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Detection of Tryptophan to Tryptophan Energy Transfer in Proteins

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Förster resonance energy transfer (FRET) studies usually involve observation of intensity or lifetime changes in the donor or acceptor molecule and usually these donor and acceptor molecules differ (heterotransfer). The use of polarization to monitor FRET is far less common, although it was one of the first methods utilized. In 1960, Weber demonstrated that homotransfer between tryptophan molecules contributes to depolarization. He also discovered that the efficiency of homotransfer becomes much less effective upon excitation near the red-edge of the absorption. This "red-edge effect" was shown to be a general phenomenon of homotransfer. We have utilized Weber's red-edge effect to study tryptophan homotransfer in proteins. Specifically, we determined the polarization of the tryptophan fluorescence upon excitation at 295 nm and 310 nm (near the red-edge). Rotational diffusion leads to depolarization of the emission excited at either 295 nm or 310 nm, but homotransfer only contributes to depolarization upon excitation at 295 nm. Hence, the 310/295 polarization ratio gives an indication of tryptophan to tryptophan energy transfer. In single tryptophan systems, the 310/295 ratios are generally below 2 whereas in multi-tryptophan systems, the 310/295 ratios can be greater than 3.

KEY WORDS: Förster resonance energy transfer (FRET); polarization; red-edge effect; tryptophan.

1. INTRODUCTION

Radiationless transfer of energy from an excited donor to an acceptor has been observed in a great number of chemical and biological systems. The underlying principles and basic theories have been available for many years, having originally been elucidated by Perrin (Perrin, 1932), Förster (Förster, 1946; Förster, 1948; Förster, 1960), and others. At the present time, the use of Förster resonance energy transfer (FRET)[5] in studies on conformations of biomolecules is well established (see, for example, Clegg, 1996; dos Remedios and Moens, 1999; Jameson *et al.*, 2003; Lakowicz, 1999; Selvin, 1995; Valeur, 2002; Van Der Meer *et al.*, 1991). Usually such studies involve observation of changes in the intensity or lifetime of the donor or acceptor molecule and usually these donor and acceptor molecules are different, i.e., the FRET process is heterotransfer. The use of polarization to monitor energy transfer is a far less common approach, although it was one of the first methods used to demonstrate the FRET phenomenon. Specifically, in 1924 Gaviola and Pringsheim (Gaviola and Pringsheim, 1924) observed decreases in the polarization of solutions of some fluorophores in glycerol as the fluorophore concentration increased. This decrease was due to homotransfer between fluorophore molecules at high concentration when the average intermolecular distance approached the critical transfer distance. Descriptive theories of this

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[5] Abbeviations: FRET, Förster resonance energy transfer.

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concentration-dependent depolarization were given by Vavilov (Vavilov, 1927) and Förster (Förster, 1948), and a quantitative treatment was given by Weber (Weber, 1954).

In 1960, Weber first demonstrated that nonradiative electronic energy transfer could occur between tyrosine and tryptophan molecules free in solution (at sufficiently high concentrations) as well as in proteins (Weber, 1960a, 1960b). Energy transfer from tyrosine to tryptophan leads to depolarization of the tryptophan emission, compared to emission from directly excited tryptophans, as a consequence of the angular dependence of electronic energy transfer function. Hence, determination of the polarization of the emission from tryptophan residues (appropriately isolated through optical filters) upon excitation at 270 nm, where both tyrosine and tryptophan absorb, and at 305 nm, where only tryptophan absorbs, can provide a qualitative demonstration of tyrosine to tryptophan energy transfer. Specifically, in the absence of such heterotransfer, the ratio of the polarizations observed upon 305 nm and 270 nm excitation (the 305/270 ratio) will be in the range of 1.4–1.5 while tyrosine to tryptophan energy transfer will increase this ratio due to the decrease in the 270 nm polarization (Weber, 1960b). This method was used in other protein systems, for example to follow the acid dissociation of lactate dehydrogenase (Anderson and Weber, 1966) and to study the binding of elongation factor Ts (which has no tryptophan residues but has five tyrosine residues) to the single tryptophan protein elongation factor Tu (Jameson *et al.*, 1987).

In his original observations, Weber (Weber, 1960a) also pointed out that self-transfer, that is, indole to indole or tryptophan to tryptophan, became much less effective upon excitation near the red-edge of the absorption. This "Weber red-edge effect" has been shown to be a general phenomenon characteristic of homotransfer (Weber and Shinitzky, 1970). Homotransfer and the failure of such transfer upon red-edge excitation was used, for example, to study intra-subunit proximity of fluorescein probes attached to specific positions on adjacent subunits in the dimeric protein L7/L12 (Hamman *et al.*, 1996). More recently, in a study of site-directed mutants of fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase, including both single and double tryptophan variants, it was noted that the tryptophan to tryptophan energy transfer could be detected between a specific pair of tryptophan residues by monitoring the polarization of the tryptophan emission upon excitation at two wavelengths, namely 295 nm and 310 nm (Helms *et al.*, 1998). At 295 nm, only tryptophan residues in a protein are effectively excited (i.e., tyrosine excitation is minimized), and the observed depolarization is due to two processes: rotational diffusion of the tryptophan moiety (due to a both "global" rotation of the protein and "local" mobility of the tryptophan) and energy transfer to another tryptophan moiety. Griep and McHenry (Griep and McHenry, 1990) also noted this fact in their study of DNA polymerase III. Excitation at 310 nm, however, greatly reduces the extent of tryptophan to tryptophan self-transfer due to the Weber red-edge effect so that, at this excitation wavelength, depolarization is primarily due to rotational diffusion processes. Hence, observation of the polarization ratio observed upon excitation at 310 nm and 295 nm (the 310/295 ratio) provides information on tryptophan–tryptophan transfer. For dilute tryptophan ($<10^{-5}$ *M*) in glycerol at low temperature, the average distance between tryptophan molecules is too great for energy transfer to occur, and rotational diffusion is effectively abolished due to the high solvent viscosity. In this case, the 310/295 ratio is near 1.7. In similar glycerol solutions of di-tryptophan and poly-tryptophan, however, the 310/295 ratio increases as tryptophan to tryptophan transfer preferentially decreases the polarization upon 295 nm excitation. Excitation polarization spectra for these three cases (tryptophan, di-tryptophan, and poly-tryptophan) were originally presented by Weber and Shinitsky (Weber and Shinitzky, 1970). To ascertain the generality of this observation, we re-investigated these systems as well as several single, double, and multi-tryptophan proteins.

2. MATERIALS AND METHODS

2.1. Materials

N-acetyl-tryptophanamide, di-tryptophan, poly-tryptophan, human and bovine serum albumin, liver alcohol dehydrogenase, and aldolase were from Sigma-Aldrich (St. Louis, MO, USA). The tryptophan peptides were dissolved in a small amount of dimethylformamide and then added to propylene glycol. The proteins were dissolved in 50 m*M* Tris-HCl, pH 7.4. All solutions were further diluted so that the optical density at 295 nm in a 1-cm path length cuvette was below 1.

2.2. Measurements

A short path length cuvette (3 mm) was used for the fluorescence measurements. Polarizations were

measured using an ISS PC1 photon-counting instrument (ISS, Inc., Champaign, IL, USA). The excitation monochromator was set to 295, 310, or 315 nm, and the samples were excited through a Corion bandpass filter centered at 300 nm with a width at half-maximum of 40 nm (sb-300-f064w). The emission was recorded through a pair of filters: a Schott KV374 cuton filter (which passes wavelengths beyond ~355 nm) and a Corning 7-60 bandpass filter (centered at 356 nm with a width at half-maximum of 60 nm). Both excitation and emission slits were set to 4 nm. The sample compartment was purged with a continuous flow of N_2 when low temperature was used. The polarization values were appropriately corrected for buffer contribution in each experiment; these corrections were typically minimal (<2%).

3. RESULTS AND DISCUSSION

Figure 1 illustrates the variation of polarization for *n*-acetyl tryptophanamide (NATA), di-tryptophan (Trp-Trp), and poly-tryptophan (Poly-Trp) excited at 295 nm and 310 nm as a function of temperature. As expected, the polarization decreases with increasing temperature due to the faster rotation of the molecules. One feature to note in Figs. 1(A) and 1(B) is the low polarization values for Poly-Trp. Because the molecular volume of Poly-Trp is much larger than that of NATA, and hence rotates more slowly, one might expect higher polarization values even allowing for rapid local mobility. Excitation of Poly-Trp at 295 nm, however, leads to depolarization due to FRET, but the extent of this homotransfer is diminished upon 310 nm excitation. The 310/295 ratio for Poly-Trp is thus, as expected, higher than that for Trp-Trp, which in turn is higher than for NATA [Fig. 1(C)]. Figure 1(C) also indicates that there is no significant dependence of the polarization ratio with temperature, which demonstrates that the process being monitored is not related to the mobility of the fluorophore.

When 315 nm is used for excitation instead of 310 nm, the polarization ratio for Poly-Trp and Trp-Trp compared to NATA increases (data not shown). We note that one must exercise extreme care in the measurement of the polarization upon 310 nm excitation—and even more so at 315 nm—where the absorption and hence emission is minimal, to ensure that the emission filter totally blocks the exciting light and that contributions to the signal due to water Raman scattering (at 347 nm) or parasitic light (Rayleigh ghosts) are negligible. All of these potential artifacts will result in higher polarization values and hence higher 310/295 or 315/295 ratios [for a discussion of polarization artifacts, see Jameson and Croney (2003)]. One effective way to check for such artifacts is to observe the signal level when the emission filter is interposed in the exciting light path (Weber, 1956); if the resulting signal is non-negligible, then scattered or parasitic light may be passing through the emission filter.

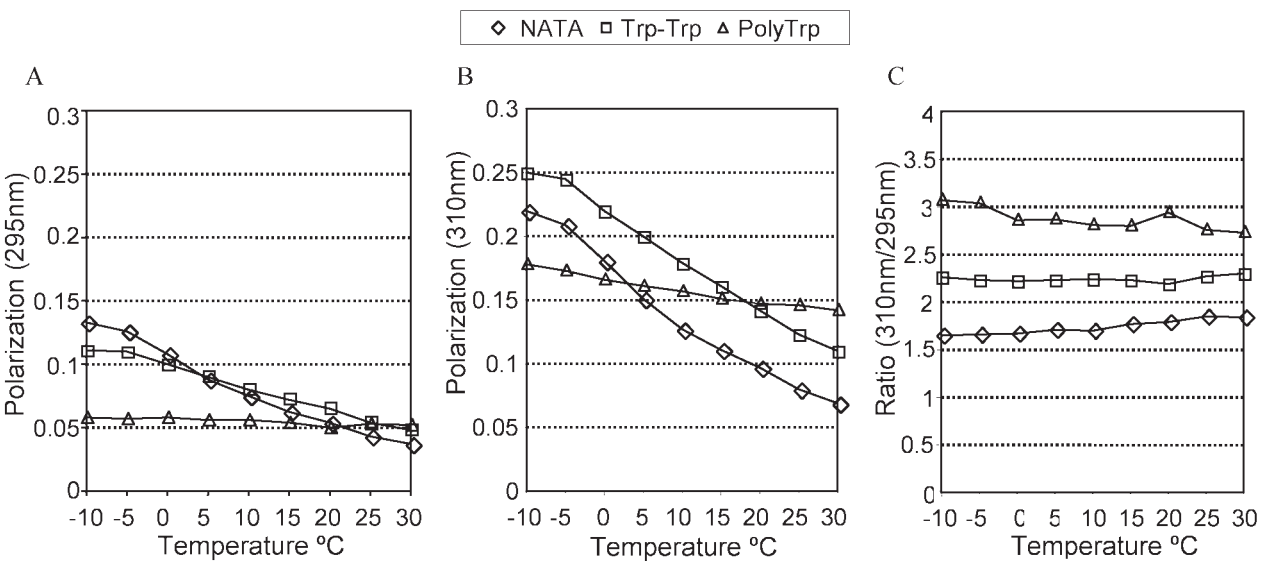

Fig. 1. Polarization and polarization ratio of tryptophan peptides as a function of temperature. (A, B) Temperature dependence of the polarization when *N*-acetyl tryptophanamide (NATA), di-tryptophan (Trp-Trp), and poly-tryptophan (Poly-Trp) are excited at 295 nm and 310 nm. (C) Polarization ratio (310/295) for NATA, Trp-Trp, and Poly-Trp as a function of temperature.

The 310/295 ratios of several proteins at 20°C are presented in Fig. 2. One notes that the single tryptophan proteins yield 310/295 ratios below 2. The tryptophan mutant W299/320 of rat testis fructose 6-phosphate 2-kinase:fructose 2,6 bisphosphatase, a two-tryptophan protein, demonstrates a 310/295 ratio significantly greater than 2 (data from Helms *et al.*, 1998). The crystal structure of this protein showed that the two mutated residues are in fact around 8 Å apart—within the R_0 value (the distance corresponding to 50% energy transfer) for Trp-Trp homotransfer, which is around 6–12Å (Van Der Meer *et al.*, 1991). Horse liver alcohol dehydrogenase (LADH) is a dimer of identical subunits with two tryptophans per subunit. The Trp15 residues are on the surface whereas the Trp314 residues are buried near the intersubunit contact region (Ramaswamy *et al.*, 1997). As illustrated in Fig. 3, the Trp15 residues are too far from each other or from the Trp314 residues for FRET to occur (>34 Å) as there is no significant energy transfer for distances greater than $2 \times R_0$ (Lakowicz, 1999; Valeur, 2002; Van Der Meer *et al.*, 1991). The two Trp314 residues, however, are less than 6 Å apart, close enough to undergo FRET, but this process is not evident due to the contribution of the Trp15 residues to the overall fluorescence of the protein dimer. Eftink and Jameson (Eftink and Jameson, 1982) demonstrated that acrylamide can selectively quench the fluorescence of the surface Trp15 residues thus allowing the fluorescence polarization ratio of the buried Trp314 residues to be evaluated. Indeed, when acrylamide is added to the solution (Fig. 4), the 310/295 ratio increases. This increase is more dramatic for the 315/295 ratio. These results indicate that tryptophan homotransfer occurs between residues 314 of the LADH

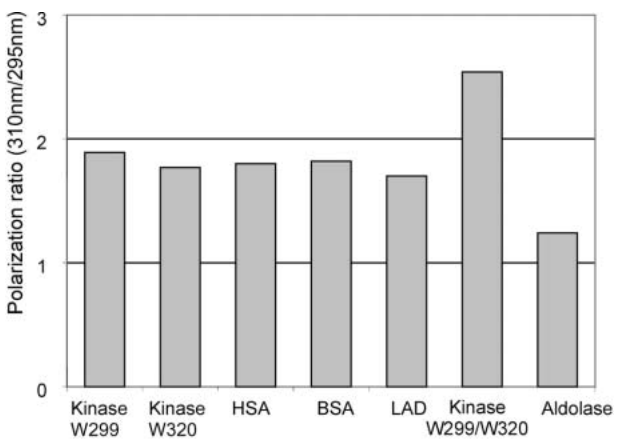

Fig. 2. Polarization ratios of various proteins at 20C. Kinase W299, W320, and W299/320. Tryptophan mutants of rat testis fructose 6-phosphate 2-kinase:fructose 2,6 bisphosphatase (data from Helms *et al*., 1998); HSA, human serum albumin; BSA, bovine serum albumin; LADH, liver alcohol dehydrogenase. The kinases are dimers with two identical subunits. Kinase W299 and kinase W320 contain one tryptophan per subunit whereas kinase W299/320 contains two tryptophans per subunit. HSA and BSA contain one and two tryptophans, respectively. LADH is a dimer of identical subunits with two tryptophans per subunit. Aldolase has three tryptophans.

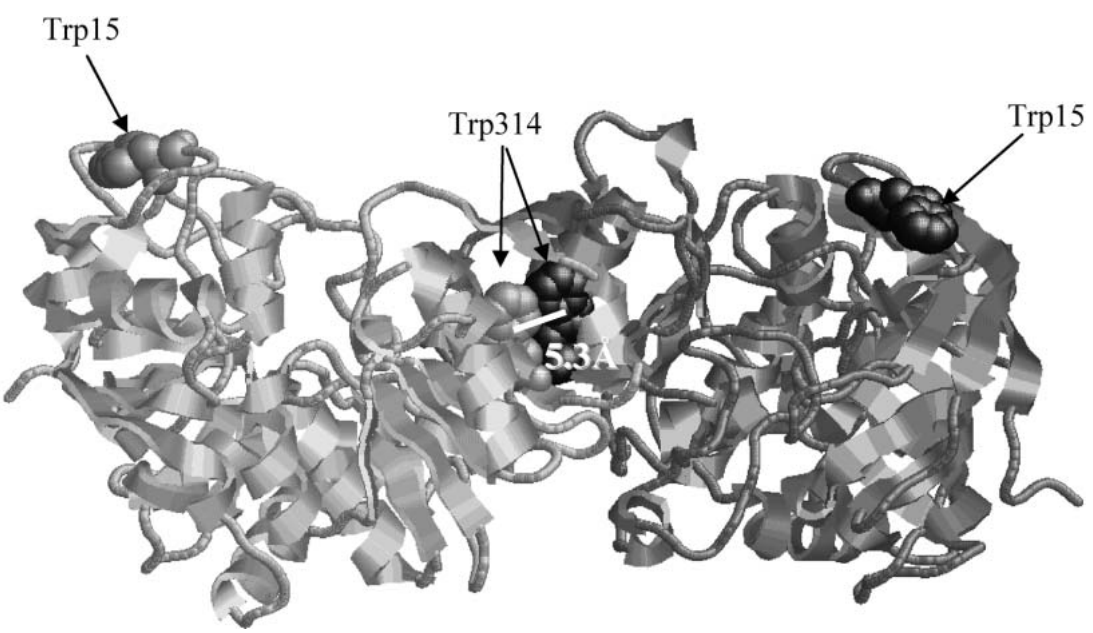

Fig. 3. Ribbon representation of liver alcohol dehydrogenase (PDB: 1LDY). LADH is a dimer composed of two identical subunits. The tryptophan residues (two per subunit) are represented as space filled objects (original structure from Ramaswamy *et al*., 1997).

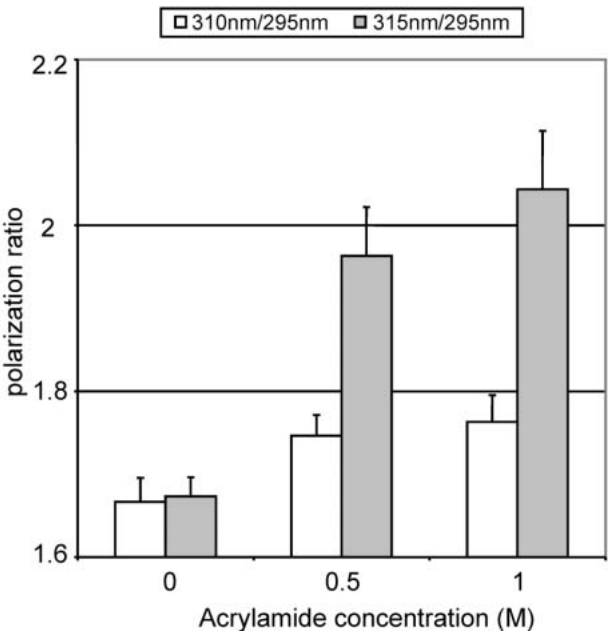

Fig. 4. Effect of acrylamide quenching on the polarization ratio of LADH. The 310/295 and the 315/295 ratio are represented for different acrylamide concentrations. The error bars are the standard deviations ($N = 3$).

dimer. In principle, precise characterization of the depolarization due to self-energy transfer between tryptophan residues can be used to determine the distance between these residues. Reliable distance determinations, however, require knowledge of the orientation of the emitting and absorbing dipoles, and the usual caveats apply. The lack of transfer in the other two proteins, BSA and aldolase, suggests that 1) the Trp residues in these proteins are separated by distances that do not result in significant energy transfer (i.e., in aldolase the three tryptophan residues are 22 Å, 28 Å, and 36 Å from other tryptophan residues (Dalby *et al.*, 1999) or 2) the relevant dipoles are not appropriately aligned. In many cases, however, the existence of self-transfer, which puts some upper limits on the separation of tryptophan residues, may be sufficient to decide between particular models. The 310/295 or 315/295 polarization ratios can provide evidence for tryptophan–tryptophan energy transfer in a protein.

310/295 or 315/295 polarization ratios greater than 2 thus may indicate that two or more tryptophan residues are roughly within 10–15 Å or closer. This information may be particularly useful in proteins whose three-dimensional structure has not been determined and may be a useful tool in protein folding studies. Quantitative distance determinations require more information on the spectral properties (absorption, emission, and orientation) of the relevant tryptophan residues.

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