formational changes. In contrast to ionic quenchers, acrylamide not only senses the existence of an indole ring on the surface of a protein, but can report how deeply a residue is buried within a protein as well. The quenching rate constants for RNase T_1, human serum albumin (HSA), and aldolase are found to be independent of the solvent viscosity. Thus, the reaction is limited by penetration of the quencher through the protein matrix. Temperature dependence studies indicate that the tryptophyl residues in aldolase and RNase T_1 are shielded by a large activation energy barrier, while the single residue in HSA is shielded by a large activation entropy barrier. These parameters characterize the nature of the protein matrix enveloping the fluorophores.

A less polar neutral quencher, 2,2,2-trichloroethanol (TCE), was examined to investigate whether it would preferentially interact with apolar regions of proteins. For most proteins studied, the degree of quenching by TCE is found to be about the same as with acrylamide. Thus, either the fluorophores are not located in large hydrophobic regions or the oily regions surrounding them are not capable of expanding to accomodate the probe. However, for HSA and bovine serum albumin hydrophobic interactions between TCE and these proteins occur, leading to an exalted quenching. The fluorescence quencher thus senses the presence of a hydrophobic domain in the vicinity of indole side chains in these proteins.

TCE is shown to be a potentially useful quencher for proteins having predominantly tyrosyl emission. The excitation wavelength dependence of the quenching constant, K, can be measured since TCE is transparent in the ultraviolet ($\epsilon_M^{240nm} = 0.29$). For example, the K for TCE quenching of *N*-acetyl-L-tryptophanamide (NATA) has a constant value of $11.7 \pm 0.7\ M^{-1}$ from 240 to 295 nm. This argues that the lifetime of NATA is the same whether it is excited into its first or second absorption band.

In summary, fluorescence quenching studies can provide information about the nature of the microenvironment and accessibility of fluorescent residues of proteins.

HETEROGENEITY AND DYNAMICS OF PROTEIN CONFORMATION REVEALED BY FLUORESCENCE DECAY KINETICS OF TRYPTOPHAN RESIDUES

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The lifetime of the excited state of proteins is in the range of 0–8 ns; thus fluorescence is unique as a tool to characterize dynamic events in the nanosecond time range. Study of the fluorescence characteristics of the aromatic amino acids in proteins offers the advantages of intrinsic probes and with the correct choice of experimental conditions one

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can study the tryptophan residues quite selectively. Measurements of the fluorescence decay of a protein possessing more than one tryptophan residue per molecule, each in a different micro-environment, yield decay kinetics that are super-position of the decay functions of the various emitting chromophores. In principle, we can differentiate between the various chromophores in a single decay measurement. Unfortunately, the analysis of decay curves to obtain the information inherent in the decay function is notoriously difficult. Nevertheless, it is still true that direct measurement of the decay kinetics may reveal information that cannot be obtained by steady-state techniques. Moreover, frequently reliable interpretation of steady-state measurement cannot be made unless the decay function is known. Progress has been made recently in the performance of nanosecond fluorimeters (1, 2) and methods of analysis of decay kinetics (3, 4), thus permitting the acquisition of data with more accuracy than previously possible, and the drawing of conclusions not warranted before.

The fluorescence decay kinetics at different ranges of the emission spectrum have been determined for 17 proteins (5). Out of eight proteins containing a single tryptophan residue per molecule, seven proteins display multiexponential decay kinetics, suggesting that variability in protein conformation may exist for most proteins. It is suggested that the observed heterogeneity in the decay kinetics is due to variability in the quenching of the tryptophan residues, resulting from differences in their interaction with neighboring groups that may originate in slow structural fluctuations (6). These results cannot be taken too lightly; they have implications for the interpretation of fluorescence studies of proteins in general!

As expected, the decay kinetics of proteins containing more than one tryptophan residue is heterogeneous, and usually it comprises at least three decay components. Different decay kinetics are observed for the blue and the red spectral ranges of the emission, the general trend being that the decay is relatively slower for light emitted at the red part of the spectrum. Exposed tryptophan residues whose fluorescence spectrum is red-shifted may have lifetimes longer than 7 ns. Such long lifetimes have not been detected in any of the denatured proteins studied, indicating that in the native state the tryptophans having a red-shifted spectrum are markedly affected by the tertiary structure of the protein, although they are "exposed" residues (7).

The fluorescence decay kinetics of all 10 denatured proteins studied obey multiexponential decay functions. The emission spectrum of the group characterized by longer lifetimes of about 4 ns is red-shifted relative to the emission spectrum of the group characterized by shorter lifetimes of about 1.5 ns. A comparison of the decay data with the quantum yield of proteins suggests that a subgroup of the tryptophan residues is fully quenched. It is noteworthy that despite this heterogeneity in the environment of tryptophan residues in each denatured protein, almost the same decay kinetics has been obtained for all the denatured proteins studied in spite of the vastly different primary structures, or the number of tryptophans. It is concluded that each tryptophan residue interacts more or less randomly with other groups on the polypeptide chain, and on the average, the different tryptophan residues in denatured proteins have a similar type of environment. However, since the decay kinetics of the denatured proteins is not mono-

exponential, the equilibration time between the various interactions must be longer than ~ 10 ns (5).

A few proteins deserve special attention. The decay of hen egg white lysozyme is heterogeneous and about 50% of the tryptophan residues are fully quenched. The picture changes dramatically upon binding of the inhibitor tri-*N*-acetyl chitotriose to the enzyme, the fluorescence decay becoming nearly monoexponential and the fraction of nonfluorescent residues dropping to a small value. A major conformational change that greatly enhances extensive energy transfer between all the residues may explain this observation (5).

Apoazurin is the only protein studied that exhibits perfectly monoexponential decay kinetics. To the extent that the tryptophan fluorescence is a reflection of the environment of this residue, all apoazurin molecules in solution have identical conformations at that site. The decay kinetics of the protein fluorescence in a quenching experiment is heterogeneous. Careful analysis suggests that a static rather than a dynamic quenching mechanism is involved (8). Such critical analysis was not described by Eftink and Ghiron in their interesting report about the dynamics of a protein matrix revealed by fluorescence quenching, assumed to be dynamic (9). Native azurin does not decay monoexponentially. In addition it could be shown that for about 60% of the molecules, the fluorescence is fully quenched. The heterogeneity displayed may be due to differences in the mode of interaction of the Cu^{2+} ions with their binding site, one possibility being ligand exchange with a rate slower than $\sim 10^8\ s^{-1}$.

The fluorescence decay of chicken pepsinogen is not monoexponential throughout the emission spectrum. For light emitted at the long wavelength region of the fluorescence spectrum, the decay can be described by two exponential terms, one of them exhibiting a negative amplitude. This behavior shows that the fluorescence builds up before it decays, indicating that the electronically excited species involved has been created during the fluorescence lifetime. This subnanosecond relaxation process may reflect reorientation of various groups around the excited chromophore or the formation of a more specific excited state complex, e.g. an exciplex. The process is conformation-dependent and disappears upon denaturation. Similarly, chicken pepsin at neutral pH fails to show it. This seems to be the first case in which a relaxation process in the nanosecond time range has been directly demonstrated with a native chromophore in a protein (7).

These observations should not come as a surprise, and may be considered complementary to the indirect observations of subnanosecond fluctuations in protein structure (10), and the slower structural fluctuations extending into the microsecond time range that must be postulated to explain the results of hydrogen exchange experiments (6).

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HETEROGENEITY AND SOLUTE QUENCHING OF PROTEIN FLUORESCENCE

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The early studies on lysozyme (1) provided clear-cut examples of spectral heterogeneity of tryptophyl fluorescence in a protein. This heterogeneity was observed in difference fluorescence spectra produced by certain mild perturbations that did not alter the conformation of the protein. The results were interpreted as due to selective quenching of the fluorescence of one (or more) tryptophyl(s) whose Stokes shift depended upon the polarity of its immediate environment. Increasing the pH from 7.5 to 11.5 resulted in the selective quenching of the fluorescence of "blue" tryptophyls for both the free enzyme and the lysozyme—tri-*N*-acetyl-D-glucosamine (triNAG) complex due to energy transfer to ionized tyrosyl. Quenching of a "reddish" tryptophyl and of a blue tryptophyl was also observed as the pH was decreased in the pH 7.5–5.5 region and the 5.5–2.0 region, respectively, only for the lysozyme-triNAG complex. Selective quenching of the fluorescence of red tryptophyls in the free enzyme resulted from the addition of iodide (2, 3). Similar selective quenching effects were obtained due to energy transfer to solute (4). These studies suggested that protein fluorophors in general can be grouped into classes.

Solute quenching studies can help to separate fluorescence contributions of classes of fluorophors with differing accessibility. In these cases the resultant heterogeneity of quenching can be noted by the shifting spectra to the blue for tryptophyl or, more generally, by downward-curving Stern-Volmer plots. By replotting these data as fluorescence quenched rather than fluorescence remaining, information on those residues whose fluorescence is quenched most readily is obtained. Thus, the limiting straight