Resonance Raman Spectra of Heme Proteins at Low Temperature

Sir:

There has been much interest recently in the resonance Raman scattering of heme proteins.[1-4] Using argon and tunable dye lasers to excite in the region of the α and β absorption, the Raman scattering from vibrational modes involving the heme group is strongly enhanced. Strong signals can be obtained from concentrations of order 10^{-4} M in aqueous solution. The vibrational frequencies have been shown to be sensitive to spin and oxidation state and to ligand and peripheral substituents. The rapid development of this new spectroscopic technique for heme proteins has been reviewed by Spiro.[5]

In the work done so far, most of the Raman spectra have been measured for heme proteins in liquid solution at or near room temperature. On the other hand, several spectroscopic techniques, among them electron spin resonance and Mössbauer spectroscopy, are effective only at low temperature and must therefore operate with frozen solutions. In this note, we wish to report Raman spectra of ferrocytochrome *c* and oxyhemoglobin measured at low temperatures in optically clear aqueous/ethylene glycol (40/60) solutions. The most important findings are that the Raman vibrational frequencies are unchanged upon freezing and that, in the case of cytochrome *c*, resonance effects are more pronounced as the absorption structure becomes better resolved at lower temperatures.

Samples of horse heart cytochrome *c* were obtained from Sigma Chemical Company (Type VI). Reduction was accomplished with an excess of sodium dithionite under anaerobic conditions. The oxyhemoglobin sample was prepared from lysed human red blood cells and was a gift from the laboratory of Professor Q. Gibson.

The sample cell was a standard 1-mm path length quartz absorption cell, cut down to a height of ~2 cm. The cell was spring-mounted against the conduction tail of a variable temperature optical cryostat and the open top of the cell was vacuum sealed using an indium gasket and brass cap. The cell mount was thermally coupled, via an exchange gas, to the helium or nitrogen reservoir of the cryostat. The temperature was monitored with a Keystone Carbon Company thermistor. Samples were cooled over several hours and clear glasses were produced that had relatively large (several square millimeters) areas free of cracks. All optical measurements were performed in such regions.

Raman and absorption spectra were both measured with a Spex 1401 double monochromator and photon counting detection. For the former, CW argon or tunable dye lasers were used in a back-scattering geometry. For the latter, a weak white light source (tungsten lamp) was used in a single-beam transmission geometry.

Figure 1 shows the optical absorption spectra of a ferrocytochrome *c* sample measured at 280 and at 68 K. The low-temperature spectrum shows a splitting of the α band (Q_{00} transitions) and a partially resolved vibronic structure in the β band (Q_{01} transitions) observed earlier by Elliott and Margoliash[6] and more recently by Wagner and Kassner.[7] Measurements made at temperatures above 68 and at 4.2 K indicate that little further improvement in resolution takes place below liquid nitrogen temperature.

Figure 2 shows the Raman spectra of ferrocytochrome *c* excited with the 5145-Å argon laser line at 280 and 68 K. The signal-to-noise ratio is clearly better at low temperature. The low temperature spectra show polarization effects at least as pronounced as those at room temperature, and there is no indication of any polarization scrambling. The extra features at 862 and 1089 cm^{-1} in the low temperature spectrum arise from the glassy solvent, ethylene glycol. As shown in Figure 3, some

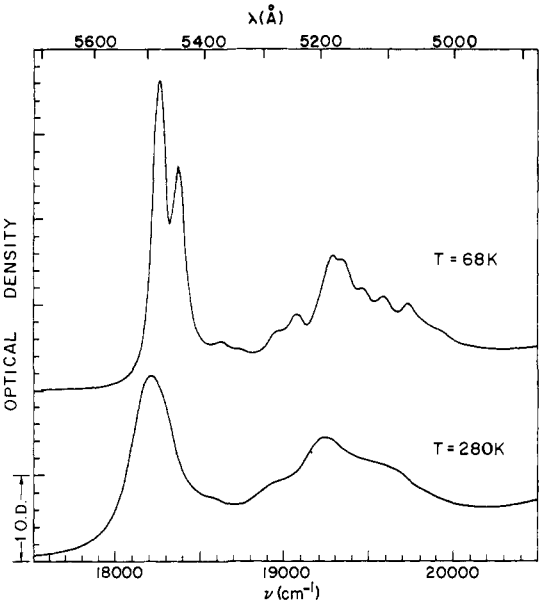

Figure 1. The optical absorption spectra of ferrocytochrome *c* taken at 68 and 280 K.

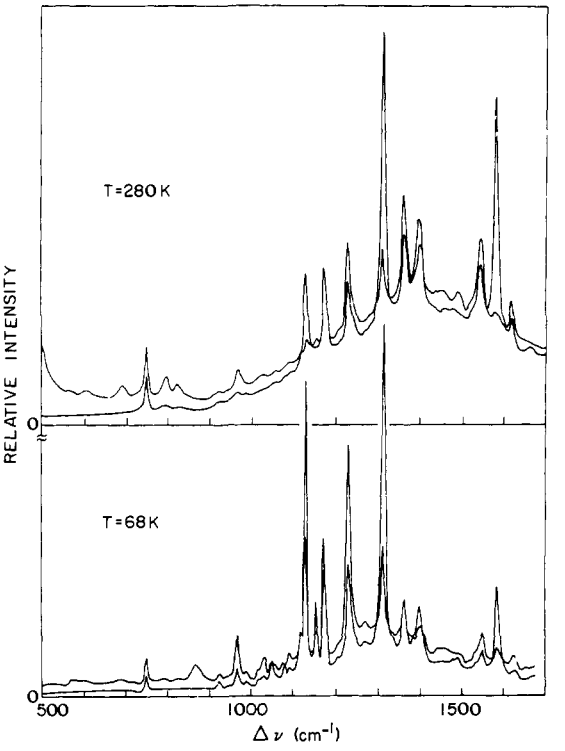

Figure 2. The resonance Raman spectra of ferrocytochrome *c* taken with 15 mW of 5145 Å laser excitation. these spectra were obtained from the same sample as the absorption spectra in Figure 1. In each pair of spectra the upper curve corresponds to parallel polarized scattering and the lower curve to perpendicular polarized scattering, except at the anomalously polarized peaks (1132, 1150, 1310, 1397, 1585 cm^{-1}) where perpendicularly polarized scattering becomes dominant. The differences in peak intensities are attributed to narrowing and shifting of the Q_{01} absorption bands at low temperature which results in a more selective mode enhancement.

of the Raman lines are narrower at 68 K than at 280 K. A detailed comparison of peak positions indicates that in no case do the positions at room and low temperature differ by more than the instrumental uncertainty of about 2 cm^{-1}.

Figure 4 shows unpolarized Raman spectra of oxyhemoglobin taken at 110 and 280 K using 5817-Å excitation (near the α band) from a Rhodamine 6G tunable dye laser. Although

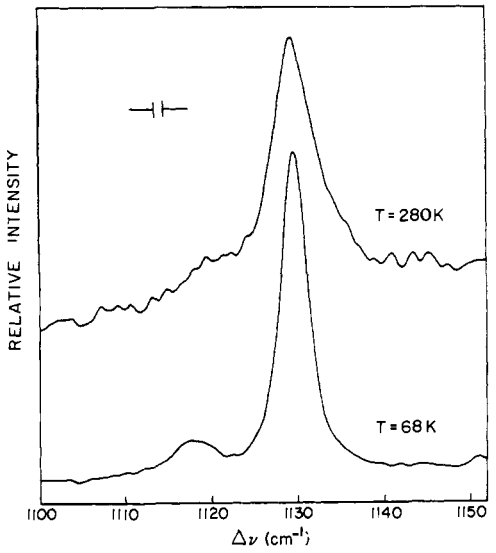

Figure 3. High resolution (1 cm^{-1}) unpolarized Raman spectra of the 1130-cm^{-1} line of ferrocytochrome *c*.

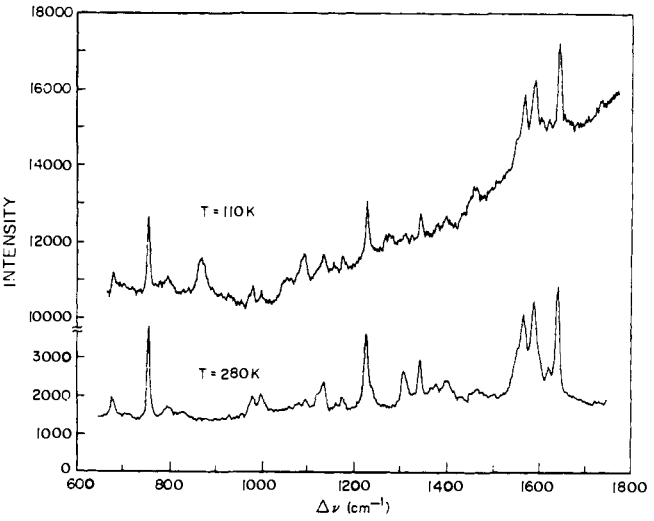

Figure 4. Raman spectra (unpolarized) of oxyhemoglobin using 5817-Å laser excitation at 15-mW incident power. The excitation frequency is quite close to the α band, which shows no sign of splitting but does shift from 5775 to 5720 Å as the temperature is lowered to 110 K. The extra features in the low temperature run are due primarily to the glassy solvent, ethylene glycol. Intensity differences are due to the shift of the Q_{00} band.

the signal to noise is decreased in this low temperature measurement due to increased fluorescent emission, the Raman peak positions can still be determined accurately and compared with the spectrum of oxyhemoglobin in the liquid state. The data show that, as with ferrocytochrome *c*, there is no apparent shift of line positions as the sample is frozen. This is strong evidence that the heme group is perturbed very little, if at all, in going from liquid to solid solutions.[8] This result should be quite encouraging to those who routinely perform spectroscopic measurements at cryogenic temperatures with little knowledge of the effects on the heme site produced by the liquid-to-solid transition.

One of the most interesting aspects of resonance Raman scattering in heme proteins is the strong variation in intensities and polarization properties as the laser frequency is scanned through the absorption bands. A rise in the scattering cross section occurs when either the incident or scattered photon is in resonance with the Q_{00} transition. As the transition sharpens up at low temperature, this dispersion in the scattering efficiency becomes more pronounced and more abrupt. This is the principal explanation for the changes in Figure 2. The depolarization ratio, $\rho_l = I_\perp/I$, also varies with laser frequency for several Raman bands. This dispersion has been noted by several workers and is not well understood. For at least some of the bands, it appears to reflect interferences between Q_{00} and Q_{01} resonances, and between nondegenerate Q_{00x} and Q_{00y} resonances. The low temperature spectra for cytochrome *c* show both these effects. In particular, there is a very pronounced dip in ρ_l near the α band for the 1585-cm^{-1} mode. This dip is in quantitative agreement with our earlier predictions.[9] It becomes more pronounced as the widths of the α band components decrease relative to their splitting (cf. Figure 1).

A more complete account of the low temperature Raman spectra of several cytochromes will be published elsewhere. In this report, we merely wish to point out that excellent Raman spectra can be obtained at low temperature, that the dispersion effects near resonance are more pronounced at low temperature, and that the heme group vibrational frequencies are not detectably modified upon freezing.

References and Notes

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Allylic Cyanobis(methylthio)methylation. Insertion of a Functionalized Carbon in an Allylic Carbon–Hydrogen Bond

Sir:

A procedure that would allow the unambiguous insertion of a functionalized carbon atom into an allylic carbon–hydrogen bond would be of great synthetic value. At present procedures exist which lack regiospecificity since an allylic carbanion, carbonium ion or radical is an intermediate.[1] A method that proceeds by an ene reaction[2] followed by a [2,3] sigmatropic rearrangement as does the selenium dioxide oxidation of olefins[3,4] would allow an unambiguous functionalization of an alkene without double bond migration. Since allylic sulfides are known to undergo [2,3]-sigmatropic rearrangements readily on deprotonation,[5] our goal was to convert an alkene to an allylic sulfide by an ene reaction. Hexafluorothioacetone reacts readily with a variety of alkenes at −78 °C to give ene adducts which are allylic sulfides.[6] Unfortunately, these are of limited synthetic value. Methyl cyanodithioformate (**1**) is readily available[7] and is known to function as a dienophile in Diels–Alder reactions.[8] Furthermore, the expected ene adduct **2** should possess a relatively acidic hydrogen which could be easily abstracted to give an anion which