Resonance Raman Study of Mollusc and Arthropod Hemocyanins Using Ultraviolet Excitation: Copper Environment and Subunit Inhomogeneity

Sir:

Resonance Raman spectroscopy has been shown to be a sensitive structural probe for biomacromolecules with intense chromophores.[1] While most studies have involved excitation in visible absorption bands, the availability of laser lines at 363.8 and 351.1 nm from high-powered argon lasers permits extension of the technique to near-UV chromophores. An attractive candidate is the oxygen binding protein of molluscs and arthropods, hemocyanin.[2,3] The oxy form has an intense absorption band at 345 nm (ϵ 10 000 M^{-1} cm^{-1}) as well as weaker bands (ϵ 600 M^{-1} cm^{-1}) in the visible region. Excitation in the latter produces resonance enhancement of a Raman band at 742 cm^{-1}, assignable to O–O stretching on the basis of its $^{18}O_2$ isotope shift.[4,5] The frequency is characteristic of peroxide, and establishes the mode of O_2 binding as $Cu_2^{2+}O_2^{2-}$.

Figure 1 shows Raman spectra obtained with 351.1-nm excitation for oxyhemocyanin from *Busycon canaliculatum* (mollusc) and *Limulus polyphemus* (arthropod). Similar spectra are obtained with 363.8-nm excitation. Several bands are observed, in a frequency region characteristic of metal–ligand vibrations. All are resonance enhanced, as evidenced by their disappearance, along with the 345-nm absorption band, on deoxygenation of the proteins. Depolarization ratios are within experimental error of 0.33, the value expected for resonance enhanced totally symmetric vibrations of a low-symmetry chromophore.[6]

The most intense of these Raman bands, near 280 cm^{-1}, can be observed with visible wavelength excitation, and its excitation profile was shown to be consistent with preresonance enhancement via the 345-nm transition.[5] It was assigned[5] to Cu^{2+}–imidazole stretching,[7] on the basis of vibrational studies of simple Cu^{2+}–imidazole complexes, including metal-isotope shifts.[7] Chemical evidence suggests binding of up to four histidine side-chains to each copper ion,[8] while the EPR spectrum of nitrosyl-hemocyanin implicates two nitrogen ligands per copper ion.[9] The spectra in Figure 1 suggest considerable complexity in the copper coordination groups, and detailed assignments must await further structural characterizations of hemocyanin.

What can be ascertained at present is that these low frequency modes are the only ones in resonance with the 345-nm transition. Apart from a remnant of the 742 cm^{-1} O–O stretching band, no other features could be detected in the Raman spectra. In particular, a careful search failed to show detectable Raman emission associated with imidazole ring modes (1322, 1253, 1165 cm^{-1}). These should be enhanced if the character of the resonant electronic transition is imidazole → Cu^{2+} charge transfer, as has been suggested,[5] since bonding in the imidazole ring would be significantly perturbed in the excited state.[11] Indeed, Yoshida et al.[10] have shown for $Co(imidazole)_2Cl_2$ that these modes undergo substantial preresonance enhancement with visible excitation, presumably via imidazole → Co^{2+} charge-transfer transitions in the ultraviolet region. An analogy is provided by Cu^{2+} transferrin, for which strong resonance scattering from phenolate ring modes is observed on excitation in a phenolate → Cu^{2+} charge transfer band.[13] The lack of UV enhancement of the O–O stretch also weighs against assigning significant O_2^{2-} → Cu(II) charge-transfer character to the resonant transition.[14]

Alternatively, the 345-nm absorption band may be associated with a simultaneous pair excitation (SPE), as has been proposed for the 370-nm band in dimeric cupric acetate.[15,16] The intensity of a Cu^{2+} SPE transition, which involves simultaneous excitation of a "d-d" transition on each of the Cu^{2+} ions by a photon of about twice the energy, depends on the strength of the Cu^{2+}–Cu^{2+} interaction. The Cu^{2+} ions in oxyhemocyanin have been shown to be strongly antiferromagnetically coupled, with an exchange constant in excess of 625 cm^{-1}.[17] Resonance with a SPE transition can be expected to enhance vibrational modes which are localized on the Cu^{2+} pair, since such modes should couple most strongly to a Cu^{2+} centered electronic transition. Thus the observation of enhanced low-frequency modes, with no detectable enhancement of imidazole, or other ligand modes, favors assignment of the 345-nm absorption band to a SPE, rather than a charge-transfer transition.

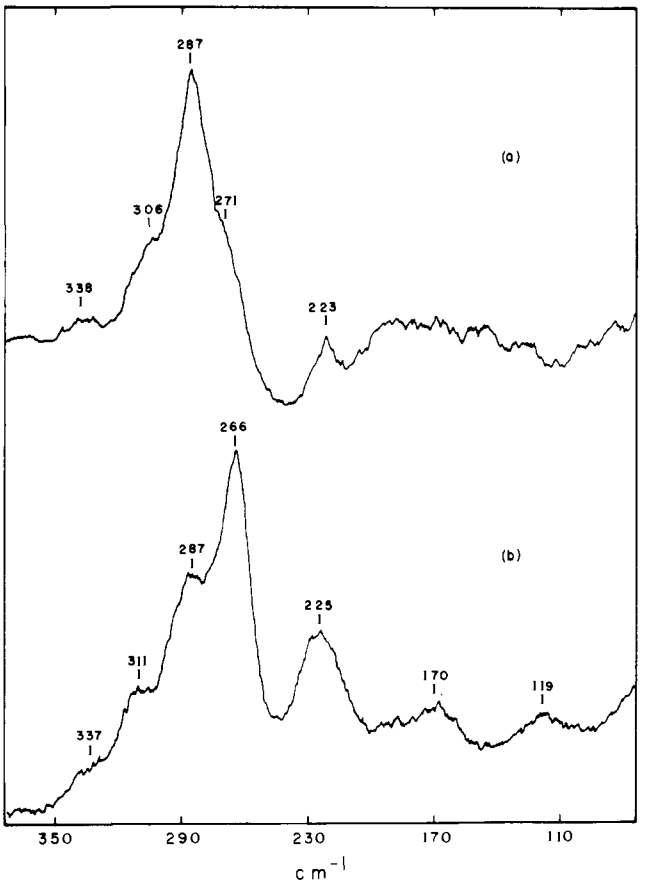

Figure 1. Raman spectra of Limulus (a) and Busycon (b) oxyhemocyanin, recorded with 351.1-nm excitation (150 mW, Spectra Physics 170 Ar^+ laser). Spex 1401 double monochromator with UV optics and grating filter, spectral slit width 7 cm^{-1}; ITT FW 130 photomultiplier with photon counting detection: sensitivity (Hz) 5.0×10^3 (a) and 7.4×10^3 (b); rise time 3 s (a) and 1 s (b). The specimens were obtained from Marine Biological Labs, Woods Hole, Mass., and the hemocyanin was separated from the cardiac hemolymph by standard procedures:[18,21] (a) *Limulus polyphemus*, C_{Cu} = 1.5 mM, pH 7.0 (phosphate buffer); (b) *Busycon canaliculatum*, C_{Cu} = 1.0 mM, pH 9.8 (carbonate buffer).

The two spectra in Figure 1 show appreciable differences. Particularly noteworthy is the marked intensity reversal of the 287 and 266 cm^{-1} features. A change in relative intensities reflects changes in Franck-Condon overlaps of the vibrational modes, between the ground and resonant excited states. The likeliest cause is a change in imidazole coordination geometry for the Cu^{2+} sites between mollusc and arthropod hemocyanin.

Subtler differences are observed among the Raman spectra of the five chromatographic fractions of Limulus hemocyanin, which are shown in Figure 2. The most intense peak (287 cm^{-1}) remains at the same frequency for all five fractions, but the two shoulders at 271 and 306 cm^{-1} which are evident in fraction 1 gradually coalesce with the main peak in fractions

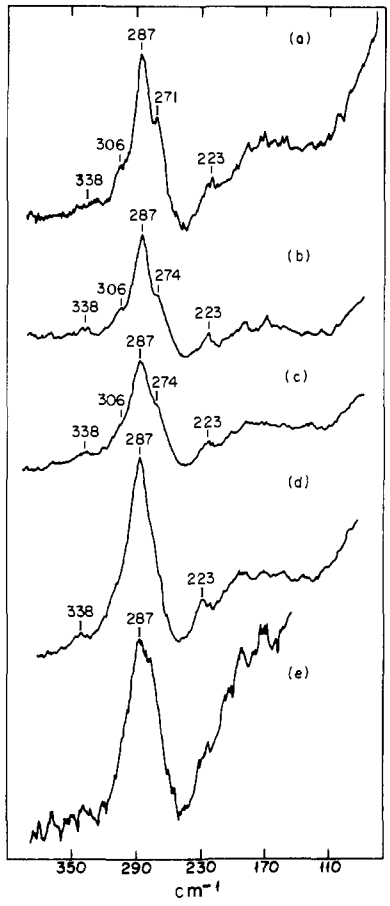

Figure 2. Raman spectra of successive fractions[20-22] of Limulus oxyhemocyanin, eluted from DEAE cellulose with a three-bottle concave gradient of 0-0.5 M NaCl in 0.05 M HN_4HCO_3, 2 mM $MgCl_2$, then concentrated by vacuum dialysis in the cold and dialyzed against distilled water. Their molecular weights (SDS gel electrophoresis) ranged from 70 000 to 78 000 daltons: (a)-(e) Fractions 1-5, C_{Cu} = 0.4, 1.0, 1.0, 1.1, and 0.35 mM, respectively, pH 7.8. Spectrometer conditions as in Figure 1, sensitivity 1.1-2.4 × 10^3 Hz.

2-5. This coalescence is accompanied by a shift in the visible absorption maximum from 560 to 590 nm. Subtle changes in copper coordination geometry might be responsible for the spectral changes which are observed in the five Limulus fractions.

On the other hand, the chromophore structure is independent of the state of protein aggregation. The same spectra are observed at pH 9.8, 8.7 and 7.0 for both Busycon and Limulus hemocyanin, although aggregation is known to increase with decreasing pH over this interval.[18,19]

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References and Notes

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Photochemical Generation of $Ru(bpy)_3^+$ and O_2^-

Sir:

From low temperature emission measurements Crosby and his co-workers have concluded that there is considerable charge separation in the luminescent excited state of $Ru(bpy)_3^{2+}$ (bpy is 2,2′-bipyridine) and that the absorption of visible light by the ion leads to the transition:

$$Ru(bpy)_3^{2+}\ (d^6) \rightarrow [Ru^{III}[(bpy)_3^{-}\cdot]]^{2+}\ (d^5\pi^*)^{1-3}$$

The excited state has simultaneously both oxidizing (Ru(III)) and reducing [$(bpy)_3^{-}\cdot$] sites. Experimental studies based on the electron transfer quenching of $Ru(bpy)_3^{2+*}$ have allowed formal reduction potentials for the excited state acting as either oxidant or reductant to be estimated.[4-6]

$$Ru(bpy)_3^{3+} + e \rightarrow Ru(bpy)_3^{2+*} \qquad E = -0.81 \pm 0.02\ V$$

$$Ru(bpy)_3^{2+*} + e \rightarrow Ru(bpy)_3^{+} \qquad E = 0.77 \pm 0.02\ V$$

(in 0.1 M $[NEt_4]ClO_4$-CH_3CN vs. the SSCE)

Oxidative quenching of $Ru(bpy)_3^{2+*}$, which has been demonstrated using flash photolysis,[7,8] gives $Ru(bpy)_3^{3+}$ which is a strong oxidant ($Ru(bpy)_3^{3+} + e \rightarrow Ru(bpy)_3^{2+}$ $E = 1.29$ V) capable of oxidizing water to O_2.[9] Reduction of the excited state, which is suggested by the results of emisson quenching experiments[5] and more recently shown by Creutz and Sutin using flash photolysis,[10] gives $Ru(bpy)_3^{+}$ which is a powerful reductant ($Ru(bpy)_3^{2+} + e \rightarrow Ru(bpy)_3^{+}$ $E = -1.33$ V), thermodynamically capable of reducing water to H_2.[11]

Sprintschnik, Sprintschnik, Kirsch, and Whitten have reported that monolayers formed from long-chain derivatives of $Ru(bpy)_3^{2+}$ act as catalysts for the photodissociation of water.[12] The operation of the photocatalytic system may depend on the electron transfer properties of the excited state or