Photo-induced processes in aromatic biochromophores

H. Kang¹, C. Charrière¹, C. Dedonder-Lardeux¹, C. Jouvet¹, S. Martrenchard¹, C. Desfrançois², G. Grégoire², J-P. Schermann², M. Barat³ and J.A. Fayeton³

Laboratoire ELYSE, Bât 349, Université Paris-Sud, 91405 Orsay, France
¹Laboratoire de Photophysique Moléculaire du CNRS, Bât. 210. Université Paris-Sud, 91405 Orsay, France. e-mail christophe.jouvet@ppm.u-psud.fr
²Laboratoire de Physique des Lasers du CNRS, Institut Galilée, Université Paris-Nord, 93430 Villetaneuse, France.
³Laboratoire des Collisions Atomiques et Moléculaires du CNRS, Université Paris-Sud, 91405 Orsay, France.

Abstract. The measurement of protein lifetimes in vivo is a tool to follow the dynamics of conformational changes. The natural fluorophore in proteins is the tryptophan residue and its lifetime can vary by two orders of magnitude. We have developed a model inferring that the tryptophan lifetime is controlled by the coupling between the initially excited state and another state, dissociative along an NH coordinate. We have undertaken experiments in the gas phase in order to test this model. Two cases will be investigated: the case of the neutral species like indole (chromophore of tryptophan) and that of protonated species. In that latter case, we will show that protonated tryptamine ions, after excitation in the UV are losing a hydrogen atom in the femtosecond regime. This hydrogen-atom loss produces a radical cation that further fragments in a non statistical way.

1 Introduction

The fluorescence of the tryptophan amino acid (Trp) is highly sensitive to its environment, making it an ideal choice for reporting protein conformation changes and interactions with other molecules [1]. The properties used are changes in the fluorescence intensity, wavelength maximum (λmax), band shape, anisotropy and fluorescence lifetimes.

The power of this probe has been considerably amplified since Trp can often be substituted for other amino acids by site-directed mutagenesis, with minimal effect on structure and activity.

The emission spectrum of Trp in proteins varies from a structured band to a broad, diffuse band with wavelength maximum spanning a 40-nm range. The fluorescence quantum yield ranges from 0.35 to near zero [2]. The environmental sensitivity of the wavelength of the emission maximum seems to be well understood [3]. It has been clearly shown that these shifts are due to the electric field imposed by the protein and the solvent on the indole chromophore of Trp. They may be termed as an internal Stark effect, by analogy to the familiar shifting of energy levels via an applied (external) field. The agreement of prediction and experimental observation presented in reference 3 seems to be very good, so one can assume that the local electric field direction and magnitude determines primarily the sign and magnitude of the fluorescence shifts relative to vacuum. The relative orientation and intensity of the electric field on the indole ring determines whether there will be a red shift, a blue shift, or no shift at all.

The variation of the fluorescence lifetime of Trp or its indole chromophore has also been the subject of many investigations in both condensed and gas phases [4-17]. However, no clear picture emerges from these works that allows to understand why the fluorescence lifetime (or the fluorescence quantum yield) is varying by more than two orders of magnitude, depending on the environment. To account for this effect, it is necessary to anticipate the existence of very fast nonradiative decay channels that efficiently quench the fluorescence.

Many single-Trp proteins also display double or triple exponential decays. One obvious origin of these multi-exponential decays is the existence of multiple
protein conformations. Since the electric field generated by nearby amino acids on the indole chromophore depends on the conformation, one expects that the electric field will change the indole lifetime, as it will be established in the following.

2 Neutral aromatic chromophores

- In pyrrole [19], the $1\pi\sigma^*$ state is lower than the $1\pi\pi^*$ and its excitation leads to the formation of an H atom with a narrow kinetic energy distribution, related to the direct population of a repulsive state [20,21].

- In clusters of phenol and ammonia, PhOH-(NH$_3$)$_n$, or indole and ammonia, calculations predict that the H transfer leads to the formation of hydrogenated ammonia clusters NH$_4$(NH$_3$)$_n$ through an avoided crossing between the $1\pi\pi^*$ and the $1\pi\sigma^*$ states [22]. In this system the reaction seems to proceed via tunneling, since the process is fast in the hydrogenated species and considerably slower for deuterated species [23-25]. It has been shown, in good agreement with this mechanism, that H atoms are produced when tryptophan within a protein is irradiated with ultraviolet light [26].

In indole, at the ground state equilibrium geometry, the $1\pi\pi^*$ state has a strong dipole moment of nearly 10 Debyes due to an electron transfer from the ring to the H atom of the NH bond [27,28]. The dipole moment of the $1\pi\sigma^*$ potential-energy function intersects not only the bound potential-energy functions of the $1\pi\pi^*$ excited states, but also that of the electronic ground state. These symmetry-forbidden intersections for the planar systems are converted into conical intersections when out-of-plane modes are taken into account [18]. Via predissociation of the $1\pi\pi^*$ states and a conical intersection with the ground state, the $1\pi\sigma^*$ state triggers an internal-conversion (IC) process. The lifetime of the optically excited $1\pi\pi^*$ state is governed by the first intersection which determines the barrier for IC process, and varies from one molecule to the other depending largely on the energy gap between the $1\pi\pi^*$ and the $1\pi\sigma^*$ states.

This model has been already substantiated by several ab-initio calculations and agrees well with experimental observations.
3) Protonated molecules

![Protonated Tryptamine](image)

Figure 3. Protonated tryptamine (m/z=161). The proton is located on the terminal amino group.

It is known \(^1\) that tryptophan in solution has a very low fluorescence quantum yield at very low pH, i.e. when it is protonated. This means that a fast non-radiative process occurs in this protonated species. Once more one can wonder what is this non-radiative process. We present the first direct demonstration of the existence of an ultrafast fragmentation channel, hydrogen atom loss, and we investigate its branching ratio with respect to internal conversion (I.C.) by monitoring, on a femtosecond time-scale, the different fragmentation channels of photo-excited protonated ions containing an indole chromophore. By considering an isolated ion as the investigated species, we have experimental access to the H-atom loss by means of mass-spectrometry in the absence of any solvent effect. Among naturally occurring indole compounds, we have chosen the protonated form of tryptamine (TrypH\(^+\)), an intermediate derived by decarboxylation of tryptophan in various metabolic pathways. The optical properties of the neutral tryptamine parent are well established as well as the fragmentation properties (collision-induced dissociation) of the protonated ion, which are very close to those of protonated tryptophan. As in the case of tryptophan, the most stable structure corresponds to protonation on the amino group, the structure with protonation on the pyrrolic nitrogen of the indole ring being less stable by 1.0 eV \(^{29}\) (see fig: 3 and 6).

Experimental

The protonated species are produced by an electrospray source. After the skimmer the ion are guided by an hexapolar trap. The exit plate of the hexapole is maintained at a high potential most of the time and is ground just before the laser shot. The laser is interacting on the ion beam just at the output of the hexapole. The fragmentated ion are the focused between to plate of a wiley/Mc Laren time of flight mass spectrometer. The ion are flying in a field free region and are detected with channels plates and the ion signal is recorded on a digital oscilloscope. For time resolved experiment, the delay between the pump and the probe laser is realized by a standard delay line with step of 10fs.

![Mass Spectrum](image)

Figure 4. Top: difference mass spectrum (mass spectrum with the 266 nm pump laser minus the mass spectrum obtained without the dissociation laser : the parent peak (m/z=161) is depopulated while fragment ions are produced. Bottom: mass spectrum obtained by electron impact ionization of the neutral molecule (m/z=160), adapted from the NIST reference data base.
Results

Figure 5. Femtosecond pump (266 nm)/probe (800 nm) signal observed on the different products issued from the photoexcitation of protonated tryptamine: a depopulation of the tryptamine radical cation signal (m/z=160) is observed as well as a time dependent signal consisting of an exponential decay of 400 fs followed by a plateau for fragments at m/z=131 and 132; no time evolution is observed for fragments at m/z=132 and 144.

In figure 5 is presented the difference mass spectrum of protonated tryptamine (TrypH+) obtained by subtracting the mass spectra obtained from the electrospray source with and without the 266 nm laser. One sees a depopulation of the parent mass (m/z=161) and the appearance of different fragments at m/z=160, 144, 132, 131, 132.

The m/z=160 channel corresponds obviously to the H-atom loss. It should be mentioned that this channel is not observed when this molecule is fragmented by collision impact. This channel is specific to the electronic excitation and, as in the neutral molecule, it suggests a dissociative state which lead to the H loss.

How fast is this dissociation?

This can be studied through femtosecond pump/probe techniques. In such an experiment, the first laser beam at 266 nm (4.6eV) leads to the optically accessible excited state of the parent ion and the dynamics of this excited state is probed by a second photon at 800 nm (1.5eV) delayed in time by steps of 10 fs. The intensity of the fragment ion is recorded as a function of the pump/probe delay.

As can be seen on figure 5, each fragmentation channel has a different pump/probe signal behavior. For two channels no dynamics is observed (in red), whereas for three other ones a femtosecond dynamics is observed: a depletion is observed on the m/z=160 fragment, whereas the dynamics for m/z=130 and 131 show a fast rising signal followed by a 400 fs decay and by a plateau which lasts at least 100 ps.
Interpretation.

Figure 6. Scheme of the photo-induced dissociation in protonated tryptamine. -middle: the TryptamineH+ S\textsubscript{1} state is excited by the pump photon at 266 nm (4.6 eV). This state decays in 400 fs. Before the decay a probe photon can promote the system in a higher electronic state. These two states decay either through internal conversion (left hand side) or through the H-atom loss. -Left: after internal conversion, the molecule further fragments into 2 channels (1 and 2) with the rate constants k\textsubscript{1} and k\textsubscript{2}. The rate constants are not necessarily the same when IC occurs from S\textsubscript{1} or from S\textsubscript{n} since the internal energy is not the same (4.6 eV if IC occurs from S\textsubscript{1} and 6.1 eV if it occurs from S\textsubscript{n}). -Right: the H-atom loss can be produced either from S\textsubscript{1} or from S\textsubscript{n}. The radical cation produced has an open shell electronic structure, and thus possesses low lying excited states that can absorb a photon in the 800 nm region. This radical cation undergoes fragmentation either before the absorption of the 800 nm photon or after. The internal energy in the radical cation is much less than in the ions issued from internal conversion since energy has been lost to break the N-H bond.

Let us put forward a few remarks which can be helpful in understanding the observed signal.

a) In the NIST reference data of mass spectrometry the main fragment obtained when tryptamine is dissociated by electron impact is m/z=130 and 131, precisely those on which a nice pump/probe signal is observed in the present work. After electron impact, a hot radical cation (which is a doublet) is produced which further dissociates. Neither the m/z=144 nor the m/z=132 fragments are observed in this case.

b) After the H loss the ion obtained (m/z=160) is a radical cation, the same than the one obtained in the electron impact ionization, and therefore it will follow the same dissociation channel.

c) In the radical cation one electron of the highest occupied molecular orbital is removed, and therefore this cation has low lying electronic states. These low lying electronic states can be excited by the probe photon at 800nm (1.5eV).

d) After the excitation of the tryptamine S\textsubscript{1} state with the 266 nm laser, a 800 nm photon can be absorbed through a S\textsubscript{1}-S\textsubscript{n} transition.
With this in mind, we can make a model to understand why some peaks show a clear femtosecond pump/probe dynamics and others do not. The overall mechanism is depicted on figure 6.

The basic of the mechanism is the following. The first photon populates the S1 state which is coupled to a state dissociative along a NH coordinate leading to the H loss. The lifetime of S1 is then short. When the NH distance increases there is a crossing with the ground state surface where the system can choose to stay on the dissociative state, leading to the H loss or to jump from the dissociative state to the ground state where the NH distance will shorten leading to a hot ground state protonated molecule (this internal conversion process IC is represented on the left side of figure 6). The hot ground state protonated molecule is a singlet molecule with a first absorption region in the UV. One should notice that, due to our mass spectrometric detection method, a dynamic can only be observed if there is a fragmentation of the parent ion and more precisely if the fragmentation pattern changes with the internal energy in the ion. This fragmentation is not necessarily fast but must occur during the detection time window, which is the time between the absorption of the photon and the time of analysis of the resulting fragmentation. In our experimental conditions this time window is typically 10 s (tobs).

Femtosecond signal from internal conversion. Let us examine what would be the signal observed if the parent protonated ion undergoes solely internal conversion. For simplicity, we will assume that only two fragmentation channels (1 and 2) are open. After absorption of the first photon, there is internal conversion resulting in a hot ion containing 4.6 eV of internal energy that will undergo fragmentation in the two channels, the branching ratio between channel 1 and 2 being given by

\[
N_1 = \frac{k_1}{(k_1 + k_2)} [1 - \exp(-(k_1 + k_2)t_{obs})]
\]

and

\[
N_2 = \frac{k_2}{(k_1 + k_2)} [1 - \exp(-(k_1 + k_2)t_{obs})]
\]

assuming that the reaction time is of the same order as the observation time: t_{obs}. A probe photon can be absorbed before the internal conversion occurs, leading to a similar system in which the internal energy is larger (4.6 + 1.5 eV). This can change the branching ratio between the two fragmentation channels.

\[
N'_{1} = \frac{k'_{1}}{(k'_{1} + k'_{2})} [1 - \exp(-(k'_{1} + k'_{2})t_{obs})]
\]

and

\[
N'_{2} = \frac{k'_{2}}{(k'_{1} + k'_{2})} [1 - \exp(-(k'_{1} + k'_{2})t_{obs})]
\]

Let us assume that the lifetime of the excited state is \(\tau = 400\) fs and that \(\sigma\) is the probability for the initially excited molecules (N) to absorb a second photon. The signal observed for fragment i will be:

\[
S_i = \sigma \exp(-t/\tau) [N_i - N] \exp(-(k_1 + k_2)t_{obs})
\]

\[
S_i = \sigma N \exp(-t/\tau) [k'_{1}/(k'_{1} + k'_{2}) [1 - \exp(-(k'_{1} + k'_{2})t_{obs})] - k_{1}/(k_1 + k_2) [1 - \exp(-(k_1 + k_2)t_{obs})]].
\]

From this equation it appears clearly that:

a) if the branching ratio or/and the reaction time does not change with the internal energy of the hot ion, absolutely no dynamics is observed on the ion signal.

b) if one channel is clearly dominant \(k_1 > k_2\), then also no dynamics is observed.

c) a clear dynamics can only be observed if the branching ratios between the two fragments change drastically when the internal energy increases from 4.6 eV to 6.1 eV.

2) Fragmentation from the tryptamine' radical cation (m/z= 160).

The situation is slightly different in this case since the 800 nm photon can also be absorbed in the cation and the internal energy after the H-atom loss is decreased because some energy is necessary to break the NH bond (typically 3.5 eV). The 800 nm photon can thus be absorbed either in the parent ion S1 state (channel a) or in the daughter radical cation (channel b). We will make the assumption that the percentage of excited molecules (absorption cross section) is \(\sigma\) for channel a and \(\sigma\) for channel b and that the effect of this photon absorption is to increase the internal energy in the ion. This increase in internal energy may not be the same in the two channels since for channel a some of the energy brought by the 800 nm extra photon can be carried off by the H atom. When the system follows the hydrogen atom loss channel, the radical cation (m/z=160) and its fragments (m/z=130 and 131) are detected. The time evolution of the population for each ion is the same, and only their formation/detection efficiency \(N_i\) changes. For the radical cation m/z=160,

\[
N_{160} = \exp(-(k_3 + k_4)t_{obs})
\]

and

\[
N'_{160} = \exp(-(k'_{3} + k'_{4})t_{obs})
\]

for its fragments 130 and 131 produced with rate constants \(k_3\) and \(k_4\) respectively,

\[
N_3 = N_3/k_3 \exp(-(k_3 + k_4)t_{obs})
\]

and

\[
N'_{3} = N'_{3}/k'_{3} \exp(-(k'_{3} + k'_{4})t_{obs})
\]

The signal due to channel a is similar to the previous IC case

\[
N \sigma \exp(-t/\tau) [N_i - N]
\]

For channel b the signal should be a rising signal as the time increases.
The total signal $S_i$ being the sum of channel a and b.

The depopulation observed at $m/z=160$ is independent of the variation of branching ratio and reflects the lower efficiency of its production/detection from $S_a$ than from $S_b$. Accordingly, its two fragments 130 and 131 gain in intensity when the $m/z=160$ ion is promoted to its electronic excited state.

These equations point out that the observed signal is a complicated sum of decreasing and increasing exponentials with the same time constant.

**Comparison with the experimental data**

From the above description and the observed signal, one can differentiate the channels which are produced through internal conversion and those subsequent to initial H-atom loss. The $m/z=132$ and 144 fragments are produced through internal conversion, for the following reasons:

* they are **not** observed when tryptamine is ionized and dissociated via electron impact, which produces hot radical cations.
* they do not exhibit any observable dynamics. This is explained by the fact that when the energy content in a system is large as compared to the reaction barrier, the reaction time constant is short with respect to the observation time ($k_1+k_2>>1/t_{obs}$ and the branching ratio does not change much with the energy.

In contrast, the $m/z=131$ and 130 ions are produced after H-atom loss for the following reasons:

* they are observed in the electronic impact ionization of neutral tryptamine which produces the hot radical cation.

* after H-atom loss, the tryptamine$^+$ radical cation can absorb a 800 nm photon. The two fragments ($m/z=131$ and 130) indeed show the evidence of a change of reactivity at very long time (10 ps), which is the sign of the absorption of the 800 nm by the radical cation.

*after the H-atom loss, the energy in the system is decreased by the NH binding energy. Therefore the energy content of the radical cation is much smaller than that of the protonated ion produced through internal conversion, so that the effect of the probe 800 nm photon (1.5 eV) changes strongly the energy of the system which, in turn, changes strongly both the reaction time ($1/(k_3+k_4)$) and the branching ratio. Then some dynamics can be evidenced.

**quantum yield for H-atom loss**

It is possible now to deduce the efficiency of the H-atom loss channel under the assumption that the fragmentation channels are really specific of the initial path, i.e. internal conversion and H-atom loss do not lead to the same fragments. This seems to be justified by the fact that $m/z=144$ and 132 are not observed in the mass spectrum obtained by electron impact ionization of neutral tryptamine$^{[30]}$ (see figure 3). On the other hand, the $m/z=144$ fragment corresponds to NH$_3$ loss from Tryptamine$^+$, which is the lowest energy channel observed in the collision induced fragmentation of the similar compound, protonated tryptophan.

Thus the branching ratio between the two primary processes – H-atom loss and internal conversion - can be directly deduced from the ratio of the peaks due to internal conversion ($m/z=144$ and 132), i.e. 54% and those issued from the H loss at $m/z=160$, 131 and 130, i.e. 46%.

**Which H atom is lost?**

By comparison with the neutral molecule, one could think that the leaving H atom could come from the pyrrolic ring.

![Figure 6. Potential energy functions along the amino-group NH dissociation coordinate calculated using Gaussian98 package (using TD/DFT, B3LYP/6-31G**).](image-url)

It is however not the case. *Ab-initio* calculations have shown that the lowest excited state of protoned tryptamine (or tryptophan) is a dissociative state on the amino group resulting from the promotion of a π electron of the indole ring to a Rydberg orbital on the terminal ammonium group (see figure 6). This orbital is collapsing into an antibonding orbital when the NH distance is increased, leading to a direct and fast dissociation. In the way out, the excited state potential energy function crosses the ground state potential energy function and at this second crossing the H atom can either recombine on the ground state potential.
surface or be ejected. From our experimental data, the branching ratio at this second crossing can be estimated to be 50%.

4 Conclusions

The excited dynamics of several aromatic molecules of biological interest has been investigated to understand the non-radiative process occurring in these systems. The non-radiative process is controlled by the coupling between the first excited state and an upper dissociative state. The role of dissociative excited states is outlined by the observation of a fast hydrogen-atom loss channel.

\textit{Ab initio} calculations substantiate the experimental data: the excited state that bears the oscillator strength is coupled to dissociative states leading to a ‘non-ergodic’ hydrogen loss, in other words, the dissociation is fast and direct.

In neutral molecules, the excited state leads to the H-atom loss from the aromatic ring (linked to an oxygen or a nitrogen atom). In ionic systems protonated on the terminal amino group, another excited state is involved which leads to the H-atom loss from the amino group.

This H-atom loss then results in the formation of a radical cation in which subsequent fragmentations proceed via the available thermal energy. This mechanism opens new insight on the optical properties of protonated tryptophan in biological environments; in particular it brings up the question of the fate of the H atom in the surrounding protein.

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