Ultraviolet resonance Raman excitation profiles of nucleic acid bases with excitation from 200 to 300 nanometers

(excited electronic states/vibrational spectroscopy)

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ABSTRACT Raman spectra are presented for dilute aqueous solutions of the four ribonucleotides AMP, GMP, UMP, and CMP obtained with laser excitation at 299, 266, 253, 240, 229, 218, 209, and 200 nm. Distinct evidence of strong, selective resonance enhancement is obtained. Low-resolution excitation profiles have been constructed for the strongest bands by using the phosphate band at 994 cm^{-1} as an internal reference. The excitation spectra for many of the vibrational bands are dominated by a peak corresponding to the lowest-energy electronic transition near 260 nm. Smaller peaks are seen for higher-energy electronic transitions. For some modes, the resonance enhancement is dominated by the higher-energy transitions. It is clear from these new data that a full description of the resonance Raman profiles of the nucleic acids will have to include several excited electronic states. Two examples are given of cases where ionic species can be distinguished easily by using far-UV excitation, but these species are indistinguishable with 266-nm excitation. This demonstrates the utility of far-UV resonance Raman spectroscopy for obtaining structural information.

The vibrational frequencies of a macromolecule are often dependent on specific conformation. Consequently, useful structural information about biological macromolecules can be obtained by using Raman or infrared spectroscopy. The Raman technique is well suited for studying biological molecules because it is applicable to aqueous solutions and does not damage the sample. Unfortunately the classical Raman effect is weak, making high sample concentrations necessary. Additional problems arise in the study of large molecules when the Raman spectrum becomes crowded with many bands from different vibrational modes that have similar frequencies. Many of these problems can be circumvented by taking advantage of the resonance effect in Raman spectroscopy. This technique involves generating Raman spectra with an excitation frequency that is in the region of an electronic absorption of the macromolecule.

The intensity of Raman light scattered from an absorbing molecular chromophore changes considerably as the excitation wavelength of the incident light is varied over the range of the absorbing frequencies (1–3). This resonance effect can be appreciated when one examines the expression describing the intensity of the Raman scattered light. Clearly, when the excitation frequency ω approaches the frequency of an electronic absorption, the denominator, $E_v - E_0 - i\Gamma_v$, (where E_v is the energy of a vibronic level of the excited electronic state, E_0 is the laser energy, and Γ_v is the half-width of the vibronic level) becomes small and the intensity of Raman scattered light for a given vibrational mode approaches infinity. This enhancement permits drastic reductions in sample concentrations, often by several orders

of magnitude. The particular excited electronic state that will give rise to resonance enhancement of particular vibrational frequencies depends on the vibrational overlap factors $\langle 0|v\rangle\langle v|r\rangle$ between both the initial state, $\langle 0|$, and the vibronic states, $|r\rangle$, with the intermediate vibronic state, $|v\rangle$, of the excited electronic states that have excitation energies close to that of the laser radiation. This condition reduces to the statement that resonance Raman activity for a particular vibration requires the resonant excited electronic state to have a geometry that differs from that of the ground state along the normal coordinate direction corresponding to this normal mode of vibration.

The study of variations in Raman intensities as a function of excitation wavelength serves several useful purposes compared to a resonance Raman spectrum at a single wavelength. As the incident wavelength is changed, particular vibrations may exhibit more resonance Raman enhancement and, hence, appear more intense in the Raman spectrum. Resonance Raman profiles of specific vibrations can be generated by plotting intensities of a given vibration as a function of wavelength. Consequently, if one is interested in the frequency and intensity of a certain Raman band, the excitation wavelength can be chosen to maximize the intensity of that band relative to other bands. This effectively simplifies the Raman spectrum enabling one to study specific vibrational modes of interest without interference from other modes with similar frequency. Observing changes in Raman band intensities as a function of wavelength adds a second dimension to the resonance Raman spectrum, vastly increasing the specificity and amount of vibrational information for a sample. Furthermore, the profiles of all of the bands provides a rich source of information from which knowledge of the excited electronic states can be obtained. At the simplest level of interpretation, a set of resonance Raman excitation profiles is equivalent to N_e absorption spectra where N_e is the number of enhanced vibrational frequencies. This additional information is particularly important for the nucleic acid bases because of the great complexity of the electronic spectra of these chromophores (4).

Resonance Raman studies of chromophores with visible excitation are well known (1–3), but it is only recently that studies in the ultraviolet region (below 300 nm) have been made for a number of species (5–9) including nucleic acids (9–17). The resonance Raman excitation profiles of both fundamental and overtone vibrations of the nucleic acid bases have been studied in the region of the first absorption band near 260 nm (10–15). In this report we give excitation curves of the nucleic acid bases in the region of the two (or more) absorption bands that occur in the 200- to 300-nm region. Eight excitation frequencies were used to construct resonance enhancement profiles for the four 5'-monophosphates UMP, CMP, AMP, and GMP in dilute aqueous solutions. The spectra and intensity profiles provided by these eight excitation frequencies show many new and interesting features even with the low resolution provided by eight excitation wavelengths.

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The importance of Raman spectroscopy as a structural tool in the determination of nucleic acid structure is well known (1-3, 9-17), and it has been shown recently that base vibrations that are resonance-enhanced in the first major absorption band are sensitive to the conformation of nucleic acids in solution (17). It is becoming increasingly evident that DNA conformation information can be obtained from the enhanced vibrations of the chromophoric aromatic portions of nucleic acids. As a consequence, ultraviolet resonance Raman spectroscopy may become an important technique in the study of these macromolecular conformational changes.

MATERIALS AND METHODS

The experimental apparatus is essentially that which has been described earlier for taking resonance Raman spectra in the ultraviolet range by using the higher harmonics of a repetitively pulsed Nd:YAG (yttrium/aluminum garnet) laser (5-9, 16; see also 18, 19). In this study we examine the resonance enhancement profile of several vibrational modes in the four ribonucleotides by using eight different excitation frequencies between 200 nm and 300 nm. The excitation wavelengths were 200, 209, 218, 229, 240, 253, 266, and 299 nm.

5'-Monophosphates were purchased from P-L Biochemicals and used without further purification. Except as noted in the figure legends, all spectra were obtained in a 1 M potassium phosphate solution (pH 7) at 1 mM nucleotide concentration. Spectra were collected from a flowing 15-ml sample using a backscattering geometry. The scattered Raman light was collected and dispersed by a 1-m single monochromator and was detected by a solar-blind photomultiplier and a Stanford Research Systems box car/integrator connected to a minicomputer (Digital Equipment, Maynard, MA; PDP 11/34). Potential sample degradation and laser power drift was carefully monitored by collecting several scans in succession and comparing the peak intensities of those scans.

The band areas were compared by using the area of the phosphate band at 994 cm^{-1} as an internal standard. The phosphate peak was chosen as an internal standard because it exhibits essentially no resonance enhancement as a function of excitation wavelength, at least relative to that of the nucleotides. Also, there are few nucleotide bands that would be obscured or that obscure this phosphate band. Furthermore, the phosphate peak at 994 cm^{-1} is relatively sharp, providing a reliable intensity standard. Absorption of the Raman scattered light by the nucleotide ("inner filtering") was treated in the fashion described by Shriver and Dunn for

FIG. 1. The resonance Raman spectra of the nucleotide bases. The concentration was 1 mM in each case except for the 299-nm spectra, which were obtained for a 10 mM solution. These spectra have been appropriately scaled for concentration and to a constant intensity for the 994-cm^{-1} phosphate band present at 1 M concentration.

a back-scattering geometry (20). Under our experimental conditions with phosphate as an internal standard, no correction for self-absorption was necessary. This is because the extinction coefficients of the chromophore at the wavelength of phosphate scattering and sample scattering are essentially identical. The only correction necessary is that for the increase in sample concentration in the 299-nm case when a concentration of 10 mM was used.

RESULTS

The Raman spectra of the four ribonucleotides obtained with excitation at 299, 266, 253, 240, 229, 218, 209, and 200 nm radiation are shown as three-dimensional plots in Fig. 1. The general appearance of these spectra changes with the excitation wavelength. For example, for GMP the strong band at 1495 cm^{-1} observed with long-wavelength excitation is replaced by bands at 1375 , 1600 , and 1676 cm^{-1} with shorter wavelengths. For CMP the strong peaks at 1250 and 1300 cm^{-1} at long wavelengths disappear at intermediate wavelengths, leaving only the 1540 and 1650 cm^{-1} bands. Then, at short wavelengths, the 1300 cm^{-1} band returns and becomes the dominant spectral feature. For AMP the strong band at 1344 cm^{-1} is replaced by features at 1486 or 1585 cm^{-1} , depending on the specific wavelength. For UMP the spectrum at long wavelengths is dominated by a peak at 1238 cm^{-1} but this is replaced at shorter wavelengths by a peak at 1438 cm^{-1} that is a weak feature with longer-wavelength excitation. These resonance Raman spectra show that the vibrational modes couple distinctively to different electronic states in this region—i.e., that there are different shapes for different states.

The strong enhancement of these nucleotide Raman spectra is clearly evident with the magnitude of the cross sections. At the point of maximum enhancement, the strongest mode for each mononucleotide has an intensity ranging from 1500 to 8500 times that for the strong totally symmetric mode of phosphate at 994 cm^{-1} . This enhancement was selective so that only a few of the vibrational modes of the mononucleotide were observed in these spectra. These modes are all associated with the nucleotide rings, as expected. This greatly simplified the spectra.

Raman spectra for GMP and AMP obtained with 266- and 217-nm radiation at pH 3, 7, and 11 are shown in Fig. 2. For GMP the spectra obtained with 266-nm excitation at all three pHs are virtually identical. However, the 218-nm spectra are quite distinct for the pH 7 and 11 cases. For AMP the spectra obtained with 266-nm excitation are again very similar at all three pHs, but the 217-nm spectra are very different for the neutral and protonated forms. This demonstrates the utility in obtaining resonance Raman spectra at a variety of wavelengths when looking for particular structural or ionization effects. (The spectra of CMP at pH 3, 7, and 11 were very similar at both 266 and 218 nm; the spectrum of UMP at pH 11 differed from that at pH 7 for both 266- and 218-nm excitation.)

DISCUSSION

The excitation profiles deduced from these spectra are presented in Fig. 3. The profiles are grouped by mononucleotide, and are all shown on the same scale with the 994-cm^{-1} mode of phosphate as the internal reference. The absorption spectra of the mononucleotides are also shown in this figure. A number of features of these profiles are of interest. The first is the general dominance of the profile by the *ca.* 260-nm band, at least for the strongest transitions of GMP, AMP, and UMP. This dominance by the 260-nm band occurs despite the fact that the extinction coefficient for the nucleotides is higher, in most cases, at the shorter wavelengths. This implies that the higher-energy electronic transitions are more nearly vertical than those near 260 nm, resulting in a smaller change in the nucleotide ring geometry. It is interesting to note that for GMP, which has two low-energy transitions near 255 and 290 nm, it is the higher-energy band that seems to dominate the intensities. On the other hand, some of the bands, such as the 1650-cm^{-1} band of CMP, appear to obtain most of their intensity from transitions at wavelengths shorter than 200 nm. Furthermore, there are significant contributions to the intensities of some of the vibrational bands from states in the 200- to 240-nm region. This is most dramatic for the 1529-cm^{-1} band of CMP, which seems to gain intensity from two bands in the 210- to 250-nm region. By taking these excitation profiles

FIG. 2. Resonance Raman spectra of GMP (Upper) and AMP (Lower) obtained with 266-nm (Left) and 218-nm (Right) radiation at pHs 3, 7, and 11. The phosphate buffer concentration was 200 mM for the pH 3 and 11 samples and was 1 M for the pH 7 sample.

FIG. 3. Resonance Raman excitation profiles for the four bases studied. All panels are on the same intensity scale. The curves are labeled by the vibrational band in question (in cm^{-1}).

together, it is clear that the nucleotides have a rather complex pattern of electronic excitations but that, at least qualitatively, these data are in accord with deductions made on the basis of absorption data [including single-crystal polarized absorption and theoretical calculations (4)].

Previous studies of resonance Raman enhancement of nucleotides have emphasized fitting of preresonance data to A-type terms in order to determine which excited state dominates the resonance behavior (10–12). This procedure involves a large number of approximations, and it is not surprising that it may fail in some cases. It is interesting to examine these new data to see if these extrapolations are valid. This type of analysis was attempted in our previous publication (9), but the present results make the arguments much more incisive because of the availability of a reliable internal intensity standard and many more excitation frequencies.

For UMP, preresonance data indicates that the bands at 1232, 1396, and 1475 cm^{-1} are associated primarily with the transition at 260 nm. Examination of the excitation profiles of Fig. 3 shows that this conclusion is essentially correct for the 1232- and 1396-cm^{-1} bands but that it fails for the 1475 cm^{-1} band as previously noted (9). For CMP, preresonance studies indicate that the 1296-cm^{-1} and 1533-cm^{-1} bands activities

are associated with the 260-nm transition. The data of Fig. 3 show that the intensity of the 1294 cm^{-1} band may be dominated by the 260-nm transition at long wavelengths but that there are significant contributions from some state(s) at higher energy. The behavior of the 1529-cm^{-1} band is very complex and probably requires contributions from at least three states in the 200- to 300-nm region. For AMP the preresonance behavior has been used to conclude that the 1580- , 1480- , and 1340-cm^{-1} bands derive their intensity primarily from the 260-nm band. The excitation profiles for this nucleotide demonstrate that this conclusion is substantially correct for the 1480- and 1340-cm^{-1} modes but that, as noted previously (9), the 1583-cm^{-1} band derives considerable intensity from a state near 215 nm. Finally, for GMP, preresonance behavior partitions the bands into those at 1580-, 1488-, and 1322 cm^{-1} as being associated with the low-energy transitions near 260 nm and those at 1415 and 675 cm^{-1} as deriving intensity from higher states near 210 nm. The data for GMP (Fig. 3) show that this is a correct association for the strong 1488-, 1585-, and 1322-cm^{-1} bands but that the weak 1415- and 670-cm^{-1} bands have flat excitation profiles in the region so far investigated.

It seems clear that a full description of the Raman excitation profiles of any of the bases in this spectral region will involve contributions from several excited states. An extension of the Kramers–Kronig transform method (21–23) to the case of several absorption bands may be a useful way of treating this data theoretically.

Another point of interest is the rather large difference in the maximum intensity of the strongest bands for each nucleotide. The general order for the intensities at the excitation maxima of the strongest band is $\text{GMP} > \text{AMP} > \text{UMP} > \text{CMP}$. However, the dominance of, say, GMP over CMP does not hold at all wavelengths. With excitation at 230 or 200 nm, the bands of CMP are stronger than those of GMP. This base-specific differential excitation permits selective enhancement of particular bases. It should be realized, however, that interesting secondary structure changes may have significant effects on the Raman excitation profiles because of shifts in electronic excitation energies. The resonance Raman excitation profile is, as a first approximation, the trace of the absorption spectrum of the component of the macromolecule having a particular vibrational band. Thus, in the weak coupling limit, this type of experiment permits the determination of the location of the electronic transitions corresponding to a particular base type (G, C, A, or T) or a particular base if it has a unique vibrational spectrum.

The dominance of the 260-nm band for the largest peaks of the excitation profile implies that the optimum wavelength for obtaining high-quality spectra is in this region. This is reinforced by the fact that the highest intensity radiation is also available in this region. However, one cannot conclude from this that the most information will be obtained by concentrating on this region. Some of the weaker bands, which may be the ones most sensitive to secondary structure changes, do not show particular enhancement in this region but are stronger at very short wavelengths. This results in the kind of behavior shown in Fig. 2, where ionization states can be distinguished easily with excitation at 218 nm but not with 266-nm excitation.

Finally, it would be of some interest to associate the strongly and selectively resonance-enhanced vibrations of the nucleotides with motions of particular components of the ring bases. This will be particularly interesting when it is determined which of these bands are sensitive to secondary structural changes and interactions with other molecules because this “spatial” information will help localize these structural and bonding changes. This requires reliable normal mode calculations. Considerable progress is being made in this area at the present time, especially using combinations of *ab initio* calculation methods with empirical data (24,

25). It seems premature, however, to insert a discussion of the specific forms of these normal modes at the present time. One conclusion that does seem certain is that many of the modes that are strongly enhanced by resonance with the 260-nm band are delocalized ring modes. These may be correlated with secondary structure and bonding changes, but they will provide little in the way of "spatial resolution" because changes at any point in the ring can alter their vibrational frequency. On the other hand, some of the modes enhanced at shorter wavelengths are probably localized in particular functional groups because they involve localized electronic excitations. These modes will probably provide information with significant spatial resolution.

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