Structure Elucidation

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Exploring the Mechanism of IR–UV Double-Resonance for Quantitative Spectroscopy of Protonated Polypeptides and Proteins**

*Natalia S. Nagornova, Thomas R. Rizzo, and Oleg V. Boyarkin**

Determination of intrinsic three-dimensional structures of biomolecules challenges spectroscopy to provide detailed, conformer-selective fingerprints of these large species in the gas-phase. Infrared–ultraviolet (IR–UV) double resonance (DR) spectroscopy has proven to be a powerful tool for measurement of conformation-selective vibrational spectra of polyatomic molecules,[1–7] ions,[8–12] and their clusters.[9,13–16] The technique is based on the change of the vibrational population of the molecule by an IR laser pulse with subsequent detection of this change by a UV laser pulse. DR spectroscopy, when combined with UV photofragmentation, electrospray ionization, and cryogenic cooling, has allowed the detection of IR spectra of large protonated species in the gas phase.[8,9,11,12,17] The opened questions remain, however, how truly these spectra reflect absorption for large species and what are the limitations of this technique. Here we analyze the mechanism of IR–UV DR photofragmentation spectroscopy in its application to large, protonated molecules cooled to cryogenic temperatures. We demonstrate the use of depletion spectroscopy for the unambiguous assignment of vibronic transitions to different conformers of a protonated decapeptide and for the measurement of absolute absorption cross-sections of vibrational transitions in the same species. Finally we extend DR spectroscopy to an intact, protonated protein.

Our experimental setup has been described in detail elsewhere.[18,19] Briefly, we produce protonated gas-phase molecules directly from solution using electrospray ionization. The ions of interest are selected by a quadrupole mass-filter, guided to a cold (6 K), 22-pole ion trap, where they are cooled by collisions with He. The cold ions are then interrogated by an IR optical parametric oscillator (OPO) and a UV laser. Charged UV-induced photofragments are detected in a quadrupole mass spectrometer.

Figure 1 (blue trace) shows an electronic spectrum of the doubly protonated decapeptide gramicidin S, $[GS+2H]^{2+}$, with several peaks that have been previously assigned to three different conformers by IR–UV depletion spectroscopy.[17,20] In those experiments we fixed the wavenumber of the UV photodissociation laser on different peaks of the blue trace in Figure 1, each time recording depletion of the ion signal as a function of wavenumber of the preceding IR pulse. Difference in the IR spectra measured for some UV peaks (labeled A, B, and C in Figure 1) allowed their conformational assignment. To assign the numerous remaining peaks we reverse the experiment and scan the UV laser wavenumber whereas the wavenumber of the IR OPO is fixed to excite only the two most abundant conformers, labeled A and B. Figure 1 (pink trace) illustrates the changes induced by this vibrational pre-excitation. All sharp peaks, including those assigned to the conformers A and B, are almost completely suppressed, indicating that the IR pulse significantly depletes the vibrational ground states of these two conformers. We thus assign all the previously unassigned UV peaks that are suppressed to these two conformers. Tuning the OPO to a vibrational transition of conformer C produces the green trace, where the remaining sharp peaks of the pink trace are strongly reduced. We therefore assign these reduced peaks to the conformer C. To distinguish peaks of the conformers A and B we tune the OPO to a transition in the A species only. This reveals electronic transitions of the conformer B (red trace), some of which were not fully resolved in the UV-only experiments even at the lowest achievable ion temperature of 12 K (black trace). A comparison of all the traces in Figure 1

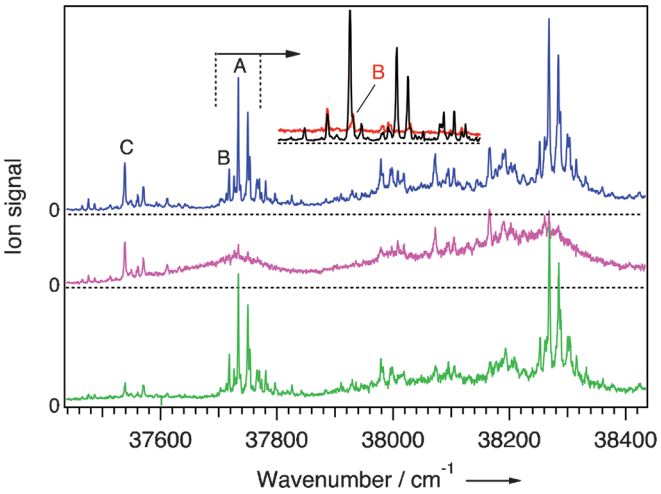

Figure 1. Photo-fragmentation spectra of $[GS+2H]^{2+}$, measured at $T=30$ K with the OPO blocked (blue trace), tuned to 3331 cm^{-1} (pink trace) and to 3429 cm^{-1} (green trace) to excite IR transitions in the conformers A/B and C, respectively. The insert shows the same spectra measured at $T=12$ K with the OPO blocked (black trace) and with the OPO tuned to a transition (3409 cm^{-1}) of conformer A only (red trace). The spectra are measured by detecting fragments with $m/z=550$ and 557.The assignment of IR transitions is from Refs. [20, 21].

[*] N. S. Nagornova, T. R. Rizzo, O. V. Boyarkin
Laboratoire de Chimie Physique Moléculaire, École Polytechnique Fédérale de Lausanne
CH-1015 Lausanne (Switzerland)
E-mail: oleg.boiarkin@epfl.ch
Homepage: http://lcpm.epfl.ch/

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reveals no peaks that do not change upon IR excitation of the conformers A, B, and C. We therefore rule out the presence of other conformer families of significant intensity in our experiment.

In addition to the conformational assignment of the vibronic transitions the experiment described above provides evidence to explain the depletion mechanism in large species.

A key difference in the use of DR spectroscopy for small and large molecules is in the localization of vibrational excitation. The high density of vibrational states (DVS) in large molecules ensures that there is always a large number of vibrational eigenstates excited within the 0.7 cm^{-1} incoherent bandwidth of our OPO. The remainder of states within this energy interval is populated either during the 5 ns pulse of the OPO or shortly after it through intramolecular vibrational energy redistribution (IVR). This is supported by the lack of any detectable change in the IR spectra of $[GS+2H]^{2+}$ measured at 10 and 200 ns delay between the IR and UV pulses. In small molecules where the DVS is low, vibrational excitation can be localized in a few eigenstates, allowing selective detection of the ground species. IVR in large molecules makes such detection impossible, because absorption by IR "preheated" ions also contributes to the measured photodissociation. The detected depletion must therefore occur because of a difference in the UV absorption by the cold and "preheated" ions, which arises from differences between vibrational frequencies in the electronic ground and excited states and/or reduced Frank-Condon (FC) factors for the recorded UV transitions in the preheated species. The former may result in the broadening of vibronic transitions in IR-preheated molecules (see Section S1 in the Supporting Information). This can be considered as "statistical inhomogeneous" broadening, and it is fundamentally different from thermal congestion, which arises from a Boltzman distribution of vibrational energy. Figure 1 (pink trace) provides evidence of such broadening for vibrationally excited conformers A and B of gramicidin S. We do not see, however, any experimental evidence of reduced FC factors, as the spectra of the excited and cold ions in Figure 1 appear of nearly the same integral absorption intensity. We therefore suggest that the key reason for IR–UV depletion in our experiments is the difference between vibrational frequencies in the two electronic states, rather than different FC factors. This understanding of the depletion mechanism would imply that IR–UV depletion spectra of a species should reflect all its IR-active vibrations, regardless of their location relative to the UV chromophore.

In using infrared spectra to determine the structure of a large molecule, one typically compares with calculations only the vibrational frequencies and relative intensities of IR transitions. Absolute absorption intensities, if available, could serve as an additional stringent constraint in validating calculated structures. The model of the IR–UV DR process proposed here allows us to elaborate a procedure for measuring absolute absorption cross-sections of vibrational transitions (Section S2). The main idea of the procedure lies in the fact that in IR–UV depletion experiments the signal because of UV absorption by the IR-preheated ions can be determined under conditions of high saturation of an IR transition of the ions. Provided that the UV beam within the ion trap is much smaller than the IR beam such that the IR fluence is nearly constant over the volume probed by the UV beam, the cross-section of an IR transition of a particular conformer A, $\sigma_A^{IR}(\nu)$, can be expressed as Equation (1).

$$\sigma_A^{IR}(\nu) = \ln\left(\frac{I_A^0 - I_A^\infty}{I_A(\nu) - I_A^\infty}\right)\frac{1}{\Phi^{IR}} \quad (1)$$

Here I_A^0 is the photofragmentation signal because of absorption by the ground-state conformers A only, measured with a UV laser fixed on a transition of conformer A without IR-preheating; $I_A(\nu)$ is the double-resonance signal, measured as a function of the IR wavenumber with the UV laser fixed on a transition of conformer A; I_A^∞ is the signal because of UV absorption by only the IR-excited species A, measured similar to $I_A(\nu)$, but with the OPO fixed on an IR transition that is fully saturated; and Φ^{IR} is fluence of the IR OPO.

We use Equation (1) to derive cross-sections of vibrational transitions in the 3 μm region of $[GS+2H]^{2+}$. Figure 2a shows depletion spectra of the $[GS+2H]^{2+}$ conformer A measured at low (2.5–3 mJ cm^{-2}, blue trace) and high (40–60 mJ cm^{-2}, pink trace) fluence of the IR beam in the ion trap. In both experiments we measured profiles of the UV and IR beams and ensured that the latter fully overlaps the former over the entire length of the trap. The high fluence spectrum exhibits apparent saturation to 85% depletion level for all prominent IR transitions, including the NH-stretch transition of phenylalanine residues in conformer A. This transition is well separated from transitions of the other identified conformers, allowing us to assign the remaining 15% of UV fragmentation signal to I_A^∞/I_A^0. Figure 2b shows the absorption spectrum of $[GS+2H]^{2+}$ conformer A derived from the blue trace of Figure 2a and the measured IR beam power curve (not shown here) using Equation (1). As an alternative to the fluence measurements, one can record $I_A(\nu)$ at different relative (low) values of Φ^{IR} and then apply Equation (1).[22]

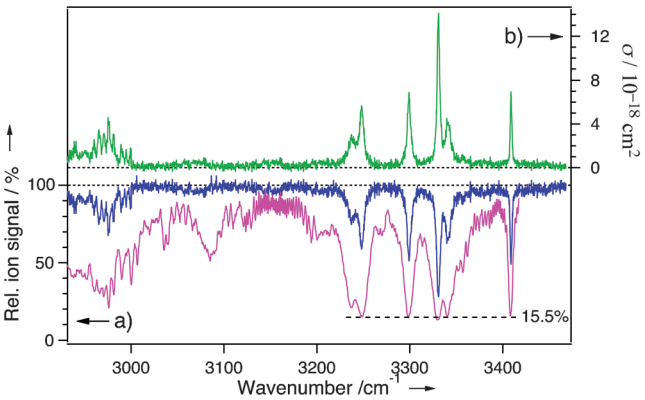

Figure 2. a) Depletion spectra of $[GS+2H]^{2+}$ conformer A, measured at low (blue trace) and high (pink trace) fluence of the OPO, when the UV laser is tuned to 37733.5 cm^{-1} (peak A1 in Figure 1). The spectra are normalized to the UV laser-only signal I_A^0. b) Absorption spectrum of the same species derived from the blue trace using Equation (1) and plotted in units of cross-section.

Compared with the original depletion spectrum (Figure 2a, blue trace), the derived absorption trace exhibits different relative peak intensities [because of the logarithmic dependence in Eq. (1)] and it is plotted in absolute units of cross-section. The integrated intensity of the peak at 3409 cm^{-1}, for instance, is (108 ± 10) km mol^{-1}, in agreement with the calculated value of 114 km mol^{-1} for the two near degenerate NH stretches of Phe backbones, reinforcing the previous assignment of the peak, which was based on its position.[20] The 2 cm^{-1} width of this peak implies, at least, 2.6 ps lifetime of the first excited state of Phe NH-stretch. The observed depletion level of above 50% points to the irreversible character of incoherent IR excitation from the vibrational ground level directly to the quasi-continuum of vibrational states with a rate, which must be much slower than the rate of IVR.[23] The estimated rate of Phe NH-stretch excitation in our experiments is $4 \cdot 10^8$ s^{-1} (Section S2). This allows us to bracket the IVR time for the first excited level of this vibration between 2 ps and 2.5 ns.

The capability of the IR–UV depletion for measurements of vibrational spectra is limited by molecular size. Indeed, for an absorbed IR photon to alter the UV spectrum significantly, its energy must be at least comparable with the internal thermal energy of an ion, $E_{vib}(T)$. For example, for gramicidin S our calculations yield $E_{vib}(300\ K) = 18.5 \times 10^3$ cm^{-1}, $E_{vib}(120\ K) = 3.5 \times 10^3$ cm^{-1}, and $E_{vib}(12\ K) = 27$ cm^{-1}. These numbers underline the difficulty of depletion experiments on $[GS + 2H]^{2+}$ at room temperature and justify the need for cooling of the ions. A linear extrapolation of these numbers (by the number of residues) suggests that $E_{vib}(12\ K) \approx 3 \times 10^3$ cm^{-1} for a protein as large as a thousand residues. This implies that excitation of an NH-stretch in cold biomolecules of up to this size may noticeably influence their UV absorption. To verify this assessment we performed IR–UV DR spectroscopy of a yet small natural protein, cytochrome c ($M = 12396$ Da, 104 residues), cooled and stored in our 6 K ion trap. The UV fragmentation spectrum of $[Cyt\text{-}c + 12H]^{12+}$ shows an unstructured band, which spans the range 265–290 nm. While such a broad spectrum inhibits conformer-selective IR–UV depletion spectroscopy, IR-preheating of the protein shifts its UV absorption band to the red, which affects its slope on the low-energy side. Tuning the UV laser to this edge therefore allows the detection of the increased fragmentation yield of the protein upon IR excitation. Figure 3 shows such an IR "gain" spectrum measured for $[Cyt\text{-}c + 12H]^{12+}$. To our knowledge this is the first vibrational absorption spectrum of a bare, intact protein in the gas phase. The spectrum contains three bands of about 100 cm^{-1} width in this region where one expects absorption by CH-, NH- and OH-stretch vibrations. The width of the bands is similar to that in the infrared multiphoton dissociation spectrum of $[Cyt\text{-}c + 11H + K]^{12+}$ clusters, measured in the 6 μm region at room temperature.[24] In contrast to those measurements, the shapes of the bands in our spectrum are, apparently, non-Gaussian, implying their non-thermal broadening. The broad absorption even by cooled cytochrome c is not surprising, given that there are more than a hundred NH oscillators alone, which all absorb within a range of a few hundred cm^{-1}, as well as broadening arising from conformational heterogeneity of the protein. Nevertheless, even an enveloped spectrum such as this still may provide an important test of theory.

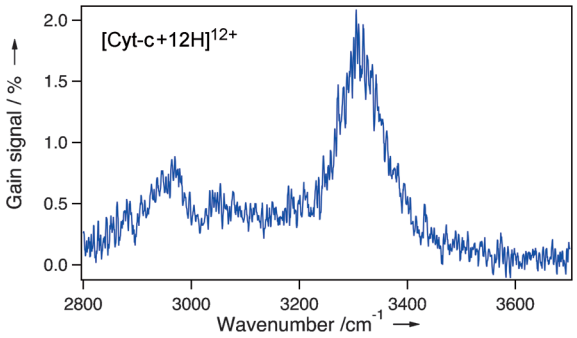

Figure 3. IR–UV gain spectrum of $[Cyt\text{-}c + 12H]^{12+}$ protein (from horse heart, > 95% purity) cooled in a 6 K ion trap. The gain corresponds to the increase of photo-fragmentation signal at $m/z = 1022$ because of IR pre-excitation, and it is normalized to the parent signal at $m/z = 1033$. The UV laser is tuned to 288.75 nm.

In conclusion, we have demonstrated the use of IR–UV photofragmentation depletion spectroscopy for conformational assignment of vibronic transitions in UV spectra of protonated decapeptide gramicidin S, revealing all three conformers of this species. The proposed model of depletion mechanism suggests that depletion spectra will reflect all absorbing vibrations of large molecules and allows us to elaborate a procedure for measuring absolute absorption cross-sections of vibrational transitions. We show that cryogenic cooling of ions is essential for IR–UV DR spectroscopy of large molecules and illustrate this by measuring the IR absorption spectrum of an intact gas-phase protein, cytochrome c.

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