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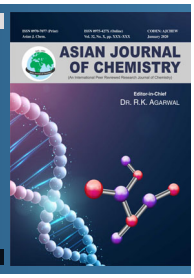

REVIEW

Study of Organization and Dynamics of Multi-Tryptophan Protein Molecules Utilizing Red Edge Excitation Shift Approach

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A shift in the fluorescence emission maxima with gradual increase in excitation wavelength is termed as red edge excitation shift (REES). Tryptophan residues are widely utilized as intrinsic fluorescence probe to investigate the protein structures. Wavelength selective tryptophan fluorescence can explore the dynamics of surrounded water molecules, the ubiquitous biological solvent. Thus REES experiment of various protein conformational states can provide significant input to the study of protein folding pathway and it can also be useful to study interaction of proteins with others. In this review article, we shall focus on red edge effect of various multi-tryptophan proteins in their respective native, intermediate and denatured state.

Keywords: Fluorescence, Multi-tryptophan protein, Red edge excitation shift, Red edge effect, Protein structure.

INTRODUCTION

Proteins, the ubiquitous biological macromolecules, are workhorse of the living cell. They regulate numerous biological processes *in vivo* by acting as enzymes, antibodies, hormones, neurotransmitter, nutrient storage and many more. Polypeptide chains, build up from naturally occurring amino acids, fold into unique native structure of protein, in post translational period. Protein molecules are often organized in highly ordered conformation in the crowded milieu of the cell. These conformations show characteristic dynamic properties which enable the interaction of protein molecule with its counter parts and owes significantly to protein function [1]. Thus the interplay between structure and dynamics of protein molecules is crucial for its function. Crystallization and thereafter X-ray crystallographic diffraction analysis of many soluble proteins provides detailed and precise information about their structure [2]. But all these information about protein structure obtained from X-ray crystallography, are necessarily static in nature and can't give insight into the dynamic properties of protein molecules which is intricately related with its function [3]. Again in case of membrane proteins, we have lesser crystallographic information since crystallization of the membrane proteins are extremely challenging [4]. Further, apart from its native form protein molecules can also exist in different conformations like molten globule, denatured or misfolded states depending on environmental demand. It is not often possible to characterize these states by crystallographic technique because of the transient nature of these non native states. In this context, spectroscopic techniques are very much appreciated because they can provide subtle information about the structure as well as organization and dynamics of protein molecules.

Fluoroscence spectroscopy is a widely used technique for analysis of protein structure, dynamics and function because of its intrinsic sensitivity, suitable time scale, non-invasive nature and minimum perturbation [5]. Tryptophan, the essential amino acid, serves as the intrinsic fluorescence probe of protein molecules. Tryptophan fluorescence is widely used spectroscopic technique for extracting information about protein structure in solution. A shift in the emission maximum of fluorescence spectra towards higher wavelength due to shift of the excitation wavelength towards the red edge of the absorption spectrum, is termed as red edge excitation shift (REES) [5]. The study of REES is a novel approach of fluorescence spectroscopy to

monitor the immediate environment of the fluorophore and dynamics of the surrounded solvent layer [6,7]. This review is aimed to discuss the theoretical background of REES for tryptophan in short and mainly to archive some recent applications of REES studies to elucidate organization and dynamics of multi-tryptophan soluble proteins.

Tryptophan fluorescence and protein structure: Intrinsic fluorescence of proteins arises due to presence of three amino acid residues having aromatic side chain namely tryptophan, tyrosine and phenylalanine (Fig. 1). Table-1 represents absorbance and fluorescence parameters of the three aromatic amino acids [8,9]. Fluorescence from phenylalanine is very weak due to low molar extinction coefficient and small fluorescence quantum yield, and hence is not generally used experimentally [5]. Though tryptophan has greater absorption coefficient than tyrosine, both have almost same fluorescence quantum yield (Table-1). Tyrosine fluorescence is less affected by polarity of the immediate environment surrounding the fluorophore whereas the emission of tryptophan is highly sensitive to its local environment. If all three amino acids are present in a protein, it is difficult to observe tyrosine fluorescence because of energy transfer to tryptophan. Tyrosine fluorescence is also quenched by its interaction with the peptide chain [5,10]. Among these three, tryptophan fluorescence is most widely used for fluorescence analysis of proteins. In tryptophan, the indole side chain is responsible for the characteristic UV absorption and fluorescence emission. Tryptophan is present at 1 mol % of protein. This low content of tryptophan in protein is advantageous because interpretation of spectral data becomes less complicated due to absence of inter tryptophan interaction [5].

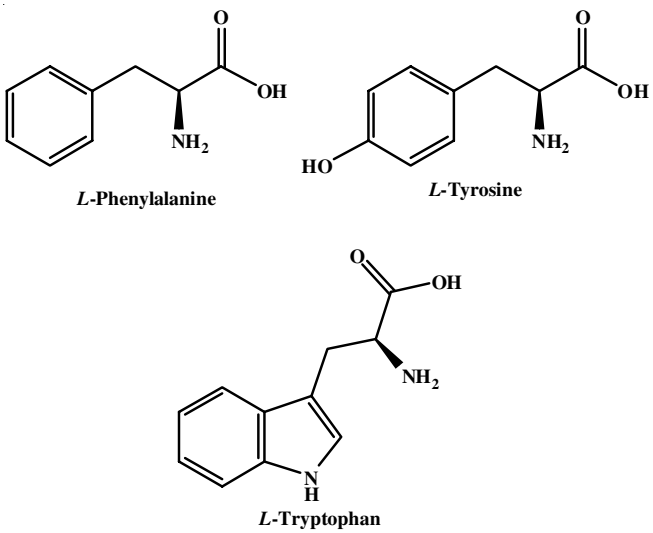

Fig. 1. Naturally occurring amino acids with aromatic side chain

TABLE-1
ABSORPTION AND EMISSION DETAILS OF FREE AROMATIC AMINO ACIDS AT pH 7.0

Amino acid	Absorption maxima (nm)	Extinction coefficient (M^{-1} cm^{-1})	Emission maxima (nm)	Quantum yield
Phenylalanine	257	195	282	0.02
Tyrosine	275	1405	303	0.13
Tryptophan	279	5579	353	0.12

Fluorescence emission typically occurs at longer wavelength than the absorption wavelength. This phenomenon, first observed by Sir G.G. Stokes in 1852 at University of Cambridge, is known as Stokes shift. One common cause of the Stokes shift is the rapid non-radiative decay to the lowest vibrational level of 1st electronic excited state (S_1), followed by fluorescence emission and the molecule returns to the ground state (S_0). In addition, fluorophores can display further Stokes shifts due to solvent effects, excited-state reactions, complex formation, and/or energy transfer [5]. These Stokes shifts, which are most dramatic for polar fluorophores in polar solvents are due to interactions between the fluorophore and its immediate environment. The indole group of tryptophan residues in proteins is one such solvent-sensitive fluorophore. The indole group has two aromatic rings in fused condition. Tryptophan has two overlapping $S_o \rightarrow S_1$ transition, denoted as ^1L_a and ^1L_b in Fig. 2 [5,11]. ^1L_a transition has higher dipole moment because it is directed through the -NH group of the indole ring. In polar solvent, energy of ^1L_a is lowered due to dipole-dipole interaction with solvent molecules and it becomes the lowest energy state [12]. Therefore, the emission spectra of indole can reveal the location of tryptophan residues in proteins. The emission from an exposed surface residue, which can interact with polar solvent molecules, will occur at longer wavelengths than that from a tryptophan residue buried in the protein's interior [13]. Absorption maximum of free tryptophan is at 279 nm and emission occurs at 353 nm in water (Table-1). But in proteins, intrinsic emission maximum varies from ~310 nm to ~350 nm (Table-2) depending on the environment of the tryptophan in the protein structure [13-17]. For example azurine, a small copper containing protein, shows lowest fluorescence emission

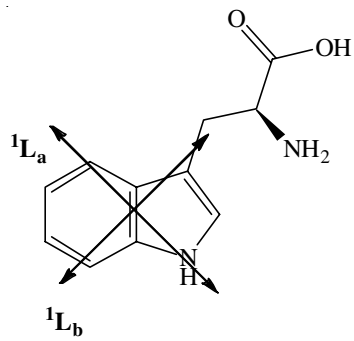

Fig. 2. Two overlapping $S_o \rightarrow S_1$ transition of tryptophan, denoted as ^1L_a and ^1L_b. In polar solvent, energy of ^1L_a is lowered than that of ^1L_b

TABLE-2
TRYPTOPHAN EMISSION MAXIMA OF FEW PROTEINS

Protein	Emission maxima (nm)
Azurin	308
Asparaginase, *E. coli*	319
Ribonuclease T1	322
Staphylococcal nuclease	330
Human serum albumin	330
Bovine spleen galectin-1	340
Monellin	342
Thioredoxin	345
Adrenocorticotropine hormone	352
Glucagon	352

maxima at 308 nm, whereas glucagon and adrenocorticotropine hormone exhibit most red shifted fluorescence spectra with emission maxima of 352 nm similar to free tryptophan. In azurine, the sole tryptophan residue located in a hydrophobic pocket making the microenvironment extremely non-polar as the case of 3-methylindole in cyclohexane and in glucagon or adrenocorticotropine hormone the environment surrounding the tryptophan residue is exactly opposite [10,14,18].

Theoretical background on red edge effect: Fluorescence emission spectrum is generally independent of the excitation wavelength, which is known as Kasha's rule [8,19]. When the fluorophore is excited to higher electronic and vibrational level, the excess energy is quickly dissipated leaving the fluorophore in the lowest vibrational level of S_1. Because of this rapid relaxation same fluorescence emission spectrum is generally observed irrespective of the excitation wavelength [20]. But when polar fluorophore is surrounded by solvent of restricted mobility then Kasha's rule is not obeyed [21]. Under such conditions, when the excitation wavelength is gradually shifted to the red edge of the absorption band, the maximum of fluorescence emission shows a concomitant shift toward higher wavelengths [22-24]. Such a shift in the wavelength of maximum emission toward higher wavelengths, caused by a corresponding shift in the excitation wavelength toward the red edge of the absorption band, is termed the red edge excitation shift (REES). Upon excitation of the polar fluorophore, if the dipole moment increases appreciably then the polar solvent molecules reorient themselves accordingly around the excited state fluorophore. But the scenario is changed when the solvent is viscous in nature. Now the solvent molecules have mobility restriction and can not rearrange themselves in consistent with the changed dipole within the life time of the excited state. In case of the ground as well as the excited states of a polar fluorophore, there exists a statistical distribution depending on the interactions with the immediate environment. Excitation with higher energy light results in excitation of the entire ensemble of the chromophores and no site selection occurs. But if excited with sufficiently lower energy light (*i.e.* at longer wavelength), instead of excitation of whole population, there occurs selective excitation of those fluorophores, which can interact with the environment in the excited state much stronger than the average and have minimum interaction in the ground state. This type of photoselection results in the origin of REES [25,26]. Overall the essential criteria to observe REES [12,27,28] are as follows: (i) the fluorophore should be polar, (ii) solvent molecules surrounding the fluorophore should be polar; (iii) solvent relaxation time around the excited state fluorophore should be longer or comparable than the fluorescence lifetime, and (iv) there should be a large change of dipole moment of the fluorophore when it is excited. Tryptophan, the intrinsic fluorophore of protein molecules, is an eligible candidate in this category since it is polar and shows a change of 6D diplole moment upon excitation [29]. So tryptophan may show significant red edge effects in suitable solvent environment as mentioned above. Thus, REES study of intrinsic tryptophan fluorescence of protein molecules provides reliable information about the rigidity or flexibility of tryptophan environment.

History of wavelength selective tryptophan fluorescence studies in proteins: Red edge effect was first studied in solid chromophore solutions in 1970 [30,31]. Later Demchenko and Ladokhin [7] have investigated the excitation wavelength dependence of indole and tryptophan fluorescence emission maxima in glycerol and glucose glasses and observed a significant red shift of the emission maxima for excitation in the range from 290 to 310 nm. In viscous medium, the magnitude of this effect is shown to be directly related to the dipolar relaxation of solvent molecules in the environment of the excited fluorophore. Human serum albumin (HSA) was the first protein for which REES was observed. It is a single tryptophan protein and the tryptophan is located in the ligand binding pocket of the protein. The native protein shows the fluorescence emission maxima at 340 nm and shows a small REES of 2 nm only when the excitation wavelength is shifted from 295 to 305 nm [32]. At acidic pH on isomerization when the ligand binding pocket is closed, the emission maximum is blue shifted indicating more hydrophobic nature of the surroundings. At this condition, the fluorescence emission maxima is red shifted by 11 nm with concomitant red edge excitation (295-305 nm). From these results, it was realized that the REES effect is very sensitive to tryptophan environment. Following this, studies with a variety of single tryptophan proteins established the fact that REES is observed only for those proteins which shows fluorescence emission maxima within the range 325-340 nm when excited at absorption maximum [28,33,34]. Peptides and denatured proteins having emission maximum above 340 nm does not exhibit REES because the tryptophan residue is exposed to rapidly relaxing solvent [35]. Again REES was not observed for myelin basic protein and β-casein, which have emission maximum beyond 341 nm characteristics of solvent exposed tryptophans [12]. On other hand, proteins (such as azurin, parvalbumin and ribonucleases T1 and C2) having emission maximum between 307-323 nm also do not exhibit REES [12,14]. In these cases, tryptophan environment is completely non polar and lack of polar surrounding molecules resulted no REES. Another interesting result was observed for the single tryptophan protein melittin. The monomeric form of melittin is a flexible chain where the sole tryptophan residue is exposed to solvent and does not show any REES. But melittin tetramer has ordered structure and shows REES [36].

Application of REES in study of organization and dynamics of multi-tryptophan proteins: For multi-tryptophan proteins different tryptophan residues generally experience dissimilar environment. Some of the tryptophans are in solvent exposed position and others are in protein interior. These differently positioned tryptophans have different photophysical properties, fluorescence spectra [37] and emission decays [38]. But the indole fluorophore is highly sensitive to excited state distribution on interaction with the environment but has very low sensitivity to ground state distribution. The fluorescence spectra of differently located tryptophans vary within a broad range but the variation in absorption spectra is within 3 nm [39]. For this unique property of indole, REES for tryptophan residues in multi-tryptophan proteins is not influenced by the photoselection of the differently positioned tryptophans in the

ground state rather the red-edge effect reflect mainly the site distributions in the excited state. REES serves as useful approach to probe the mobility parameters of the environment (the otherwise "optically silent" water molecules) and the dynamic properties of the protein matrix surrounding the tryptophans. Usually for mutitryptophan proteins REES is observed at the same level of single tryptophan protein [34].

Crystallins are the major constituent protein of eye lens. The eye lens in its normal state is transparent and highly refractive in nature, which mainly depends on the protein crystallins [40]. Three types of crystallin proteins are present in the eye lens *viz*. α-crystallin, β-crystallin and γ- or δ-crystallin. Change in physico-chemical properties of crystallines would affect lens function and lead to cataract. In an interesting study, red edge effect has been used to relate the properties of the lens with those of its constituent proteins, their homo- and hetero-aggregates [41]. Different REES values were observed for isolated crystallines from eye lens of different species. The REES of α-crystallin from bovine lens was 6 nm, while it was 11 nm for the same protein from the chick lens, which suggests different extents of hydration, differing subunit arrangements and different tryptophan environments in the two molecules. The REES values for α-crystallin were 9 and 6 nm for bovine and chicken eye source respectively. Other crystallins [γ- for bovine and δ- for chicken] exhibited smaller REES value of 3 nm and 5 nm respectively, probably because of monomeric forms of these crystallins differing from the aggregated state of the α/β-type. Another interesting point mentioned in that study REES value for intact chick lens (9 nm) differed from α-crystallin in solution (11 nm) due to distinct packing of the crystallins. Again 1:1 mixture of α and β-crystallins showed similar REES value (9 nm) with weight average value (7.5 nm) discarding any inter-crystallin interaction in the dilute solution.

Tubulin is a heterodimeric protein composed of two closely related 55 kDa proteins called α and β tubulin and there exists eight tryptophan residues per dimer [42]. Tubulin shows REES of 7 nm when the excitation wavelength is increased from 280 to 305 nm [35]. Though the tryptophan(s) responsible for the observed REES effect could not be localized, this result indicated that the average tryptophan environment in tubulin is motionally restricted. A REES of 7 nm was also observed in the case of tubulin complexed with colchicines, an antimitotic drug. Interestingly, such REES was not observed when tubulin is complexed with structural analogues of colchicines [35]. Analyzing the structure of the two additives along with the opposite outcome REES measurement, proposed model of the tubulin-drug complex could be rationalized.

α-Toxin, a soluble hemolytic protein secreted by *S. aureus*, is heptamer in its water soluble native form. It has eight tryptophan residues per monomer with molecular mass 33,400 per monomer [43]. The emission maximum of native toxin was observed at 333 nm. Red edge experiment with the native α-toxin showed paltry 2 nm red shift of emission maximum. *N*-Bromosuccinimide (NBS) can selectively oxidize the solvent exposed tryptophans [44]. Fluorescence intensity of α-toxin was lost by 80% following the NBS oxidation and the NBS modified toxin exhibited emission maxima of 328 nm. The modified toxin showed significant increase in the REES value (~6 nm). The REES observed in modified toxin was attributed to deeply buried tryptophans located in very hydrophobic environment since the solvent exposed tryptophans were eliminated [45].

Wavelength selective fluorescence approach was also successfully applied in the large dimeric protein, spectrin. Spectrin shows a REES of 4 nm in the native state [46]. This indicates that tryptophans in spectrin are localized in a microenvironment of restricted mobility. The study with spectrin was more significant for the denatured protein. Generally, denatured proteins do not exhibit red edge effect since the tryptophans are fully exposed to the solvent and consequently motional restriction of solvent molecules surrounding the fluorophore is absent. But surprisingly, spectrin shows a REES of 3 nm even when denatured in 8 M urea. This was the first report of a denatured protein displaying REES. Observation of REES in the denatured state implies that some of the structural and dynamic features of this microenvironment around the spectrin tryptophans are retained even when the protein is denatured [46,47]. Thus wavelength selective fluorescence approach excelled over the other techniques like circular dichroism (CD) spectroscopy, normal fluorescence measurement which can not reveal the residual structure in denatured state. Variation in ionic strength of the medium changed the conformation of spectrin dimer. At physiological ionic strength spectrin dimer is a flexible rod but lower ionic strength results in elongation and stiffening of spectrin. In another study wave length selective fluorescence was used to assess the influence of different ionic strength on the environment and dynamics of tryptophan residues of spectrin [48]. The results shows that microenvironment of spectrin tryptophan remains unaltered in low and high ionic strength condition and give insight into molecular details underlying the conformational flexibility of spectrin.

'Molten globule' state of the protein has special significance in field of protein structure and folding as a key intermediate in the folding pathway [49]. Chattopadhyay *et al*. [50,51] pioneered one of the early REES study upon molten globule state of proteins. They reported red edge effect on molten globule state of bovine α-lactalbumin (BLA) generated at acidic condition [50] and apo-state after removal of Ca^{2+} [51] Acid-induced molten globule state of BLA showed 3 nm REES whereas apo-BLA fluorescence red shifted by 8 nm when excitation wavelength increased from 280 to 307 nm. At similar red edge excitation, native BLA associated with 7 nm REES and urea denatured BLA with 3 nm REES. The findings indicated that the tryptophan residues of BLA experience motionally restricted environment in both native and molten globule states. Presence of REES in denatured state confirmed presence of some residual structure around tryptophan residues even in the denatured protein.

Lectins represent an important and diverse protein family, which are recognized by its specific carbohydrate binding capacity [52]. First member of this family which was studied for red edge effects was the lentil lectin, *Lens culinaris* agglutinin (LCA). It is a dimer with each monomer having two chains (α and β) and five tryptophan residues [53]. Emission maximum

of tryptophan fluorescence for LCA is at 330 nm and independent of excitation wavelength (295, 300 and 305 nm). Absence of REES implies that the microenvironment of the tryptophan residues is not rigid [54].

Lectin for which first significant amount of REES was observed is the GalNAc/Gal specific legume lectin, soybean agglutinin (SBA). SBA exists as tetramer in native state and it has six tryptophan residues per monomer. Wavelength selective fluorescence studies with various states of SBA in the folding/unfolding pathway exhibit interesting results [55]. SBA tetramer shows a REES of 10 nm whereas the monomer shows a REES of 5 nm only. This happened because two tryptophans (Trp8 and Trp203), which were located at monomer-monomer interface were buried in tetramer but became solvent exposed in monomer. Unlike spectrin or α-lactalbumin, SBA showed no red edge effect in the denatured state. Since REES effect of multi-tryptophan proteins reveals average tryptophan environment, additional experiment can decide the subset of tryptophans contributing predominantly to the effect. In this study, selective oxidation of solvent accessible tryptophans by NBS did the trick. NBS oxidized two and four tryptophan residues of tetramer and monomer respectively and post oxidation both the tetrameric and monomeric form showed remarkable REES (21 nm and 19 nm, respectively). Based on these results, it was concluded that REES effect was mainly contributed by the buried tryptophans in multi-tryptophan proteins.

Again localization and environment of tryptophan residues in different structural states of the lectin Concanavalin A (ConA), (a glucose/mannose specific legume lectin) was depicted nicely by wavelength selective fluorescence approach [56]. ConA exists as a tetramer in native state and the folding/unfolding pathway of Con A has shown presence of dimer and monomer of the protein as folding intermediate [57]. Each ConA monomer contains four tryptophan residues and one each of Mn^{2+} and Ca^{2+} ion. The native tetramer, dimer and demetallised ConA exhibited a REES of 6-8 nm. Analogous REES effect confirmed about similar average tryptophan environment for these species and indicates that immediate vicinity of at least some of the tryptophans must be highly rigid. ConA monomer showed REES of only 3 nm indicating exposure of some of the buried tryptophans in the bulk of the solvent. As in the case of SBA, oxidized ConA monomer displayed significant increase in REES value to 11 nm. In another study, native pea lectin (PSL) dimer, specific for D-Man/D-Glc showed a REES of 6 nm [58]. In contrast no REES was observed for any of the species in unfolding equilibrium in GdnHCl. This result implied that all five tryptophan residues became solvent-exposed in these folding intermediates.

Conclusion

Red edge excitation shift (REES) effect are extensively studied for different structural forms of the multi-tryptophan proteins and provides key information about dynamics of the 'optically silent' surrounding solvent molecules *i.e.* water molecules in case of proteins. Knowledge of hydration dynamics in the molecular level will help in understanding of the large number of important cellular events including protein folding, lipid-protein interactions, and ion transport [59]. Other fluorescence techniques like fluorescence quenching, energy transfer and polarization measurements gives information about the fluorophore itself but REES provides insight into relative rates of solvent (water in biological system) relaxation dynamics and this makes REES so significant. In this review, REES effect is found to be very sensitive to various native protein structures as well as to a range of conformational change in a particular protein. Table-3 displays a summary of observed REES value of different multitryptophan proteins in different experimental conditions and in their different structural forms. Results indicate about different degree of structural rigidity of tryptophan environments and structural rigidity gradually looses with unfolding of protein structures. Interestingly, few denatured states still have some residual structure left in the strong denaturing condition and exhibited modest red edge effect [46,51]. These residual structures may have important clue to the protein folding. One constraint of the REES study is lack of information about specific tryptophan contribution in multi-tryptophan proteins. This challenge is somewhat overcame by chemical modification of selective tryptophans and it was found those

TABLE-3
REES VALUE OF VARIOUS PROTEINS IN NATIVE, INTERMEDIATE AND DENATURED STATE

Protein name	REES value of various conformational states (nm)		
	Native	Intermediate	Denatured
α-Crystallin (chicken)	11	–	–
α-Crystallin (bovine)	6	–	–
β-Crystallin (chicken)	6	–	–
β-Crystallin (bovine)	9	–	–
Tubulin	7	–	Nil
α-Toxin	2	–	–
Oxidized α-toxin	6	–	–
Spectrin	4	–	3
α-Lactalbumin	7	3 (acid-induced MG) 8 (apo MG)	3
Lentil lectin (LCA)	Nil	–	–
Soybean agglutinin (SBA)	10 (tetramer)	6 (monomer)	Nil
Oxidized SBA	21 (tetramer)	19 (monomer)	–
Concanavalin A (ConA)	6 (tetramer)	8 (dimer)	Nil
Oxidized ConA	–	3 (monomer), 11 (monomer)	–
Pea lectin (PSL)	6	–	Nil

tryptophans contribute more which located in relatively interior part of the protein structure [55,56]. Site specific incorporation of tryptophan analogues, like 7-azatrytophan, can serve this purpose satisfactorily. These analogues have distinct absorption and emission maximum than tryptophan and can be selectively excited in a pool of other tryptophans present in their native environment [60]. In a recent study, it is proposed that quantified REES data can potentially be used as a 'fingerprint' of the structure and stability of a protein. Quantified REES data can find immediate application in biopharmaceutical industries [61]. Overall this review studies have shown that REES can be an excellent tool to monitor the protein folding pathway and there lies provision for more promising applications.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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