

Understanding Fluorescence Decay in Proteins

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Understanding the origin of heterogeneous fluorescence intensity decays in single tryptophan-containing proteins has intrigued fluorescence spectroscopists for some time. Its detailed interpretation is highly desirable, as such behavior is reflective of the dynamic and structural properties of proteins in solution, and could in principle inform upon factors affecting function. A large number of studies of the time-resolved fluorescence of proteins have been reported in the literature in the past 5-10 years. The increased sophistication of the instrumentation (due to the appearance of reliable pulsed laser excitation sources and ultrafast detectors) and data analysis methodologies, along with the availability of three-dimensional structures for many of the systems studied, has allowed for an increasingly detailed interpretation of intrinsic fluorescence decays in proteins. For an appreciation of how our understanding of intrinsic fluorescence of proteins has increased one should compare the review by Beechem and Brand (1) from 1985 to that of Eftink (2) which appeared in 1991. Even since the latter was written, a large number of important papers have appeared, many of which take advantage of site-directed mutagenesis techniques, to further clarify the origin of fluorescence lifetime heterogeneity. The manuscript in the current issue by Kim et al. entitled "Time-resolved Fluorescence of the Single Tryptophan of *Bacillus stearothermophilus* Phosphofruktokinase" (pp. 215-226) represents one of the most thorough studies to date on the origins of nonexponential intensity decay of tryptophan fluorescence in proteins.

Nonexponential fluorescence intensity decay behavior has generally been interpreted as arising from conformational heterogeneity of the protein in solution (3-5), although it has been difficult to demonstrate definitively that such is the case. Within the framework of this interpretation, the shortest life-

time components are thought to arise from conformational states in which interactions during the lifetime of the excited state between the tryptophan residue and functional groups on neighboring amino acid residues lead to quenching of the fluorescence. Excited state electron transfer has been suggested as the main mode of quenching in proteins (6). Since these quenching interactions compete with the fluorescence decay, they necessarily imply motion of the tryptophan and/or the quenching moiety on the timescale of the fluorescence decay, that is in the picosecond to nanosecond range. Thus, fluorescence decay heterogeneity has been interpreted as reflecting the existence of conformational substates which have different dynamic properties.

While such interpretations in terms of conformational heterogeneity are reasonable, direct proof, as noted above, has been elusive. In studies of RNase T1, Chen and co-workers (5) demonstrated that the fluorescence heterogeneity was dependent upon pH and that the bimolecular quenching rates (using externally added quenchers) of the two lifetime components were different. Such differential quenching has been reported for other single tryptophan proteins, as well (7-9). Harris and Hudson (6) determined from the Arrhenius behavior of the lifetimes of the single tryptophan mutants of T4 lysozyme, that the activation energies and frequency factors for the quenching were consistent with repeated encounters of the tryptophan with quenching moieties requiring prior alteration of the amino acid side chains. Dipolar relaxation also appeared to be involved in the causing the heterogeneity of the decay.

While all of these previous studies combine to provide a substantial experimental basis for the conformational substate framework for interpreting lifetime heterogeneity, the work by Kim and co-workers from Mary Barkley's laboratory presented in this issue represents one of the most exhaustive attempts to identify the basis for the heterogeneity of fluorescence decays in single tryptophan proteins. The intensity decay of the single tryptophan Bs-

PFK was measured as a function of wavelength and the emission spectra corresponding to two lifetime components (decay-associated spectra or DAS) were resolved using standard global analysis techniques. The recovered DAS were quite similar in shape, and no evidence for dipolar relaxation was observed. The fact that the shorter lifetime component represented up to 40% of the molecules rules out the possibility that it may reflect a small amount of impurity. The temperature dependence of the emission was also probed and analyzed for the individual frequency factors, activation energies, and decay rates. These experiments revealed a difference in the temperature behavior of the two components. To test for differences in solvent accessibility of the two lifetime components, Kim and co-workers tested for deuterium isotope effects on the decay, as well as differential quenching rates by both acrylamide and iodide. There was no heavy water effect, indicating no solvent accessibility. Acrylamide quenching was much more efficient than iodide quenching, consistent with no solvent exposure and a through space-quenching mechanism such as dipolar energy transfer to the acrylamide. In addition, there were differences between the quenching rates by acrylamide for the two lifetime components supporting the conformational substate theory. Since Bs-PFK is a tetramer in solution, it was possible that the heterogeneity in fluorescence decay could arise from a ground state equilibrium between tetrameric and dimeric forms of the enzyme. In order to eliminate this possibility, time-resolved fluorescence anisotropy and dynamic and static light scattering experiments were performed. All of these experiments demonstrated conclusively that the enzyme is entirely tetrameric under the conditions of the fluorescence decay experiments. It is also known that Bs-PFK is an allosterically regulated enzyme whose functional properties have been interpreted as arising from an equilibrium between a relaxed (R) and a tense (T) form of the tetramer. Experiments in which the allosteric inhibitor, phosphoenolpyruvate or PEP, was added

demonstrated that the tryptophan fluorescence was insensitive to the allosteric state of the enzyme and that this equilibrium could not be at the origin of the fluorescence lifetime heterogeneity.

The differences in the quenching constants for acrylamide and the differential temperature dependence of the two components of the tryptophan emission of Bs-PFK provide very strong evidence for the existence of two conformational states of the enzyme which interconvert on a timescale which is slow compared to fluorescence. Dynamic quenching of the tryptophan fluorescence by the surrounding protein matrix is more efficient in one of these states than in the other, hence the two decay components. Large-scale motions of the tryptophan do not occur, and thus cannot be responsible for the dynamic quenching. These conclusions are quite similar to many other studies which have been carried out. In addition to the quenching studies which provide evidence for the conformational heterogeneity, Kim and co-workers systematically eliminated all other possible origins of the ground-state heterogeneity and thus provided very strong

evidence for dynamic conformational heterogeneity as the basis for the double exponential decay of the single tryptophan residue in Bs-PFK. While this interpretation has gained broad acceptance within the fluorescence community, the basis for the observed fluorescence decay behavior of proteins is not widely known by researchers in related fields concerned with protein structure and dynamics. One reason is that the body of evidence in favor of conformational substates has only grown appreciably in the past 5 years. Another may stem from the somewhat cryptic manner in which fluorescence data have been reported and discussed. For those interested in what novel information about protein structural dynamics fluorescence can provide, the work by Kim and co-workers is a good place to start. Besides being one of the most exhaustive studies available to date, it is also one of the most straightforward and clearly written.

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