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Case Report

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Insights on Structural, Environmental and Dynamical Behaviour of Multi-Tryptophan Proteins by Analysis of Their Low Temperature Phosphorescence Spectra: A Review

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Abstract

The individual amino acids of proteins can experience a variation in their local environments because of the folded tertiary/quaternary structure of the former. These microenvironments are regions that differ from one another with respect to polarizability, polarity and lability of the residues. The three intrinsic fluorescent probes are Tyrosine (Tyr), Tryptophan (Trp) and Phenylalanine (Phe) which form a part of protein backbone. Fluorescence of Phe is weak and rarely used in protein studies. On the other hand, Trp fluorescence is strong. Therefore, most studies of intrinsic protein fluorescence focus on Trp residues, and it provides specific and sensitive information on the structure of proteins and their interaction with probes. In steady-state fluorescence spectra, the shift in λ_{max} of Tryptophan to blue or red regions of the spectrum indicates the solvent accessibility of the former – a red-shifted band indicating significant solvent exposure. Though Trp fluorescence in proteins is an important tool to understand the structural and dynamical evolution of a protein, it is difficult to ascertain the contribution of individual Trp to the total fluorescence of the former. Usually steady-state fluorescence spectra are broad and even a protein with a single Trp residue can have a multi-exponential fluorescence decay upon time resolution. So, low temperature phosphorescence studies (LTP) at 77K in a suitable cryosolvent is an important tool from structural and biological aspects. LTP gives a structured spectra with definite (0-0) band characteristics of the Trp environment. In phosphorescence, a blue-shifted maximum is an indication of solvent exposed Trp whereas a red-shifted maximum indicates a buried Trp. Several proteins containing more than one Trp residues are known to exhibit multiple (0,0) bands. Usually, the no. of bands should correspond to the no. of Trp residues present in the protein. Non-correspondance between the two (no of Trp residues and no of bands) may be attributed to energy transfer to another residue, interaction with neighbouring residues or electron transfer from excited state. This review is an attempt to focus and analyze the low temperature phosphorescence spectra of multi-tryptophan proteins which will eventually throw light on the structure and function of such proteins or protein-substrate complexes. LTP of mutated proteins have also been reported. We have also documented the theoretical modeling studies which help us to understand the assignment of multiple (0-0) bands corresponding to different Trps in multi-Trp proteins.

Keywords: Multitryptophan proteins; Fluorescence; Low-temperature phosphorescence; Probes; Blue-shifted maxima; Red-shifted maxima

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Introduction

Proteins form an integral part of all bimolecular entities, their functioning being an interplay of structural, conformational and dynamical aspects. Fluorescence studies on intrinsic fluorophores of the polypeptide like tryptophan and tyrosine have enriched our knowledge on the conformations adopted by the former in addition to providing important insights into the region surrounding such fluorophores. In this review, we have reported several studies on the fluorescence of both single and multi-tryptophan proteins [1-3]. However tryptophan fluorescence spectra are broad with multi exponential time resolved decay curves, which makes data analysis and interpretation rather difficult [5-7]. This difficulty can be resolved if low temperature phosphorescence (LTP) spectra (at 77K) in a suitable cryosolvent is analyzed, since such spectra are much more well resolved with one or more sharp 0-0 bands and the band positions can be directly correlated with the tryptophan environment, especially in case of multi tryptophan proteins [7-12]. Low temperature phosphorescence has also shown its versatility in case of biopolymer interactions with small molecules, particularly with respect to monitoring both short- and long-range interactions [13-17]. In LTP spectra, individual Trp residues and additionally, Trp and Tyr residues can be well- resolved [18,19]. Some proteins have more than one Trp residue, but they exhibit a single (0,0) band in the respective LTP spectra. This non-correspondence can be attributed to the following factors: (i) energy transfer to another residue, (ii) interaction with neighboring residues like formation of charge-transfer complex, or (iii) electron transfer from the excited state. We have documented cases where multi tryptophan proteins exhibit multiple 0-0 bands although there may not be a correspondence between number of tryptophan residues and number of bands [9-11,20-24]. Such observations indicate the inhomogeneity of the tryptophan environment (between the different tryptophans) preventing efficient energy transfer between them. For the triplet state, a reverse Stokes shift pattern has been indicated via studies of LTP proteins having Trp residues [19,25-28]. In phosphorescence, a blue-shifted maximum is an indication of solvent exposed tryptophan and red-shifted maximum corresponds to a buried tryptophan [24]. However buried tryptophans having blue shifted 0-0 band can be a result of specific interactions with polar residues [24,25]. In general, the LTP spectra is more structured and redder shifted with respect to the fluorescence spectra (Fig 1) and the λ_{max} values of the (0,0) phosphorescence bands have direct correlation with the polarity of the environment surrounding the chromophore. Additionally, the homogeneity of such environments corresponds directly to the sharpness of the bands enabling identification of tryptophan residues residing in different surroundings [24,25,29-38]. It is worth mentioning in this context that phosphorescence spectroscopy of such polypeptide or polypeptide ligand complexes often give rise to spectra that represents structures obtained from XRD or NMR data analysis [9,23,39-41]. This is because at low temperatures specific lower energy conformations will predominate over other conformations at ambient temperatures. If the crystal structure of the protein is unknown, then the structure is derived finding a maximum homology with a known crystal structure protein with the help of BLAST search in the PDB with the full sequence of that structure unknown protein. This approach will give a reasonably good backbone and buried residue side chains even though surface side chains and loops may be somewhat less accurate. This crystal structure is used to calculate the accessible surface area (ASA) [using available software i.e. NACESS, VADAR etc.] and to find the neighboring residues of each Trp.

These crystal structural data correlate well with the experimental LTP data and are sometimes used to interpret multiple (0,0) bands in LTP. Some representative examples of LTP of multiple tryptophan containing proteins having known and unknown crystal structures are presented in this article. Phosphorescence at room temperature has also been carried out for many proteins but proper degassing of the system must be done. Observation of Room Temperature. Phosphorescence (RTP) does indicate the presence of tryptophan in a somewhat rigid environment. Although the lifetime of RTP (microsecond-millisecond) observed in many cases could be rationalized with the presence of tryptophan in rigid and homogeneous environment, the spectra are not well resolved as in the case of LTP. Furthermore, there is no way to resolve the (0,0) bands using RTP in the case of multi tryptophan proteins [36].

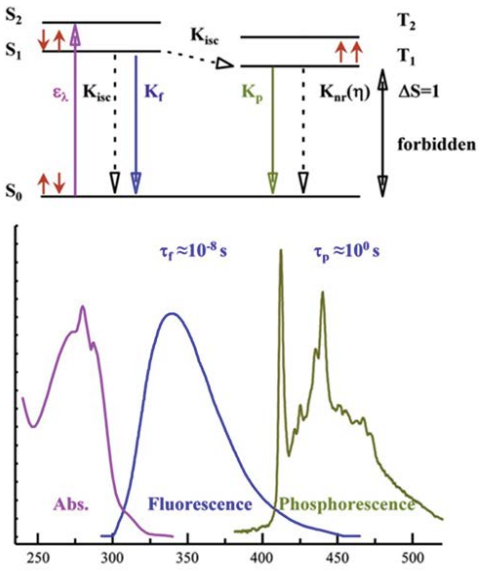

Figure 1: Jablonski energy diagram. (S_0: ground state, S_1: excited singlet, T_1: triplet state respectively. Small red arrows: spin multiplicity of the electronic state dashed black arrows: radiation less transitions) [4].

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Studies of Ltp of Multitryptophan Proteins

Case study I: Phosphorescence of proteins containing two Trp residues

Mutant Bovine Odorant-Binding Proteins

- **Function:** Vertebrate odorant-binding proteins have an important role in odor detection by means of receptor events. To do this work, they carry, deactivate and/or select odorant molecules [42-43].
- **Phosphorescence studies:** D'Auria et al. [31] showed that the (0-0) vibronic bands corresponding to two Trp residues are optically resolved and peak at 403.8 (one of the bluest till date) and 410.0 nm respectively. Analysis of solvent accessibility from crystallographic data for each Trp and its neighbouring residues reveals that Trp 133 is surrounded by highly charged residues and has higher solvent accessibility than Trp 17 which is surrounded by hydrophobic side chains. So, the observed (0,0) bands at 403.8 nm.and 410.3 nm can be duly assigned to Trp133 (solvent-exposed) and Trp17 (buried), respectively.

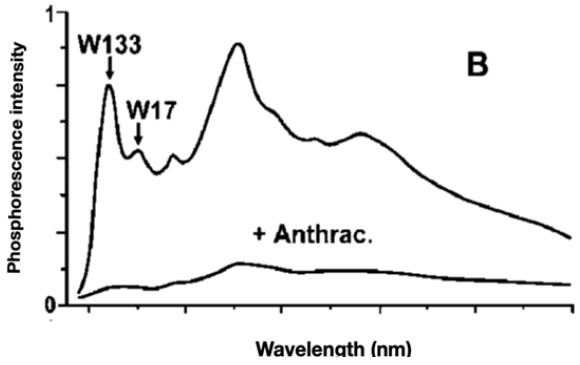

Figure 2: Phosphorescence spectra of bOBP in a glycerol/buffer (10 mM Tris-HCl, pH8) (77K). λ_{ex} = 290 nm [31].

Horse liver alcohol dehydrogenase (HLAD)

- **Function:** This zinc-dependent enzyme catalyzes the reversible oxidation of various alcohols to aldehydes and ketones and utilizes NAD+ as its coenzyme [44].
- **Phosphorescence studies:** Horse liver alcohol dehydrogenase (mol. wt. 80,000) is a dimeric enzyme containing Trp residues at positions 15 and 314 in each monomer [45]. Purkey and Galley [19] showed the (0,0) LTP band of HLAD is split into a distinct doublet with maxima at 405 and 410 nm. This indicates that the band at 405 nm corresponds to the Trp which is more exposed to the solvent and therefore has a greater solvent accessibility than the Trp having a band at 410 nm.

This is also substantiated by crystal structure data which throws additional insight into the micro-environment of the Trp residues. Trp 15 being solvent exposed can be assigned to the band at 405 nm and Trp 314 (ASA=0) being buried is responsible for the experimental peak at 410 nm. So, the bands obtained at 405 nm and 410 nm could be assigned without any site-directed mutagenesis.

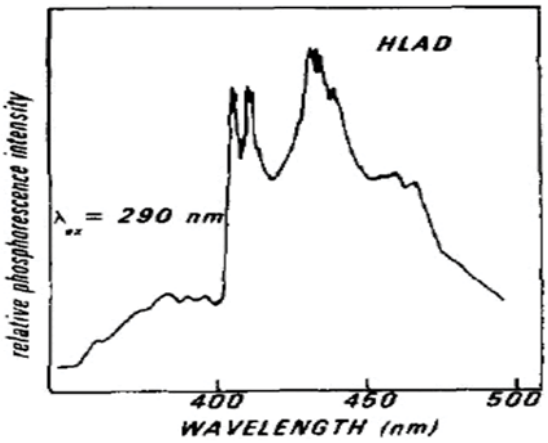

Figure 3: Phosphorescence spectra of 10^{-4} (M) HLAD in 1: 1 ethylene glycol-buffer glasses at 77°K [19].

Tryptophan repressor from E.coli (trpR)

- **Function**: From the study of the E. coli trp operon, it has been found that five polypeptides comprise three enzymes which mediate the biosynthesis of tryptophan from chorismite. It has established that the genes of trp operon are coordinately expressed under the control of the trpR [46]. It is a dimeric protein of identical 107-residue subunits. It is expressed from trpR gene. The trp repressor binds L-tryptophan [47], which is the product of the pathway. It forms a complex with trp operator (trpO) thereby reducing the rate of transcription of trp operon by 70-fold [48-49].
- **Phosphorescence studies:** trpR has two Trps at 19 and 99 positions and experimentally two (0-0) vibronic bands are observed at 407 nm and 415 nm.
- **Mutational studies:** In this case, however, site-directed mutagenesis is required in addition to LTP studies. Eftink et.al [21] explored the use of LTP of these tryptophan residues for wild-type (WT) and single mutants to probe the structure and dynamics of trpR with other photophysical studies. Fig 4 is the low-temperature (77K) phosphorescence spectrum of wildtype (WT), Trp 19 substituted with Phenylalanine (W19F) and Trp 99 substituted with Phenylalanine (W99F). The mutant form of the protein exhibits only one (0,0) vibronic band. Mutation studies show that in W99F mutant, Trp 19 contributes to this band at 407 nm and in W19F mutant, the (0,0) band is a contribution of Trp 99.
- **Analysis from crystal structure data:** Studies show that Trp l9 is located as part of the A α-helix that forms a hydrophobic core at the subunit interface of this protein. It also

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- highlights that Trp 99 is part of the F α-helix and is more solvent exposed. Therefore, the latter should be bluer shifted compared to Trp 19. However, the environment of Trp 99 stabilizes the exciting triplet state. This occurs due to the polarizability of groups around this indole ring. This can also be due to conformational changes in protein that occur upon freezing which occludes solvent molecules from the environment of Trp 99. As a result, a very red-shifted (0,0) band appears [47,49-51].

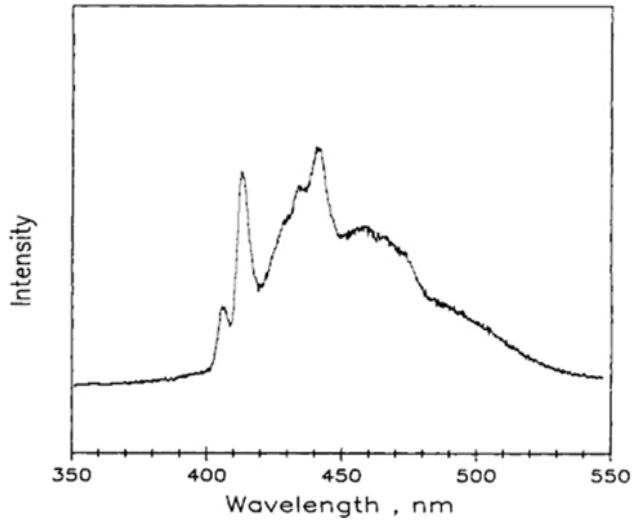

Figure 4: Phosphorescence spectra of trpR and its mutants WT [21].

Phosphoglycerate kinase from yeast

- **Function:** This enzyme catalyzes the seventh step of glycolysis. It converts 1,3-bisphosphoglycerate to 3-phosphoglycerate with the concomitant generation of ATP [52].
- **Phosphorescence studies:** this enzyme from yeast has Trps positioned at 308 and 333 and the LTP is optically resolved with two (0-0) vibronic bands located at 408 and 412.5 nm. From the crystal structure data, it is known that Trp residues are present in the C-domain but in different micro-environments. Trp 308 resides in mobile region whereas the other Trp 333 is surrounded by the α-helical rods and remains buried. Therefore, the blue component at 408 nm can only be due to Trp-308 which is surface accessible and exhibits greater mobility as it is present in the unstructured region of the molecule, the band at 412.5 nm band is due to solvent inaccessible highly immobile Trp 308 [53].

Allergen Ra5 of the pollen of Ambrosia elatior

- **Function:** Allergen Ra5, also known as Amb a 5, is a major allergen found in the pollen of short ragweed (Ambrosia elatior). On inhalation of the pollen, an allergic response is triggered in susceptible individuals [54].

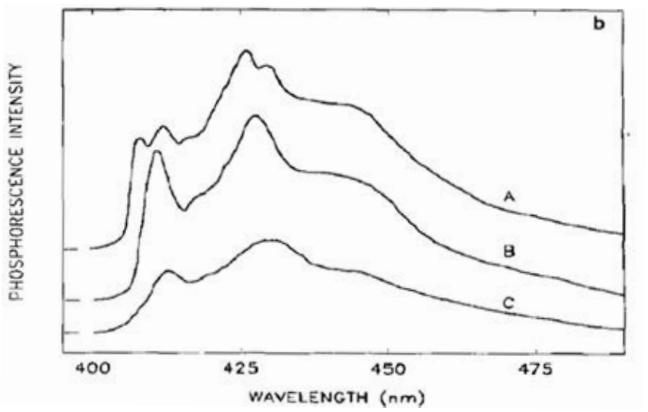

Figure 5: Phosphorescence spectrum of PGK at different temperatures and excitation wavelength. (a) PGK at 160K; (b)PGK (λex = 295nm) at 220K (A) and 274K(C) [53].

- **Phosphorescence studies:** The LTP spectra of Allergen Ra5 of the pollen of Ambrosia elatior shows two optically resolved (0-0) band at 403 and 409.5 nm. This indicates that the micro-environment of both Trp residues is distinct and they can be independently studied. The peak at 403 nm denotes an extremely blue shifted solvent exposed Trp residue. The band at 409 nm falls at the interface of exposed and buried residues.

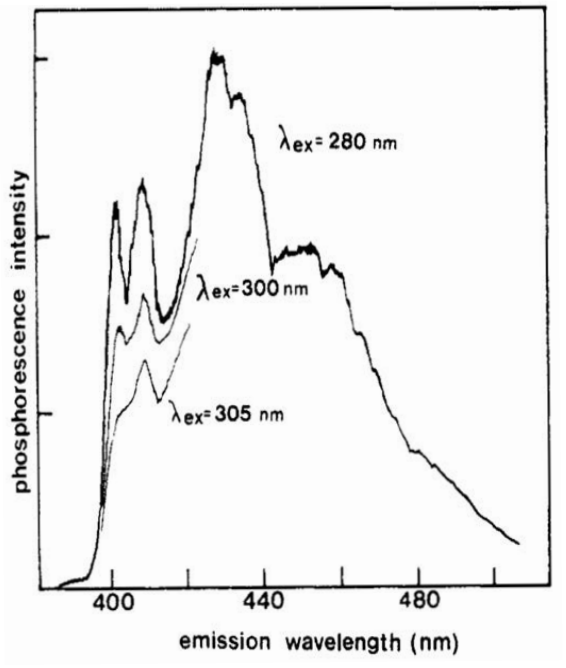

Figure 6: Phosphorescence spectrum of Ra5 at -165°C. [55].

The blue-shift is due to the large solvent exposure of the Trp residue which is further stabilized by the strong polar interaction with adjoining charged residues. The location of a acidic amino acids like aspartic acid or glutamic acid side chain with respect to the aromatic ring of Trp could be one of the reasons for the anomalous phosphorescence spectrum [55].

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Phosphorescence of proteins containing two Trp residues but with single (0,0) bands

1. Bovine serum albumin (BSA)

- **Function:** Albumin helps in the transport of other proteins in the body. The Trps are in different domains and therefore experience differing environments as outlined in the next section. Optical resolution, based on LTP studies has not been observed for these Trps and a single (0-0) band at 412.6nm has been observed. Possibly, Forster energy transfer occurs between two Trps.
- **Phosphorescence studies:** Despite having two tryptophan residues, the (0-0) band at

412.6 nm is observed [58]. Hence, the two tryptophan moieties are not optically resolved and are possibly located at energy transfer distances. Crystal structure analysis indicates the differential environment of the Trps:

(i) Trp 134 experiences a relatively more hydrophobic neighborhood.

(ii) Trp 213 is partially buried with sufficient lability.

However, the corresponding oleic acid complex of the protein (figure 7) splits the single peak into two well resolved (0,0) bands, corroborating points (i) and (ii) mentioned above.

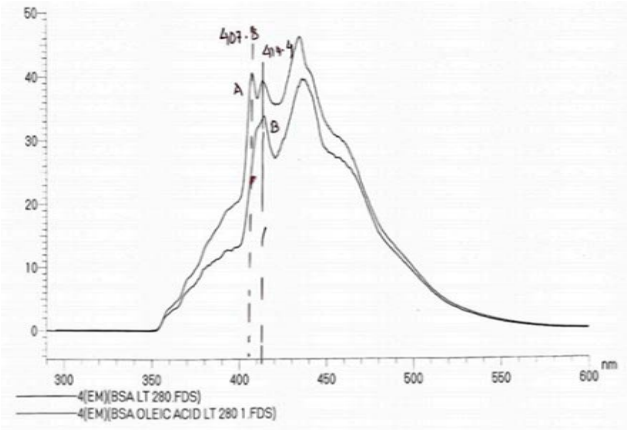

Figure 7: Phosphorescence spectra of BSA with and without oleic acid [59].

Phosphoprotein (P protein) of Chandipura virus (CHPV)

- **Function:** The primary function of this negative-stranded RNA virus has been identified to be involved in the processes of transcription and replication. Crystal structure data has been analyzed for this virus [60-62].
- **Phosphorescence studies:** Characterization of the two Trps has been carried out in both the native and mutant forms of the virus. [63]. The primary focus of the study was on the interaction of the protein with other nucleocapsid protein. ASA calculations also contributed to the knowledge of protein structure.

LTP spectra of wild-type, Trp 105 mutated to Phe and Trp 135 mutated to Phe conclusively showed that the band at 412.6 nm is a contribution from Trp 105 while the Trp 135 appears at 414.8 nm like wild-type. Difference in the band widths at half maxima [20] for all the (0,0) bands in Fig. 8 nullifies the possibility of overlapping of the (0-0) bands of both Trps [63].

The (0,0) band of WT can be assigned to Trp 135. Trp 105 is much more blue-shifted since it has higher ASA due to the presence of neighbouring polar residues.

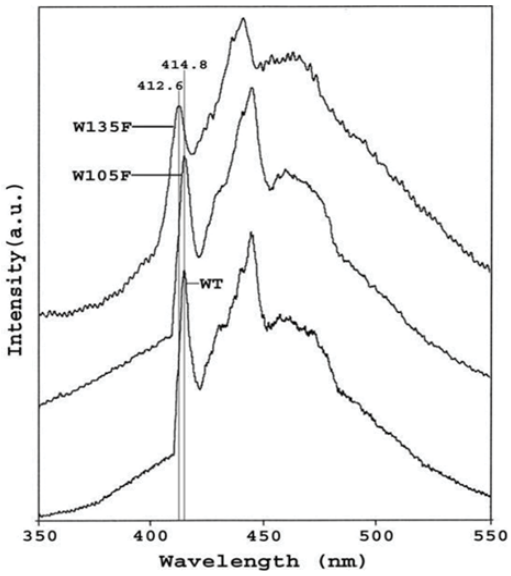

Figure 8: Phosphorescence spectra of WT, W135F and W105F in TET buffer þ 40% EG matrix at 77 K, exc 295 nm, Excitation bandpass = 10 nm and emission bandpass =1.5nm. Concentration of wild-type protein and both the mutants = 750 nM [63].

Case study II: Phosphorescence of proteins containing three Trp residues

Glyceraldehyde-3-phosphate dehydrogenase of rabbit, pig and yeast (GAPD)

- **Function:** GAPD is a glycolytic enzyme which catalyzes Step 6 of Glycolysis thereby converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate using NAD+ as coenzyme [64-66].
- **Phosphorescence studies:** GAPD has three Trp residues. The phosphorescence spectra of GAPD from rabbit, pig, and yeast are compared in Fig.9A [67]. GAPD from pig and rabbit muscle shows two well-resolved (0,0) bands at 406 and 415 nm while GAPD from yeast exhibits well-resolved (0,0) bands corresponding to all the three Trps (409nm, 415nm, 420nm). As has been discussed previously, the (0-0) band positions in LTP spectra throw

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light on the immediate neighbourhood of the Trps, and this is very well-exemplified in the case of rabbit muscle GAPD, where two overlapping (0,0) bands at 406.4 and 415.4 nm have been observed. Corroborating with ASA values, Trps 310 and 84 are buried (ASA 0.0) with the corresponding band at 415.4 nm in contrast to Trp 193 (ASA 64.0) which has more solvent accessibility [68]. The response of the LTP spectrum to the quencher acrylamide is rather informative – the peak at 415.4 nm corresponding to Trp 310 is relatively more quenched and therefore accessed by the quencher (λexc at 280 nm) as per Figure 9B, Table 1.

Table 1: Phosphorescence Data at 77K in 40% Ethylene Glycol Matrix.

Systems	(0,0) band position(nm)*	Width of the (0,0) band phosphorescence at half maxima (cm^{-1})**	Assignment of (0,0) band	Ratio (Iw193/ Iw310)
Free GAPD	406.4	247	Trp193	1.09a
				1.06b
	415.4	240	Trp310	0.91c
GAPD +Acrylamide	406.4	245	Trp193	1.57a
				1.53b
	415.4	240	Trp310	1.45c

* Error - ± 0.2 nm.

**Error - ± 5 cm.

-1‡ Intensity ratio of the (0,0) bands at 406.4 nm and 415.4nm.

a λexc=280nm,b λexc=295nm, c λexc=305nm.

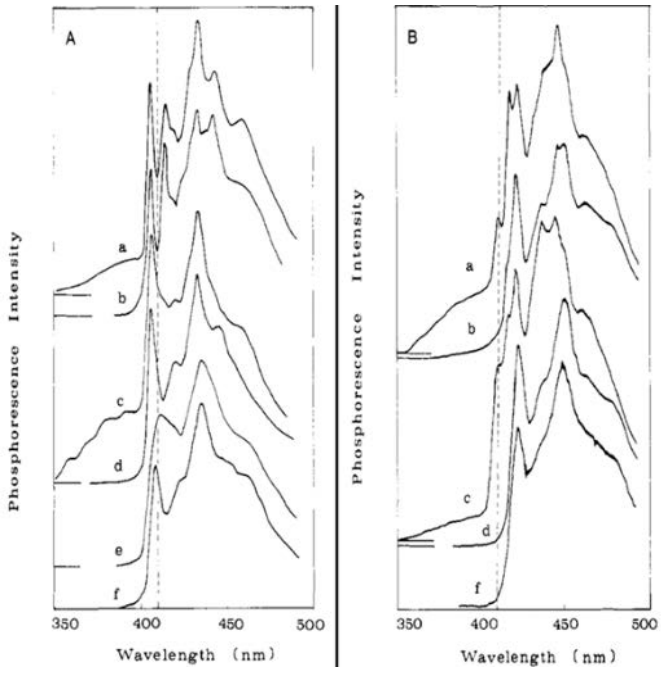

Figure 9A: Phosphorescence spectra of (A) GAPDHs from pig and rabbit muscle and (B) GAPDH from yeast [67].

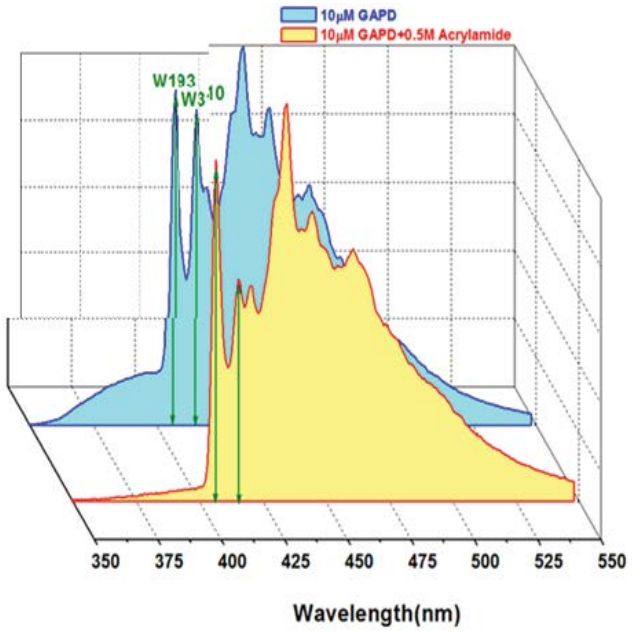

Figure 9B: Phosphorescence spectra of 10μM GAPD and it's complex with 0.5 M acrylamide in aq buffer + 40% EG matrix at 77K, λexc = 280 nm [68].

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- **Molecular docking studies**: These studies were initiated to throw light on the binding characteristics of GAPD with acrylamide. It was found that the latter could access the interior of the protein with binding affinity -3.3 kcal/mole (theoretical). Docking studies also reveal the immediate surroundings (within 5Å) and stabilization of ligand acrylamide and its favourable interaction with Trp 310 [68].

Lysozyme from bacteriophage T4 (T4 lysozyme)

- **Function:** T4 lysozyme plays an important role in the lytic cycle of bacteriophage T4. It is an enzyme that catalyses the hydrolysis of the β-1,4 glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of bacterial cell walls [69].
- **Phosphorescence studies:** The correspondence between the no. of Trps (three) and triplet (0,0) bands is broken with only two peaks at 408.0 and 413.8 nm showing up. Hence site-directed mutagenesis studies had to be evoked [23].

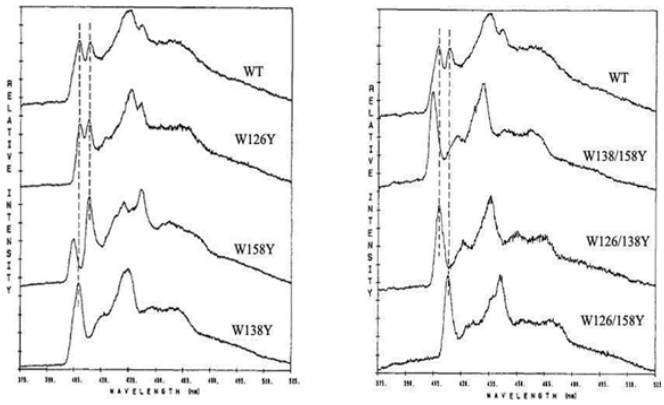

Figure 10: Phosphorescence spectra of WT T4 Lysozyme and its single and double mutants [7].

- **Mutagenic studies**: Comparison with the phosphorescence spectra of double mutants containing a single Trp residue demonstrate that these (0-0) band originate from Trp 158 (W126/138Y) and Trp 138 (W126/158Y). Among the three Trp residues, the phosphorescence of Trp 138 is the most red-shifted while the (0-0) band of Trp 126 is the most blue-shifted. Trp 126 does not contribute strongly to LTP spectra. Studies on single mutants (W126Y, W138Y, W158Y) containing two Trps by Ghosh et.al. [7] showed that LTP of Trp 126 is quenched due to selective energy transfer between Trp 126 and Trp 158 residues selectively. The solvent exposed nature of the Trp residue 126, the partial solvent exposure of 158, the buried nature of Trp 138 and the selective ET between Trp 126 and Trp 158 are consistent with the inter Trp distances, orientation factor and neighbouring residues as obtained from the crystal structure data [7].

Alkaline Phosphatase (AP) from Escherichia Coli

- **Function:** Alkaline phosphatase is a metalloenzyme which catalyzes the hydrolysis of phosphate groups from a wide variety of phosphate monoesters. It is a homodimer with an appropriate mass of 94 KDa [70].
- **Phosphorescence studies:** Each subunit of AP has three tryptophan (Trp) residues at positions 109, 220, and 268. An exceptional case of AP is that phosphorescence is observed at room temperature also [71] with a lifetime of about 2 sec. This is the longest as far as reports highlight [72]. At 77K, AP exhibits a band near 350 nm which is a contribution from Tyr. Reasonably resolved (0,0) bands are obtained at 411.4 and 414.5 nm, respectively [10].

The contribution from Trp 220 is identified in that mutant of Alkaline phosphatase where Trp at position 220 is mutated to Tyr (W220Y). LTP spectra of Tb-AP (where Mg2+ and Zn2+ ions are replaced by Tb3+) clearly indicates that the (0,0) band at 414.5 nm originates from Trp 109. The line width and the position of the (0,0) band clearly suggested a rigid and almost quasi-crystalline hydrophobic environment for Trp 109. This also suggested that Trp 109 is the origin of RTP of AP with longest lifetime [10]. From the crystal structure of AP, a disulphide bond between Cys 286 and Cys 336 lies within a proximity of 6Å to Trp 268 [73], which results in efficient quenching of the latter's triplet state. This is the reason behind the LTP quenching of Trp 268.

Recombinant human erythropoietin

- **Function:** Recombinant human erythropoietin (rHuEPO) is used for treating certain diseases related to kidneys and certain cancers as well [74].
- **Phosphorescence studies:** rHuEPO has Trps at 51, 64, and 88 and contributes well to the phosphorescence of

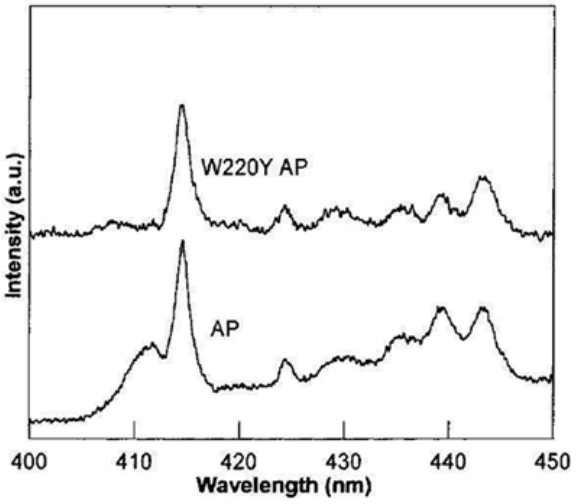

Figure 11: High-resolution phosphorescence of AP (2.5X 10 M), W220Y (3.0 X10 M) in 30% EG-buffer at 1.2 K. λex=280 nm, 0.5 nm emission bandwidth [10].

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rHuEPO and hence with peaks at 406.4 and 410.4 nm. The LTP of wild type rHuEPO are compared with single mutants obtained by Trp substitution by Phe at positions 51, 64 and 88 (W51F, W64F and W88F) [75]. This is done to assign the (0-0) band to a particular Trp residue.

In cases of W51F and W64F, the LTP spectrum was characterized by a doublet, at 406.2 and 410.5 nm, respectively.

Since the mutant W88F does not yield any characteristic band, it can be concluded that Trp 88 is non-emmisive in both rHuEPO and mutant proteins and therefore the bands at 406.2 and 410.5 nm can be ascribed to Trp 64 and Trp 51 respectively.

- **Analysis of band positions of Trp 88 and Trp 64:**

  - Trp 88 is located at a convenient ET distance of 0.42 nm from the disulphide linkage in C29-C33 and as a result, the triplet state is deactivated quite efficiently.
  - The dipole-dipole interaction between Trp 64 and Arg 110 stabilizes the former. Also, attractive contributions from other distant amino acids like Glu 62, Arg 103, Glu 117 lying parallel to the charge transfer axis of Trp 64 also leads to further stabilization of the triplet state of the latter, leading to observed blue shift.
  - Trp 51, however, has no such stabilizing interaction as it is buried within the protein core. However, other interactive sources have been identified – (i) H-bonding interactions between H2O and Pro 60 (ii) H-bonding interactions between N-H group of Trp 51 and water molecule [76].

Case study III: Phosphorescence of proteins containing four Trp residues

MPT63

- Function: MPT63 is a secreted protein of Mycobacterium tuberculosis. It acts as a significant drug target [77].

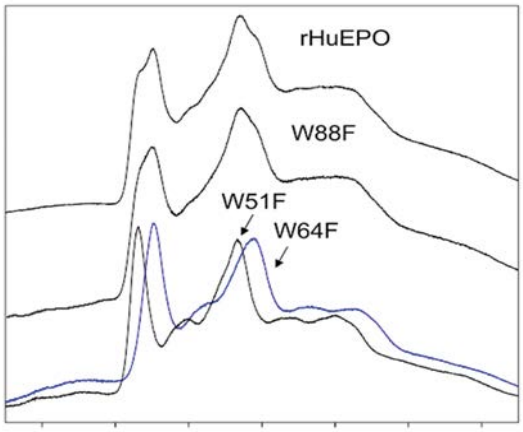

Figure 12: Comparison of phosphorescence spectra of recombinant human erythropoietin (rHuEPO) and analogs [76].

- **Phosphorescence studies**: Phosphorescence studies show a correspondence between the number of Trps and the number of experimental (0-0) vibronic bands (407, 412.2, 417, 421.6 nm)

Assignment of four well-resolved (0-0) bands to four Trp residues become possible with the help of:

(ι) calculation of ASA of different Trp residues,

(ιι) investigation of immediate environment of Trp residues,

(ιιι) comparison of LTP of WT MPT63 and W26F mutants.

Table 2: The table for calculation of ASA has been shown below:

	WT		W26F	
Trp residue	ASA(Å²)	% accessibility	ASA(Å²)	% accessibility
W26	4.3	1.7		
W48	31	12.4	31	12.4
W82	149.8	60.2	137.9	55.4
W129	0	0	0	0

- Trp 82 is rather solvent-exposed with a 60.2% accessibility and hence should correspond to the most blue-shifted band at 407nm, followed by Trp 48 at 412.2nm (12.4% accessible).
- On the other hand, as indicated by ASA calculations (Table 2) both Trp 26 and Trp 129 are immobile and buried and could correspond to the red-shifted bands at 417.4 and 421.6 nm but precise assignment is rather difficult [78].
- **Mutational analysis**: The phosphorescence of W26F mutant was studied and it has been observed that the first three bands are present at the same position. The absence of band at 421.6 nm indicates that the band at 417.4 nm is a contribution from the Trp 129 and the band at 421.6 nm is a contribution from Trp 26.

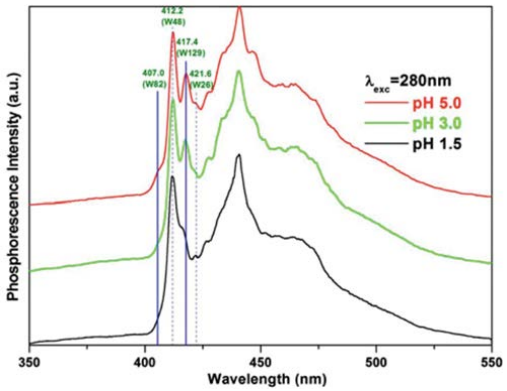

Figure 13: Phosphorescence spectra of MPT 63 in 40% EG-buffer matrix at 77K, [78].

Citation: Priyanka Mukherjee, Sanjukta Chatterjee, Sourav Banerjee, Sudeshna Shyam Chowdhury, Rina Ghosh. Insights on structural, environmental and dynamical Behaviour of multi- tryptophan proteins by analysis of their Low Temperature Phosphorescence spectra: A review. Journal of Bioinformatics and Systems Biology. 9 (2026): 28-42.

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Esterase from Pseudoalteromonas haloplanktis

- **Function:** Pseudoalteromonas haloplanktis produces 4-hydroxybenzoic acid (4-HBA), and it inhibits the proliferation of A549 lung epithelial cancer cells with an IC50 value ≤ 1 μg ml−1 [79].
- **Phosphorescence studies:** This multi-tryptophan enzyme (molar mass 60KDa) possesses four residues at positions 14, 150, 181, 197. In contrast, two peaks centered at 407.5 nm and 411.7 nm are obtained, corresponding therefore to two different and well-distinguished micro-environments. The red-shifted narrow band peaks at 411.7 nm are suggestive of a single residue entrenched in a compact hydrophobic site. The blue-shifted band at 407.5 nm results from greater solvent accessibility of the corresponding Trps. To identify the Trp positions and the corresponding bands, crystal structural data gives a detailed insight, pointing to the following facts:

(i) Trp 14, following the central β-sheet of each monomer, is highly solvated, with accessibility around 46% (subunits A and B).

(ii) Trp 50 is buried and rendered almost immobile by certain hydrophobic side chains.

(iii) Trp 181 is also relatively buried with a solvent exposure of 7%.

(iv) Trp 197, is at an energy transfer distance of Trp 181 (16Å separation) and has a fair amount of accessibility of 29.5%.

Thus, the unique environment of Trp 50 renders it a candidate for the red-shifted band at 411.7 nm [80], as assigned by D.Auria et al. The broad, blue-shifted shoulder is therefore a contribution from all the solvent-exposed Trps, viz. 14,181 and 197 of varying degrees of accessibility.

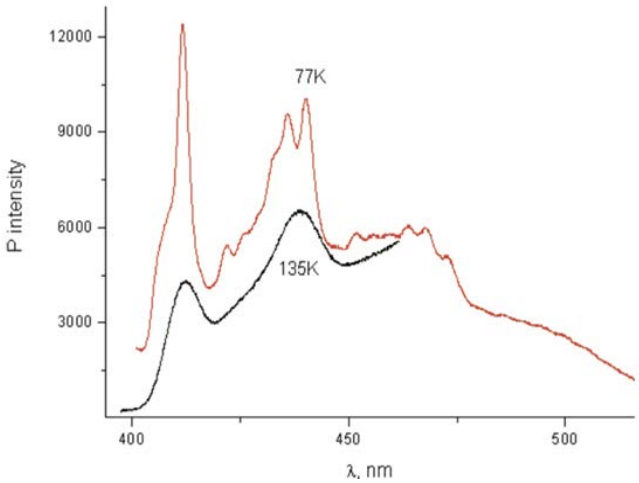

Figure 14: Trp phosphorescence spectrum of PhEST in glycerol/ buffer with λex= 290 nm [80].

F-Actin from Rabbit Muscle

- **Function:** Actin is a protein, which in conjugation with myosin, is essential for muscle contraction and other cellular processes [81].
- **Phosphorescence studies:** Strambini et al. [82] characterized the LTP of Globular action and Filamentous action. They have four Trp residues namely Trp 79, Trp 86, Trp 340 and Trp 356. At low temperature they exhibit (0-0) vibrational bands centered at 405 nm and 415.5 nm. However, under high resolution, energetically distinct (0-0) vibrational bands centered at 404.5 nm and a broad and composite red band, with peaks at 414.1 and 416.7 nm were revealed. Trp 79 and Trp 356 are largely exposed to the solvent while Trp 86 and Trp 340 are more buried and Trp 356 is shielded from the solvent. So Trp 79 is responsible for LTP at 404.5nm and triplet emissions at 414.1 and 416.7 nm are generated from Trp 356 and Trp 340. Trp 86 emission is found to be severely quenched by Cy's residues (4 Å distance) and energy transfer may take place from Trp 86 to Trp 79 due to their proximity [83]. The fluorescence data for single and triple Triple Trp mutants by Doyle et al [84] is well-corroborated with phosphorescence studies and structural data.

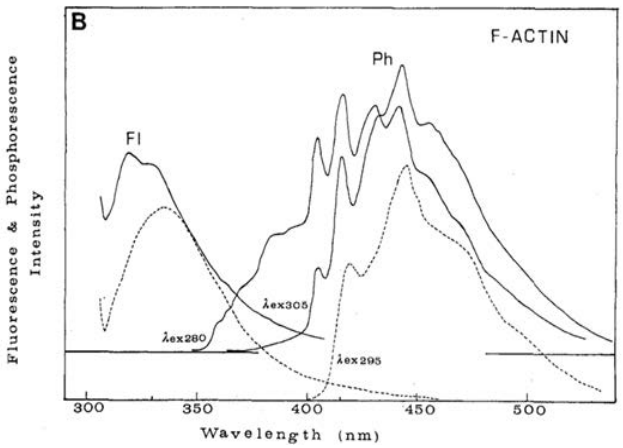

Figure 15: Phosphorescence spectra of F-actin in glycerol/buffer at 140 K and in buffer at 275 K with ex = 280 nm, 295 nm and305 nm [82].

Case study IV: Phosphorescence of proteins containing five Trp residues

Aspartate aminotransferase from E. coli.

- **Function:** This enzyme catalyses the transamination reaction i.e., the transfer of an amino group from glutamate to oxaloacetate yielding a keto-acid and a new amino acid α-ketoglutarate and Aspartate [85].
- **Phosphorescence studies:** Aspartate aminotransferase is a dimeric enzyme. Each monomeric subunit has a large and a small domain. The active sites are present in between the two monomers. It has five Trp residues at the

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position 134, 140, 205, 217, 319 in each subunit. Cioni et al. [86] characterized the LTP properties of the five Trp residues/monomers of the enzyme from E.coli.

- **Modified phosphorescence spectra in case of different prosthetic groups:** The LTP spectra of Aspartate-aminotransferase is characterized by three distinct (0-0) vibrational bands centered at 408, 415 and 417 nm when it uses Pyrido- xamine-5-phosphate as its prosthetic group. When Aspartate aminotransferase uses pyridoxal-5-phosphate as its prosthetic group, the triplet emissions are strongly quenched, and a single (0-0) vibrational band is observed at 415 nm.

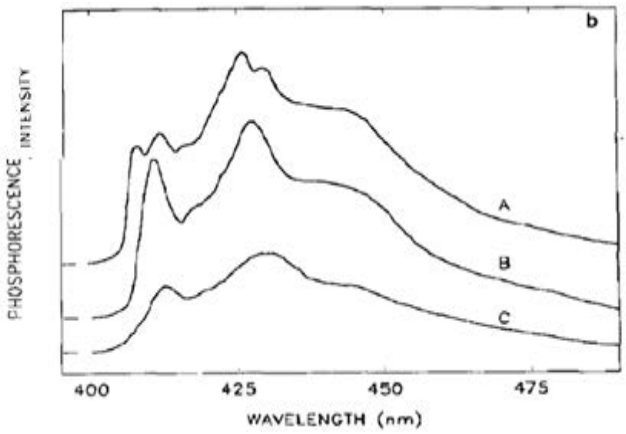

Figure 16: Phosphorescence spectra of apo-A Atase and pyridoxamine-5P-AATase(A) and pyridoxal-5P-AATase(B) in propylene glycol [87].

Here, the chemical environment is much more homogeneous compared to case (i). Further, an anomalous shoulder in the spectrum of Trp is evident around 470 - 480 nm. This can be due to Trp-sensitized delayed fluorescence of bound pyridoxal phosphate.

D-galactose/D-glucose-binding protein from E. coli.

- **Function:** The D-galactose/D-glucose-binding protein (GGBP) from E. coli. serves as a component for chemotaxis towards both galactose and glucose. It helps in active transport of sugars [88].
- **Phosphorescence studies:** The enzyme contains five Trp residues at positions 127, 133, 183, 195, 284. GGBP exhibits an LTP spectra having two energetically distinct (0-0) vibrational bands at 404.4nm and 409.6 nm. The former is less intense than the latter, which is indicative of the fact that more Trp residues i.e., presumably three (Trps 127, 133, 195) out of five contribute to this red band.
- **Analysis from crystallographic structure:**
  - The crystallographic structural data indicates electrostatic interaction between the indole ring and the surrounding side chains and hence it is possible to assign Trp 183 and Trp 284 to the blue band at 404.4 nm and the remaining Trp 127, Trp 133, and Trp 195 to the red band at 409.6 nm.
  - Of the above three Trps, the pair Ttp 127-Trp 133 are in proximity with a distance between the two rings as 6.79Å and contribute to the red-shifted band.
  - On examination of the micro-environment of the above pair, it was inferred that their phosphorescence in fluid solutions is bound to be heavily quenched. Trp 133 is partially solvent-exposed. It is in contact with His 126 and Phe 233. The distances between the Trp 133 ring and the His 126 and Phe 233 rings of 7.13 and 6.92 Å, respectively. Trp 127 is more protected from the solvent and both singlet-singlet and triplet-triplet energy transfer processes with Trp 133 makes the pair behave as single chromophore.
  - Out of the Trps at positions 183 and 284, the latter is superficial and very well solvent-exposed and therefore the emission has been found to be very weak, and the lifetime is short in fluid solutions. Trp 183 is internal, but it is in Vander-Waals contact with His-152. The latter is on a mobile site and is an effective quencher [89].

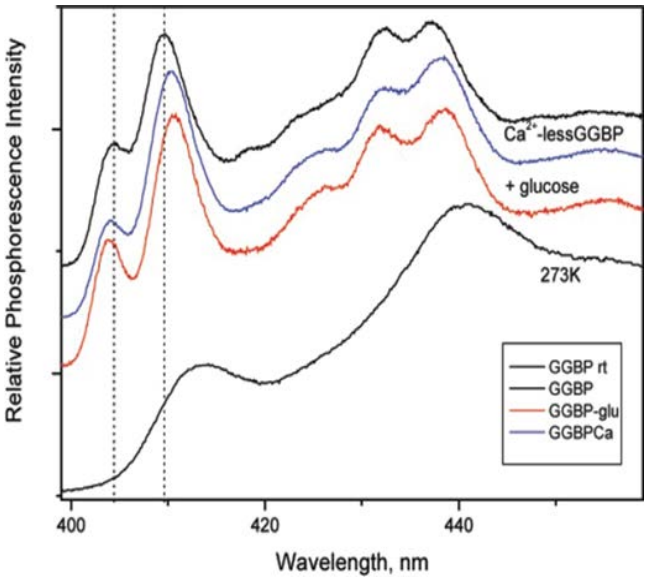

Figure 17: Trp Phosphorescence spectrum of GGBP in a glycerol / buffer (60/40, w/w) glass, at 77 K, and in buffer, at 273 K. The effect of glucose binding and of Ca2+ removal, on the low-temperature spectrum, are also indicated [89].

Case study V: Phosphorescence of proteins containing six Trp residues

Human placental ribonuclease inhibitor (hRI)

- **Function:** The ribonuclease inhibitor is present in the cytosol of mammalian cells and functions as a pancreatic-type ribonuclease inhibitor [90].

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- **Phosphorescence studies:** In poly-tryptophan protein, a Trp cluster involving those at 261, 263, 318 and 375 positions are present with 19 and 431 being separated from the cluster [20]. The LTP spectra (Fig 18C) detected the presence of two (0-0) bands at the 409.0 and 415.2 nm for free hRI and at 409.8 and 416 for the bound form of the inhibitor.

Based on calculation of inter-Trp distances, orientation factors and ASA values, the allocation of Trp residues to the phosphorescence bands are summarized as follows:

(i) Trp 19 has the lowest ASA (7.92 Å2) and hence most buried compared to the others

a. the band at 415.2 nm can be ascribed to this Trp.

(ii) Trp 375 is exposed (ASA – 86.28 Å2) along with other Trps in the cluster (261, 263, 318) and hence contributes significantly to the band at 409.0 nm.

These results have been also corroborated by other methods and support the previous work of Shapiro et al [92].

Case study VI: Phosphorescence of proteins containing eight Trp residues

Tubulin

- **Function:** Tubulin, a heterodimeric (αβ) protein, the main constituent of microtubules [93].
- **Phosphorescence studies:** It contains eight Trp residues at 21, 346, 388, 407 in the A subunit and at positions 21, 103, 346, and 407 in the B subunit respectively. Though Tubulin contains eight Trp residues, a single (0-0) band at 407.6 nm appears in the phosphorescence spectra indicating that the emitting Trps should have polar environments with high solvent exposure.

Comparison of quantum yield values of free Trp (0.14) and eight Trp containing Tubulin (0.06) suggests that the emission of the free protein is a contribution from at least three or four Trp residues which are not appreciably quenched by interaction with the surrounding residues.

- **Analysis from crystallographic data:** Calculations carried out by Sardar et al [94] revealed that significant energy transfer is possible between the pairs Trp B103-Trp B407 and Trp A407-Trp B346. The ASA values of Trp A346 and Trp B407 indicate that they are solvent exposed. Similarly, the ASA values suggest that Trp A388 is in a buried environment. Trp A407 is more or less solvent exposed. The ASA values of Trp B103 are also indicative of a buried residue. Based on the calculated ASA values, the environment of the Trps are summarized as:

i. A388 and B103: buried

ii. A407: rather solvent-exposed

iii. A346 and B407: solvent exposed

The candidates contributing to phosphorescence emission of WT Tubulin are therefore A346, A407, B407, B21.

Conclusion

From a study of all documented proteins, we see that the resolved (0,0) bands in LTP could occur between 403nm and 421.8 nm (the bluest shifted at 403nm for OBP and the reddest shifted at 421.8 nm for. MPT63 so far). This comprehensive review helps us to understand how LTP spectroscopy could be correlated with the environment of Trp residues in proteins and how such studies could be correlated with the microenvironment of a particular Trp residue. We have tried to analyse and highlight cases where there is non-correspondence between the actual no of Trp residues and the no. of resolved (0,0) vibronic bands. The reason for quenching of particular Trp residues has been sought from crystal structure analysis and explained wherever applicable. Over and above the crystal structure predictions and analysis, we have penned cases where site-directed mutant varieties (of the corresponding wild-type protein) can throw additional insights into the microenvironment of a particular Trp, its energy transfer efficiency and binding characteristics.

Credit Authorship Contribution Statement

Priyanka Mukherjee: Review of literature, draft preparation, framing the write-up

Sanjukta Chatterjee: Draft preparation, compilation of used figures and identification of sources

Sourav Banerjee: Draft preparation, compilation of used figures and identification of Sources

Sudeshna Shyam Chowdhury: Editing

Rina Ghosh: Supervision, Conceptualization, Framing the write-up, review and editing

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