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Direct Observation of Acrylamide Fluorescence*

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Acrylamide is a widely used quenching agent in UV/VIS spectroscopic studies of biopolymers. It is believed to interfere with the chromophore luminescence by electron capture, resonance energy transfer or through collisional deactivation. The agent itself has been presumed optically silent, due to the low magnitude of the lowest UV absorption band. We have postulated that the dynamics of the semi-rigid acrylamide, at room temperature, can provide an efficient dissipative channel of the optical energy. Consequently, by restricting the acrylamide by a glass environment in the 10 – 95 K range, we were able to record flourescence emission and excitation spectra associated with the lowest UV absorption band. This observation, corroborated by the electronic structure calculations, further supports the photon exchange as the mechanism of the acrylamide-induced quenching.

INTRODUCTION

Monoacrylamide is the most widely used quenching agent in the UV/VIS optical spectroscopic studies of biopolymers.[1] It has been established as a quencher of both the singlet-singlet and triplet-singlet radiative relaxations.[2] In most spectroscopic studies of tryptophan containing proteins, acrylamide is added, usually in high concentrations (0.1 to 2 mol/L), to induce decrease in the fluorescence emission yield. In addition to interference with the tryptophan luminescence, acrylamide was observed to quench fluorescence of protein labels (*e.g.* benzo[*a*]pyrene tetrol, BPT)[3], nucleic acids labels (*e.g.* benzo[*a*]pyrenediol epoxide, BPDE, label),[4] and labeled protein:nucleic acid complexes. The extent of fluorescence quenching is directly correlated with the degree of exposure of the fluorophore to the quencher. Consequently, in the case of strong acrylamide-induced quenching, the fluorophore-containing domains in proteins, DNAs, and their complexes are classified as solvent accessible (or type II domains[3]). The opposite, however, does not quite hold true; the fluorescence of inaccessible tryptophan residues (*e.g.* in LADH[2] or in alkaline phosphatase[5]) coud also be quenched by acrylamide, though to a lesser extent. »Reso-

* Dedicated to the memory of Professor Tibor Škerlak

nance energy transfer« was invoked to explain this observastion.[5] In most other studies, electron transfer from the excited chromophore to acrylamide has been the assumed mechanism of quenching. Semiempirical electronic structure calculations, however, provide no support for the latter hypothesis.[6] Whatever mechanism has been implicated in a specific case of acrylamide-induced luminescence quenching, the agent has been considered optically silent; no acrylamide luminescence has ever been reported. We recently forwarded the idea that acrylamide itself can act as a chromophore in the near UV domain.[6] Electronic transition calculations predict the existence of a weak $\pi^* \leftarrow n$ singlet-singlet transition in the 284 – 296 nm domain. The band is predicted to be too weak to be observed as a distinct feature in the absorption spectrum. However, as it has been observed in carbonyl compounds,[7] some fluorescent emission could take place. We hypothesized that acrylamide is a flexible molecule that can dissipate the excited state energy at room temperature. Cooling it down should reduce the molecular dynamics. On the other hand, partial rigidity of the carbonyl-ethene backbone in the molecule should restrict the number of conformational substates. These two conditions could, in principle, enable observation of the acrylamide fluorescence. In this paper we show that this is indeed true.

METHODS AND PROCEDURES

Highest purity commercially available acrylamide (Schwarz-Mann Biotech., ultrapure, mass fraction 99.99%, lot # 19181) was used as such or recrystallized from a high purity methanol (Aldrich, spectrophometric grade). The methanol from the same batch was used to correct the luminescence base line. Acrylamide samples were $1 \cdot 10^{-2} - 1 \cdot 10^{-3}$ mol/L, dissolved in a glycerol/water mixture of volume ratios 3:2 to 4:1. Glycerol was of spectrophotometric grade (Aldrich, lot. # 05902BX) and water was de-ionized, doubly distilled (Corning Mega-Pure System MP-6A). Prior to the preparation of glass-phase the solution was sonicated 1 – 2 hrs at 0 °C to prevent acrylamide self-association and possible onset of polymerization. A stream of ultrapure Ar (Scott Specialty Gases, volume fraction 99.9995%) was passed through the solution to decrease the dioxygen content and reduce the dioxygen-induced fluorescence quenching. The sample was prepared between two pre-cooled optical windows (fused quartz or synthetic sapphire), separated by lead spacers (0.025 to 0.05 mm), and mounted on a two-stage closed-cycle helium refrigerator (APD Cryogenics). By slow cooling to 215 – 205 K in a steady stream of high purity helium the solution was turned into a glass. High vacuum ($< 1 \cdot 10^{-3}$ Pa; reading of a degasssed Bayard-Alpert ionization gauge) was applied and the sample cooled down to 10 – 8 K (Chromel/Au(Fe) thermocouple reading). The vacuum line consisted of an upgraded Alcatel 86615 L high vacuum pumping station, equipped with a high capacity LN_2-cooled trap.

The UV absorption spectra were recorded by a Varian CARY-2200 spectrophotometer. Luminescence was measured in a front-face geometry (21° off the incident beam) on a SPEX AF125-I spectrofluorometer, equipped with a Peltier-junction-cooled Hamamatsu R928 PM tube detector. The monochromators were calibrated with reference to the mercury lines in the laboratory fluorescence light (365.02 and 435.83 nm) and the Gd(III) solvent-induced $^8S_{7/2} \leftarrow {}^6P_{7/2}$ transition (excitation at 272.94 nm and emission at 310.95 nm).

Mass-spectra were obtained by either a direct inlet probe or following a GC separation, in a Kratos MS25 spectrometer, by the electron impact method (70 eV).

Calculations of the ground electronic state molecular geometries were done with the *ab initio* Gaussian-90 program package.[8] Full geometry optimization calculations were carried out with the triple split valence shell HF/6-311G** basis set. Electronic transition properties were calculated with our version of Zerner's[9] INDO/S1 program. Details of the one-electron parameters, two-electron integral approximations and configuration selection criteria were described elsewhere.[10] All calculations were carried out on a cluster of Digital Equipment Corporation VAX computers running the VMS operating system.

RESULTS AND DISCUSSION

The near UV absorption spectrum of mono-acrylamide is dominated by a single band at 197 ± 1 (ε = 13370 ± 200 L/(mol · cm) in glycerol/water mixture); its red end extends beyond 300 nm, Figure 1.

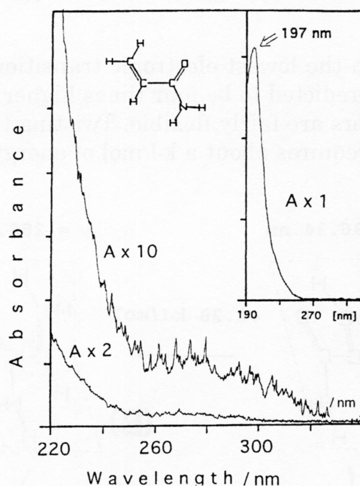


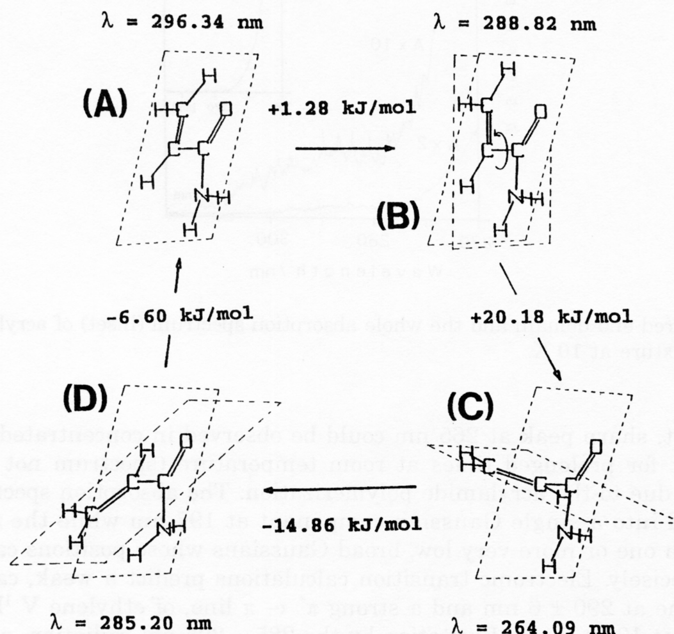
Figure 1. The red end domain and the whole absorption spectrum (inset) of acrylamide in glycerol/water mixture at 10 K.

A distinct, sharp peak at 265 nm could be observed in concentrated acrylamide solutions left for prolonged times at room temperature (spectrum not shown); we think this is due to the acrylamide polymerization. The absorption spectrum can be deconvoluted into a single Gaussian component at 197 nm while the red tail can be fitted with one or more very low, broad Gaussians whose positions cannot be determined precisely. Electronic transition calculations predict a weak, carbonyl-type $\pi^* \leftarrow$ n(O) line at 290 ± 6 nm and a strong $\pi^* \leftarrow \pi$ line, of ethylene V $^1B_{1u} \leftarrow$ N ^1A_g parentage,[11] at 197 ± 2 nm. Excitation by the 285 – 295 nm radiation, at room temperature, fails to entice measurable fluorescence emission even in highly concentrated acrylamide solutions and at high sensitivity photon counting. This is not very surprising in view of the (i) very low molar absorption coefficient (ε = 70–250 L/mol cm,

estimated from the INDO/S1-CISD oscillator strengths, calibrated with reference to the molar absorption coefficient of the 197 nm band) and (ii) the notoriously poor emission in chromophores containing the lowest $\pi^* \leftarrow n$ bands (*e.g.* nucleic acids).

In molecules of the size of acrylamide, fluorescence emission can be interfered with or completely corrupted by the dynamics of the excited state. No data are available on the potential energy surface of the lowest excited state of acrylamide; however, given the low excitation energy and the localized character of the $\pi^* \leftarrow n(O)$ transition, the ground state electronic structure properties provide a good approximation. The molecule could exist in two principal forms. The form in which ethene is in *cis*-position to carbonyl (A, Scheme I) is predicted to be planar and more stable than the other forms. In the following analysis we will consider it as the reference structure. The *trans*-isomer (D, Scheme I) is helicoidal due to the repulsion between the terminal ethene and the amino hydrogens (N-C-C-C- dihedral angle is –25.1°). While the *cis-trans* isomerization barrier is predicted at 21.46 kJ/mol (C, Scheme I), the difference between the ground state energies in two isomers (6.60 kJ/mol) is in the range of the solvent kinetic energy at room temperature (kT = 2.49 kJ/mol), Scheme I.

The difference between the lowest electronic transition energies for the *cis*- and *trans*-isomer, however, is predicted to be four times higher than the ground state energy difference. Both isomers are fairly flexible. Twisting the *cis*-isomer by 16° along the saturated C – C bond requires about a kJ/mol of energy. The corresponding tran-



Scheme I. Differences in the ground state energies (HF/6-311G**, full optimization) of the acrylamide conformers and the corresponding variations in the transition energies (INDO/S1-CISD).

sition energy change into the lowest lying excited state of the twisted acrylamide is eight times higher. Increased twisting decreases the mixing of the carbonyl and ethene low-lying molecular orbitals and the transition energies become higher. The same geometry change brings certain centers closer and induces changes in the corresponding one-electron terms. As a consequence, oscillator strengths of certain transition lines increase several times. This is a crude model, based on semiempirical INDO/S1 results but the trend is correct. Experimentally observed increases of molar asbsorptivities of the carbonyl bands in sterically hindered α,β-unsaturated ketones are well known.[7]

The following picture is suggested by these calculations. Acrylamide is a flexible molecule with a bifurcated, asymmetric (due to the energy difference beween the *cis*- and *trans*-isomers) distribution of conformers. Due to the small energy differences, the conformers within either sub-population are exchanging at a rate of the order of 10^{12} s^{-1}. However, due to the differences in orbital mixing, the absorption energies of the conformers are predicted to vary over nearly 2000 cm^{-1}. The exchange rate between the conformers spanning this excitation energy band is $10^3 - 10^4$ higher that the average radiative rate of an electronically excited state. With a comparable molecular dynamics for the ground and the lowest excited state, the latter means that, in acrylamide, photon exchange could occur over a virtual continuum of states covering a 2000 cm^{-1} wide energy band. *Cis – trans* isomerization occurs at a rate of $4 \cdot 10^8$ s^{-1}, which is comparable to the average singlet-singlet radiative rate. Though a relatively small molecule, acrylamide is likely to act as a very efficient sink of the excited state energy. Cooling the molecule down to 100 K should slow the isomerization rate to 2/10 of a second; at 10 K, isomerization is nonexistent. At this temperature, the exchange between conformers should slow down to $2 \cdot 10^6$ s^{-1}, well below the threshold for the interference with fluorescence emission. Indeed, by cooling acrylamide in the glycerol/water glass ($1 \cdot 10^{-2}$ mol/L) down to 10 K, we were able to observe the acrylamide fluorescence. The maxima of the excitation and emission spectra are at 284 ± 4 nm and 344 ± 4 nm, respectively; both bands are very weak, Figure 2.

The low temperature itself was not a sufficient condition; the sample had to be prepared by a very slow cooling down and, once the bottom temperature was

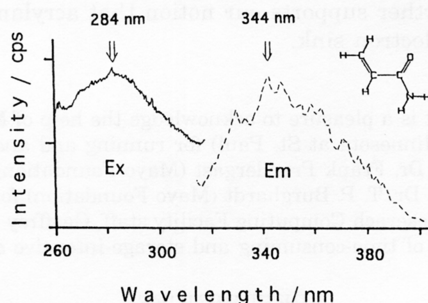


Figure 2. Fluorescence excitation (λ_{em} = 346 ± 2 nm) and emission (λ_{ex} = 284 ± 2 nm) of acrylamide in glycerol/water glass at 55 K. The intensities are normalized to equal heights. Both spectra are corrected for the solvent residual luminescence and the signal nonlinearities.

achieved, it had to be annealed at 55 – 95 K for at least half an hour. Prolonged sample preparation and repeated freeze-thaw cycles are conducive to the formation of acrylamide oligomers. The latter exhibit, as said before, a prominent peak in the 262 – 270 nm domain and, as noticed previously by the author,[6] they fluoresce weakly even at temperatures above 150 K. The mass-spectral analyses, run subsequently to each spectroscopic measurement, revealed no traces of polymerization in the samples we prepared.

There is a number of small-to-medium size molecules that fluoresce weakly or not at all. Cooling them down will not necessarily improve the chances to observe the light emission. Propionamide, the »saturated variant« of acrylamide, is an illustrative example. The saturated carbon chain in propionamide is very flexible. Full energy optimization calculations (*ab initio* HF/6-31G*) predict a rotational barrier at 3.7 kJ/mol, less than two kT quanta at room temperature. The molecule, therefore, exist in a virtually continuous distribution of conformational states. Furthermore, due to the absence of the xy-directionality of the saturated carbon σ-orbitals, the transition energy changes depend primarily on the distance-dependent one-electron interactions. Cooling the propionamide sample to 10 K slows down the rate of the conformer interchange below the emissive lifetime but by no meas narrows the conformational distribution. When excited into the $\pi^* \leftarrow$ n(O) transition, the molecule (probably) fluoresces but the emission is distributed over so many substates that no fluorescence band can be observed by convention data collection methods.

CONCLUSION

The ground electronic structure properties, calculated at the HF/6-311G** level, suggest that acrylamide exists in a bifurcated distribution of flexible *cis*- and *trans*-conformers. Electronic transition energies of these conformers are predicted to vary over 2000 cm^{-1}, and oscillator strengths could vary over an order of magnitude. High flexibility and a broad excitation band suggest a very low intensity of the lowest excitation and emission bands. Cooling the acrylamide and its environment to 10 K stops the *cis–trans* isomerization and slows down the rate of the conformational exchange below the emissive rate. Due to a fortuitous combination of flexibility and partial rigidity, the slowing down of the molecular dynamics results in a considerable narrowing of the distribution of conformational sub-states. In this way we were able to record, for the first time, fluorescence excitation and emission of acrylamide monomer. This data further supports our notion that acrylamide acts as a chromophore rather than an electron sink.

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SAŽETAK

Izravno mjerenje fluorescencije akrilamida

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Akrilamid se često upotrebljava u UV/VIS spektroskopskim proučavanjima biopolimera za gašenje svjetlosne emisije. Pretpostavlja se da akrilamid interferira sa svjetlosnim zračenjem kromofora mehanizmom prijelaza elektrona prema akrilamidu, putem rezonantnog prijenosa energije, ili deaktivacijom sudarima. Pretpostavljalo se da akrilamid sam po sebi ne emitira svjetlo, uglavnom zbog vrlo male absorpcijske snage u najnižoj UV vrpci. Pretpostavivši da gibanje polu-krutog akrilamida pri sobnoj temperaturi može značajno pridonijeti rasipanju i gubitku svjetlosne energije, hlađenjem akrilamida i onemogućavanjem molekulskog gibanja, u krutom staklu na (10 – 95) K, uspjeli smo izmjeriti spektre fluoresentne emisije i pobude. Ti eksperimentalni podaci, zajedno s kvantno-kemijskim proračunima elektronske strukture molekule, dodatni su dokaz da je mehanizam izmjene fotona važan za gašenje svjetlosne emisije izazvane akrilamidom.