Reply to Comment on "Vibrational Assignments of *trans-N*-Methylacetamide and Some of Its Deuterated Isotopomers from Band Decomposition of IR, Visible, and Resonance Raman Spectra"

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In their *revised* Comment,[1] Jordan and Spiro (JS) take issue with our recent assessments[2,3] of their interpretations[4−6] of the nature and the resonance Raman enhancement of two bands of aqueous *N*-methylacetamide (NMA), viz., the ∼1380 cm^{-1} CCH_3 symmetric bend (sb) and the amide I normal modes. In this *revised* Reply, we respond in turn to the two specific points raised in their Comment.

1. Enhancement Mechanism of Amide S. The enhancement of the ∼1380 cm^{-1} CCH_3 sb mode has two aspects that need consideration: the nature of this mode and the origin of its enhancement. We note first that there is no question that this mode in aqueous NMA is predominantly CCH_3 sb with a significant contribution of CCH_3 antisymmetric bend[7] and a small contribution of CC stretch (s).[2,8] Therefore, it is not only desirable, as is common practice, that it should be referred to by its major contributing local symmetry coordinate but it is also inappropriate to refer to it as an "amide" mode, which historically[9] has been reserved for characteristic normal modes of the CONH group. The amide S designation[4] should be dropped.

With respect to the enhancement mechanism of this mode, Spiro *et al.*[4−6] have stated that it arises from vibrational mixing with the nearby amide III mode. We have taken exception to this explanation,[2] on grounds of both its formulation and its factual basis.

We start out with the belief that there is merit in precise formulations of concepts. To imply that there is vibrational mixing between normal coordinates[4−6] is indeed "inaccurate".[1,2] In their revised Comment, JS state that they "meant the sharing of internal coordinates between normal modes of similar energy". While this is closer to the truth, in that a given internal coordinate can contribute to different normal modes, we note that these need not be of "similar energy": for example, in aqueous NMA[8] the CCN deformation coordinate makes small contributions to amide I at 1626 cm^{-1} and to the CN s mode at 883 cm^{-1} while its major contribution is to the normal mode at 446 cm^{-1}.

With respect to the origin of the enhancement of the CCH_3 sb mode, this has been said to be "dependent primarily on the C−N displacement in the excited state" [4] and due to "vibrational mixing between the CN stretch of amide III and $(C)C_\alpha$ hydrogen bending displacements".[6] In fact, CN s makes a negligible contribution to the potential energy distribution of the CCH sb mode,[7,8] and in terms of the eigenvector components of the enhancement,[3] CC s is 60% larger than CN s.[10] That the CC s contribution is the crucial one is evidenced by "The lack of resonance enhancement for CCH_3sb in [NMA−ND, which] is caused by the reduction and the sign change of the CC s contribution." [2] It is, thus, clear that CN s, which is a dominant component of amide III, does not play the major role in the resonance Raman enhancement of the CCH_3sb mode. In their revised Comment, JS "do not contest" our analysis.

2. Enhancement Mechanism of Amide I. There is a fundamental disagreement between Spiro *et al.* and our group concerning the origin of the resonance Raman enhancement of the amide vibrations, especially the amide I vibration. These conflicting views result in different conclusions concerning the nature of the amide $\pi\pi^*$ excited state and also in different conclusions about the excited state geometry differences between NMA in aqueous solution versus that in non-hydrogen-bonding solvents and in the gas phase. The correct interpretation is needed in order to develop a deep understanding of amide electronic and vibrational spectroscopy and to utilize UV resonance Raman spectroscopy for protein secondary structural investigations.

The disagreement is summarized succinctly: Spiro *et al.* claim that their experimental data for NMA in aqueous solution, in either H_2O or D_2O, indicate that amide I enhancement does not arise from the lowest energy amide $\pi\pi^*$ excited state, which gives rise to the absorption band at ca. 190 nm, even when excitation occurs directly within this absorption band. In contrast, we claim that the previous experimental data simply and directly force the conclusion that the amide I band is resonance enhanced by this $\pi\pi^*$ excited state.

The ability to reconcile these views was previously clouded by disagreement over the experimental excitation profile results for NMA in H_2O and D_2O. Although our preresonance excitation profile data above 220 nm[3] were similar to those of Wang *et al.*,[5] their Raman cross sections were ca. 2-fold larger than ours for excitation within the amide $\pi \rightarrow \pi^*$ transition (below 200 nm). This disagreement has now been reconciled, and JS now concede that their data were not corrected for the $\pi \rightarrow \pi^*$ transition self-absorption.

Our excitation profile data[3] demonstrated that in the region between 192 and 220 nm the amide I Raman cross sections are relatively constant compared to the amide II and III cross sections. In fact, we directly plotted the amide I′/amide II′ ratio in the region between 192 and 235 nm and found the ratio to be essentially constant. JS have now similarly found that the amide I′/amide II′ as well as amide I/amide II intensity ratios are constant in this region.

Both groups agree that the amide II and III bands are enhanced dominantly by the 190 nm $\pi\pi^*$ excited state. The constant ratio between the amide I and amide II Raman cross sections indicates that these bands have the same frequency dispersion over the region excited; thus, we simply concluded that the amide I and I′ bands are enhanced by the 190 nm $\pi\pi^*$ excited state. Any other conclusion requires that the dispersions fortuitously coincide.

Spiro *et al.* continue to argue that the second $\pi \rightarrow \pi^*$ transition at ∼165 nm is the only source of enhancement of the amide I band.[4] We argued that it is impossible for the preresonance Raman dispersion of amide I from a ca. 165 nm transition to show the same dispersion as the amide II band, which is only enhanced by the 190 nm $\pi \rightarrow \pi^*$ transition. In

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fact, our preresonance Raman Albrecht *A* term fits for amide I for excitation between 211 and 235 nm are essentially identical to those of amide II and III.[3] In addition, we showed excitation profile data down to 192 nm, which is well within resonance.

The issue of whether the amide I band is enhanced by the 190 nm amide $\pi \rightarrow \pi^*$ transition can also be independently decided from the dispersion of the depolarization ratios. We reported earlier that the amide I, II, III, II′, and I′ bands had depolarization ratios close to 0.33 with excitation at 244 nm in preresonance with the 190 nm amide $\pi \rightarrow \pi^*$ transitions.[2] This clearly indicates that the enhancements of these bands are dominated by a single (not necessarily the same) electronic transition. We have recently measured the depolarization ratio of NMA in D_2O (NMAD) at very high signal to noise with a CW 206 nm laser source and have found depolarization ratios close to 0.33 for the amide I′ and II′ bands. In view of the preresonance Raman excitation profiles, this comes very close to proving that the amide I′ enhancement is dominated by a single 190 nm amide $\pi \rightarrow \pi^*$ transition. The only qualification in this argument is if both transitions have identical transition moment orientations (which is extremely unlikely in view of the recently measured orientation of the amide $\pi \rightarrow \pi^*$ transition[11,12]). In this case the depolarization ratio will always equal 0.33.

Wang *et al.*[4] argue that the amide I band is enhanced only by the 165 nm transition because they claim their data claim to show a monotonic increase in the amide I′ Raman cross sections as excitation occurs down to 184 nm, past the maximum of the 190 nm amide $\pi \rightarrow \pi^*$ transition. In contrast, the amide II and III bands seem to show maxima at ca. 188 nm, and 184 nm excitation shows decreasing Raman cross sections.

Because of the importance of this issue for the fundamental understanding of amide excited states, we have measured the 184 nm excited Raman spectrum of NMA in H_2O and in D_2O. We found this to be an extraordinarily difficult experiment due to the fact that 184 nm light is absorbed by atmospheric oxygen and because of the need to Raman shift in hydrogen four anti-Stokes harmonics from the quadrupled YAG at 266 nm. We utilized a Coherent Inc., Infinity 100 Hz YAG laser which has the highest power and best temporal and spatial mode qualities of any commercial laser available. The fourth YAG harmonic at 266 nm (~60 mJ/pulse) was focused into a 1 m H_2 Raman shifter. Use of any but the highest quality fused silica optics in a dust-free environment resulted in destruction of lenses, prisms, and windows. The Raman-shifted light was collimated and dispersed by using a crystalline quartz Pellin Broca prism. Approximately 4 μJ/pulse of 184 nm light was generated to give an average power of 0.4 mW. The entire laser assembly and sample compartment was flushed with purified N_2 gas. The sample was enclosed in a rapidly stirred cuvette and illuminated in a backscattering geometry. The scattered light was collected by a quartz lens and imaged through a polarization scrambler onto the entrance slit of a Spex 1701 single spectrograph. Relatively new UV enhanced Al mirrors and an aluminized 3600 groove/mm holographic grating blazed at 220 nm dispersed the light onto a Princeton Instruments intensified CCD detector which had a >10% quantum efficiency at ~184 nm. The absorption of oxygen by the air efficiently rejected the Rayleigh scattering but transmitted the amide I′ and II′ frequencies.

Figure 1 shows the 206, 200, and 184 nm resonance Raman spectra of N-deuterated NMA in D_2O (NMAD). The NMAD 206 and 200 nm excited Raman spectra are very similar to those reported previously by our laboratory[3] and to the 200 nm spectrum of Spiro *et al.*[5] The amide band relative intensities are essentially identical to that excited from 190 to 244 nm; however, the NMA and NMAD absolute Raman cross sections dramatically increase as excitation approaches resonance.

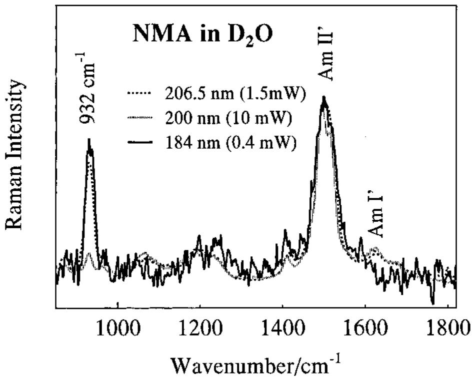

Figure 1. UV resonance Raman spectra of *N*-methylacetamide in D_2O (NMAD). The sample solution was contained in a rapidly stirred 1 cm Supracil cuvette with a 100 μm thick window. The Raman measurement utilized a 135° backscattering geometry. (a) Excitation wavelength 206 nm, NMAD concentration, 10 mM, an 1800 groove/mm 200 nm blazed grating was used in second order in the Spex Triplemate Spectrograph stage. The entrance slit was 100 μm (8 cm^{-1} resolution). Accumulation time was 5 min. Excitation derived from a CW doubled Kr^+ laser,[14] 2 mW. (b) Excitation wavelength 200 nm, NMAD concentration conditions same as in (a) above, except excitation is at 100 Hz from a hydrogen-shifted quadrupled YAG laser (3 ns pulse width). The low intensity of the 932 cm^{-1} band in the 200 nm spectra results from the low efficiency of the Triplemate in this spectral region for the optics used in this experiment. (c) Excitation wavelength 184 nm, NMAD concentration, 4 mM, 3600 groove/mm grating used in first order in a Spex 1701 0.75 m single monochromator, entrance slit is 300 μm (24 cm^{-1} resolution), accumulation time is 1 h. The 184 nm spectrum does not show the amide I′ band. (Note: the excitation power was sufficiently low that no *cis*-NMA was formed, nor was there any evidence of photochemistry.)

The strongest band at ~1500 cm^{-1} is a Fermi doublet and derives from the amide II′ band, which is dominated by C−N stretching.[3] The 1626 cm^{-1} band, which dominates the IR spectrum, derives from the amide I′ band, which is mainly carbonyl stretching.[2,3] The assignment of the ~1660 cm^{-1} is uncertain, and we expect that it derives from a combination or overtone. It has a decreasing intensity ratio to the amide I′ band as the excitation moves to longer wavelength.

Our 184 nm excited Raman spectrum clearly shows that the amide I′ band does *not* show a 5-fold intensity increase relative to the amide II′ band, compared to that excited at 200 nm suggested by Spiro *et al.*;[4,5] instead, it disappears. We see a similar loss in intensity in the 184 nm spectra of the amide I band of NMA in H_2O. We conclude that the amide I′ band is deenhanced, probably due to destructive interference arising from additional enhancement from a higher excited state, possibly at 165 nm. This alone proves that the amide I′ band is enhanced by the ~190 nm amide $\pi \rightarrow \pi^*$ transition. We previously found evidence for enhancement by states in addition to the 190 nm $\pi\pi^*$ state from the amide I′ preresonance Raman cross-section dispersion.[3]

We have tried to rationalize the fact that our spectra are totally inconsistent with results reported by Spiro *et al.* Unfortunately, Spiro *et al.* did not show their 184 nm Raman spectra.[5] Although our 184 nm NMAD spectrum shows acceptable signal to noise (S/N), it was only obtained after careful optimization of our spectrometer and careful purging with N_2. Our laser source had at least a 10-fold larger average power than theirs. (For example, we utilized 10 mW average power (100 μJ/pulse) for our 200 nm, 100 Hz spectral measurements, while Spiro *et al.* utilized 0.6 mW (60 μJ/pulse) for their 10 Hz spectral measurements.) Our detector was superior to the one they used

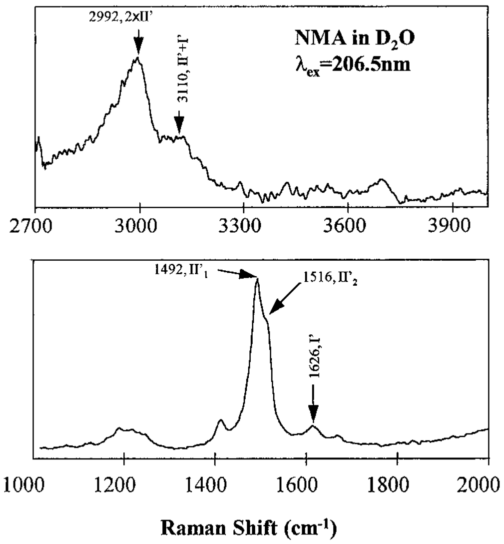

Figure 2. UV resonance Raman spectra of *N*-methylacetamide in D_2O. Excitation was at 206.5 nm from an intracavity, CW frequency-doubled Kr^+ laser, power ~2 mW. The upper panel shows the overtone Raman spectrum of NMAD, the D_2O band having been numerically subtracted. The lower panel shows the fundamental Raman spectrum of NMAD.

at that time. In fact, our 200 nm excited spectrum (Figure 1) has far superior S/N than the spectrum they displayed. We can only conclude that their measured spectrum was spurious. We will report on the quantitative excitation profile in a forthcoming publication.[11]

A final proof comes from the resonance Raman study of the fundamental and overtone spectra of NMAD (Figure 2). The spectra were excited at 206 nm, well within resonance with the 190 nm amide $\pi \rightarrow \pi^*$ transition. The amide II′ Fermi split doublet[2] is observed at 1492/1516 cm^{-1}, while the amide I′ band occurs at 1626 cm^{-1}. We observe the amide II′ overtone at 2922 cm^{-1} and the combination between amide I′ and amide II′ at 3110 cm^{-1}. Although Mayne *et al.*[12,13] have observed the overtones of amide II and amide II′ in aqueous solution, this is the first report of the observation of the amide I′ + II′ combination in the aqueous phase. It is well-known[11,12] that a combination band cannot be resonance enhanced unless both fundamentals are enhanced by the same electronic transition; since amide II′ is enhanced by the 190 nm amide $\pi \rightarrow \pi^*$ transition, amide I′ must also be enhanced by this transition. The presence of strong overtones of NMA in aqueous solution,[12] in CH_3CN, and in the gas phase[13] offers us an opportunity to compare the displacements of the excited states in these separate environments. We will report on these results elsewhere.

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