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(52) Professor Deslongchamps (private communication) and Professor Masamune (private communication) have both encountered molecules in which the epoxide moiety is so severely shielded on the backside that any trans addition (e.g., iodohydrin formation) is inconceivable. Reduction with the tungsten reagent[50b] gave excellent yields in both cases. We feel that these results and those of Professor Parker[51] suggest that the tungsten epoxide deoxygenations proceed by a unique mechanism involving direct frontal assault upon and extraction of the oxygen atom (i.e., the mechanism outlined in Scheme VII).
(53) The mechanisms shown in Scheme VIII all involve direct attack of the olefin on the heteroatom ligands. They are the type of mechanism favored in the literature (ref 2–7) and are shown here for comparison with the alternative approaches which we have been espousing. Actually the cis dichlorination of olefins by these reagents (i.e., CrO_2Cl_2 in this work and $MoCl_5$[18a,18b]) is a rather new observation and a mechanism analogous to entry 3 in Scheme VIII has, to the best of our knowledge, not yet appeared in the literature. The mechanism shown in entry 3 is what we imagine those (ref 2–7) who support direct ligand attack processes would favor to explain the cis addition aspect of this new transformation.
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Near-Ultraviolet-Excited Raman Spectroscopy of Lysozyme and the Lysozyme–Glucose Complex

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Abstract: A study is reported of the Raman spectrum of lysozyme excited by the near-UV argon ion laser line at 363.8 nm. Compared to the Raman spectrum excited by visible lines, the spectrum is simplified due to a strong preresonance enhancement of the Raman spectrum of tryptophan. This preresonance Raman enhancement by the UV excitation is approximately as expected from the simple theory, but not all normal Raman lines of lysozyme are affected the same way. The tryptophan spectrum is strongly enhanced, with the vibrations of the indole ring of tryptophan enhanced greatly in the UV-excited Raman spectrum. The net effect of the UV excitation is a simplification from the normal Raman spectrum of lysozyme, permitting easier study of the changes in the Raman spectrum of tryptophan in lysozyme when it interacts with a substrate. The procedure is illustrated by a study of both visible and UV-excited Raman spectra of the weak lysozyme–glucose complex.

The Raman spectrum of proteins is normally complicated by the presence of a large number of bands, due both to the amide backbone and to the amino acids that compose the protein.[1,2]

We expect that the Raman bands of a molecule will be enhanced when the frequency of the exciting line is near one of the allowed electronic absorption regions of the molecule, due to the preresonance Raman effect.[3] Three of the common amino acids in proteins (tryptophan, tyrosine, and phenylalanine) absorb in the near-UV region of the spectrum (250–300 nm), so we might expect the Raman lines from these three species in the protein to be selectively enhanced by use of a near-UV excitation line.[4,5] If the enhancement is sufficient we might expect to see Raman bands from only these three residues (or perhaps from only one of these three) when we study the Raman spectrum of a protein such as lysozyme using a UV exciting line. Since tryptophan absorbs more strongly at lower frequencies than do either tyrosine or phenylalanine, we expect that the Raman spectrum of most proteins excited by a line longer than 300 nm would contain, for the most part, only

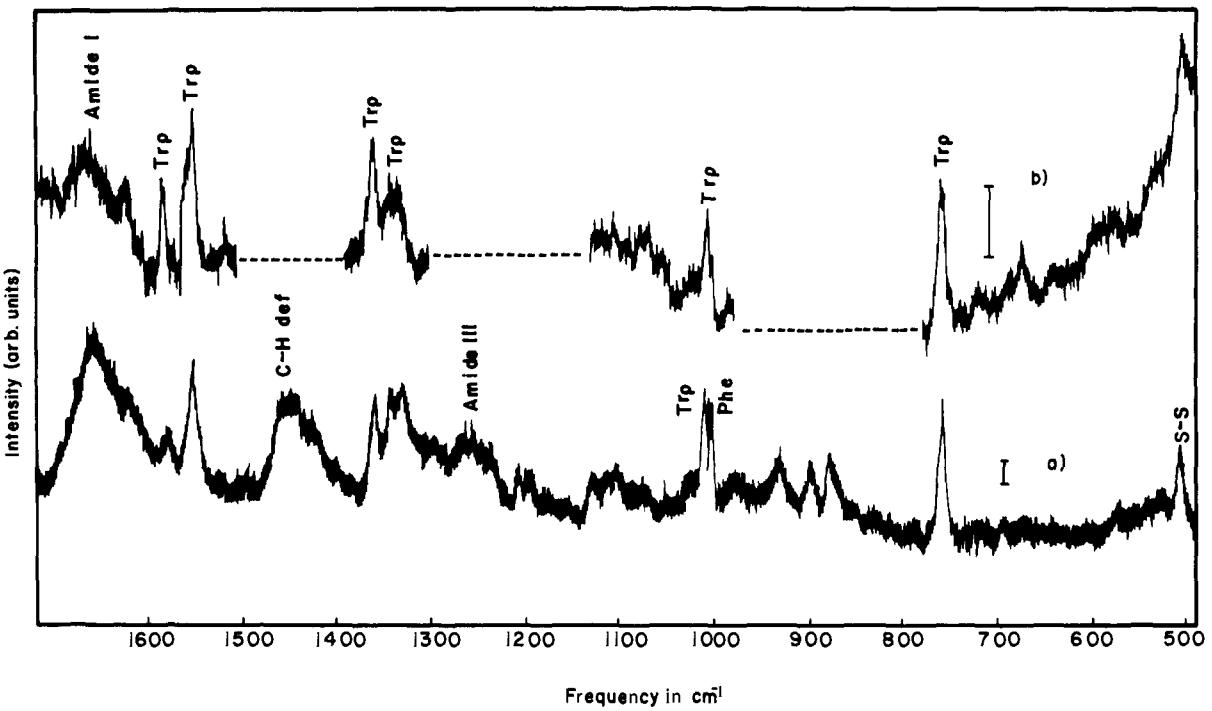

Figure 1. Raman spectra of aqueous solutions of 29% (by weight) egg-white lysozyme (Sigma grade 1): (a) 488.0-nm excitation with 200 mW of power at the sample; the spectral slit width was 4 cm^{-1} and the vertical line represents 1000 counts/s; (b) 363.8-nm excitation with 20 mW of power at the sample; the spectral slit width was approximately 4 cm^{-1} and the vertical bar represents 100 counts/s. Only the portions of the spectrum that correspond to significant bands observed with visible excitation are shown here since the sample was observed to decompose with the longer exposures required for a complete scan.

bands that are due to tryptophan, if the simplified preresonance theory is applicable. One of the purposes of this study is to determine whether this effect does occur.

Several studies of enzyme–substrate interactions involving the preresonance Raman spectrum have already appeared.[6-8] However, these studies have usually involved a *substrate* whose absorption maximum was near the frequency of the Raman excitation line. Hence, the observed Raman spectrum was characteristic of the substrate, with no direct information about the effect of the interaction on the amino acid residues of the enzyme. If our hypothesis presented above concerning the preresonance Raman effect for tryptophan is correct, we may take advantage of it, utilizing substrates with weak near-UV absorption, to study changes in the tryptophan absorption due to complex formation involving tryptophan and the substrate. If the tryptophan is on an active site for the enzyme action, it may be possible to study this action by the changes in the preresonance Raman spectrum.

Lysozyme is an enzyme which contains an active site involving tryptophan in the binding of the substrate. Its crystal structure is well known, as is that of some lysozyme–substrate complexes.[9,10] We report here some studies of the preresonance Raman effect of lysozyme to test the first hypothesis advanced above and some preliminary results from a study of its interaction with the weakly interacting substrate, glucose, to indicate how the second kind of study can be made.

Experimental Section

A Spex Ramalog 5 spectrometer was adapted for use in the UV by Spex using quartz optics and reflecting mirrors, particularly for the source optics. This prototype Ramalog 6 spectrometer was employed in all experiments. Laser light from a specially selected Coherent Radiation CR5 argon ion laser for visible and UV excitation was directed to the sample by means of a mirror. The two argon ion UV lines at 363.8 and 351.1 nm were separated by means of a Pellin-Broca prism, and the 363.8-nm line was employed as the exciting line in all our UV experiments. The scattered Raman light was collected at 90° to the incident light by means of a specially designed elliptical mirror that has the sample at one focus and the entrance slit of the monochromator at the other. The monochromator was calibrated by means of argon plasma lines and from the known Raman spectra of standard samples, such as CCl_4. The spectral slit width was determined from tables supplied by the manufacturer. All of the solution spectra were taken using a Spex spinning cell. The UV power at the sample ranged from 5 to 10 mW and the visible power ranged from 200 to 400 mW.

Results

Raman spectra of lysozyme in water, utilizing first the visible 488.0-nm and then the UV 363.8-nm laser lines for excitation, are shown in Figure 1. These spectra were determined under comparable conditions (except for the laser power in the two exciting lines) and so provide a representative survey of the differences between the Raman spectra excited by the lines from the two different regions. One difficulty with the UV line is that fluorescence from the sample causes more interference with the Raman spectrum. We have corrected the background in Figure 1 for the fluorescence. We have studied each region of the spectrum in more detail and we find that there is no Raman scattering in those regions shown with the dashed lines in Figure 1. The results for the Raman bands which are assigned (at present) are summarized in Table I.

There are two sources of intensity enhancement expected in this experiment comparing excitation by visible and UV lines—the preresonance enhancement of the aromatic amino acid residues and the enhancement of the entire spectrum caused by the dependence of Raman intensity on the exciting line frequency. The intensity of Raman-scattered light is proportional to the fourth power of the difference between the frequencies of the incident light and the vibrational mode frequencies; this difference is approximately equal to the fourth power of the frequency of the incident light, $\nu_i^4 = (c/\lambda_i)^4$. Thus we expect the entire Raman spectrum to be enhanced by the factor $[488.0/363.8]^4$ when the excitation changes from the visible to the UV laser line. This factor of 3.2 is approximately cancelled in the comparison given in Figure 1 between the

Table I. Observed Frequencies and Peak Heights of Aqueous Solutions of 29% by Weight Egg-White Lysozyme and Mixtures of 29% by Weight Lysozyme and 20% by Weight Glucose[f]

Assignment[a]	Lysozyme				Lysozyme + glucose			
	Position, cm^{-1}	Intensity			Position, cm^{-1}	Intensity		
		$I(488.0)$[b]	$I(363.8)$			$I(488.0)$	$I(363.8)$	
S–S	505	5.5	4.6	(18.7)[d]	508	*c*	*c*	
Trp	757	10.0	10.0	(24.3)	759	10.0	10.0	(18.8)[d]
Phe	1002	9.5	~3.3	(~8.0)	1003	7.8	sh	(*e*)
Trp	1010	10.0	7.7	(18.7)	1012	9.3	7.6	(14.3)
Glu					1122	9.1	4.3	(8.1)
Amide III	1261	5.6	0.0	(0.0)	1260	6.1	0.0	(*e*)
Trp	1330	9.8	5.2	(12.6)	1335	10.0	6.5	(12.2)
Trp	1360	8.8	8.3	(20.1)	1362	9.3	5.6	(10.5)
Trp	1426	5.2	0.0	(*e*)				
C–H def	1450	8.6	0.0	(*e*)	1458	9.0	0.0	(*e*)
Trp	1552	10.0	10.0	(24.3)	1554	8.4	6.7	(12.6)
Trp	1582	4.8	6.1	(14.8)	1581	4.1	6.0	(11.3)
Amide I	1657	13.1	5.4	(13.1)	1662	12.2	6.5	(12.2)

[a] Lord and Yu, ref 1. Abbreviations: Trp = tryptophan, Phe = phenylalanine, Glu = glucose, def = deformation. [b] Here $I(488.0)$ designates the peak heights of the Raman bands excited by the 488.0-nm visible exciting line; $I(363.8)$ is the UV-excited Raman peak height. [c] Possibly overlapped by glucose bands. [d] Numbers in parentheses are relative intensities corrected by the factor required for the assumption that the amide III band intensity remains unchanged by the change in laser exciting line. See text for further explanation. [e] Not able to estimate. [f] The peak heights of the 757- and 759-cm^{-1} Raman line have been arbitrarily set equal to 10.0.

visible and UV-excited spectra by differences in operating laser power and photon sensitivity selection. The UV-excited spectrum was obtained with $1/10$th the laser power and measured at photon counting sensitivity 3.3 times greater than conditions for the visible excitation, resulting in scaling by a factor of $3.3/10$ or $1/3$ relative to the visible-excited spectrum. Further intensity enhancements, which vary for the different bands within the spectrum, are attributed to preresonance enhancement and are measured relative to a selected reference band within each spectrum.

Lysozyme. One striking result of this comparison in Figure 1 for lysozyme is that not all bands for which enhancement is expected in the UV-excited spectrum are enhanced equally. This result is found both for the comparison of bands from different aromatic amino acids and also for the comparison of different bands from one single amino acid. In the second and third columns of Table I the relative Raman intensities of bands observed for lysozyme with the two exciting lines [$I(488.0)$ and $I(363.8)$] are compared, according to a previously established reference system,[11] by arbitrarily assigning the 757-cm^{-1} band a peak intensity of 10.0 for each exciting line and listing the relative peak intensities from the other bands in each spectrum. However, the results can best be appreciated by examining the amide I band at 1657 cm^{-1} and the changes in the amino acid bands relative to the amide I band. The 1657-cm^{-1} amide I band is the most intense Raman band in lysozyme excited by the visible (488.0 nm) line, as can be seen in Figure 1 and in Table I. In the UV-excited Raman spectrum, it is relatively less intense than the tryptophan Raman band at 757 cm^{-1}, which was used as the reference line. It has been previously shown that the intensity of the amide I Raman band is enhanced as the excitation line is changed from red toward the UV.[12] We show by a qualitative calculation in the Discussion section that the intensity enhancement $I(363.8)/I(488.0)$ for the amide I band expected from the simple preresonance theory is about 2.6. If this enhancement factor is correct, we conclude that the UV intensity enhancement for the tryptophan 757-cm^{-1} band [for which the peak height from UV excitation (Figure 1) is about twice as great as for the amide I band] is about 6.0 or an enhancement about 2.4 times that for the amide I band. The numbers in parentheses in the fourth column [labeled $I(363.8)$] in Table I indicate the peak intensities relative to the amide I intensity taken arbitrarily to be 13.1, the value from the visible excitation. Since we believe the amide I band actually shows some preresonance enhancement, we think the values in parentheses represent lower limits to the true intensities relative to the visible-excited Raman spectrum.

Our first observation from these values is that there has been a strong enhancement of the tryptophan (Trp) bands in the UV-excited spectrum of lysozyme relative to the spectrum of lysozyme excited by the 488.0-nm line. Trp bands at 757, 1010, 1330, 1360, 1552, and 1582 cm^{-1} are enhanced by factors varying from about $4/3$ to 4. The only Trp band we observe which is not enhanced is the 1426-cm^{-1} band.

The second observation is that the enhancement of the lysozyme spectrum is almost entirely for the Trp bands. The amide III band at 1290 cm^{-1}, even though known to be enhanced as the exciting line is changed from red to UV,[12] is so weak in comparison to the Trp 757-cm^{-1} band (Figure 1 and Table I) that it was not observed at all in the UV-excited spectrum. The S–S bridge stretching vibration at 505 cm^{-1} still appears in the UV-excited Raman spectrum of lysozyme with nearly the same intensity relative to the Trp 757-cm^{-1} band (see Table I) as in the visible-excited spectrum; since we believe the 757 cm^{-1} is enhanced as indicated by the adjusted intensities in parentheses, this bridge band is also apparently strongly enhanced. The phenylalanine (Phe) band observed at 1002 cm^{-1} is approximately equal in intensity to the Trp 1010-cm^{-1} band in the visible-excited spectrum but is merely a shoulder on the Trp 1010-cm^{-1} band in the UV-excited spectrum. The Phe 1002-cm^{-1} band is thus apparently not enhanced. The overall result is a definite simplification of the Raman spectrum of lysozyme, approaching that from Trp alone.

The third point that can be drawn from the comparison in Figure 1 and Table I is that there is considerable variation in the enhancement observed for the different Trp bands. The greatest enhancement occurs for the 1582-cm^{-1} band. The order of enhancement observed for the Trp bands is $I(1582) > I(757) \simeq I(1552) > I(1360) > I(1010) > I(1330) > I(1426)$. Apparently the intensity of the last band listed (the 1426-cm^{-1} band) is not enhanced at all by the near-UV excitation.

In addition to the above-mentioned bands, there are several other weak bands in the spectrum of lysozyme, which are not at this time definitely assignable, but which show intensity changes in the UV-excited spectrum. A band at 675 cm^{-1}

emerges in the UV-excited spectrum that is not observed in the visible-excited spectrum. Distinct bands at 877, 898, and 930 cm^{-1} appear in the visible-excited spectrum but are not observed in the UV-excited spectrum. A band at 978 cm^{-1} in the visible-excited spectrum appears to be slightly enhanced in the UV-excited spectrum. Bands in the 1100-cm^{-1} region appear in both spectra in Figure 1 and are therefore presumed to be enhanced. A band at 1615 cm^{-1} appears stronger in the UV-excited spectrum.

Lysozyme–Glucose Complex. Glucose is one of many sugars that have been shown to bind to lysozyme.[13] Although its binding coefficient ($K_a < 0.1$) has been found to be quite a bit lower than for some other substrates, it is a readily available material with sufficient binding capability to demonstrate our technique. The Raman spectra of the lysozyme–glucose mixtures are shown in Figure 2 for excitation by the 488-nm and by the 363.8-nm laser lines. One feature of the spectrum taken with visible excitation is immediately obvious; namely, a great deal of complexity is introduced [cf. Figures 1 (a) and 2 (a)] because of the appearance of bands due both to glucose and to lysozyme. This problem is reduced in the UV-excited Raman spectrum [cf. Figures 1 (b) and 2 (b)] where the only glucose band remaining with any appreciable intensity in the UV-excited Raman spectrum is the band at 1127 cm^{-1}.

Detailed comparison of the visible-excited spectra of lysozyme and the lysozyme–glucose mixture [Figures 1 (a) and 2 (a) and Table I] reveals that there are few appreciable frequency shifts in any of the bands of lysozyme as a result of the interaction with glucose, whereas there are considerable differences found in the relative intensities. At the concentrations employed less than 10% of the lysozyme is complexed[13] which means that the intensity changes observed are even more dramatic when the limited extent of enzyme–substrate complex formation is taken into consideration. We shall consider as relevant only those intensity changes that are greater than 10%. We see in Table I, for example, that the visible-excited Raman Trp band at 1362 cm^{-1} is observed to be stronger in the glucose–lysozyme mixture relative to the 757-cm^{-1} Trp reference band than it is for pure lysozyme. Since there are no glucose bands at this frequency, this intensity increase is attributed to the interaction between Trp residues and glucose. Other lysozyme visible-excited Raman bands are also altered in intensity by the interaction; for example, the 1552-cm^{-1} Trp band apparently decreases and the Phe band at 1003 cm^{-1} apparently also decreases in intensity relative to the 757-cm^{-1} Trp reference band. The amide III and amide I visible-excited Raman bands remain essentially unchanged with addition of glucose, suggesting that there is no serious alteration in the backbone structure. The 978-cm^{-1} lysozyme band virtually disappears upon the addition of glucose, while the lysozyme 1615-cm^{-1} band may increase slightly in intensity.

There appear to be no significant intensity changes in the Raman spectrum of glucose as a result of the interaction. A band characteristic of α-glucose is observed at 840 cm^{-1} and a band characteristic of β-glucose[14] lies under the lysozyme band at 898 cm^{-1}. Both bands are very weak in the visible-excited spectrum in comparison to the lysozyme bands. However, the very intense band at 1122 cm^{-1} and the broad band at 517 cm^{-1}, which we attribute to glucose, are proportionately much greater in the lysozyme–glucose mixture than in published spectra of solid-state glucose.[14,15] Therefore, Raman spectra of glucose solution were measured with 488.0- and 363.8-nm excitation. Comparison of the glucose solution and lysozyme–glucose solution spectra showed that the intensity pattern of the glucose bands in the latter mixture is that of glucose in solution. The relative intensity differences between glucose bands in the lysozyme–glucose mixture and the published solid-state glucose Raman spectra are due to solvation and not to interaction with lysozyme. We expect to see

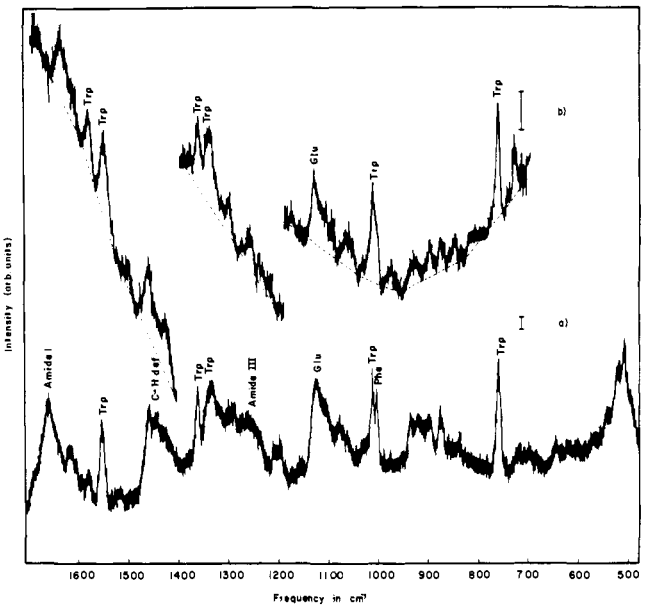

Figure 2. Raman spectra of mixtures of 29% lysozyme and 20% glucose solutions: (a) 488.0 nm; the conditions are the same as for Figure 1 (a); (b) 363.8 nm; the power at the sample is approximately 5 mW and the sample was not observed to decompose. Other conditions were the same as Figure 1. The vertical discontinuities are instrumental corrections due to rising background from fluorescence by the sample.

no intensity changes in the glucose bands in the Raman spectrum of the lysozyme–glucose mixture since less than 0.1% of the glucose is complexed with lysozyme.

The intensity changes discussed above for the visible spectra are similar to the changes observed in the UV-excited Raman spectra [Figures 1 (b) and 2 (b)] with the exception of the 1362-cm^{-1} Trp band in lysozyme. This band does not exhibit as much enhancement in the UV-excited spectrum in the presence of glucose as it did in pure lysozyme solution.

Discussion

Lysozyme. The Trp bands were observed to dominate the Raman spectrum of lysozyme. Some quantitative estimates of the Raman intensity enhancements are useful in explaining this and other observations noted in the Results section.

We will focus on one of the time-ordered terms of the third-order perturbation time-dependent perturbation expression for the Raman polarizability.[16]

$$R \propto \sum_{E,S} \frac{\langle 0|\hat{r}|E\rangle\langle E|\mathcal{H}'|s\rangle\langle s|\hat{r}|0\rangle}{(\omega_E - \omega_0 \pm \Omega - \omega_i)(\omega_s - \omega_0 - \omega_i)} \qquad (1)$$

The notation used is essentially that of ref 16, with $\mathcal{H}' = \partial H/\partial Q_a$ and ω_i = circular frequency of the incident laser beam. When the laser exciting line frequency is lower than (but reasonably close to) the frequency associated with the first electronic transition of the molecule, we assume that the Raman polarizability tensor simplifies to

$$R \propto \frac{\langle 0|\hat{r}|1\rangle\langle 1|\mathcal{H}'|1\rangle\langle 1|\hat{r}|0\rangle}{(\omega_i - \omega_1)^2} \qquad (2)$$

Since we are not considering the case when we have exact resonance Raman, and we are attempting a semiquantitative calculation, we have neglected Ω ($\Omega \ll \omega_i - \omega_1$).

The values for the transition moments deduced from the observed absorbances for the first electronic transitions for the three aromatic amino acids, used in eq 2 with the observed frequencies, explain why nearly all of the assigned amino acid bands in the Raman spectrum of lysozyme are Trp bands. If similar band shapes are assumed in the electronic spectra of

the three acids, the Raman polarizibility tensors are related to the first electronic transition extinction coefficients, ϵ_1, for the three amino acids. In fact, the integrated intensities of the Raman bands are proportional to the square of the polarizability tensor and, hence, to the square of ϵ_1. [The values of ϵ_1^2 are Phe $\epsilon_1^2 = 8.0 \times 10^3$, $\lambda_1 = 267.1$ nm; Tyr $\epsilon_1^2 = 2.02 \times 10^6$, $\lambda_1 = 274.6$ nm; and Trp $\epsilon_1^2 = 2.26 \times 10^7$, $\lambda_1 = 288.5$ nm.][17] All other factors being approximately equal [such as the first electronic transition energies (ω_1 or λ_1) and equal concentrations for the three amino acids], the Trp band intensities would be expected to be an order of magnitude greater than the Tyr band intensities and several orders of magnitude greater than the Phe band intensities. Thus, consideration of this simplified polarizability tensor suggests that the Trp bands should dominate the spectrum of a protein in which the three residues are distributed equally. The fact that one Phe Raman band is observed with intensity comparable to the Trp bands reflects the inadequacy of the simplified expression in eq 2 for some bands. This point is amplified in the following discussion.

If we assume for the moment that eq 2 is a good approximation for the interpretation of our experiments, the preresonance enhancement expected for excitation by the 363.8-nm exciting line is

$$\frac{I(363.8)}{I(488.0)} = \frac{R^2(363.8)}{R^2(488.0)} = \left[\frac{\omega_1 - (1/488.0)}{\omega_1 - (1/363.8)}\right]^4 = \left[\frac{1/\lambda_1 - 1/488.0}{1/\lambda_1 - 1/363.8}\right]^4 \quad (3)$$

Substituting an approximate value of 280 nm for λ_1 for Trp, we predict a preresonance enhancement of approximately 11.5. We may test this prediction by repeating it for the amide I band. The enhancement factor for that band is predicted to be 2.6, using eq 3 with $\lambda_1 = 190$ nm, a typical value for the first electronic transition wavelength of amides. Therefore, with the assumption that all of the Raman intensity is derived from the first excited electronic state, the preresonance enhancement for Trp is predicted to be about four times that for the amide I enhancement for the UV-excited spectrum. The figures in Table I reveal that many of the observed enhancements for Raman bands of Trp are 2–3 times the amide I enhancement. This observation suggests that many of the Trp bands do derive a large proportion (but not all) of their visible-excited Raman intensity from the lowest lying excited electronic state, and that eq 2 and 3 predict nearly the correct preresonance enhancement.

We have already noted in the Results section that there is a broad range in the observed preresonance enhancement of the different Raman bands of lysozyme. The contribution of a particular electronic state to the Raman polarizability tensor is dependent on the electronic–vibronic mixing (given by the value of $\langle 1|\mathcal{H}'|1\rangle$ in eq 2) allowed by the symmetries of the particular vibrational mode and of the electronic state, as well as on the magnitude of the electronic transition moment. Hence, it is probable that those bands which exhibit a value for the preresonance enhancement with the 363.8-nm excitation that is less than predicted may derive their intensity not from the first electronic transitions (since $\langle 1|\mathcal{H}'|1\rangle$ may be small) but from a vibronic interaction with a higher electronic state. The first three electronic transitions of Trp occur at $\lambda_1 = 288.5$ nm ($\epsilon_1 = 4.8 \times 10^3$), $\lambda_2 = 279.8$ nm ($\epsilon_2 = 5.6 \times 10^3$), and $\lambda_3 = 219$ nm ($\epsilon_3 = 3.5 \times 10^4$).[16] The transitions at λ_1 and λ_2 are sufficiently close that contributions from electronic states $|1\rangle$ and $|2\rangle$ are expected to give rise to approximately the same enhancement (about 12). However, λ_3 is sufficiently removed from λ_1 and λ_2 that the preresonance enhancement $[I(363.8)/I(488.0)]$ from eq 3 is expected to be considerably less for a Raman band which derives its intensity chiefly from vibronic mixing only with electronic state $|3\rangle$ than it would be for one with the same extent of vibronic mixing with $|1\rangle$. Using λ_3 in eq 3 predicts $I(363.8)/I(488.0) = 3.6$, about 1.5 times the calculated enhancement for the amide I band. The results (which showed that preresonance enhancement for the Trp bands occurs in the order $1582 > 757 = 1552 > 1360 > 1010 > 1330 > 1426$ cm^{-1}) may indicate that those bands which show greater preresonance enhancement derive intensity from the electronic excited states $|1\rangle$ and $|2\rangle$, while those with lesser enhancement may derive intensity from $|3\rangle$.

It is very reasonable that a Raman band for Trp derived from vibronic perturbation with the excited electronic state $|3\rangle$ should have sufficient intensity to be observable for visible excitation (488.0 nm). Although the transition energy of $|3\rangle$ is further from the laser excitation frequency [so that $(\omega_1 - 1/488)$ is larger in eq 2], the absorbance, hence, $\langle 0|\hat{r}|3\rangle$, for electronic transition to $|3\rangle$ is greater than that to $|1\rangle$. If we compare the Raman intensity $I_3(488.0)$ expected for a band involving vibronic perturbation solely of $|3\rangle$ at 219 nm with the intensity $I_1(488.0)$ involving perturbation solely of $|1\rangle$ at 288.5 nm, assuming $\langle 3|\mathcal{H}'|3\rangle = \langle 1|\mathcal{H}'|1\rangle$, the frequency dependence of the denominators of the Raman polarizability tensor decreases the intensity of I_3 relative to I_1.

$$\frac{I_3(488.0)}{I_1(488.0)} = \left[\left(\frac{1}{488.0} - \frac{1}{288.5}\right) \Big/ \left(\frac{1}{488.0} - \frac{1}{219.0}\right)\right]^4 = 0.09$$

However, the transition dipole moment dependence of the numerator of the polarizability tensor increases the predicted intensity of I_3 relative to I_1.

$$\frac{I_3(488.0)}{I_1(488.0)} = [\langle 0|\hat{r}|3\rangle / \langle 0|\hat{r}|1\rangle]^4 \simeq \frac{\epsilon_3^2}{\epsilon_1^2} = 53.2$$

The net effect is $I_3(488.0)/I_1(488.0) = 4.8$ so the visible-excited Raman band deriving intensity solely from $|3\rangle$ is expected to be more intense than one deriving intensity from $|1\rangle$, even though ω_3 is much larger than ω_1. Thus, we have another example which demonstrates the oversimplification of eq 2.

The Trp 1426-cm^{-1} band, which shows no preresonance enhancement at 363.8-nm excitation, may be due to vibronic perturbation of even higher electronic states than those considered here. The Phe band at 1002 cm^{-1} also does not show preresonance enhancement with 363.8-nm excitation and probably derives its intensity from vibronic interaction with higher excited electronic states. In the case of Phe, the absorbances for the lower energy electronic transitions ($\lambda = 267.1$–241.2 nm) are all relatively small, with molar extinctions less than 0.2. By the Thomas–Reich–Kuhn theorem, we may expect in general that higher electronic transitions have much greater absorbances, making it very probable that a Raman band such as the 1002-cm^{-1} band in Phe could derive observable intensity from perturbations by higher electronic states, while those Phe Raman bands arising from perturbation by the lower electronic states with small transition moments may not appear in the spectrum.

The differences in preresonance enhancement cited for the different lysozyme bands in Table I may be related to the geometries of the aromatic amino acid in the contributing excited electronic states[4] and will be the subject of a later communication.

Lysozyme–Glucose. Intensity changes observed in the Raman spectrum of lysozyme upon addition of glucose are believed to be indicators of interactions between the complexed glucose substrate and the amino acids residues located at the surface of the lysozyme active site. Chicken egg-white lysozyme contains six Trp residues of which three occupy active-site positions. One of the three Phe residues and one of the three Tyr residues occupy active-site positions. Since only about 10%

of the lysozyme is complexed with glucose (according to the association constant provided in ref 11) in our study, the observed intensity changes (Table I and Figure 2) are quite large.

The visible-excited Raman Trp band at 1362 cm^{-1}, which was observed to become stronger in the presence of glucose, has been shown by Lord and Yu[1] to involve directly the N–H group, since this band exhibits a pronounced shift upon deuteration of Trp. Perhaps some kind of hydrogen-bond interaction between lysozyme and glucose has affected this intensity. Model building experiments[18] have shown it is spatially plausible for the N–H groups of Trp residues 62 and 63 to form hydrogen bonds to hydroxyl group oxygen atoms in the carbohydrate substrates. The results of this study of the Raman spectral changes are consistent with the hypothesis from model building[18] for such a hydrogen bonding mechanism in positioning the substrate on the active site.

The 1552-cm^{-1} Trp band and the 1003-cm^{-1} Phe bands also apparently (Table I) decrease in intensity relative to the 757-cm^{-1} Trp reference band. A band at 720 cm^{-1}, which appears in the lysozyme solution spectra only with preresonance enhancement by 363.8-nm excitation, appears in the lysozyme–glucose spectrum with visible excitation and appears with strong intensity with UV excitation. The assignment of this band is presently under study.[4] The very pronounced intensity changes which appear for glucose in the 1120-cm^{-1} region require further study in experiments with varying glucose concentration.

In general, preresonance intensity enhancements in the UV-excited spectrum of the lysozyme–glucose mixture are similar to the enhancements observed in the UV-excited spectrum of pure lysozyme solution, for the vibrations mentioned. The net effect is to reinforce the conclusions drawn above for the visible-excited Raman spectrum. However, there is one very obvious deviation from the pattern. The 1362-cm^{-1} band for Trp, which involves the N–H group, does not show the UV enhancement in the presence of glucose that it shows in pure lysozyme solution. This effect may be due to a change in the pattern of excited electronic states caused by hydrogen bonding. We conclude that the UV-excited Raman spectrum reinforces the suggestion given above that tryptophan is involved in hydrogen bonding to the substrate.

Recently, the preresonance Raman effect has been utilized to simplify the Raman spectrum of tRNA.[19] It was demonstrated that the spectrum of only one type of base residue was observed, with the obvious implication that it would be possible to determine structural properties of the tRNA in solution since these bases are known to appear in critical structural regions of the tRNA. This study[9] illustrates the same principle that we have demonstrated here, namely, simplification of the lysozyme spectrum observed with the UV-excited Raman spectrum to the spectrum due only to the tryptophan residues. We believe that this technique will prove to be of great importance in the study of enzymes, particularly those in which tryptophan plays an important role in the binding properties. The Raman effect has proved here to be unusually sensitive to substrate effects, and we believe it provides corroborative evidence for the previously suggested[18] mechanism for lysozyme–substrate interaction.

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