Absorption and luminescence spectroscopy of mass-selected Flavin Adenine Dinucleotide mono-anions

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We report the absorption profile of isolated Flavin Adenine Dinucleotide (FAD) mono-anions recorded using Photo-Induced Dissociation action spectroscopy. In this charge state, one of the phosphoric acid groups is deprotonated and the chromophore itself is in its neutral oxidized state. These measurements cover the first four optical transitions of FAD with excitation energies from 2.3 to 6.0 eV (210–550 nm). The $S_0 \rightarrow S_2$ transition is strongly blue-shifted relative to aqueous solution, supporting the view that this transition has significant charge-transfer character. The remaining bands are close to their solution-phase positions. This confirms that the large discrepancy between quantum chemical calculations of vertical transition energies and solution-phase band maxima can not be explained by solvent effects. We also report the luminescence spectrum of FAD mono-anions \textit{in vacuo}. The gas-phase Stokes shift for $S_1$ is 3000 cm$^{-1}$, which is considerably larger than any previously reported for other molecular ions and consistent with a significant displacement of the ground and excited state potential energy surfaces. Consideration of vibronic structure is thus essential for simulating the absorption and luminescence spectra of flavins.

I. INTRODUCTION

Flavin Adenine Dinucleotide (FAD) is a ubiquitous redox cofactor serving many key metabolic roles for example as an electron acceptor in the citric acid cycle. FAD is a member of the flavin family which also includes Flavin Mononucleotide (FMN) and riboflavin (RF). These molecules all share the tri-cyclic iso-azoxyazine chromophore, whose high reduction potential and multiple redox states give a versatility which is exploited in autofluorescence imaging applications, the perception of magnetic fields by some migratory birds, and the protein micro-environment may alter the electronic absorption and emission spectrum of flavin cofactors. For example, the cellular redox equilibrium may favor one or another resting redox state of flavin, which have rather different optical properties. The redox-specificity of flavin fluorescence has been exploited in autofluorescence imaging applications, where flavins serve as a non-invasive intrinsic biomarker of metabolic activity. Even for a given redox state, significant differences in the optical spectra and excited state lifetimes of flavins in different proteins have been observed. In order to quantitatively understand such effects, the intrinsic optical spectra of isolated flavins are useful as a baseline for comparison. Such studies are readily compared to high-level theoretical calculations and eliminate the potentially confounding influence of solvent. The electronic structure of flavins has been called “a difficult case for computational chemistry,” and many authors have bemoaned the dearth of experimental benchmarks. To date, only a few optical spectra of isolated flavin-related compounds have been published, including fluorescence and fluorescence excitation spectra of lumilavlin in helium nanodroplets, and action spectra of protonated lumichrome (a flavin derivative lacking a N-10 substituent) and anionic FAD in vacuo. All of these studies examined only the lowest singlet excited state of the system in question.

Here, we give the full UV-Vis absorption profile of FAD mono-anions in vacuo, covering the first four bright transitions with excitation energies from 2.3 to 6.0 eV (210–550 nm). In addition, we report the luminescence spectrum of FAD mono-anions in vacuo. In this charge state, FAD is deprotonated on one of the phosphoric acid groups linking the flavin and adenine moieties, and the flavin chromophore is in its neutral, oxidized form. These new experimental results, in conjunction with previously reported solution-phase data, are used to critically evaluate the state of the art in modeling the electronic structure of flavins.

II. EXPERIMENTS

Absorption profile measurements were performed at two different instruments, the SepI accelerator mass spectrometer complex, and the ELISA electrostatic ion storage ring, both located at Aarhus University. In both cases, photo-absorption was measured indirectly by Photo-Induced Dissociation (PID) action spectroscopy. Flavin adein dinucleotide disodium salt hydrate was purchased from Sigma Aldrich and dissolved in methanol. FAD anions were transferred to the gas phase via electrospray ionization and stored in a multipole ion trap which was emptied every 25 ms (SepI) or 40 ms (ELISA). Ion bunches extracted from the trap were accelerated to kinematic energies of 50 keV (SepI) or 22 keV (ELISA) and the ions of interest were selected using a bending magnet according to their mass-to-charge ratio. A high-intensity pulsed laser system (EKSPLA OPO) was used to excite the mass-selected ion bunches in vacuo. In action spec-
troscopy, it is usually assumed that the electronically excited system ultra-rapidly crosses over to a highly vibrationally excited level of the ground electronic state (Internal Conversion), and that this vibrational energy is redistributed over all internal degrees of freedom in a matter of picoseconds (Internal Vibrational Redistribution). During the ∼5 ns irradiation time, the ions may (or may not) absorb multiple photons sequentially, i.e. the ion returns to its electronic ground state in between each photon absorption. The deposited energy leads to unimolecular dissociation and/or thermionic electron emission on timescales ranging up to several milliseconds. By monitoring the yield of the photo-products (daughter ions or neutral fragments) as a function of excitation wavelength (corrected for the variation in laser power/photon flux across the spectral range), the so-called action spectrum is constructed. It should be kept in mind that the action spectrum may not perfectly reflect the absorption cross section due to limitations such as sampling time or alternative relaxation channels such as photon or electron emission. Additional experimental details are presented in the Supplementary Material.

At SepI, daughter ions were separated using an electrostatic energy analyzer (mass resolving power ∼100) positioned after the laser-ion interaction region and counted with a channeltron detector. Every second ion bunch was irradiated with the laser and the difference in counts between the "laser-on" and "laser-off" injections is the photo-induced signal. The low background rate and high detection efficiency of daughter ions provides superior signal-to-noise than measurement of the depletion of parent ions, particularly for large molecules like FAD with many internal degrees of freedom and correspondingly low dissociation yields. The depletion of the parent FAD mono-anion ion beam measured with 210 nm excitation was 0.8±0.5%. The SepI instrument samples photo-induced dissociation occurring during the ∼10 µs it takes for the ions to travel from the laser interaction region to the electrostatic analyzer. This limited sampling time could in principle skew the absorption profile towards the blue, an effect known as a kinetic shift. The PID mass spectrum recorded with 250 nm (5 eV) excitation is shown in Figure 1. The dominant daughter ion is that with 542 m/z. This corresponds to the loss of neutral lumichrome (the flavin rings plus a hydrogen atom), which is the main product of normal photolysis of flavins in solution.

Figure 2 shows the ELISA electrostatic ion storage ring. FAD mono-anions circulate around the race-track like ring with a revolution time around 60 µs. After being stored for 11 ns the laser pulse is overlapped with the ion bunch in the upper straight section. Laser-excited ions may then continue to circulate for several ms before decaying. If they dissociate while in the lower straight section (i.e. after at least one half revolution) the neutral fragments will no longer be affected by the electrostatic confinement fields and fly to the microchannel plate (MCP) detector mounted on this section.

The luminescence spectrum of gas-phase FAD mono-anions was recorded using the LUNA luminescence spectrometer in Aarhus. Ions were again produced by electrospray ionization and accumulated in a cylindrical Paul trap. The amplitude and DC offset of the radio frequency trapping voltage applied to the cylinder electrode were set to apply a low-mass cutoff of approximately 600 m/z i.e. higher than any of the daughter ions observed in the PID mass spectrum (Figure 1). The luminescence signal rate was insufficient to further optimize the mass selection parameters. The trapped ions were excited at 445 nm by an EKSPLA OPO laser system. The laser power was reduced to 50 µJ/pulse to reduce multiple-photon absorption. Luminescence was collected through one of the end caps of the Paul trap, which is made of a wire mesh. An aspheric condenser lens mounted directly
ple different dissociation timescales, indicates that our measurement techniques. The excellent agreement in the produced here to show consistency between the two monitoring the total neutral fragment yield. The SepI data from 420 to 550 nm was reported previously with ions in the trap followed by 100 cycles with no ions experiment was repeated in alternating sets of 100 cycles. The difference between the "ions-on" and "ions-off" acquisitions is the luminescence signal.

III. ABSORPTION RESULTS AND DISCUSSION

In the upper panel of Figure 3, PID action spectra of FAD mono-anions, recorded in three overlapping spectral regions, are shown. SepI was used for the wavelength ranges 210–350 nm and 420–550 nm, monitoring the yield of the daughter ion with 542 m/z (lumichrome loss). ELISA was used in the range 309–550 nm, monitoring the total neutral fragment yield. The SepI data from 420 to 550 nm was reported previously, and is reproduced here to show consistency between the two measurement techniques. The excellent agreement in the low-energy range between the two datasets, which sample different dissociation timescales, indicates that our results are not strongly affected by any kinetic shift. The lower panel of Figure 3 shows the absorption cross section of FAD in neutral aqueous solution adapted from Islam et al. With the exception of the $S_0 \rightarrow S_2$ transition, which is red-shifted by 0.22 eV (23 nm) in solution, the band maxima are identical within experimental accuracies. Hints of vibronic structure are present in the gas-phase spectrum (upper panel), such as a minor peak at 411 nm, but the bands are generally broad and featureless, consistent with the solution-phase measurements (lower panel).

In Table I, our experimental results are compared with a survey of previously published calculations of gas-phase transition energies for various flavins. Most electronic structure calculations of flavins focus on lumiflavin in vacuo. The stick spectrum is the consensus of DFT calculations of vertical transition energies for lumiflavins and absorption band maxima of FAD in neutral aqueous solution. All in eV.

| $S_0 \rightarrow$ | FAD$^a$ | FMN$^a$ | LF$^{12,14,15,24–27}$ | RF$^{29}$ | FMN$^{30}$ | FAD$^{23}$ |
|------------------|---------|---------|----------------|--------|----------|---------|
| $S_1$            | 2.74    | -       | 3.00           | 2.56/2.89 | 2.74     |
| $S_2$            | 3.50    | -       | 3.84           | 3.68   | 3.26/3.3 | 3.37    |
| $S_{3a}$         | -       | -       | 4.70           | 4.83   | -        | -       |
| $S_{3b}$         | 4.75    | 4.69    | 4.88           | 4.89   | -        | 4.73    |
| $S_4$            | 5.8     | 5.6     | 5.81           | 5.79   | -        | 5.78    |

TABLE I. Band maxima of action spectrum of FAD and FMN mono-anions in vacuo compared to electronic structure calculations of vertical transition energies for various flavins in vacuo and absorption band maxima of FAD in neutral aqueous solution. All in eV.

$^a$Present work, experiment in vacuo, uncertainties implied by the number of significant digits

$^b$Consensus of TD-DFT values from various authors, see Supplementary Material

$^c$Experiment in aqueous solution
gives this transition a significant degree of charge transfer character\textsuperscript{13}, which is widely thought to be responsible for the solvatochromic behavior of $S_0 \rightarrow S_2$. The HOMO and LUMO, in contrast, are both spread across the entire chromophore\textsuperscript{13,14,25} and this transition shows little solvatochromism in theory\textsuperscript{25} or experiment\textsuperscript{12,29,32}. The present experimental results qualitatively support this view, with a large blue-shift (0.22 eV) upon desolvation for $S_0 \rightarrow S_2$, but no such shift for $S_0 \rightarrow S_1$.

Several authors have investigated whether solvent effects can explain the large deviation between calculated vertical excitation energies and experimentally measured absorption band maxima in solution\textsuperscript{11,13,25,27}. The present results, however, show that such effects are small. As has been pointed out earlier\textsuperscript{33}, the absorption spectra of flavins are hardly affected by the solvent environment\textsuperscript{12,29,34–36}, except of course for the $S_0 \rightarrow S_2$ transition. Moreover, calculations\textsuperscript{13} and measurements\textsuperscript{37} find only a small increase in the permanent dipole moment of the flavin chromophore upon excitation to $S_1$. Solvatochromism measurements\textsuperscript{32} actually imply a slight decrease in the dipole moment upon excitation, but with a large uncertainty. There is thus no reason to expect large solvent effects for $S_0 \rightarrow S_1$, and indeed none are found in most calculations\textsuperscript{13,25}, or from the present gas-phase experiments.

Setting aside solvent effects, the vibronic structure of flavins must seriously be taken into account. As the density of vibrational levels in an electronically excited state increases strongly with energy, the absorption band maximum is usually observed to the blue of the 0-0 transition energy. The band maximum often roughly coincides with the vertical transition energy calculated in TD-DFT from the ground state equilibrium geometry, although clearly not in the case of flavins. Full calculations of broadened vibronic excitation spectra reported for flavins\textsuperscript{33,38–40} come closer to reproducing the profile and position of the present gas-phase results than “simple” TD-DFT. There remains some discrepancy in the 0-0 energy (0.05 eV\textsuperscript{38} to 0.5 eV\textsuperscript{40}, depending on the calculation) compared to that measured in He droplets for lumiflavin\textsuperscript{15}, which is presumed to be due to limitations in the chosen functionals. These methods have struggled to include micro-solvation effects, and are rarely attempted for excited states higher than $S_1$. We hope the present contribution will serve as a benchmark for further refining these methods, as it is clear that careful consideration of vibronic activity is essential in modeling absorption by flavins.

Solvent effects are evidently important for modeling the $S_0 \rightarrow S_2$ transition. The $S_2$ absorption band maximum of riboflavin varies from 332 nm (3.75 eV) in apolar dioxane to 367 nm (3.38 eV) in water\textsuperscript{29,34}. The present value for the gas phase is 346 nm (3.59 eV). Calculations\textsuperscript{13} and measurements\textsuperscript{37} find that the permanent dipole moment of the $S_2$ state is significantly higher than that of $S_0$, implying bulk polarization effects may be important. In addition, significant differences between polar aprotic solvents such as DMSO and polar protic solvents like water suggest that hydrogen bonding plays a role as well\textsuperscript{32}. Calculations including both of these effects do well at reproducing the magnitude of the solvent shift of $S_0 \rightarrow S_2$\textsuperscript{13,14}.

A few TD-DFT calculations have been performed on more complex flavins including the ribityl sidechain. The addition of the sidechain appears to have little influence on the HOMO and LUMO orbitals\textsuperscript{11,29,38,41}. However, the sidechain may participate in other orbitals, notably including those involved in the $S_0 \rightarrow S_2$ transition\textsuperscript{11,30}. This could affect the degree of charge transfer character in these transitions. Sikorska and coworkers reported theoretical spectra for both LF\textsuperscript{12} and RF\textsuperscript{29} and found $S_0 \rightarrow S_2$ to be nearly 0.2 eV lower for RF, while the other transition energies agreed with the consensus for LF.

Interpretation of the UV portion of the absorption profile is complicated by the presence of the adenine moiety in FAD and the relative lack of calculations and solution-phase data in this spectral region. Most calculations find the $S_0 \rightarrow S_3$ band of the flavin chromophore to be composed of two transitions (labeled $S_{3a}$ and $S_{3b}$ in Table I), which are not resolved in the present experiment. Adenine absorbs at wavelengths similar to those of flavins, with band maxima near 250 and 200 nm in the gas phase\textsuperscript{42}, but with lower absorption cross sections (in solution)\textsuperscript{23}. We are aware of no modern quantum chemical calculations of the full FAD system. To add another point of comparison, we recorded a PID action spectrum of FMN (which lacks the adenine part) mono-anions at SepI. Figure 4 shows the PID action spectra of FAD and FMN mono-anions recored at SepI. The FAD spectrum is the same data presented in Figure 3, the solid line is a 5-point moving average. The action spectrum for FMN...
In aqueous solution (adapted from Islam et al.\textsuperscript{23}). Both spectra represent $S_1 \rightarrow S_0$ fluorescence. To our knowledge, Figure 5 is the first reported gas-phase fluorescence spectrum of a completely bare (tag-free), naturally occurring biomolecular ion in its physiological charge state. The light grey line is the raw data from the CCD and the solid black line is a fit to the functional form

$$y = A \times \exp[-\exp(-(x-x_0)/w) - (x-x_0)/w],$$

as recommended by Greisch et al.\textsuperscript{44} as an empirical tool for characterizing asymmetric luminescence bands. The fit gives the position of the band maximum $x_0 = 525 \pm 2$ nm ($2.37$ eV). The position of the fluorescence maximum in water is $541$ nm\textsuperscript{23}, which is consistent with other flavins in water\textsuperscript{32,36}. In contrast to $S_0 \rightarrow S_1$ absorption band maximum, the fluorescence band maximum of flavins varies significantly with solvent polarity, varying from 542 nm in water to 509.5 nm in benzene for riboflavin\textsuperscript{32}. This suggests that the fluorescence solvatochromism is due to stabilization of the more polarizable excited state by solvent dipole rearrangement. The gas phase Stokes shift of $3000$ cm$^{-1}$ (0.37 eV) is consistent with that of riboflavin in the least polar solvents such as chloroform\textsuperscript{32}. In polar, protic solvents, Stokes shifts close to $4000$ cm$^{-1}$ are observed\textsuperscript{32,36}. We note that the emission maximum and Stokes shift for riboflavin in DMSO are more similar to those in apolar solvents than polar protic ones\textsuperscript{32}, again suggesting that micro-solvation effects (e.g. H-bonding) play some role.

Notably, the gas-phase Stokes shift for FAD is significantly higher than that measured for other complex molecular ions such as xanthene\textsuperscript{15-47} and phenoxazine\textsuperscript{48} laser dyes. Gas-phase stokes shifts for rhodamine dyes, for example, have been found to range from $900$ cm$^{-1}$ to less than $500$ cm$^{-1}$. The analysis of Klaumünzer et al.\textsuperscript{38} indicates that several stretching modes of the iso-axloazine chromophore with frequencies in the range $1400-1600$ cm$^{-1}$ dominate the vibronic spectra of flavins and predicts a Stokes shift of $3400$ cm$^{-1}$. As has been observed for other complex molecules\textsuperscript{48}, these vibrational frequencies correspond to about half the value of the gas-phase Stokes shift. This helps us understand the discrepancy between calculated vertical transition energies and observed absorption band maxima, the correspondence between which relies on the assumption of high vibrational excitation upon absorption such that the vibrational wavefunctions peak at the classical turning points\textsuperscript{50}. Although the ground and excited state structures of flavins may be significantly displaced from each other, the difference in energy between the vertical and adiabatic (0-0) transition is covered by only a few quanta of the most strongly coupled vibrational modes and thus fails to meet this criterion.

The fluorescence signal detected from FAD mono-anions is very weak. Determination of absolute fluorescence quantum yields of trapped ions is experimentally challenging as key parameters such as the number of ions
in the trap and the overlap between the laser beam and the ion cloud are difficult to measure precisely. Instead, the “brightness” (the total integrated fluorescence signal per laser shot) is often used to compare the luminescence from different ions recorded under similar experimental conditions. The brightness of FAD mono-anions is at least an order of magnitude lower than that of resorufin, an anionic xanthene dye. If we assume that the fluorescence quantum yield of gas-phase resorufin is the same as in aqueous solution (0.75), we can roughly estimate the gas-phase quantum yield of FAD to be about 0.1 (for additional details, see Supplementary Material).

While the quantum yields of riboflavin and FMN are reasonably high (around 0.26), neutral solutions, FAD is thought to exist in a stacked conformation where electron transfer from the adenine moiety quenches the flavin excited state. This leads to a reduced fluorescence quantum yield of 0.03. At reduced pH, unstacked conformation becomes dominant and the quantum yield rises to 0.13.

While our estimate of the gas-phase quantum yield is too crude to distinguish between stacked and non-stacked conformations, it is interesting to note that the quantum yield does not appear to be significantly lower than in solution. In contrast, no fluorescence was detected for FAD di-anions or, more remarkably, from FMN anions, using the LUNA instrument. Using the same brightness comparison with resorufin, we can estimate an upper limit for the gas-phase quantum yield of FMN of 0.04, much less than in solution. This suggests that these ions may decay through some non-radiative channel (e.g. electron detachment or inter-system crossing) that is not competitive for FAD mono-anions. Changes in fluorescence quantum yield upon desolvation have been reported for other molecules and may be a more sensitive indicator of changes in photophysics than transition energies.

V. CONCLUSION

We have reported the photo-induced dissociation action spectrum and the luminescence spectrum of FAD mono-anions in vacuo. These results confirm that the vertical transition energies calculated using various electronic structure methods overestimate the intrinsic absorption band maxima. Neglect of vibronic structure, rather than solvent effects, is the cause of this discrepancy. Bulk polarization and micro-solvation effects appear to be important only for the $S_0 \rightarrow S_1$ transition, in agreement with calculations which indicate that this transition has significant charge-transfer character. Luminescence emission occurs at a rather high Stokes shift compared to previously reported studies of complex molecular ions in vacuo in what again appears to be a vibronic effect. No emission was seen from two other flavin anions. The observed micro-environmental sensitivity of fluorescence portends a prominent role for gas-phase luminescence spectroscopy studies in unraveling the intrinsically complex photophysics of complex biomolecules.

SUPPLEMENTARY MATERIAL

The Supplementary Material includes additional experimental details, a tabulation of previously published calculations of transition energies of lumiflavin, and a description of our approach to estimating the gas-phase quantum yield of FAD.

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