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# Tryptophan photoionization from prefluorescent and fluorescent states

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**Abstract**

Contributions into tryptophan ionization from non-relaxed prefluorescent $S^*$ and relaxed fluorescent $S_1$ states are separated by measurement of the ionization quantum yield in neutral and extremely acidic solutions. The rate constant of the ionization from the prefluorescent $S^*$ state is temperature independent, the rate constant of ionization from $S_1$ state has frequency factor $A_{ion} = (2.3 \pm 0.9) \times 10^{15}$ s$^{-1}$ and activation energy $E_{ion} = 50 \pm 5$ kJ/mol. For L-tryptophan at room temperature the ionization from $S_1$ state is completely suppressed by other processes of $S_1$ state decay, in particular – by the intramolecular proton transfer (IPT) from the protonated amino group to the indole ring ($A_{ipt} = (6.1 \pm 1.9) \times 10^{10}$ s$^{-1}$, $E_{ipt} = 14.0 \pm 1.8$ kJ/mol).


## 1. Introduction

Tryptophan (TrpH) is the strongest chromophore among the natural amino acids. Photochemical reactions of tryptophan and tryptophan residues in proteins play an important role in nature. The fluorescence and phosphorescence of tryptophan residues are widely used for the study of structure and dynamic processes in proteins [1–5]. Due to these reasons, the complex photochemistry and photophysics of tryptophan has been intensively studied over last decades [2,6–11]. It is established that under UV irradiation the following intermediates are formed in nanosecond time scale: (a) excited singlet state $S_1$ with the lifetime of about 3 ns [7]; (b) solvated electron $e^-_{aq}$ and tryptophan cation radical $TrpH^{\cdot+}$, the later rapidly deprotonates in neutral solutions ($pK_a = 4.3$ [6,12]) yielding neutral radical $Trp^{\cdot}$; (c) triplet tryptophan $^TTrpH$ formed due to intersystem crossing in $S_1$ state (lifetime about 10 μs) [6,11,13]; and (d) protonated triplet $^TTrpH_2^+$ formed after intramolecular proton transfer from the protonated $NH_3^+$ group to the C-4 position of indole ring ($\tau \approx 30$ ns) [6,7,11].

Photoionization of tryptophan is believed to be a major pathway of photo-oxidation of many proteins [10]. The reported quantum yield of the monophotonic ionization is about 0.04–0.08 [9,11,14–16], and significant biphotonic contribution into ionization has been revealed under intensive pulse laser irradiation. However, despite the numerous studies, the fundamental question about the precursor state for monophotonic ionization is still not resolved completely. Flash photolysis and fluorescence measurements show that with the temperature rise the photoionization yield significantly increases, whereas both fluorescence lifetime and fluorescence quantum yield decrease [6,7,16–18]. This observation is strong evidence that the precursor for ionization is thermally relaxed fluorescent singlet state $S_1$. At the same time, ultra-fast laser flash photolysis experiments [19–21] demonstrated that at room temperature the solvated electron is formed within 200 fs, and its absorption remains unchanged at pico- and nanosecond time scales. That testifies that the photoionization takes place from the non-relaxed prefluorescent state. The last conclusion was recently confirmed in our work [11]: the photoionization quantum yield was measured in neutral and in extremely acidic solutions, where the fluorescent $S_1$ state is completely quenched due to the protonation of the

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indole ring. It has been revealed that in both cases the yields of monophotonic ionization are approximately equal. Thus, it is natural to presume that at room temperature the photoionization of tryptophan occurs mostly from the prefluorescent state $S^*$, while at higher temperatures the relaxed state $S_1$ also participates in the ionization. The main goal of the present work is to investigate the temperature dependence of the tryptophan photoionization from the prefluorescent $S^*$ and relaxed $S_1$ states separately, which is performed by the following way. The temperature dependence of the ionization from $S^*$ state is determined by the quantitative measurements of the photoionization quantum yield under extremely acidic conditions. The obtained results are compared with the measurements in neutral solutions, where the photoionization from both $S^*$ and $S_1$ states is possible. Finally, the temperature dependence of the intramolecular proton transfer is taken into account by the comparing of the results obtained during the photolysis of L-tryptophan (L-TrpH) and *N*-acetyl tryptophan (NATrpH), in which the presence of the acetyl group prohibits the proton transfer reaction.

## 2. Experimental

A detailed description of the LFP equipment has been published earlier [22,23]. Solutions, running through a rectangular cell (inner dimensions 3 mm × 10 mm), were irradiated with a Lambda Physik EMG 101 excimer laser (308 nm, pulse energy up to 100 mJ, pulse duration 15–20 ns). The dimensions of the laser beam at the front of the cell were 3 mm × 8 mm. The monitoring system includes a DKSh-150 xenon short-arc lamp connected to a high current pulser, a home-made monochromator, a 9794B photomultiplier (Electron Tubes Ltd.), and a LeCroy 9310A digitizer. The monitoring light, concentrated in a rectangular of 3 mm height and 1 mm width, passed through the cell along the front (laser irradiated) window. Thus, in all experiments the excitation optical length was 1 mm, and the monitoring optical length was 8 mm. All solutions were bubbled with argon for 15 min prior to, and during, irradiation. The temperature of solutions was varied with the use of hot air flowing around the cell, and measured by a thermocouple inserted inside the cell just above the irradiated zone.

Actinometry was performed using naphthalene in cyclohexane. The incident laser energy was determined by triplet naphthalene absorption at 414 nm (absorption coefficient $2.45 \times 10^4$ $M^{-1}$ $cm^{-1}$ [24], triplet quantum yield 0.75 [25]).

L-Tryptophan and *N*-acetyl tryptophan (Sigma/Aldrich) were purified by re-crystallization. Neutral solutions were prepared with the use of phosphate buffers, below pH 1 the acidity of solutions was adjusted by addition of hydrochloric acid.

## 3. Results

### 3.1. Dependence of the photoionization yield on laser energy

In the study of the tryptophan photoionization, the contribution from biphotonic processes can significantly distort the obtained results [9,16]. Therefore, the measurements of the photoionization yield dependence on laser pulse energy were performed in order to determine the conditions at which the biphotonic processes can be neglected. The experiments were performed in neutral solution (pH 7.1), the photoionization yield was measured by the initial absorption of tryptophanyl radical Trp· at the absorption maximums 330 and 510 nm, and of solvated electron at 580 nm. As an example, Fig. 1 shows the absorbance of Trp·, formed in the 308 nm photolysis of $2.0 \times 10^{-2}$ M L-TrpH (pH 7.1), monitored at 510 nm and extrapolated to the zero time point. Similar results have been obtained with the measurements at other wavelengths. The obtained results were fitted according to Eq. (1)

$$\mathrm{OD} = C \times (E + \alpha E^2), \tag{1}$$

where OD is the intermediate absorbance, $E$ is the laser energy in mJ, $C$ and $\alpha$ are the numerical coefficients. The value $\alpha$, averaged from the measurements at different wavelengths, was determined as $\alpha = (8.2 \pm 2.1) \times 10^{-3}$ $mJ^{-1}$. Thus, in our experimental conditions the influence of biphotonic processes becomes noticeable at $E > 30$ mJ. In our experimental conditions the area of the laser beam is about 25 $mm^2$, the duration of the laser pulse is about 20 ns. Thus, the laser energy $E = 30$ mJ corresponds to the light intensity $6 \times 10^{10}$ $W/m^2$, which is in a good agreement with the previously reported threshold for biphotonic ionization [9]. All next following experiments were performed with the laser pulse

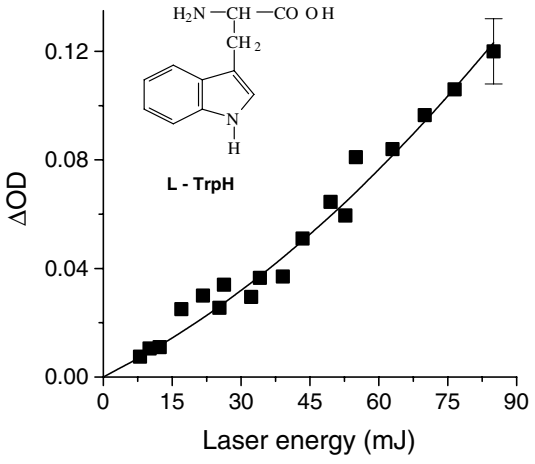


Fig. 1. Plot of Trp· radical absorbance at 510 nm versus the laser pulse energy. Solution is $2.0 \times 10^{-2}$ M L-TrpH, pH 7.1. The solid line is a least-squares fit to Eq. (1).

energies 5–20 mJ, i.e., under conditions when only monophotonic ionization takes place.

### 3.2. Temperature dependence of photoionization of N-acetyl tryptophan (NATrpH)

Fig. 2 shows the temperature dependence of the quantum yield of radical formation in the photolysis of NATrpH in neutral (phosphate buffer, pH 7.1) and extremely acidic (1 M HCl, pH 0.1) solutions. In neutral solution the quantum yield was determined by the absorption of NATrp$^{\cdot}$ radical at 510 nm ($\varepsilon = 1800$ M$^{-1}$ cm$^{-1}$ [12,26,27]), extrapolated to the zero time point; in acidic solution the measurements were performed at 570 nm, the absorption coefficient of the cation radical NATrpH$^{\cdot+}$ was taken $\varepsilon = 2500$ M$^{-1}$ cm$^{-1}$ [12,27]. Since the absorption coefficient of tryptophan $\varepsilon_{\mathrm{TrpH}}$ at 308 nm significantly changes with the temperature variation (inset in Fig. 2), the temperature dependence of $\varepsilon_{\mathrm{TrpH}}$ was taken into account in the quantum yield calculations. The absorption coefficients of tryptophanyl radicals at 510 and 570 nm were presumed to be temperature independent.

As it follows from Fig. 2, the temperature dependences of tryptophan photoionization under neutral and acidic conditions are quite different. In neutral solution the quantum yield of tryptophanyl radicals increases from 0.06 at room temperature to about 0.50 at 355 K, whereas under extremely acidic conditions in the same temperature range it slightly decreases from 0.024 to 0.019. Similar results have been obtained when the radical yield was determined by the measurement of the absorption at 330 nm.

### 3.3. Temperature dependence of photoionization of L-tryptophan (L-TrpH)

Temperature dependences of the radical yield in the photolysis of L-TrpH in neutral and acidic solutions, presented in Fig. 3, are similar to that obtained in NATrpH photolysis. At room temperature the radical quantum yields in both types of solution are similar and equal to about 0.035–0.040. With the temperature growth, the quantum yield of L-Trp$^{\cdot}$ radicals at pH 7.1 increases and reaches the value 0.18 at 360 K, and in acidic solution the yield of L-TrpH$^{\cdot+}$ remains almost unchanged, with the minor decrease to approximately 0.030 at high temperatures. The most significant differences between results, obtained in the photolysis of L-tryptophan and *N*-acetyl tryptophan, are:

(a) At room temperature for L-TrpH the photoionization quantum yields in neutral and acidic solutions are practically the same, whereas for NATrpH the radical yield in neutral solution is more than twice as large as at pH 0.1.
(b) The temperature dependence for NATrpH is steeper than for L-TrpH. In fact, for L-TrpH the dependence of the photoionization quantum yield on temperature becomes noticeable only above 315–320 K. The last statement can be confirmed by an inspec-

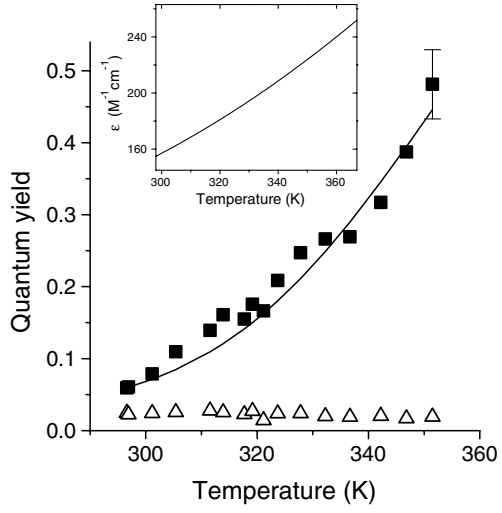


Fig. 2. Temperature dependence of NATrpH ionization quantum yield in neutral (squares: $6 \times 10^{-3}$ M NATrpH, pH 7.1, measured by the Trp$^{\cdot}$ radical absorbance at 510 nm) and extremely acidic (triangles: $1 \times 10^{-2}$ M NATrpH, pH 0.1, measured by the TrpH$^{\cdot+}$ radical absorbance at 570 nm) solutions. Solid line: calculations according to Eq. (10) with the parameters: $\Phi_{0\mathrm{ion}} = 0.024$, $A_{\mathrm{ion}} = 2.3 \times 10^{15}$ s$^{-1}$, $E_{\mathrm{ion}} = 50$ kJ/mol, $k_0 = 1.1 \times 10^{8}$ s$^{-1}$, $k_{\mathrm{ipt}} = 0$. Inset: temperature dependence of the NATrpH absorption coefficient at 308 nm.

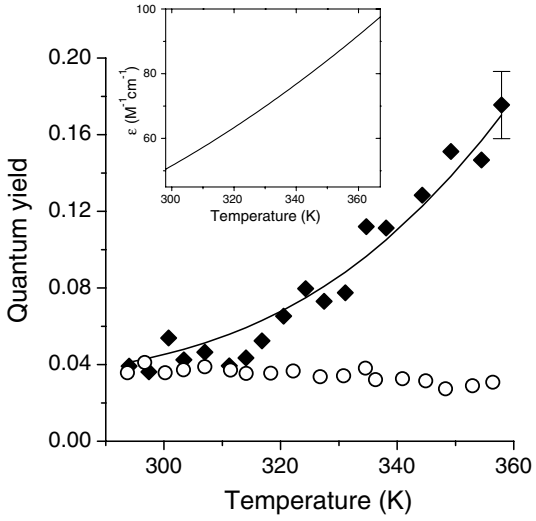


Fig. 3. Temperature dependence of L-TrpH ionization quantum yield in neutral (diamonds: $1.4 \times 10^{-2}$ M L-TrpH, pH 7.1, measured by the Trp$^{\cdot}$ radical absorbance at 510 nm) and extremely acidic (circles: $2.9 \times 10^{-2}$ M L-TrpH, pH 0.1, measured by the TrpH$^{\cdot+}$ radical absorbance at 570 nm) solutions. Solid line: calculations according to Eq. (10) with the parameters: $\Phi_{0\mathrm{ion}} = 0.030$, $A_{\mathrm{ion}} = 2.3 \times 10^{15}$ s$^{-1}$, $E_{\mathrm{ion}} = 50$ kJ/mol, $k_0 = 1.1 \times 10^{8}$ s$^{-1}$, $A_{\mathrm{ipt}} = 6.1 \times 10^{10}$ s$^{-1}$, $E_{\mathrm{ipt}} = 14$ kJ/mol. Inset: temperature dependence of the L-TrpH absorption coefficient at 308 nm.

tion of the previously reported results on the temperature dependence of TrpH ionization (Fig. 7 in [6], Fig. 2 in [16]).

### 3.4. Temperature dependence of the excited-state intramolecular proton transfer in L-TrpH

An important pathway of the tryptophan $S_1$ state decay is the intramolecular proton transfer (IPT) from the protonated amino group to the indole ring [7,28–30]. As we have recently shown [11], the IPT is followed by the fast intersystem crossing with the formation of the protonated triplet state $^{T}TrpH_2^+$. The last species absorbs in the region 300–500 nm with the maximum at 400 nm, and decays exponentially with the lifetime of about 30 ns [6,11]. In the present work, the temperature dependence of the IPT quantum yield was determined by the following procedure. The decay of the protonated triplet was measured at 400 nm and fitted to the exponential function

$$\mathrm{OD} = A\exp(-k_T t) + B, \tag{2}$$

where $A$ and $B$ are the time-independent constants. The decay rate constant $k_T$ was found to be temperature independent within the experimental error: $k_T = (3.4\pm 0.8)\times 10^7\ s^{-1}$. The calculated values $A$ were used for the determination of the initial concentration of protonated triplets $C_0$

$$A = \varepsilon_T C_0 L, \tag{3}$$

where $L = 0.8$ cm is the optical length; the absorption coefficient $\varepsilon_T$ of the protonated triplet $^{T}TrpH_2^+$ at 400 nm was assumed to be equal to the absorption coefficient of the deprotonated triplet $^{T}TrpH$ at the absorption maximum 450 nm: $\varepsilon_T = 5000\ M^{-1}\ cm^{-1}$ [13]. This assumption is based on the similarity of the absorption spectra of protonated and deprotonated forms of the triplet state [11]. The obtained values $C_0$ were used for the calculation of the IPT quantum yield; the results are presented in Fig. 4.

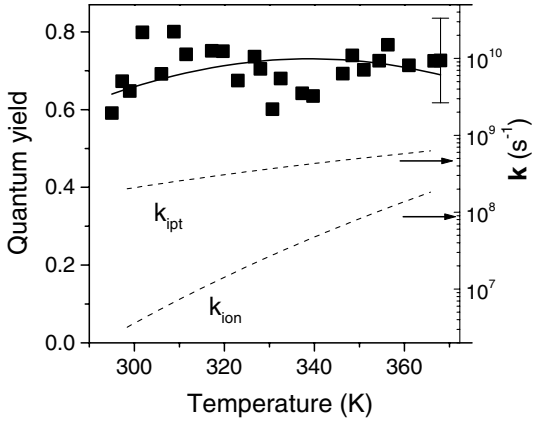


Fig. 4. Squares: temperature dependence of the protonated triplet $^{T}TrpH_2^+$ quantum yield in neutral solution ($1.4\times 10^{-2}$ M L-TrpH, pH 7.1), measured by the absorbance of fast-decaying signal at 400 nm during the photolysis of L-TrpH. Solid line: calculations according to Eq. (13) with the same parameters as in Fig. 3. Dashed lines: calculated temperature dependences of $k_{ion}$ and $k_{ipt}$.

## 4. Discussion

It is well known that high acid concentrations efficiently quench the excited singlet state of tryptophan [6,7,11,17]. The mechanism of the quenching includes the protonation of the excited indole ring followed by the fast intersystem crossing with the formation of the protonated triplet $^{T}TrpH_2^+$ [11]. At pH 0.1, this process is the main channel of $S_1$ state decay. Thus, the temperature dependence of the radical quantum yield, obtained in extremely acidic solutions, corresponds to the photoionization from the non-relaxed prefluorescent state $S^*$ only. The obtained results testify that for both NATrpH and L-TrpH the rate constant of the ionization from the prefluorescent state does not depend on temperature (triangles in Fig. 2 and circles in Fig. 3). Some decrease of the photoionization yield with the temperature increase, observed under acidic conditions, should be probably attributed to the competition between two processes, ionization and relaxation to $S_1$ state. With the temperature growth, the relaxation rate increases, and the ionization yield decreases from 0.024 at room temperature to 0.019 at 355 K for NATrpH, and from 0.04 to 0.03 for L-TrpH.

In neutral solutions, the observed photoionization originates from both prefluorescent $S^*$ and relaxed fluorescent $S_1$ states. The increase of the radical yield with temperature should be attributed to the increase of the ionization yield from $S_1$ state. The general scheme of the tryptophan $S_1$ state decay in neutral solutions can be presented as following:

$$^{S}TrpH \xrightarrow{k_{fl}} TrpH + h\nu \tag{4}$$

$$^{S}TrpH \xrightarrow{k_{ic}} TrpH \tag{5}$$

$$^{S}TrpH \xrightarrow{k_{ISC}} {}^{T}TrpH \tag{6}$$

$$^{S}TrpH \xrightarrow{k_{ion}} TrpH^{+\cdot} + e^-_{aq} \tag{7}$$

$$^{S}TrpH(NH_3^+) \xrightarrow{k_{ipt}} {}^{S}TrpH_2^+(NH_2) \rightarrow {}^{T}TrpH_2^+ \tag{8}$$

The following processes can be considered as temperature independent: the fluorescence ($k_{fl}$), the internal conversion ($k_{ic}$), and the intersystem crossing into the triplet state ($k_{ISC}$). The rate constants of two other processes, ionization ($k_{ion}$) and intramolecular proton transfer ($k_{ipt}$), depend on temperature.

The difference between kinetic behaviors of the excited states of NATrpH and L-TrpH is that the presence

of the protonated amino group $NH_3^+$ in the later allows for the intramolecular proton transfer reaction (reaction (8)), whereas in NATrpH this reaction cannot take place. At room temperature the IPT reaction is the major pathway of the $S_1$ state decay: the quantum yield of this process can be estimated as $\Phi_{ipt} \approx 0.5$–$0.7$ (Fig. 4 and [7,11]), whereas the efficiency of other channels of $S_1$ decay is significantly lower: for intersystem crossing $\Phi_{isc} \approx 0.06$–$0.1$ [7,11], for fluorescence $\Phi_{fl} \approx 0.14$–$0.19$ [7,18,31–33]. The quantum yield of photoionization of L-TrpH at room temperature is about $\Phi_{ion} \approx 0.04$ [9,11,16,34]. However, as it follows from Fig. 3, below 310 K the contribution of $S_1$ state into ionization is negligible, and radicals are formed only due to ionization from the prefluorescent state. That means that in $S_1$ state of tryptophan at temperature below 310 K the IPT reaction dominates over ionization.

According to the kinetic scheme (4)–(8), the formation of tryptophanyl radicals $R$ can be described as

$$R = R_0 + \frac{k_{ion}}{k_{ion} + k_{ipt} + k_0}(1 - \exp(-(k_{ion} + k_{ipt} + k_0) \times t)), \tag{9}$$

where $R_0$ is the fraction of radicals formed due to ionization from the prefluorescent state, and $k_0 = k_{fl} + k_{ic} + k_{ISC}$ is the sum of the temperature independent rate constants. Therefore, the temperature dependence of the radical quantum yield $\Phi_{ion}$ for L-TrpH can be written

$$\Phi_{ion} = \Phi_{0ion} + (1 - \Phi_{0ion}) \times \frac{k_{ion}}{k_{ion} + k_{ipt} + k_0}, \tag{10}$$

where $\Phi_{0ion}$ is the quantum yield of ionization from the prefluorescent state, and the rate constants $k_{ion}$ and $k_{ipt}$ are expressed in the form

$$k_{ion} = A_{ion} \exp(-E_{ion}/RT), \tag{11}$$

$$k_{ipt} = A_{ipt} \exp(-E_{ipt}/RT), \tag{12}$$

A similar expression can be written for the temperature dependence of NATrpH ionization with $k_{ipt} = 0$. And, finally, the quantum yield of the protonated triplet formation due to IPT reaction in the photolysis of L-TrpH can be written

$$\Phi_{ipt} = (1 - \Phi_{0ion}) \times \frac{k_{ipt}}{k_{ion} + k_{ipt} + k_0}. \tag{13}$$

Since in the expression for NATrpH only one rate constant, $k_{ion}$, depends on temperature, it is convenient to start the numerical modeling with the temperature dependence of the photoionization quantum yield in the photolysis of NATrpH. The value $k_0$ can be estimated as $\approx 1.1 \times 10^8$ s$^{-1}$, taking into account that tryptophan fluorescence lifetime in basic solution at room temperature is about 9 ns [7]. Indeed, in basic solution the deprotonation of the amino group makes the IPT reaction impossible, and, as it follows from Fig. 2, at room temperature the ionization of L-TrpH from $S_1$ state does not give noticeable contribution. The value $\Phi_{0ion} = 0.024$ was determined in the experiments under extremely acidic conditions (see above). Thus, the only fitting parameters are $A_{ion}$ and $E_{ion}$. The results of the calculations, shown in Fig. 2 by solid line, have been obtained with $A_{ion} = (2.3 \pm 0.9) \times 10^{15}$ s$^{-1}$ and $E_{ion} = 50 \pm 5$ kJ/mol.

The obtained values of $A_{ion}$ and $E_{ion}$ were used for the calculation of the temperature dependences of photoionization quantum yield (Fig. 3, Eq. (10)) and IPT quantum yield (Fig. 4, Eq. (13)) in the photolysis of L-TrpH. In the calculations, the values $A_{ipt}$ and $E_{ipt}$ were fitting parameters; the value $\Phi_{0ion}$ was also slightly adjusted in order to obtain the best agreement between calculations and experimental data. The best fit (solid lines in Figs. 3 and 4) was obtained with $\Phi_{0ion} = 0.030 \pm 0.006$, $A_{ipt} = (6.1 \pm 1.9) \times 10^{10}$ s$^{-1}$, and $E_{ipt} = 14.0 \pm 1.8$ kJ/mol.

The observed independence of $\Phi_{ipt}$ on temperature (Fig. 4) should be attributed to the competition between the reactions of IPT and of ionization. At relatively low temperatures the ionization does not play significant role in $S_1$ decay, and temperature increase causes the increase of $\Phi_{ipt}$. At higher temperatures, the rate of ionization becomes comparable with the rates of the other channels of $S_1$ decay, and significant increase of $\Phi_{ion}$ results in some decrease of $\Phi_{ipt}$. For the reader's convenience, the calculated temperature dependences of $k_{ion}$ and $k_{ipt}$ are shown in Fig. 4 by dashed lines.

The reported values $A_{ion}$ and $E_{ion}$ are in a good agreement with the results obtained by the measurements of tryptophan fluorescence lifetime and quantum yield [7,20,32,35]. The high value of the frequency factor $A_{ion}$ for the ionization rate constant probably indicates that it is electronic rather than nuclear motion involved.

## 5. Conclusions

At room temperature the main precursor of tryptophan ionization is the non-relaxed prefluorescent state $S^*$. The rate constant of the ionization from $S^*$ state does not depend on temperature, whereas the quantum yield slightly decreases with temperature. This effect should be probably attributed to the increase of the relaxation rate from $S^*$ to the fluorescent $S_1$ state. In this case, the measurements of the ionization of indole and its derivatives, especially at low temperatures, might be a unique tool for the studying of ultrafast relaxation processes.

The ionization from $S_1$ state depends on temperature. The Arrhenius parameters for the ionization rate constant $k_{ion}$ are $A_{ion} = (2.3 \pm 0.9) \times 10^{15}$ s$^{-1}$ and $E_{ion} = 50 \pm 5$ kJ/mol. For L-TrpH at room temperature the ionization from $S_1$ state is almost completely suppressed

by other channels of $S_1$ state decay, in particular – by the intramolecular proton transfer from the protonated amino group to the indole ring. The contribution of $S_1$ state into ionization becomes noticeable at temperature above 320 K, and grows up with the temperature increase. For NATrpH the IPT reaction is not possible, and the significant contribution into ionization from $S_1$ state can be observed already at room temperature. For tryptophanyl residues in proteins the IPT reaction can proceed only in very specific cases; thus, in real biological systems at temperatures 300–320 K the ionization should occur from both $S^*$ and $S_1$ states.

The IPT reaction is the main channel of $S_1$ state decay. Due to competition between IPT and ionization, the quantum yield of IPT reaction remains approximately constant $\Phi_{ipt} \approx 0.7$ at the entire temperature range from 300 to 360 K.

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