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Journal of Photochemistry and Photobiology A: Chemistry 89 (1995) 247–250

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY A:CHEMISTRY

Fluorescence studies of tryptophan and tryptophan–retinal Schiff base in reverse micellar matrix ☆

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Received 18 May 1994; accepted 7 February 1995

Abstract

The fluorescence properties of tryptophan and tryptophan–retinal Schiff base are investigated in a reverse micellar matrix of sodium bis(2-ethylhexyl)sulphosuccinate, a membrane mimetic system. The results are discussed in terms of energy transfer interaction between retinal and tryptophan in the native protein and the conformational aspects of tryptophan residues in the protein.

Keywords: Tryptophan–retinal Schiff base; Fluorescence; Bacteriorhodopsin; Reverse micelle

1. Introduction

Bacteriorhodopsin (bR) is a photosensitive protein in the purple membrane of *Halobacterium halobium* [1]. This protein in its light-adapted form has an all-*trans*-retinal chromophore which forms the Schiff base bond with a lysine residue in the protein. The structural and functional features of bR are a reflection of the sensitivity of the Schiff base chromophore to the environment provided by the surrounding apoprotein. Thus the Schiff base chromophore undergoes intimate interactions with the amino acid residues at the active site. The three-dimensional structure of bR reveals that several amino acid residues line the retinal pocket in bR, with tryptophan (Trp) being the maximum (four residues: Trp_{86} (C helix), Trp_{138} (E helix), Trp_{182} and Trp_{189} (F helix)) [2]. While side-chains of Asp, Arg, Tyr, etc. can be directly implicated in the active site electrostatic interactions and proton channelling in bR, the role of the Trp residues is far from clear. It is known that Trp_{182} is located above the central polyene chain whereas Trp_{189} lies below the β-ionyl ring. A sandwich is formed by Trp_{182} above and both Trp_{86} and Trp_{189} lying below the retinal chromophore.

Some early studies have indicated the involvement of charge transfer interaction between retinal and Trp residues [3,4]. Shirane [5] and Bensasson et al. [6] highlighted the role of Trp by reporting the detection of the radical cation of Trp when visual protein rhodopsin was irradiated. The retinal–Trp interaction was further underlined by Johnston et al. [7] when they noted a striking resemblance between the retinal–indole reaction products and the chromophore of bR. Fluorescence studies on bR have indicated that there can be energy transfer between retinal and Trp [8–12]. Very recently Fourier transform IR (FTIR) difference spectroscopic studies on bR labelled with indole-^{15}N-Trp have indicated the perturbation of Trp in the L intermediate of bR and suggested that Trp residues are involved in H bond interactions to Asp residues at the active site of bR [13]. Mutation of Trp_{182} has been found to decrease the rate of L-to-M reaction in bR, suggesting its importance [14].

Thus it is patently clear that Trp residues play an important role in the structure and function of bR. However, the exact nature of the interactions between retinal and Trp and between Trp and other amino acid residues is not fully understood. The present paper deals with the fluorescence properties of tryptophan (**1**) and retinal–tryptophan Schiff base (**2**) (Fig. 1) in a membrane mimetic medium of sodium bis(2-ethylhexyl)sulphosuccinate (aerosol-OT, AOT) reverse micelles.

In this study we have adopted an approach in which a suitable retinylidene Schiff base containing Trp residue is incorporated in a membrane mimetic system followed by fluorescence spectroscopic studies of the immobilized chromophore to derive information about its location and bimolecular interactions. In this regard the availability of a novel retinylidene Schiff base chromophore containing a tryptophanyl residue prompted the present study [15]. An added novelty came from the fact that the model Schiff base can be constituted in an AOT reverse micellar matrix which is

☆ Dedicated to Professor Koji Nakanishi on the occasion of his 70th birthday.

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SSDI 1010-6030(95)04063-3

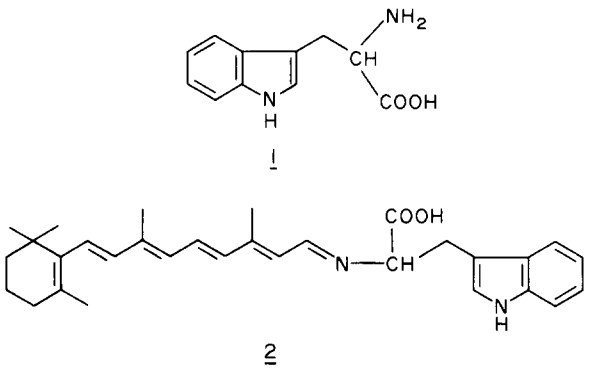

Fig. 1. Structure of compounds **1** and **2**.

known to mimic the active site of enzymes containing non-polar and polar pockets.

2. Experimental details

AOT was purchased from Fluka and purified before use. all-*trans*-Retinal was obtained from Sigma. L-Tryptophan was purchased from SRL, India and used as received. Retinal–tryptophan Schiff base was constituted in the micelle as described earlier [15]. *n*-Heptane of spectroscopic grade was obtained from Spectrochem, India and dry distilled before use. Doubly distilled deionized water was used throughout the studies.

Steady state fluorescence measurements were performed on a Spex Spectrofluorolog equipped with a 16810.22 m spectrometer as the single-grating excitation monochromator, a 16800.22 m double spectrometer as the double-grating emission monochromator and a 450 W xenon lamp as the light source. Samples were adapted to normal room temperature (25 °C) prior to measurements. All the samples were excited at 280 nm and the emission spectrum was scanned from 300 nm.

Fluorescence lifetime measurements were carried out using single-photon technique. The excitation light source was a tunable picosecond dye laser pulse derived from a cavity-dumped dye laser pumped synchronously by the frequency-doubled output (532 nm) of a mode-locked continuous wave (CW) Nd–YAG laser from Spectraphysics Inc., USA. The visible tunable excitation, 580–640 nm, was achieved with rhodamine 6G, with a typical pulse width of 4 ps and a pulse energy of 10–60 nJ. The dye output (power 20–30 mW) was further frequency doubled to get UV pulses via an angle-tuned potassium diphosphate (KDP) crystal. The UV pulses were used to excite the sample held in a quartz cuvette of 1 cm^2 cross-section. The emitted fluorescence was detected by an MCP PMT R2809 U01 (Hamamatsu) instrument and amplified by an HP 8447F preamplifier. The excitation wavelength used was 290 nm. The instrument response had a full width at half-maximum of 80 ps.

3. Results and discussion

3.1. Steady state fluorescence studies in reverse micellar system

The steady state fluorescent emission studies were carried out for tryptophan (**1**) and retinal–tryptophan Schiff base (**2**) in a reverse micellar matrix of AOT/*n*-heptane of varying water pool size ($w = [H_2O]/[AOT] = 0–20$). The results are given in Table 1.

It is observed that the fluorescence properties of both tryptophan and its retinal Schiff base are dependent on its microenvironment. While in water tryptophan showed an emission maximum at 363 nm, in AOT medium the emission spectrum was found to be blue shifted. However, as the water pool size was increased from 0 to 20, the emission maximum approached that in water. This wavelength dependence indicates that the indole ring of tryptophan is located in an environment less polar than water.

3.2. Fluorescence lifetime (τ_f) measurements in AOT/n-heptane

The lifetime measurement studies on both tryptophan (**1**) and tryptophan–retinal Schiff base (**2**) show a triple-exponential decay (Figs. 2 and 3; Table 2).

A systematic investigation of tryptophan decay kinetics in aqueous solution as a function of pH and buffer compositions has led to interesting results [16]. Below pH 7.0 the decay appears to be double exponential with a subnanosecond component, confirming earlier findings, whereas at pH > 7.0 the decay becomes triple exponential with the appearance of a long component whose contribution to the total emission intensity increases rapidly with increasing pH. In analogy with these findings it can be inferred that the appearance of τ_3 in our experiment is due to the anionic form of tryptophan. Since the lifetime measurements for the tryptophan/AOT system were carried out in basic media (pH > 7.0), the tryptophan molecules will predominantly exist in anionic form besides having the zwitterionic components. Thus the two

Table 1
Emission wavelengths (λ_{max}) for tryptophan (**1**) and tryptophan–retinal Schiff base (**2**) in AOT/*n*-heptane system [a]

w	λ_{max} (nm)			
	Tryptophan (**1**)		Schiff base (**2**)	
	0.01 M	0.025 M	0.01 M	0.025 M
0	337.0	335.0	341.5	343.5
5	353.0	349.5	355.0	356.5
8	355.5	353.0	355.0	360.0
10	355.0	353.5	355.5	360.0
15	356.0	356.0	357.5	364.5
20	357.5	357.0	359.0	365.0

[a] [AOT] = 0.01 and 0.025 M; $w = [H_2O]/[AOT]$.

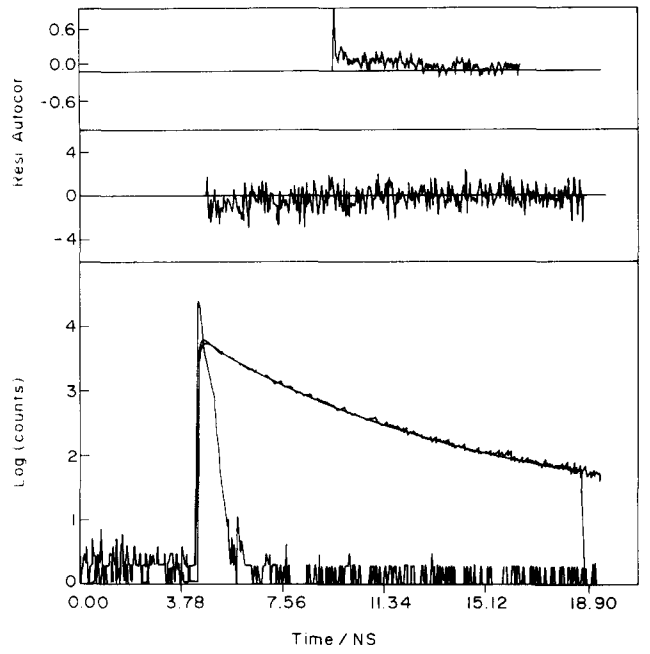

Fig. 2. Typical triple-exponential fluorescence decay curve of tryptophan (1) in AOT/*n*-heptane system ($w=20$) at 25 °C.

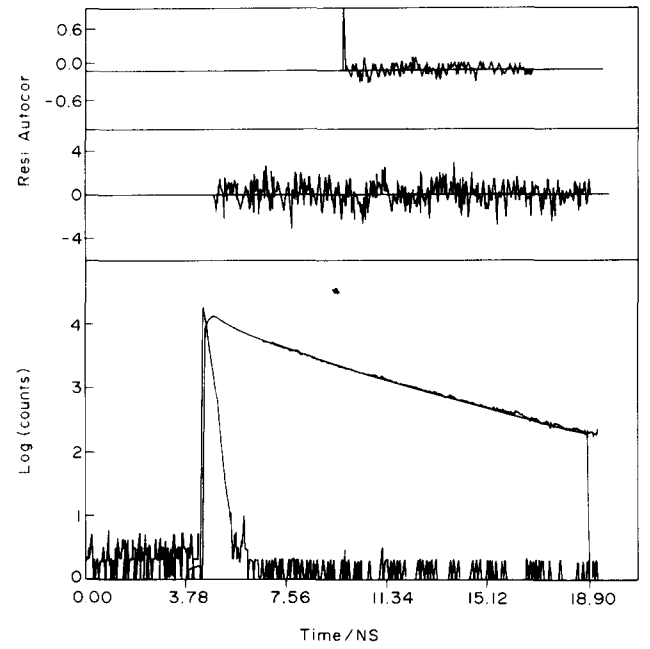

Fig. 3. Typical triple-exponential fluorescence decay curve of tryptophan–retinal Schiff base (2) in AOT/*n*-heptane system ($w=15$) at 25 °C.

shorter components τ_1 and τ_2 are due to the zwitterionic species.

Alternatively, the existence of triple-exponential decay for the fluorescence lifetime measurements for tryptophan/AOT can also be explained on the basis of another model proposed earlier [17]. The presence of multi-exponential decay is explained in terms of the rotamers of tryptophan. When tryptophan is in the zwitterionic form, it can exist in the conformers shown in Fig. 4. At pH > 7 there exists another form of tryptophan (anionic) in addition to the above-mentioned one. The rotamer for this form is shown in Fig. 5.

As has been reported earlier [18], the most electrophilic group nearer to the indole ring will show a shorter lifetime; thus rotamers **A** and **B** should have a longer lifetime than rotamer **C**. The latter has two groups (NH_3^+ and COO^-) whose contribution to the electrophilicity is more than in the case of either the **A** or **B** form. Thus the shortest lifetime τ_1 can be assigned to rotamer **C**. Rotamers **A** and **B** contribute to τ_2. Even though the electrophilicity of NH_3^+ and COO^- is not equivalent, both of them give a resultant lifetime value

Table 2
Fluorescence lifetime (τ_f) data for tryptophan (1) and tryptophan–retinal Schiff base (2) in AOT/*n*-heptane system

Sample	τ_f (ns)			
	τ_1	τ_2	τ_3	χ^2
Tryptophan (1)				
$w=0$	0.372	1.63	4.33	1.02
$w=5$	0.481	1.94	4.71	1.25
$w=8$	0.349	1.63	4.03	1.06
$w=10$	0.356	1.52	4.28	1.15
$w=15$	0.400	1.64	4.39	1.15
$w=20$	0.355	1.71	5.08	1.17
Trp–retinal Schiff base (2)				
$w=0$	0.333	1.63	4.44	0.99
$w=5$	0.562	1.82	4.42	1.13
$w=8$	0.562	1.90	4.57	1.37
$w=10$	0.350	1.59	4.13	0.95
$w=15$	0.408	1.61	4.07	1.12
$w=20$	0.366	1.70	4.62	1.12

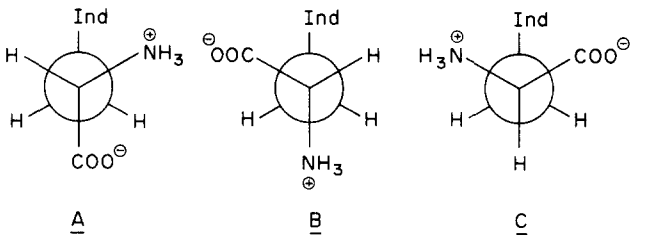

Fig. 4. Conformation of tryptophan (1) rotamers in AOT/*n*-heptane system

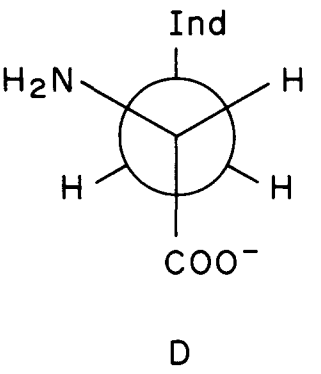

Fig. 5. Conformation of tryptophan rotamer (anionic form) in AOT/*n*-heptane system.

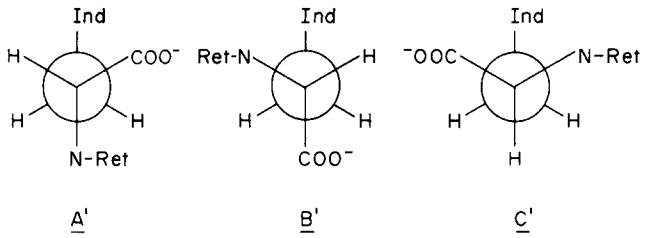

Fig. 6. Conformation of tryptophan–retinal Schiff base (**2**) rotamers in AOT/*n*-heptane system.

τ_2 due to fast exchange in the position of NH_3^+ and COO^- groups. However, at pH > 7 there exists the anionic form of tryptophan whose contribution predominates over the zwitterionic forms. Thus the component τ_3 which is observed in our experiments and which has the longest lifetime is due to this anionic form (**D**).

Both the models described above can explain the existence of three different lifetimes in the tryptophan/AOT system in basic media. Although the rotamer model reported earlier does not take into consideration the anionic form of tryptophan, it can be used here to account for the various lifetimes obtained.

The fluorescence lifetime measurements of tryptophan–retinylidene Schiff base (**2**) also showed the existence of triple-exponential decay. In this case also the rotamer model could be used for explaining the fluorescence decay profile. However, in this case the three rotamers of the Schiff base can exist irrespective of the pH of the medium (Fig. 6).

Here all the rotamers are in anionic form, since a zwitterionic form is not possible owing to the presence of the *N*-retinylidene (*N*-ret) chain. Accordingly, the existence of a new rotamer (**D′**) at higher pH as in the case of the tryptophan/AOT system can be ruled out. In fact, this unreacted rotamer **D′** is the same as rotamer **C′**.

Rotamer **C′** has $-COO^-$ as well as *N*-retinylidene groups in proximity to the indole ring, whereas in each of rotamers **A′** and **B′** only one of the substituents, $-COO^-$ and *N*-retinylidene respectively, can be adjacent to the indole ring. Accordingly, the longest lifetime τ_3 is assigned to **B′** owing to the electrophilic criteria, while the shortest lifetime τ_1 is due to the conformer **C′**. The third lifetime τ_2 is due to the remaining conformer **A′**.

4. Conclusions

The observed differences in the lifetimes obtained reflect the differences in the quenching probability of tryptophan emission by retinal as a result of differences in the relative orientation of the emitting tryptophan molecules with respect to the retinal transition moment direction.

Energy transfer interaction between retinal and tryptophan in the native protein is possible. This interaction is dependent upon the conformational aspects of tryptophan residues. It will be interesting to investigate the emission properties of bR and its photocycle intermediates by time-resolved techniques.

Acknowledgement

The authors would like to thank TIFR, Bombay for the fluorescence lifetime measurement studies.

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