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Properties of excited states of aqueous tryptophan

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Abstract

The short-lived intermediate ($\tau \approx 26$ ns) with absorption maximum at 400 nm, observed in 308 nm photolysis of aqueous tryptophan, is identified as a protonated triplet state. It is shown that the main channel of photoexcited singlet state S_1 decay is intramolecular (in neutral solutions) or through-solvent (in acidic solutions) protonation followed by a rapid intersystem crossing into the protonated triplet state. The dissociation constant of triplet tryptophan $pK_a = 3.2$ and the lower limit of the dissociation constant of singlet-excited tryptophan $pK_a \geq 2.2$ are determined from the pH dependence of the triplet lifetime and quantum yield. Ground-state tryptophan and acetone quench triplet tryptophan with the rate constants $k_{T\text{-}TrpH} = 1.2 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{T\text{-}Ac} = 1.9 \times 10^6\ M^{-1}\ s^{-1}$, respectively. The major precursor of monophotonic tryptophan photoionization at room temperature is a non-relaxed prefluorescent state.

Keywords: Tryptophan; Photolysis; Triplet state; Photoionization

1. Introduction

Among the major amino acids present in proteins, tryptophan (TrpH) has the strongest absorption in near UV, which makes it the main target for photo-oxidation of proteins. Photochemistry of aqueous tryptophan has been widely studied over last decades [1–3]. Based on the previous studies, the following general scheme of the tryptophan photolysis can be drawn. Under UV irradiation, the tryptophan molecule experiences transition into excited singlet state S_1. The main channels of S_1 decay are fluorescence, intersystem crossing into triplet state T_1, intramolecular proton transfer from amino group to the excited indole ring, and ionization with the formation of solvated electron e^- and tryptophan cation radical $TrpH^{\bullet+}$.

Since photoionization of tryptophan is a major pathway of photo-oxidation of many proteins, this process received much attention. The key questions of these studies were the nature of the ionized-state precursor (namely, non-relaxed prefluorescent state, relaxed first singlet or triplet state), and whether the photoionization proceeds via monophotonic or biphotonic mechanism [4–20]. Study of the dependence of electron quantum yield on the intensity of laser irradiation ($\lambda = 248$–266 nm) revealed that at the intensities below 10^{10} W/m^2 the major mechanism of photoionization is monophotonic, and at higher intensities it is a mixture of monophotonic and biphotonic processes [9,13–16]. The reported quantum yield of the monophotonic ionization varies significantly, from 0.015 to 0.25 [5–7,9,10,13–16,21,22], the most reliable data lie in a range $\Phi_e = 0.04$–0.08 [13–16]. Pico- and femtosecond laser photolysis experiments [18,19] demonstrated that the solvated electron is formed within 200 fs, i.e. much faster than the decay of the fluorescent S_1 state (3–10 ns [10]). The absorption of the solvated electron remains constant in time up to 15 ns. That implies that (a) no appreciable geminate recombination of the tryptophan cation-radical and solvated electron occurs, and (b) the photoionization takes place from the non-relaxed prefluorent excited state. However, the increase of the yield of monophotonic ionization with temperature [4,5,12,16], accompanied by the fluorescence decrease [4,10,12], testifies that at least partly the photoionization occurs from the relaxed S_1 state. Intermediate precursors of biphotonic ionization are S_1 and, to a lesser degree, T_1 states [9,15,16].

The quantum yield of tryptophan monophotonic photoionization depends also on the wavelength of irradiation. Under 193 nm irradiation [15], the quantum yield is as high as $\Phi_e = 0.32$. Bernas et al. [11] determined the ionization threshold for aqueous indole equal to 4.35 eV; however, Bazin et al. [14] reported direct observation of monophotonic photoionization of tryptophan excited at 300 nm. In the present work, we will demonstrate that the monophotonic ionization plays an important role even under 308 nm

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irradiation, i.e. at the very border of the aqueous tryptophan absorption.

The triplet state of tryptophan T_1 forms due to intersystem crossing from S_1 state with the quantum yield of 0.1–0.27 [8,10,12,14]. Low- and room temperature phosphorescence of tryptophan is widely used as a probe in studying the spatial structure and dynamic process in proteins [23–30]. Thus, the investigation of physical and chemical properties of triplet tryptophan is of large importance for biology and biochemistry. In absence of quenchers, the main channels of triplet decay are triplet–triplet annihilation and the decay into the ground state by phosphorescence. There is a significant discrepancy in literature concerning the triplet lifetime of aqueous tryptophan and its derivatives. Measurements of the triplet lifetime by decay of the triplet absorption give values from 12 to 40 μs, whereas the reported phosphorescence lifetimes vary from 40 μs to 1.2 ms [5,8,31–33]. It seems that the main sources of this discrepancy are, on one hand, the effect of residual oxygen, impurities, and photoproducts in solution, which may quench the triplet state [31], and on the other hand, the formation of triplet tryptophan in dark reactions of radical species [33], which increases the observed radiative lifetime.

Bent and Hayon [5] in 265 nm flash photolysis of aqueous tryptophan observed also a short-lived intermediate ($\tau \sim$ 20–45 ns), which was attributed to another triplet state (authors [5] called it T_1 state, whereas the long-lived triplet was assigned as T_2 state). Although there was no confirmation of this observation in consequent works, the supposed "T_1 state of Bent and Hayon" was often drawn to explain some features of tryptophan photolysis. In particular, Robbins et al. [10] suggested that this state originates from the intramolecular proton transfer from the protonated NH_3^+ group to the excited indole ring.

The present paper is devoted to studying of properties of intermediates, in particular of the triplet state, formed under direct 308 nm and acetone-sensitized photolysis of aqueous tryptophan in neutral and acidic solutions. The main goals of this study are: (a) to investigate the nature and properties of "T_1 state of Bent and Hayon"; (b) to clarify the contributions of prefluorescent and relaxed singlet states into tryptophan photoionization; (c) to establish a general qualitative scheme of evolution of photoexcited states of tryptophan.

2. Experimental

A detailed description of the LFP equipment has been published earlier [34,35]. Solutions in a rectangular cell (10 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, two synchronously operating monochromators, 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 L was 8 mm. Correspondingly, in all figures the absorbance values are given for $L = 0.8$ cm.

Actinometry was performed using naphthalene in cyclohexane. The incident laser energy was determined by triplet naphthalene absorption at 414 nm (absorption coefficient $2.45 \times 10^4\ M^{-1}\ cm^{-1}$ [36], triplet quantum yield 0.75 [37]).

The chemicals (L-tryptophan, *N*-acetyl tryptophan, indole, acrylamide) were used as received from Sigma/Aldrich. In pH range 1–7.1, solutions were prepared in phosphate buffers, below pH 1 the acidity of solutions was adjusted by addition of hydrochloric acid. All solutions were bubbled with the appropriate gas (Ar, N_2O, or O_2) for 15 min prior to, and during, irradiation. All experiments were carried out at room temperature.

3. Results

3.1. Spectral data

Fig. 1a (triangles) shows a transient absorption spectrum, obtained 3 μs after the laser irradiation of 10 mM TrpH in neutral aqueous solution (pH 7.0). The solution was bubbled with argon prior and during the measurements. The following intermediates appear within the laser pulse duration (15–20 ns): a triplet tryptophan ^{T}TrpH, a solvated electron e^-, and a tryptophan cation radical $TrpH^{\bullet+}$. The later rapidly deprotonates in neutral solution ($pK_a = 4.3$ [5,7,38]), giving rise to a neutral radical $Trp^\bullet$. The solvated electrons decay in the reactions of recombination and addition to the ground-state tryptophan:

$$TrpH + e^- \xrightarrow{k_e} TrpH^{\bullet-}, \quad k_e \approx 3 \times 10^8\ M^{-1}\ s^{-1} \tag{1}$$

$$TrpH^{\bullet-} \xrightarrow{+H^+} TrpH_2^\bullet, \quad \text{protonation} \tag{2}$$

Thus, in our experimental conditions the presented spectrum includes contributions from three species: ^{T}TrpH, $Trp^\bullet$, and $TrpH_2^\bullet$ (see [39–44] for reaction (1) and [39,42,45] for reaction (2)).

In the presence of 1.4×10^{-2} M acetone (Ac), the solvated electrons are scavenged in the reaction

$$Ac + e^- \xrightarrow{k_{sc}} Ac^{\bullet-}, \quad k_{sc} \approx 6 \times 10^9\ M^{-1}\ s^{-1} \tag{3}$$

$$Ac^{\bullet-} \xrightarrow{+H^+} AcH^\bullet, \quad pK_a = -2 \tag{4}$$

Thus, the second spectrum in Fig. 1a (open circles), obtained in the presence of acetone, consists of spectra of triplet tryptophan ^{T}TrpH and radical $Trp^\bullet$ (see [44,46–50] for reaction (3) and [51] for reaction (4)). One should note that the

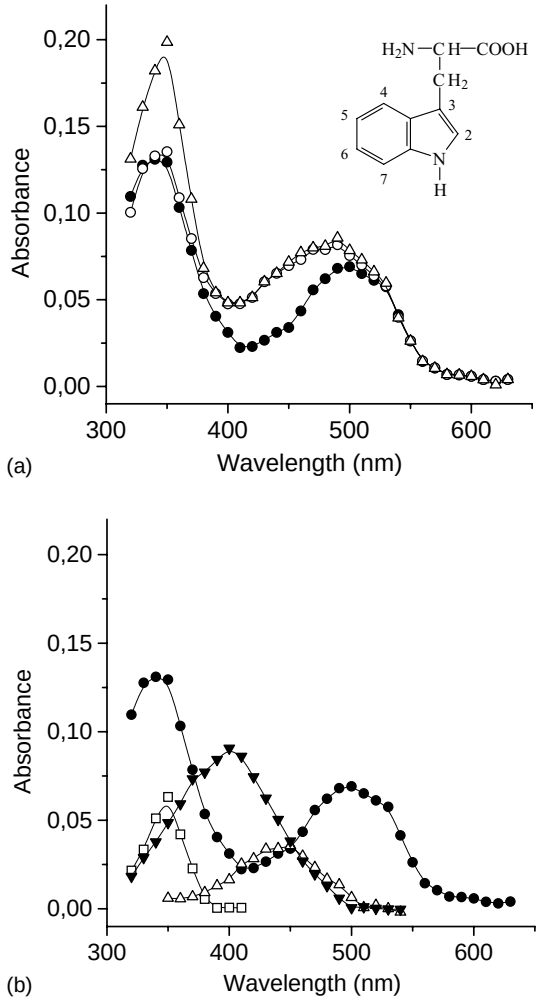

Fig. 1. (a) Transient absorption spectra, obtained 3 μs after 308 nm photolysis of 10^{-2} M TrpH at pH 7.0—triangles: under Ar; open circles: under Ar in the presence of 1.4×10^{-2} M acetone; solid circles: under O_2. (b) Absorption spectra of intermediates formed during TrpH photolysis—circles: radical $Trp^{\bullet}$; squares: adduct $TrpH_2^{\bullet}$; open triangles: neutral triplet $^{T}TrpH$; solid triangles: protonated triplet $^{T}TrpH_2^{+}$.

absorption of acetone at the concentration used is negligible (the absorption coefficient of acetone at 308 nm is $\varepsilon_{Ac} = 0.4\,M^{-1}\,cm^{-1}$). A similar spectrum was obtained when instead of acetone, the solvated electrons were scavenged by N_2O in the presence of 0.2 M t-BuOH ($OH^{\bullet}$ scavenger).

The third spectrum in Fig. 1a (solid circles) was obtained under O_2 bubbling. Oxygen quenches triplet states and scavenges solvated electrons, and, hence, this spectrum corresponds to the absorption of $Trp^{\bullet}$ only.

A simple subtraction allows the obtaining of the individual spectra of intermediates formed in 308 nm photolysis of tryptophan (Fig. 1b). In this figure, circles represent the absorption spectrum of $Trp^{\bullet}$ with the characteristic bands at 330 and 510 nm [5–7,52–54]. Squares show the difference between spectra, obtained in oxygen-free solutions in the absence (Fig. 1a, triangles) and presence (Fig. 1a, open circles) of acetone, and correspond to the absorption of $TrpH_2^{\bullet}$ radical with $\lambda_{max} = 350$ nm [8,39,42]. And, finally, the difference between the second and the third spectra in Fig. 1a shows the absorption spectrum of the triplet $^{T}TrpH$ (Fig. 1b, open triangles) with the maximum at 450 nm [5,8,55].

Fig. 1b also shows the difference between spectra, obtained under argon bubbling in the presence of acetone 50 and 250 ns after the laser flash (solid triangles). A transient has a maximum at 400 nm and decays exponentially with $k_t = 3.8 \times 10^7\,s^{-1}$, the spectrum and the lifetime of the intermediate are similar to that of "T_1 state of Bent and Hayon" [5]. The nature of this state will be discussed later.

In the absence of electron scavengers, the absorption of the solvated electron can be observed practically in the whole spectral region under study, the intensity of the signal increases at longer wavelengths. The rate of the signal decay is proportional to the initial TrpH concentration, $k_{obs} = k_e \times [TrpH]$, $k_e = 3.0 \times 10^8\,M^{-1}\,s^{-1}$, and in our experimental conditions ([TrpH] = 10 mM) at 3 μs after the laser pulse all electrons are scavenged by tryptophan.

3.2. Kinetic data

In neutral solution, the decay of $Trp^{\bullet}$ radical, monitored at 510 nm, obeys the second-order law with $2k_t/\varepsilon_R = (4.4 \pm 0.4) \times 10^5$ cm/s. Taking the absorption coefficient of $Trp^{\bullet}$ at 510 nm $\varepsilon_R = 1800\,M^{-1}\,cm^{-1}$ [6,7,52–54], one obtains $2k_t = (7.9 \pm 0.7) \times 10^8\,M^{-1}\,s^{-1}$, which is in the agreement with the previous reports [1,38,39].

The decay of the triplet state (450 nm) is more complex. At high intensities of the laser irradiation, the main channel of the triplet decay is triplet–triplet annihilation with $2k_{T\text{-}T}/\varepsilon_T = (1.9 \pm 0.3) \times 10^6$ cm/s with an admixture of the first-order component. At sufficiently low initial triplet concentration the first-order decay becomes dominant; with the TrpH concentration increase from 1.1 to 18.0 mM the apparent first-order rate constant k_{obs} increases from 9.7×10^4 to $3.2 \times 10^5\,s^{-1}$:

$$k_{obs} = k_0 + k_{T\text{-}TrpH} \times [TrpH] \quad (5)$$

Linear plot of k_{obs} versus [TrpH] (not shown) gives $k_0 = 8.0 \times 10^4\,s^{-1}$ and $k_{T\text{-}TrpH} = 1.2 \times 10^7\,M^{-1}\,s^{-1}$.

With pH decrease, both the triplet lifetime and the triplet yield decrease. Fig. 2 (squares) shows the pH-dependence of the rate constant k_0 of triplet decay. For each pH value, three–four kinetic curves at 450 nm have been obtained with different laser energies in order to separate the bimolecular component of decay. The solid line corresponds to the simulation of the titration curve according to the Eq. (6)

$$k_0 = \frac{k_{0a}[H^+] + k_{0b}K_a}{[H^+] + K_a}, \quad (6)$$

where k_{0a} and k_{0b} are the rate constants of triplet tryptophan decay in extremely acidic and neutral solutions, correspondingly, and K_a is the dissociation constant. The best fit was obtained with $pK_a = 3.2$.

The spectrum of the short-lived ($k_t = 3.8 \times 10^7\ s^{-1}$) intermediate with the maximum at 400 nm (Fig. 1b, solid triangles) does not change upon pH variation from 7.1 to −0.3, although the intensity of the signal in extremely acidic solutions is about 20–30% higher than that in neutral solution. In order to check whether this intermediate, indeed, represents the triplet state of tryptophan, the quenching experiments have been performed. The decay kinetics of both short-lived intermediate and "long-lived" triplet tryptophan were measured at 450 nm in neutral solution with the addition of different concentrations of acrylamide. The observed decay rate constant of the long-lived component of the signal at 450 nm increases linearly with acrylamide concentration, the calculated second order rate constant of the quenching of the long-lived triplet species by acrylamide is $k_{q1} = (1.3 \pm 0.2) \times 10^9\ M^{-1}\ s^{-1}$. The lifetime of the short-lived intermediate is also affected by the presence of 0.01–0.1 M acrylamide. However, the obtained value of the quenching rate constant $k_{q2} = 1.6 \times 10^8\ M^{-1}\ s^{-1}$ should be considered as a crude estimation only, since the temporal resolution of our setup is of the same order as the lifetime of the short-lived intermediate.

We also tried to quench the triplet states by oxygen. The lifetime of the long-lived triplet state linearly depends on the O_2 concentration with $k_q = (5.3 \pm 0.8) \times 10^9\ M^{-1}\ s^{-1}$, but the decay of the short-lived intermediate was not noticeably affected even by 100% O_2 bubbling. Apparently, the solubility of oxygen in water (about 1.4 mM) is not sufficient to quench such short-lived species.

3.3. Quantum yield measurements

Fig. 2 (circles) shows the pH-dependence of the triplet quantum yield with the apparent $pK_a = 2.2$. The absolute values of the triplet yield were determined by two methods. In the first method, the kinetic traces at 450 nm were obtained with four different laser energies varying from 4.1 to 50 mJ for 9.9×10^{-3} M TrpH solution at pH 7.0 under N_2O bubbling. The absorption of the tryptophan triplet at 450 nm was extrapolated to the zero time point; the residual absorption of tryptophanyl radical at 450 nm was subtracted. The quantum yield was calculated by using the extinction coefficient of triplet tryptophan at 450 nm $\varepsilon_T = 5000\ M^{-1}\ cm^{-1}$ [8]. No noticeable dependence on the laser energy has been detected. An average value of the triplet yield in neutral solution is equal to $\Phi_T = 0.065 \pm 0.012$.

The second method is based on acetone-sensitized photolysis of tryptophan. Since the triplet energy of acetone (337 kJ/mol [51]) is higher than that of tryptophan (296 kJ/mol [57]), and triplet acetone has long lifetime [58–60], the triplet energy transfer from acetone to tryptophan should proceed with the high efficiency. Fig. 3 demonstrates the kinetic traces, obtained at 450 nm during the flash photolysis of 1.3×10^{-2} M TrpH (tryptophan optical density at 308 nm at the excitation optical path 1 mm is 0.073) in the presence of (a) 1.5×10^{-2} M acetone (optical density of acetone (OD_{Ac}) at 308 nm is 6×10^{-4}), (b) 0.28 M acetone ($OD_{Ac} = 0.011$), and (c) 0.56 M acetone ($OD_{Ac} = 0.022$). When the acetone absorption is negligibly small, the formation of triplet tryptophan corresponds to the direct photolysis only. With the acetone concentration increase, two effects are observed: (1) an additional growth of absorption at the initial part of kinetic curves takes place, the rate and the intensity of the growth are proportional to the acetone concentration. This effect should be attributed to the energy transfer from triplet acetone to tryptophan. It is obviously from Fig. 3 that the efficiency of the triplet tryptophan production via sensitization is much higher than in the case of the direct photolysis. (2) The rate of the triplet decay increases, which corresponds to the triplet tryptophan quenching by ground-state acetone. Thus, the kinetic

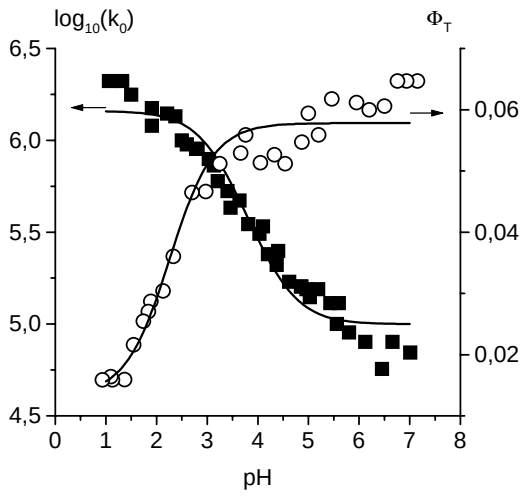

Fig. 2. pH dependence of the rate constant k_0 of triplet tryptophan decay (squares) and of the triplet quantum yield (circles).

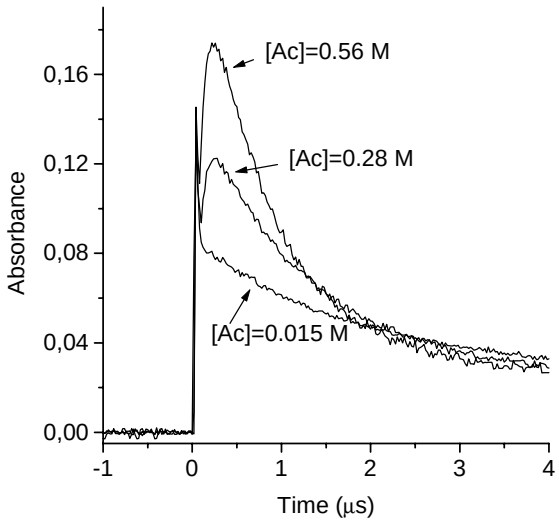

Fig. 3. Transient absorption kinetics of triplet tryptophan (450 nm) obtained during the photolysis of tryptophan at pH 7.0 in the presence of 1.5×10^{-2} M acetone, 0.28 M acetone, 0.56 M acetone.

scheme of the triplet tryptophan formation and decay in the presence of acetone can be described by the following set of equations:

$$\mathrm{TrpH} \xrightarrow{h\nu,\mathrm{ISC}} {}^{\mathrm{T}}\mathrm{TrpH} \quad (7)$$

$$\mathrm{Ac} \xrightarrow{h\nu,\mathrm{ISC}} {}^{\mathrm{T}}\mathrm{Ac} \quad (8)$$

$$2{}^{\mathrm{T}}\mathrm{Ac} \xrightarrow{k_9} 2\mathrm{Ac} \quad (9)$$

$${}^{\mathrm{T}}\mathrm{Ac} \xrightarrow{k_{10}} \mathrm{Ac} \quad (10)$$

$${}^{\mathrm{T}}\mathrm{Ac} + \mathrm{TrpH} \xrightarrow{k_{11}} \mathrm{Ac} + {}^{\mathrm{T}}\mathrm{TrpH} \quad (11)$$

$$2{}^{\mathrm{T}}\mathrm{TrpH} \xrightarrow{k_{\mathrm{T\text{-}T}}} 2\mathrm{TrpH}, \quad 2k_{\mathrm{T\text{-}T}} = 1.0 \times 10^{10}\,\mathrm{M^{-1}\,s^{-1}} \quad (12)$$

$${}^{\mathrm{T}}\mathrm{TrpH} \xrightarrow{k_0} \mathrm{TrpH}, \quad k_0 = 8.0 \times 10^{4}\,\mathrm{s^{-1}} \quad (13)$$

$${}^{\mathrm{T}}\mathrm{TrpH} + \mathrm{TrpH} \xrightarrow{k_{\mathrm{T\text{-}TrpH}}} \text{quenching},$$

$$k_{\mathrm{T\text{-}TrpH}} = 1.2 \times 10^{7}\,\mathrm{M^{-1}\,s^{-1}} \quad (14)$$

$${}^{\mathrm{T}}\mathrm{TrpH} + \mathrm{Ac} \xrightarrow{k_{\mathrm{T\text{-}Ac}}} \mathrm{TrpH}^{\bullet+} + \mathrm{Ac}^{\bullet-} \quad (15)$$

Since the acetone triplet lifetime is about 20–50 μs [58–60], reaction (10) can be neglected. At sufficiently high TrpH concentration and low intensity of laser irradiation the reaction of acetone triplet–triplet annihilation (9) can also be disregarded. Thus, in the reaction scheme (7)–(15) two rate constants, k_{11} and $k_{\mathrm{T\text{-}Ac}}$, are unknown. They were determined by varying the acetone concentration in the range from 5.6×10^{-2} to 1.4 M and the laser power from 1.85 to 70 mJ, followed by computer simulation of kinetic traces according to the scheme (7)–(15). The quantum yield of triplet acetone formation was taken 1.0, the fitting parameters were the quantum yield of triplet tryptophan formed in the direct photolysis Φ_{T} and the rate constants k_{11} and $k_{\mathrm{T\text{-}Ac}}$. The best fit of 24 obtained kinetic traces was obtained with $k_{11} = (2.0 \pm 0.4) \times 10^{9}\,\mathrm{M^{-1}\,s^{-1}}$, $k_{\mathrm{T\text{-}Ac}} = (1.9 \pm 0.5) \times 10^{6}\,\mathrm{M^{-1}\,s^{-1}}$, and $\Phi_{\mathrm{T}} = 0.09 \pm 0.02$. Perhaps, the relative actinometry (second method) gives somewhat overestimated value of Φ_{T}, since it presumes the quantitative triplet acetone formation and energy transfer. Thus, we suppose that the value of $\Phi_{\mathrm{T}} = 0.065$, obtained by the first method is more reliable.

The quantum yield of the tryptophan ionization was determined for four values of laser energy varying from 4.1 to 50 mJ by monitoring the initial absorption of $\mathrm{Trp}^{\bullet}$ at 510 nm ($\varepsilon = 1800\,\mathrm{M^{-1}\,cm^{-1}}$ [6,7,52–54]) or of $\mathrm{TrpH}^{\bullet+}$ at 570 nm ($\varepsilon = 500\,\mathrm{M^{-1}\,cm^{-1}}$ [7,53]) for four pH values: 7.1, 3.5, 3.0 and 0.02. The first three solutions were prepared in buffers, and for the last solution 1 M HCl in water was used as a solvent. The results, obtained under neutral (pH 7.1) and moderately acidic (pH 3.5 and 3.0) conditions are essentially the same within 10% experimental uncertainties, they are shown in Fig. 4 by squares. In extremely acidic solution, the biphotonic contribution to the tryptophan ionization disappears, and for all laser energies used in the experiment the same value $\Phi_i \approx 0.04$ has been obtained (circles in Fig. 4).

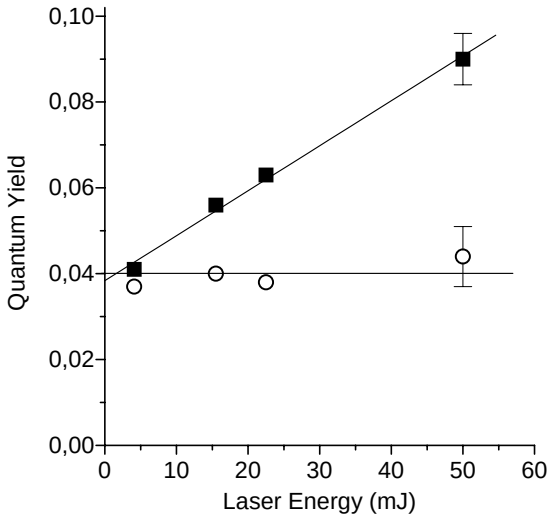

Fig. 4. Dependence of tryptophan photoionization quantum yield on the intensity of laser irradiation—squares: average values for pH 7.1, 3.5, and 3.0; circles: data for pH 0.02.

4. Discussion

4.1. Rate constants and quantum yields

The majority of the obtained in this work kinetic data, related to the decay of triplet tryptophan, are consistent with previous findings. The value of the rate of triplet–triplet annihilation $2k_{\mathrm{T\text{-}T}}/\varepsilon_{\mathrm{T}} = (1.9 \pm 0.3) \times 10^{6}$ cm/s can be compared with the previously reported: $2k_{\mathrm{T\text{-}T}} = 1.0 \times 10^{10}\,\mathrm{M^{-1}\,s^{-1}}$, $\varepsilon_{\mathrm{T}} = 5000\,\mathrm{M^{-1}\,cm^{-1}}$ [5,8]. The obtained value of the triplet lifetime $\tau = 12.5$ μs is also in the agreement with the values $\tau = 14$ μs published by Bent and Hayon [5] and $\tau = 20$ μs reported by Volkert et al. [8]. The obtained rate constant of the triplet quenching by ground-state tryptophan $k_{\mathrm{T\text{-}TrpH}} = 1.2 \times 10^{7}\,\mathrm{M^{-1}\,s^{-1}}$ differs significantly from that reported by Volkert et al. [8] $k_{\mathrm{T\text{-}TrpH}} = 1.1 \times 10^{8}\,\mathrm{M^{-1}\,s^{-1}}$ and by Song et al. [56] $k_{\mathrm{T\text{-}TrpH}} = 3.7 \times 10^{8}\,\mathrm{M^{-1}\,s^{-1}}$. However, our value is in agreement with the observation of Bent and Hayon [5] that the decay of triplet indole ($\tau = 12$ μs) is independent of indole concentration up to 10^{-3} M. If the rate constant $k_{\mathrm{T\text{-}TrpH}}$ were indeed about $10^{8}\,\mathrm{M^{-1}\,s^{-1}}$ or higher, in our typical experimental conditions ([TrpH] = 5–10 mM) the triplet lifetime would be shorter than 1 μs, which is definitely not the case. A possible source of the discrepancy is that in works [8] and [56] the excitation was performed with lasers operating at 265 and 248 nm. Since at these wavelengths the tryptophan absorption is much higher than at 308 nm, much lower tryptophan concentrations (10^{-5}–10^{-4} M) were used.

Under such conditions, the rate of the triplet quenching by ground-state tryptophan $[TrpH] \times k_{T\text{-}TrpH}$ is comparable with the rate of triplet–triplet annihilation $2 \times [^{T}TrpH] \times k_{T\text{-}T}$. Thus, it is possible that in works [8,56], the increase of the triplet decay rate with the tryptophan concentration increase, attributed to the triplet quenching by ground-state tryptophan, in fact should be explained as an increase of the initial triplet concentration $[^{T}TrpH]_0$.

Previous measurements of the triplet quantum yield Φ_T were performed mostly by indirect methods. Our result $\Phi_T = 0.065 \pm 0.012$ is significantly lower than the value $\Phi_T = 0.18$ estimated by Volkert and coworker, based on the experiments of Br^- promoting of intersystem crossing in photoexcited tryptophan [8]. Estimations of Robbins et al. [10] $\Phi_T = 0.09$ (calculated by a model based on fluorescence decay measurements) and $\Phi_T = 0.10$ (calculated from the experimental data of Bent and Hayon [5]) are more close to our results.

The linear dependence of the radical quantum yield on laser energy in neutral and moderately acidic solutions (Fig. 4) shows that both monophotonic and biphotonic mechanisms of tryptophan photoionization take place. According to the results of Nikogosyan and Gorner [15], for 248 nm photolysis of tryptophan the monophotonic ionization becomes the major source of solvated electrons with the laser intensities below 10^{10} W/m^2. We can estimate the density of the laser power in our experimental conditions by using the known area of the laser beam $S = 25$ mm^2 and roughly representing the time profile of the laser pulse as a rectangle of 20 ns width. The laser intensity for the lowest energy used (4.1 mJ) in this case is about 8×10^9 W/m^2, meaning that our results for 308 nm photolysis confirm the data obtained by Nikogosyan and Gorner [15].

Extrapolation of the data presented in Fig. 4 to zero laser energy gives the quantum yield of monophotonic ionization $\Phi_i = 0.039$. In earlier works [5–7,10], somewhat higher values were published; however, in these works the careful separation of mono- and biphotonic contributions not always has been done. In more recent works, where a special attention has been paid to avoid biphotonic processes, the quantum yields of monophotonic ionization 0.04 [9], 0.05 [15], 0.037 [16] have been reported, which is similar to our result.

4.2. Nature of the short-lived intermediate ($\lambda_{max} = 400$ nm, $\tau \approx 26$ ns)

The observation of a very short-lived intermediate with lifetime 30–45 ns in the photolysis of aqueous tryptophan was first reported by Bent and Hayon [5]. According to this work, the spectrum of this intermediate (assigned as T_1 state) in neutral solution is very similar to the spectrum of long-lived triplet state of tryptophan with the maximum at 450 nm. In acidic solutions at pH < 3.0 its yield decreases, and below pH 1.5 another transient with the similar lifetime is formed, with the absorption maximum at 400 nm and a distinct shoulder at 350 nm. Our measurements testify that the spectra of the short-lived intermediate in neutral and acidic solutions are practically identical, which means that these spectra belong to the same species. Unfortunately, the data in literature on the spectral properties of the discussed short-lived intermediate are very limited. Besides the work of Bent and Hayon [5], we have found only one reference where this transient was detected [16]. In this work, the absorption maximum of the short-lived intermediate was found at 420 nm, which is between our value (400 nm) and the data of Bent and Hayon (450 nm). It has been noticed [5] that the formation of the short-lived intermediate is associated with the presence of the protonated NH_3^+ group: the signal disappears at pH > 9.5, and this species has not been observed in the photolysis of indole, *N*-acetyl tryptophan, and the peptide glycyl tryptophanyl glycine. Robbins et al. [10] studied pH and temperature dependence of aqueous tryptophan fluorescence and revealed that the fluorescence lifetime decreases from about 9 ns in alkaline solutions to about 3 ns in neutral solutions. At pH 1 the fluorescence lifetime becomes as short as 0.66 ns. Based on this observation, authors [10] concluded that the observed decrease of the fluorescence lifetime in neutral solution corresponds to the intramolecular quenching. They suggested that this process is the intramolecular proton transfer from the NH_3^+ group to the excited indole ring, and that "T_1 state of Bent and Hayon" is the triplet state formed in this process.

The quenching of the singlet exited state T_1 of tryptophan due to inter- and intramolecular proton transfer was previously studied by fluorescent methods and by monitoring photochemical hydrogen–deuterium exchange reactions [10,61–66]. It has been shown that fluorescence can be quenched not only by acids, but also by other hydrogen donors, such as trifluoroethanol [66]. Significant deuterium incorporation into the C-4 position of indole ring, observed under irradiation of TrpH solution in D_2O [62], testifies that intramolecular proton transfer in neutral solutions occurs from the protonated NH_3^+ group to the C-4 position of tryptophan.

In the present work, we obtained strong pieces of evidence that the short-lived intermediate formed due to intramolecular (in neutral solutions) or intermolecular (under acidic conditions) proton transfer in the first singlet state S_1, is indeed the protonated at the indole ring triplet state of tryptophan $^{T}TrpH_2^+$. (i) The quenching of the short-lived intermediate by acrylamide confirms that this species corresponds to the triplet state; (ii) the similarity of the spectra and lifetimes obtained in neutral and acidic solutions testifies that the same short-lived transient appears in the whole region pH < 9.5; (iii) protonation of S_1 state through solvent does not require the presence of NH_3^+ group. Indeed, we obtained the same spectrum of the short-lived intermediate (Fig. 1b, solid triangles) in the photolysis of both indole and *N*-acetyl tryptophan under highly acidic conditions (1 M HCl), although in neutral solution this transient has not been detected.

We suggest that the first step of $^{T}TrpH_2^+$ formation is the protonation of indole ring of the singlet excited state S_1

followed by the fast intersystem crossing. In neutral solution the main channel of S_1 protonation is the intramolecular proton transfer from the NH_3^+ group to the indole ring, whereas in highly acidic solutions the indole group receives proton from the solvent. The quenching of S_1 state due to proton transfer competes with other processes—fluorescence, photoionization, and intersystem crossing. With pH decrease, the protonation through solvent followed by ISC becomes the major pathway of the S_1 decay. Indeed, as it follows from Fig. 2 (circles), the quantum yield of the triplet state ^{T}TrpH formation in acidic solution decreases with the apparent $pK_a = 2.2$. This value is close to the pK_a value of the carboxilate group of ground-state tryptophan. However, since the same decrease of the triplet yield in acidic solutions was observed in the photolysis of indole [5], the obtained value should be attributed to the protonation of the excited indole ring. Robbins et al. [10] also reported the decrease of the tryptophan fluorescence under acidic conditions. Thus, it seems natural to assign the obtained midpoint of the titration curve (Fig. 2, circles) to the dissociation constant of S_1 state. However, one should take into account that the protonation can occur only when $[H^+] \times k_p > 1/\tau_f$, where k_p is the protonation rate constant and $t_f \approx 3$ ns [10] is the lifetime of S_1 state in neutral solution. Taking the typical value $k_p = 3.0 \times 10^{10}\ M^{-1}\ s^{-1}$, we conclude that the protonation of S_1 state through solvent can become important only at pH < 2. Thus, the obtained value $pK_a = 2.2$ represents only the lower limit of the dissociation constant of S_1 state.

The decrease of the triplet lifetime with pH decrease should be attributed to the protonation of the triplet state followed by the fast decay of ^{T}TrpH$_2^+$ ($\tau \approx 26$ ns). Thus, from the titration curve of the triplet tryptophan lifetime (Fig. 2, squares), the dissociation constant of the triplet tryptophan $pK_a = 3.2$ can be derived.

The intensities of "fast" and "slow" components of the signal at 400 and 450 nm can be used for the determination of the relative yields of the protonated (formed due to intramolecular quenching of S_1 state) and deprotonated (formed due to ISC in S_1 state) forms of triplet tryptophan. The extrapolation of kinetic traces to the zero time point for neutral solution of tryptophan gives the ratio of the initial absorbance $OD_{fast,400\,nm}/OD_{slow,450\,nm} \approx 8$. Since the spectra of ^{T}TrpH (open triangles at Fig. 1b) and ^{T}TrpH$_2^+$ (solid triangles at Fig. 1b) are rather similar, we can presume that the absorption coefficients of the protonated and deprotonated triplets are also similar. The value $\Phi_T = 0.065$ of ^{T}TrpH is reported above. Thus, the quantum yield of the protonated triplet can be estimated as $\Phi_{TH+} \approx 8\Phi_T \approx 0.52$. This value is in a good agreement with $\Phi = 0.65$, estimated by Robbins et al. [10] for the intramolecular quenching of tryptophan fluorescence in neutral solution at room temperature.

4.3. Precursor of tryptophan photoionization

At the moment, there are two main viewpoints concerning the precursor state for photoionization of aqueous tryptophan at room temperature. Some authors [4,10,12,14,16] suggest that photoionization proceeds via the relaxed fluorescent S_1 state. This conclusion is based mostly on the observation that with the temperature growth, the solvated electron yield increases while the fluorescence intensity decreases. At the same time, measurements of ultrafast photoionization dynamics of indole and tryptophan [18,19] demonstrate that the formation of solvated electrons is completed within first 200 fs. This result shows that in the monophotonic ionization solvated electrons originate from a non-relaxed prefluorescent state. Our results speak in favor of the second hypothesis. Fig. 4 shows that at pH 0.02, when the fluorescence is strongly suppressed by the protonation through solvent and the lifetime of the fluorescent S_1 state is very short, the biphotonic ionization ceases to exist, and quantum yield of the monophotonic ionization remains at the level $\Phi_i \approx 0.04$ for any intensity of the laser irradiation. In neutral and moderately acidic solutions, the monophotonic ionization proceeds with the same efficiency: $\Phi_i = 0.039$ (see Fig. 4). Thus, the contribution of the relaxed S_1 state into monophotonic ionization is negligible, and the major precursor of photoionization of aqueous tryptophan at room temperature is non-relaxed singlet excited state.

We should note that in the paper [5], it was reported that in the photolysis of indole and tryptophan under extremely acidic conditions (2–3 M H_2SO_4) spectra of $IH^{\bullet+}$ and $TrpH^{\bullet+}$ disappear, and photoionization does not take place. This result is inconsistent with our data: in acidic solutions only biphotonic component of ionization disappears. This discrepancy probably means that in the experimental conditions of work [5], the biphotonic processes played a significant role.

The main argument in favor of the relaxed singlet state as a precursor of photoionization is the increase of the solvated electron yield with temperature. However, the examination of data presented in [5,16] shows that in fact the electron yield becomes temperature-dependent only at $T >$ 30–40 °C. Robbins et al. [10] reported rather high activation barrier for tryptophan ionization ~50 kJ/mol. Thus, it is possible that the prefluorescent state is the main precursor for ionization only at room temperature, and above 30–40 °C the contribution from the relaxed singlet state may become important.

4.4. Qualitative model of tryptophan photolysis

Results, obtained in this work, combined with the data from literature, allow to draw a general qualitative scheme of tryptophan photolysis.

Ultrafast events:

$$TrpH \xrightarrow{h\nu} {}^S TrpH^* \tag{16}$$

$$^S TrpH^* \xrightarrow{\text{relaxation}} {}^S TrpH \tag{17}$$

$$^{S}TrpH^{*} \xrightarrow{\text{ionization}} TrpH^{\bullet+} + e^{-}, \quad \Phi_i \approx 0.04 \tag{18}$$

In non-relaxed photoexited singlet state, the ionization competes with relaxation. At room temperature, the ionization quantum yield is about 0.04. Since the same value of the quantum yield was obtained at 248 nm [15], 265 nm [9,16], and 308 nm, one can conclude that Φ_i does not depend on the excitation wavelength in this range. At the same time, under 193 nm irradiation, the ionization yield is significantly higher [15].

Singlet state processes:

$$^{S}TrpH \xrightarrow{h\nu} {}^{S2}TrpH \rightarrow TrpH^{\bullet+} + e^{-} \tag{19}$$

$$^{S}TrpH \xrightarrow{\text{fluor}} TrpH + h\nu \tag{20}$$

$$^{S}TrpH \xrightarrow{\text{ISC}} {}^{T}TrpH, \quad k_{\text{ISC}} + k_{\text{fluor}} \approx 1.1 \times 10^{8}\,s^{-1} \tag{21}$$

$$^{S}TrpH(NH_3{}^{+}) \xrightarrow{k_{\text{ipt}}} {}^{S}TrpH_2{}^{+}(NH_2), \quad k_{\text{ipt}} \approx 2 \times 10^{8}\,s^{-1} \tag{22}$$

$$^{S}TrpH + H^{+} \rightleftarrows {}^{S}TrpH_2{}^{+}, \quad pK_a \geq 2.2 \tag{23}$$

$$^{S}TrpH_2{}^{+} \xrightarrow{\text{ISC}} {}^{T}TrpH_2{}^{+} \tag{24}$$

See [10] for reactions (21) and (22).

There are three major mechanisms of the decay of singlet state S_1. The most important pathway is the protonation of the indole ring followed by intersystem crossing into the triplet state. In neutral solutions the protonation occurs due to intramolecular proton transfer from the $NH_3{}^{+}$ group (reaction (22)). The rate constant of this process at room temperature k_{ipt} can be estimated from the fluorescence lifetime in neutral solution [10]. The quantum yield of the formation of protonated triplet is 0.5–0.6 [10]. In extremely acidic solutions, the protonation proceeds through solvent with the pseudo-first order rate constant $k_p \times [H^+]$, $k_p \sim 3 \times 10^{10}\,M^{-1}\,s^{-1}$. Two other channels of S_1 decay are fluorescence and ISC into triplet state. The sum of the rate constants of these processes can be determined using the fluorescence lifetime $\tau_f \approx 9$ ns [10] in basic solutions. The quantum yield of the triplet state formation is about 0.07–0.1 [10].

Triplet state processes:

$$^{T}TrpH + H^{+} \rightleftarrows {}^{T}TrpH_2{}^{+}, \quad pK_a = 3.2 \tag{25}$$

$$^{T}TrpH \xrightarrow{k_0} TrpH, \quad k_0 = (2.5\text{–}8.0) \times 10^{4}\,s^{-1} \tag{26}$$

$$^{T}TrpH_2{}^{+} \xrightarrow{k_t} TrpH + H^{+}, \quad k_t = (2.2\text{–}3.8) \times 10^{8}\,s^{-1} \tag{27}$$

$$^{T}TrpH + TrpH \xrightarrow{k_{\text{T-TrpH}}} \text{quenching}, \quad k_{\text{T-TrpH}} = 1.2 \times 10^{7}\,M^{-1}\,s^{-1} \tag{28}$$

$$^{T}TrpH + {}^{T}TrpH \xrightarrow{k_{\text{T-T}}} 2TrpH, \quad 2k_{\text{T-T}} = 1.0 \times 10^{10}\,M^{-1}\,s^{-1} \tag{29}$$

See [5,8,33] for reaction (26), [5] for reactions (27) and (29).

The dissociation constant of triplet tryptophan is $pK_a = 3.2$. The lifetime of the protonated form $^{T}TrpH_2{}^{+}$ (about 30 ns) is much shorter that that of $^{T}TrpH$ (12–40 μs). At high triplet concentrations, the triplet–triplet annihilation with $2k_{\text{T-T}} = 1 \times 10^{10}\,M^{-1}\,s^{-1}$ becomes the main channel of the triplet decay. Triplet tryptophan can react with its ground state ($k_{\text{T-TrpH}} = 1.2 \times 10^{7}\,M^{-1}\,s^{-1}$), and with acetone ($k_{\text{T-Ac}} = (1.9 \pm 0.5) \times 10^{6}\,M^{-1}\,s^{-1}$) the reaction mechanism is most likely the electron transfer.

Radical reactions:

$$Trp^{\bullet} + H^{+} \rightleftarrows TrpH^{\bullet+}, \quad pK_a = 4.3 \tag{30}$$

$$TrpH + e^{-} \xrightarrow{k_e} TrpH^{\bullet-}, \quad k_e = 3.0 \times 10^{8}\,M^{-1}\,s^{-1} \tag{31}$$

$$TrpH^{\bullet-} \xrightarrow{+H^{+}} TrpH_2{}^{\bullet} \tag{32}$$

$$TrpH^{\bullet+} + e^{-} \xrightarrow{k_r} TrpH, \quad k_r = 7.2 \times 10^{10}\,M^{-1}\,s^{-1} \tag{33}$$

$$2Trp^{\bullet} \xrightarrow{k_t} \text{products}, \quad 2k_t = 7.9 \times 10^{8}\,M^{-1}\,s^{-1} \tag{34}$$

See [5,7,38] for reaction (30), [44] for reaction (31), [16] for reaction (33) and [1] for reaction (34).

In neutral solution, cation $TrpH^{\bullet+}$ deprotonates within 1 μs ($pK_a = 4.3$). The main channels of the solvated electron decay are recombination, addition to the ground state tryptophan, and, under acidic conditions, protonation with the formation of $H^{\bullet}$. We have not observed any difference in spectra of electron and hydrogen adducts, obtained in neutral and acidic solutions, correspondingly. Thus, it is likely that the electron adduct is a strong base and it rapidly protonates even in neutral solutions.

5. Conclusion

In the present work, it has been demonstrated that the short-lived intermediate with the maximum at 400 nm, formed in the photolysis of tryptophan, is the protonated at the indole ring triplet state. It forms due to intramolecular (in neutral solutions) and intermolecular (in acidic solutions) proton transfer in the excited singlet state S_1 followed by fast intersystem crossing. The pK_a value of triplet tryptophan, determined from the pH dependence of the triplet lifetime, is 3.2. The lower limit of the pK_a value of S_1 state is 2.2. The monophotonic photoionization of tryptophan at room temperature proceeds from the non-relaxed prefluorent state, whereas the precursor of biphotonic ionization is the relaxed S_1 state.

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