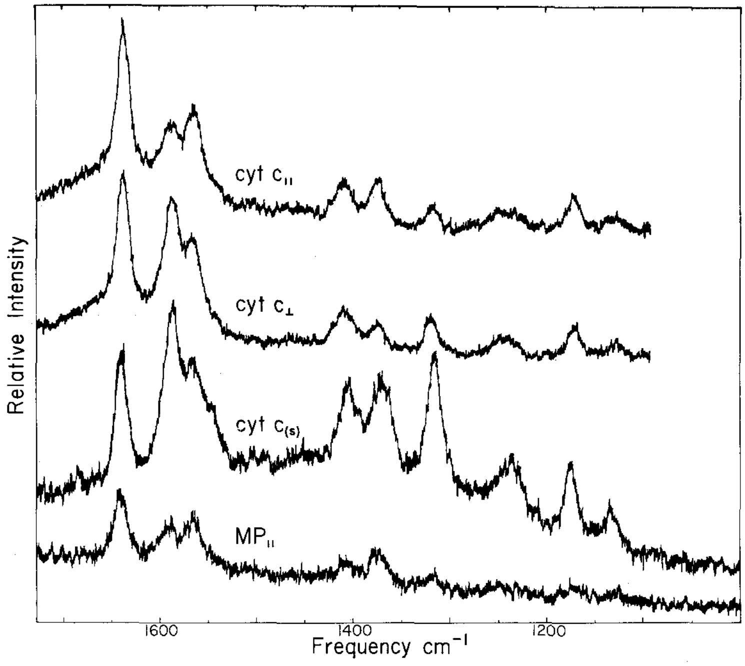

FIGURE 1: Resonance Raman spectra of ferricytochrome c in solution (cyt $c_{\parallel}$ and cyt $c_{\perp}$) and solid (cyt $c_{(s)}$) and ferrimicroperoxidase ($MP_{\parallel}$) using 5145 Å excitation.

Solutions: cytochrome c, 0.14mM, and microperoxidase, 0.1 mM, in 0.1M phosphate buffer, pH 7.3.

Solid: 5% cytochrome c in finely gound KBr; rotating sample (3600 rpm); 20° angle of inclination to incident beam. The symbols, $\parallel$ and $\perp$, have their usual meanings with regard to the direction of polarization of incident and scattered light.

From this we conclude that considerably less high-spin iron is present in the solid form than in the acid form and that the heme-iron structure in the solid is the same as in solution.

Microperoxidase, a heme undecapeptide obtained by proteolytic degradation of cytochrome c, has been characterized as a low-spin iron complex with histidine 18 and NH_2-terminal valine as axial ligands (12). Raman spectra of microperoxidase (Table 1 and Figure 1) verify that it contains low-spin iron in both oxidation states. Replacement of the axial ligands by cyanide causes no change in the microperoxidase spectra, which are essentially indistinguishable from those of cytochrome c. Thus, while the Raman spectra of hemochrome c-containing molecules are sensitive to iron oxidation state and spin state, they are insensitive to changes of axial ligands which do not affect spin state. Furthermore, the Raman spectra do not reflect changes in the protein environment around the porphyrin.

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