Exposure of Tryptophanyl Residues in Proteins. Quantitative Determination by Fluorescence Quenching Studies†

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ABSTRACT: Acrylamide is an efficient quencher of tryptophanyl fluorescence which we report to be very discriminating in sensing the degree of exposure of this residue in proteins. The quenching reaction involves physical contact between the quencher and an excited indole ring, and can be kinetically described in terms of a collisional and a static component. The rate constant for the collisional component is a kinetic measure of the exposure of a residue in a protein, and values ranging from 4×10^9 M^{-1} s^{-1} for the fully exposed tryptophan in the polypeptide, adrenocorticotropin, to $<5 \times 10^8$ M^{-1} s^{-1} for the buried residue in azurin have been found. Static quenching is readily detected in proteins that are denatured, or contain only a single fluorophor. Quenching patterns for most multi-tryptophan containing proteins are difficult to analyze precisely, but qualitative information can, nevertheless, be extracted. Applications of this probing technique for monitoring protein conformational changes, such as the acid-induced expansion of human serum albumin, and inhibitor binding to enzymes, are presented. The value of this method lies in its ability to sense not only the steady-state exposure of a residue in a protein, but also its dynamic exposure.

The folding of a polypeptide chain to form a relatively compact globular protein inevitably results in the burial of certain amino acid residues from the external, aqueous environment. Other residues, either by choice or chance, will lie on the surface, exposed to the polar solvent. A strategy often employed in studying the solution structure of proteins is to map out those residues which are exposed, vs. those which are buried (Kronman and Robbins, 1970). Since most proteins contain a relatively small number of tryptophanyl residues, this amino acid has received considerable attention in such topographical studies. To probe for the exposure of the indole ring, solvent perturbation (Herskovits, 1967; Williams et al., 1965) and chemical modification techniques (Hachimori et al., 1964; Frazier et al., 1973) are often employed. However, there are many limitations and experimental difficulties with these methods. A very promising spectroscopic technique involves the quenching of the fluorescence of tryptophanyl residues by the addition of various low molecular weight agents (Lehrer, 1975). These agents, known as quenchers, decrease the fluorescence intensity of the residues via physical contact with the excited indole ring. Hence, the ease with which a fluorophor is quenched depends upon its "exposure" to the quencher.

Molecular oxygen and a number of ionic quenchers, such as I^-, NO_3^-, and Cs^+, have been used quite extensively in studies with proteins, but their ability to estimate exposure has been rather tenuous. Ionic quenchers, being charged and heavily hydrated, should be able to quench only surface tryptophanyl residues (Lehrer, 1971a; Burstein et al., 1973). Since a protein is a polyelectrolyte, however, electrostatic effects may influence their quenching action, and lead to an under or overestimation of the exposure of a fluorophor. For example, a tryptophan that is flanked by positive charges (i.e., lysyl or arginyl residues) might be quenched to a much greater degree by an anionic quencher than would normally be expected. Such an effect has been observed by Lehrer (1971b) for the quenching of HSA[1] fluorescence by I^-.

Hoping to circumvent this electrostatic problem, Lakowicz and Weber (1973) attempted to use O_2 as a neutral quenching probe. However, they found that O_2 was very indiscriminate, being able to quench the fluorescence of supposedly buried residues (such as in aldolase) almost as easily as exposed ones (such as in pepsin). They suggested that because O_2 is so small, it is able to find its way into interior regions of a protein very rapidly. Also, there is a possibility that O_2, being a relatively hydrophobic molecule, will be accumulated into apolar regions of proteins, thus facilitating the quenching of buried tryptophans (Eftink and Ghiron, 1976). Therefore, the fact that O_2 can readily quench a residue's fluorescence does not necessarily indicate that it is solvent exposed.

We report here that acrylamide is an excellent, uncharged quenching probe that is very sensitive to the exposure of tryptophans in proteins. Since it is a phenomenologically different type of molecule, it does not share the shortcomings of the quenchers mentioned above. Therefore, it should be able to sense the exposure of residues in a purely random, kinetic fashion.

Theory

The quenching reaction between the excited state of an indole ring, M*, and acrylamide, Q, can be described by the following scheme:

† From the Department of Biochemistry, University of Missouri, Columbia, Missouri 65201. *Received August 13, 1975.* This research was supported by Research Grant URC-NSF-1241 from the University of Missouri, Oak Ridge Associated Universities Agreement No. S-1381, and by the Department of Biochemistry and School of Medicine, University of Missouri. This work is presented by M.R.E. in partial fulfillment of the requirements for the Doctor of Philosophy degree at the University of Missouri.

[1] Abbreviations used are: LBTI, lima bean trypsin inhibitor; ACTH, adrenocorticotropin; HGH, human growth hormone. Gdn-HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; HSA, human serum albumin; BSA, bovine serum albumin.

$$M^* + Q \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} (M^* \cdots Q) \xrightarrow{k_i} M + Q + \Delta \quad (1)$$

($M^*\cdots Q$) is the complex formed by diffusional encounter between M^* and Q with rate constant k_d. This encounter complex will then react to dissipate the excited state as heat with rate constant k_i. The experimentally observed rate constant for the quenching reaction, k_q, is equal to γk_d, where γ is the efficiency of the quenching process. We have previously demonstrated that the efficiency of acrylamide quenching of indole is unity (Eftink and Ghiron, 1976). This being the case, k_q is equal to k_d, the diffusion limited rate constant for collision between the reactants.

The classical relationship often employed to describe the collisional quenching process presented above is the Stern–Volmer equation (Birks, 1970):

$$F_0/F = 1 + K_{sv}\{Q\} = \tau_0/\tau \quad (2)$$

where F_0 and F are the fluorescence intensities at an appropriate emission wavelength in the absence and presence of quencher, τ_0 and τ are the fluorescence lifetimes in the absence and presence of quencher, and K_{sv} is the collisional quenching constant, which is equal to $k_q\tau_0$. By plotting F_0/F or τ_0/τ vs. $\{Q\}$, k_q can be obtained, provided τ_0 is known.

A more complete treatment of the kinetics of the quenching reaction produced by acrylamide (and other efficient quenchers) in condensed systems must include consideration of a process referred to as "static" quenching. In a randomly distributed solution there will be occasions in which quencher and chromophore molecules happen to be very close to each other at the moment that the latter becomes excited. In such a case, the probability for the reaction is so high, that quenching occurs almost instantaneously (statically). The following modified form of the Stern–Volmer relationship has been found to adequately describe the total quenching process (Birks, 1970; Eftink and Ghiron, 1976):

$$F_0/F = (1 + K_{sv}\{Q\})e^{V\{Q\}} \quad (3a)$$

$$F_0/Fe^{V\{Q\}} = 1 + K_{sv}\{Q\} = 1 + k_q\tau_0\{Q\} = \tau_0/\tau \quad (3b)$$

In this equation, the kinetics of the quenching reaction is operationally dissected into a collisional (characterized by K_{sv}) and a static (characterized by V) component. Since ($1 + k_q\tau_0[Q]$) should be a linear function, it is possible to fit quenching data to eq 3b by plotting $F_0/F \exp(V\{Q\})$ vs. $\{Q\}$ for varying V until a linear plot obtains.

There are a number of physical interpretations for the static constant. V can be considered to be a volume element surrounding the fluorophor having a radius r ($V = 4\pi N' r^3/3$, where N' is Avogadro's number per millimole). Alternatively, it can be interpreted as an association constant ($\approx k_d/k_{-d}$) for the formation of the ground state encounter complex, (M···Q). For both models, V is a parameter that is related to the probability of finding a quencher molecule close enough to a newly formed excited state to quench it immediately, or statically.

Application of the equation presented above is valid only if the fluorescence is homogeneous. For proteins containing more than one fluorescing tryptophanyl residue, the situation is quite a bit more complicated. If each of the residues fluoresce independently, the proper equation is:

$$F/F_0 = \sum_i \frac{f_i}{(1 + K_i\{Q\})e^{V_i\{Q\}}} \quad (4)$$

where f_i is the fraction of the total fluorescence corresponding to the ith tryptophan at a given wavelength, and K_i and V_i are the respective collisional and static quenching constants. In the simplest case, where only two classes of fluorophors are considered, having different K_i and approximately the same V_i, this equation becomes:

$$F_0/F = 1 + \frac{(f_aK_a + f_bK_b + K_aK_b\{Q\})\{Q\}}{1 + (f_aK_b + f_bK_a)\{Q\}} \cdot e^{V\{Q\}} \quad (5)$$

The relationship is complex indeed. In a study with binary mixtures of various indole derivatives having different collisional quenching constants and similar V, plots of F_0/F vs. $\{Q\}$ have been found which curve upward, downward, and appear linear, depending on the difference in the collisional constants for the two derivatives. When this difference is large (i.e., $K_a \gtrsim 5K_b$), a dowward curvature may be detected. If K_a and K_b differ only by a factor of ~2, a plot that is linear within experimental error is found. If the two derivatives have similar collisional constants, the Stern–Volmer plot curves upward (M. R. Eftink and C. A. Ghiron, unpublished results). The initial slope of a F_0/F plot for a system containing more than one class of fluorophors will be approximately equal to Σf_iK_i, the weighted average of the individual quenching constants (at a particular wavelength), which will be referred to as the effective quenching constant, K_{sv}(eff). By plotting $(F_0/F - 1)/\{Q\}$ vs. $\{Q\}$ and extrapolating to $\{Q\} = 0$, the initial slope can be obtained.

Experimental Section

RNase T_1 (*Asperigillus oryzae*), nuclease (*Staphylococcus aureus*), monellin (*Dioscorephyllum cumminsii*), pepsin A, α-chymotrypsin (three times crystallized), trypsin, and LBTI were obtained from Worthington Biochemical Corp. Lysozyme (egg white, three times crystallized), α-chymotrypsinogen (six times crystallized), aldolase (rabbit muscle), glucagon, HSA, papain, and trypsinogen were obtained from Sigma Chemical Company. Azurin from *Pseudomonas fluorescens* was purified by Dr. A. Finazzi-Agro, University of Rome, Rome, Italy, and was a generous gift from Dr. J. W. Longworth, Oak Ridge National Laboratory. HGH was obtained from Dr. J. Wyche, University of Missouri. ACTH (porcine, an Armour product) and BSA (three times recrystallized) were provided by Dr. B. Campbell, University of Missouri. Acrylamide was recrystallized from ethyl acetate before use, and sodium dodecyl sulfate was from ethanol. Ultra Pure guanidine hydrochloride was a Schwarz/Mann product. Cyclohexylcarboxamidine hydrochloride was synthesized by Dr. N. Ramachandran. *N*-Cbz-L-tyrosine *p*-nitrophenyl ester was obtained from Sigma Chemical Company.

Trypsin was purified by the chromatographic procedure of Schroeder and Shaw (1968). The β fraction, which possessed greater than 90% active protein (Sellers and Ghiron, 1973), was used. Trypsinogen was purified in a similar fashion. Pepsin was purified by being chromatographed through a sulfoethyl Sephadex C-25 column at pH 4.4 (Ryle, 1970). HSA was defatted by the procedure of Chen (1967). If it appeared necessary, protein solutions were filtered before being used. Glucagon was first dissolved at low pH (~3) and the solution was subsequently adjusted to the desired pH to avoid aggregation. A complex between HSA and dodecyl sulfate was prepared by simply adding a 25-fold excess of dodecyl sulfate to a HSA solution. In these proportions, HSA is believed to still be in its folded form (Steinhardt et al., 1971).

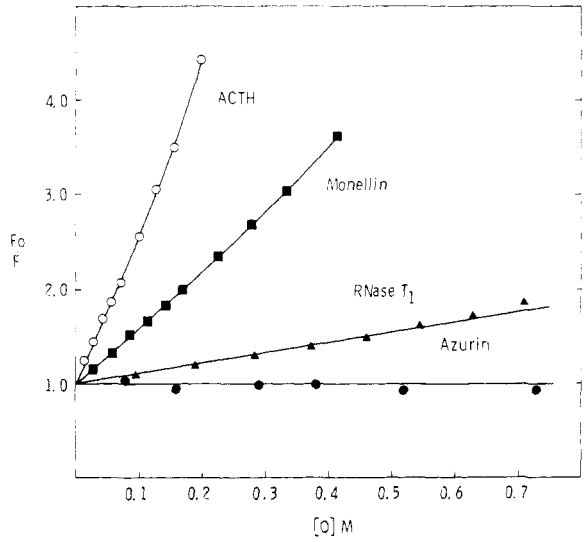

FIGURE 1: Acrylamide quenching of single tryptophan containing proteins. Experimental conditions given in Table I.

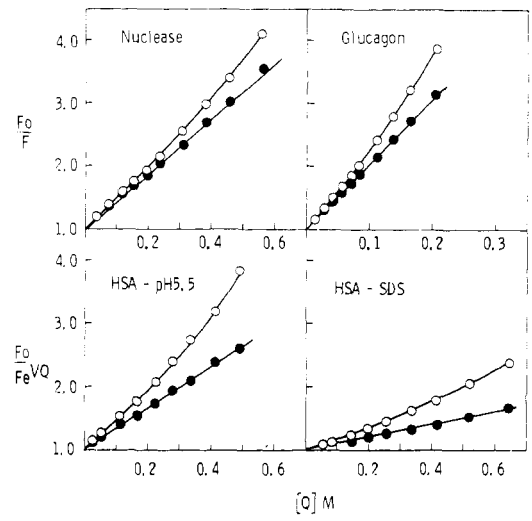

FIGURE 2: Acrylamide quenching of single tryptophan proteins—separation of static and collisional components, according to eq 3b. Open symbols, F_0/F; closed symbols, $F_0/F \exp(V[Q])$. Experimental conditions given in Table I.

A hemoglobin assay was used to measure pepsin activity (Ryle, 1970). Chymotrypsin was assayed by monitoring the increase in OD at 410 nm as *N*-Cbz-tyrosine *p*-nitrophenyl ester was hydrolyzed at pH 7.5.

Fluorescence measurements were made on a Farrand Mark I spectrofluorometer, using 5-nm bandwidths. An excitation λ of 295 nm was routinely used, to ensure that the light was absorbed almost entirely by tryptophanyl groups. Protein solutions having an OD of ~0.1 or less at 295 nm were used. The fluorescence of a protein, monitored at its emission λ_{max}, was quenched by the progressive addition of small aliquots of an 8 M acrylamide solution, as previously described (Eftink and Ghiron, 1976). A correction factor was applied for the attenuation of the excitation light intensity by the added acrylamide (Parker, 1968), which has a molar extinction coefficient of about 0.23 at 295 nm.

Fluorescence lifetime measurements were performed on an instrument resembling the Model 9200 nanosecond fluorescence spectrometer (ORTEC) constructed by S. Stevens and J. W. Longworth. Lifetimes were assigned by comparison of the raw decay data to simulated patterns which were synthesized by adding a theoretical single exponential decay curve to a measured system response. This was done using a computer program (also provided by S. Stevens and J. W. Longworth) which was designed to minimize the error between the raw and simulated curves in order to seek the best fit. For many of the proteins, including RNase T_1, nuclease, monellin, ACTH, HSA-SDS, and pepsin, single component fits were at least as good as those obtained for pure samples of indole and *N*-acetyltryptophanamide. For trypsin and HSA (at both pH 5.5 and 2.5) the decays were obviously not single exponentials (in agreement with de Lauder and Wahl (1971)). Nevertheless, they were force fitted to a single exponential in order to obtain an "average" lifetime. The protein solutions were usually excited at 290 nm in the lifetime experiments, and the light emitted at >310 nm was observed through a Corning 0-54 filter. The lifetimes were determined at 25°. For some of the proteins, the lifetimes are literature values.

The quenching experiments were performed at room temperature. This is legal since K_{sv} and V are found to be only slightly temperature dependent (Eftink and Ghiron, 1975, 1976). We may therefore consider the values of k_q to be for 25°.

Results and Discussion

Acrylamide is a polar, uncharged compound that has been shown to quench the fluorescence of indole derivatives predominately by a collisional process (Eftink and Ghiron, 1976). The quenching reaction is not affected by substitution of methyl groups at various positions on the indole ring, suggesting that there are no obvious stereochemical requirements for the quenching process. The efficiency of the reaction is near unity in both aqueous and nonaqueous (dioxane) solutions. In studies with proteins, this probe should have the ability to quench any excited tryptophanyl residues that it happens to collide with, regardless of whether the residue is located on the surface or the apolar, interior of a protein. The ease with which a given residue can be quenched should depend solely on the frequency that it encounters an acrylamide molecule. If an indole ring is sterically shielded by surrounding protein segments, the frequency of collision with the probe will be reduced, and its fluorescence will not be quenched as readily.

Single Tryptophan Proteins. The quenching of a number of single tryptophan containing proteins and polypeptides has been investigated. Some of the results are presented in Figures 1 and 2. We have emphasized these proteins in our studies, since, having only a single fluorophor, their analysis is straightforward. The most striking feature in the first figure is the wide variation in the ability of acrylamide to quench the fluorescence of these proteins. The tryptophan in ACTH is very accessible to bombarding acrylamide molecules; 57% of the fluorescence is quenched at 0.1 M acrylamide. At the other extreme, no decrease in the fluorescence of the tryptophan in azurin (*Pseudomonas fluorescens*) is detected as acrylamide is added. From these findings we would judge the residue in ACTH to be exposed and that in azurin to be buried. From other studies it is believed that ACTH is a randomly coiled peptide and would afford little steric shielding for its tryptophan (Edelhoch and Lippoldt, 1969). Azurin, on the other hand, is thought to possess a tryptophan that is extremely well insulated

Table I: Acrylamide Quenching Parameters for Single Tryptophan Containing Proteins.[a]

Protein	K_{SV} (M^{-1})	V (M^{-1})	τ_0 (ns)	k_q ($\times 10^{-9}$ M^{-1} s^{-1})	λ_{max} (nm)
Azurin[b]	~0	~0	4.0[i]	<0.05	308
HSA–SDS[c,h]	1.0	0.6	4.7	0.2	325
RNase T_1[d]	1.1	~0	3.5	0.3	324
HSA, pH 5.5[c]	3.1	0.8	6.0	0.5	342
Nuclease[f]	4.5	0.25	5.0	0.9	334
HSA, pH 2.5[e]	3.3	0.6	3.3	1.0	334
Monellin[d]	5.2	0.3	2.6	2.0	342
HGH[d]	3.0	0.2			342
Glucagon[g]	10.5	1.0	2.8	3.7	352
ACTH[d]	13.0	1.0	3.1	4.2	352

[a] Studies at room temperature; fluorescence monitored at the emission λ_{max}. Emission spectra were uncorrected. The estimated limits of error for the assigned values of V are about ±25% (except for very small V, where there is less confidence); this leads to a variability in the resulting K_{SV} of only about 10%. [b] pH 8, 0.01 M phosphate buffer. [c] pH 5.5, 0.02 M acetate buffer. [d] pH 7.0, 0.01 M Tris buffer. [e] 0.01 M formate buffer. [f] pH 7.5, 0.01 M Tris–0.05 M NaCl–0.01 M $CaCl_2$. [g] pH 6, 0.1 M NaCl. [h] ~10^{-5} M protein, ~2×10^{-4} M sodium dodecyl sulfate (SDS). [i] Value from Grinvald et al. (1975) for azurin from *Pseudomonas aeruginosa*.

from the solution based on its unusual luminescence properties (Finazzi-Agro et al., 1970; Grinvald et al., 1975).

Those proteins that are quenched to a reasonable degree do not show linear Stern–Volmer plots. Instead, they are curved upward. We have observed similar positive deviations for the quenching of indole type compounds due to static quenching. By treating the data according to eq 3b, the collisional and static quenching constants can be assigned. In Figure 2, the data for glucagon, HSA, HSA–SDS, and nuclease are analyzed in this manner and the results are presented in Table I. The static constant, V, ranges from 1.0 M^{-1} for ACTH and glucagon to practically zero for azurin. The data for RNase T_1 do not deviate enough from linearity to allow a V to be assigned; the V must be less than about 0.2 M^{-1}. Except for azurin and RNase T_1, all the other single tryptophan proteins show some degree of static quenching.

From the K_{sv} values obtained for these proteins and their fluorescence lifetimes, the rate constant for quenching, k_q, can be determined, as listed in Table I. Since quenching occurs with every collision between acrylamide and an excited indole ring, the frequency of this encounter (which is k_q) is a kinetic measure of the exposure of the residue. The measurable rate constants range from about 4×10^9 M^{-1} s^{-1} for the exposed residues in ACTH and glucagon to 2×10^8 M^{-1} s^{-1} for the HSA-SDS complex. For azurin, the limits of our experiment allow us to only estimate that k_q must be less than 5×10^7 M^{-1} s^{-1}.

The rate constants found for ACTH and glucagon are approximately the maximum value expected for the quenching of a fully exposed residue in a randomly coiled polypeptide. For small indole derivatives, a k_q of 7×10^9 has been found (Eftink and Ghiron, 1976); the value for the polypeptide is expected to be slightly lower due to the reduced diffusion coefficient of the indole ring when it is anchored to the macromolecule. Also, collision with quencher might be partially obstructed by the attached polypeptide.

The low k_q for azurin indicates that the indole ring in this protein must be almost completely protected from collision with the probe. The quenching rate for the other proteins falls in between these extremes, reflecting the intermediate topographical positioning of their tryptophanyl residues. The frequency of collision for proteins such as RNase T_1, HSA, and nuclease is very low, but their tryptophanyl residues must not be shielded nearly as much as that in azurin.

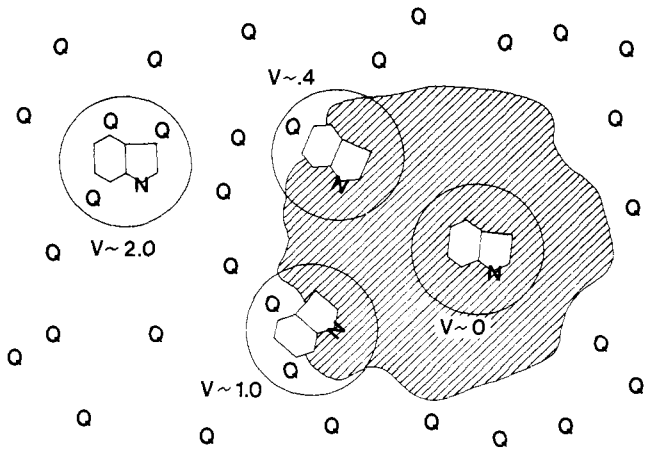

FIGURE 3: Schematic description of the relationship between the degree of static quenching and the exposure of the fluorophor.

Besides k_q, the static quenching constant can also be exploited to gain information about the exposure of the residues. Around an indole ring, one might consider an imaginary shell as depicted in Figure 3. If an acrylamide molecule happens to exist within this volume element at the instance the chromophore becomes excited, it will be quenched instantaneously. For the unsubstituted indole ring we have reported a V value of 2.5 M^{-1}. For substituted derivatives, such as tryptophan, the V is lower (1.5 M^{-1}) because the side chain sterically limits the probability of an acrylamide neighboring the ring (Eftink and Ghiron, 1976). The way in which steric shielding may determine the amount of static quenching is schematically presented in Figure 3. The more an indole ring is shielded by segments of a protein, the less it will be subject to static quenching. The trends in V from 1.0 to 0 that we find for the series of proteins can, therefore, be readily interpreted in terms of the physical exposure of the indole ring. For ACTH and glucagon, the V values of 1.0 indicate that the residues are largely exposed. However, the value is lower than that for simple indole derivatives, and some interference due to the connected polypeptide chain is apparent. The absence of static quenching for azurin and RNase T_1 must reflect the burial of their residues. In other proteins, such as nuclease and monellin, the "static" exposure of the tryptophans is intermediate.

It is of interest to note that RNase T_1 does not appear to be statically quenched to any significant degree, but that it is collisionally quenched. This argues that the indole ring is completely enveloped by protein fabric so that quencher molecules do not have an opportunity to be in steady-state contact with the residue. As discussed previously (Eftink and Ghiron, 1975), collisional quenching of such a residue that is apparently buried must require penetration of acrylamide through the surrounding protein matrix. The rate constant, k_q, in this case, is a measure of the depth of burial of the fluorophor.

For HSA, in all forms studied, a relatively large static component is observed, particularly when the collisional rate constant is considered. For example, for HSA at pH

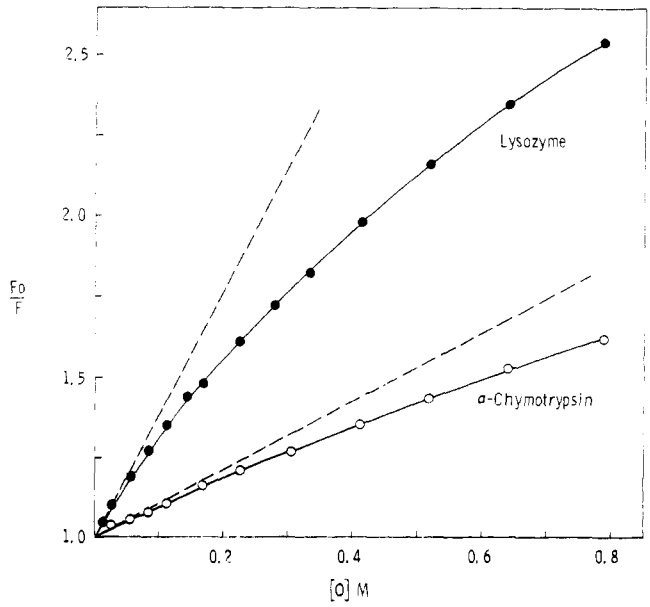

FIGURE 4: Selective quenching of the fluorescence of lysozyme and chymotrypsin by acrylamide. Dashed lines are the initial slopes, K_{sv}(eff). Experimental conditions given in Table II.

Table II: Acrylamide Quenching Parameters for the Multi-Tryptophan Containing Proteins.[a]

Protein	K_{sv}(eff) (M^{-1})	Deviation	τ_0 (ns)	k_q(eff) ($\times 10^{-9}$ M^{-1} s^{-1})	λ_{max} (nm)
Aldolase[b]	0.2	0	2.3[h]	0.1	325
Chymotrypsin[b]	1.0	–	2.1[h]	0.5	333
Chymotrypsinogen[b]	1.0	–			331
BSA[b]	3.7	+	6.2[h]	0.6	343
Trypsinogen[b]	2.3	0	2.0[h]	1.1	333
β-Trypsin[c]	2.4	0	2.2	1.1	334
Ovalbumin[d]	3.8	–			336
Pepsin[b]	9.5	0	6.0	1.6	344
Papain[e]	8.0	0			349
Lysozyme[c]	3.5	–	1.9[i]	1.8	339
Aldolase, pH 2.2[f]	6.8	+			349
Pepsin, pH 8.0[e]	8.6	+			349
β-Trypsin-Gdn·HCl[g]	4.8	+	3.2	2.7[i]	352

[a] Studies at room temperature; fluorescence monitored at the emission λ_{max}. Emission spectra were uncorrected. [b] pH 5.5, 0.02 M acetate buffer. [c] pH 3, HCl. [d] pH 7.0, 0.01 M Tris buffer. [e] pH 8, 0.01 M phosphate buffer. [f] 0.01 M formate buffer. [g] 6.7 M Gdn·HCl. [h] Values taken from Lakowicz and Weber (1973). [i] Value taken from Formoso and Forster (1975). [j] k_q normalized for the increase in the solution viscosity due to guanidine by multiplying K_{sv}/τ_0 by a factor of 1.8 (Eftink and Ghiron, 1976).

5.5 (N form) the k_q value indicates that the tryptophan is buried almost as much as that in RNase T_1, but the V suggests that there is a good chance that an acrylamide molecule might exist within the active volume of the fluorophor. We will present a detailed explanation for the unusual data for HSA, emphasizing the interpretation of V, in a subsequent paper.

Multi-Tryptophan Proteins. Since we have found a wide range of acrylamide quenching parameters, it seemed reasonable to expand our studies to include proteins containing more than one tryptophan. With these proteins, fluorescence studies are usually beset with many complications. There is a possibility that energy may be transferred between the various tryptophanyl residues. However, only in a few cases has this been unequivocally demonstrated (Longworth and Ghiron, 1974). Also, each fluorescing group may have its own characteristic fluorescence yield, lifetime, spectral position, and quenching constants (Burstein et al., 1973). As can be gathered from eq 5, the description of quenching data is rather complex for proteins with more than one fluorophor. Nevertheless, we have found that quenching studies may still reveal some useful, qualitative information concerning the fluorophors in this class of proteins.

When the fluorescence of lysozyme and chymotrypsin is quenched by acrylamide, the Stern-Volmer plots shown in Figure 4 are obtained. In contrast to the previous cases, these plots curve downward. Similar quenching patterns have been observed by Lehrer (1971), Burstein (1968), and Teale and Badley (1970). Negative deviations result because the fluorescence of certain tryptophans is selectively quenched before others in a protein. At a low concentration of quencher, the slope of the Stern-Volmer plot reflects largely the quenching of the more accessible residue(s). At higher concentrations, the easily quenched fluorescence has been greatly depleted, and those tryptophans having lower quenching constants become dominant (corresponding to the lower final slope). Selective quenching in this manner can only be detected if the quenching constants for each fraction of the fluorescence are quite different, as discussed earlier. If they are not, the data tend to collapse to give apparently linear plots. The treatment of selective quenching by Lehrer (1971) and others (Badley, 1975) does not include static quenching, which we observe for single tryptophan proteins. Static quenching causes the plots to curve upwards, and, therefore, would oppose any negative deviation due to selective quenching. The fact that for lysozyme and chymotrypsin, the Stern-Volmer plots do indeed curve downward indicates that the curvature due to selective quenching overwhelms any positive deviations caused by the static components. In these proteins, the fluorescence of certain tryptophans must be quenched much more readily than others. This suggests that some of the residues in these proteins might be almost completely buried to acrylamide. Alternatively, a large difference in lifetime for the various fluorophors, rather than the rate constant, might also explain the selectivity in quenching. Such may be the case for lysozyme, for which the fluorescence decay has at least two components (Formoso and Forster, 1975). Perusal of Tables I and II suggests, however, that as a general rule, one would expect a much greater variation in k_q than in τ_0 for a fluorophor in a protein. Any fluorophor emitting with a short lifetime will also have a low yield and might contribute negligibly to the total fluorescence of the protein. Therefore, we find it more likely that a downward curvature in a Stern-Volmer plot reflects a heterogeneity in k_q, but there may be exceptions.

When a number of other multi-tryptophan containing proteins, such as pepsin, β-trypsin, and aldolase, were studied, linear plots were observed, as shown in Figure 5. In these cases, the data appear to collapse for reasons given above. The ease of quenching of these proteins varies greatly. While pepsin is easily quenched, aldolase is only slightly perturbed.

When these proteins are denatured by the addition of 6.7 M Gdn·HCl, the variation in the quenchability is lost, as shown in Figure 5. The Stern-Volmer plots for the denatured proteins are all about the same. Denaturation appears to normalize the environment and exposure of the trypto-

phans in these proteins. In native aldolase, the tryptophanyl residues must be extremely buried, and the residues in native pepsin must be fairly exposed. When denatured, these specific structural features are lost, and the residues all become approximately equally exposed in each of the proteins. Furthermore, the plots again show the positive deviation diagnostic of static quenching. When analyzed according to eq 3b the data for denatured β-tryptin yield $V = 0.9\ M^{-1}$ and $K_{sv} = 4.8\ M^{-1}$. A similar change occurs when pepsin is unfolded by adjusting the pH to 8.0 (Ryle, 1970). Instead of the linear plot observed for the low pH, native form, an upward curving plot is found, with $V = 1.0\ M^{-1}$ and $K_{sv} = 8.6\ M^{-1}$. The only native multi-tryptophan protein for which we find an upward curving of the Stern–Volmer plot is bovine serum albumin. For this to occur, the fluorescence of both residues in bovine serum albumin must be equally quenched, or only one of the tryptophans must be fluorescent.

According to the acrylamide studies, it is possible to loosely categorize multi-tryptophan proteins in the following way. (a) Upward curving plot (+)—all residues nearly equally accessible, or fluorescence dominated by a single residue. (b) Downward curving plot (−)—heterogeneous fluorescence, residues having a widely different accessibility to quencher. (c) Linear plot (0)—heterogeneous fluorescence, residues differing slightly in accessibility. A precise distinction between these classifications is difficult to make because much depends not only on the magnitude of K_{sv} for each fluorophor, but on the V values and fractional yield of each, as well. Those proteins studied are categorized in this manner in Table II. Also listed in this table is an effective quenching constant, K_{sv}(eff).

For these proteins it is not a priori obvious to what extent quenching occurs by the two kinetic processes, collisional and static. With the single tryptophan proteins, the assignment of the collisional and static components is straightforward. It has been our experience that acrylamide is predominantly a collisional quencher. Fluorescence lifetime measurements are employed to investigate this matter, based on the grounds that a corresponding drop in the lifetime is a characteristic of collisional quenching (Pesce et al., 1971). Sellers and Ghiron (1973) have reported a simultaneous drop in the lifetime and yield on the quenching of β-tryptin by acrylamide. Eftink and Ghiron (1975) have reported similar results for the quenching of RNase T_1. Experiments with pepsin can also be added to this list. At an acrylamide concentration sufficient to drop the yield by 69%, a 63% decrease in the lifetime (from 6.0 to 2.2 ns) is observed. The fluorescence of the easily quenched tryptophans in pepsin and the largely buried residue in RNase T_1 both appear to be quenched predominately by the collisional mechanism. Although our studies with single tryptophan proteins indicate that static quenching commonly occurs, the dynamic process is the most important one. For the multi-tryptophan proteins, the static component is often masked and the K_{sv}(eff) that is found may be considered to be a collisional quenching constant, equal to $k_q(\text{eff})\tau_0$, where τ_0 is the average lifetime (or single component fit). The parameter k_q(eff) can be taken as a crude estimation of the average exposure of the fluorescing residues in the protein. Although the interpretation of the data for these proteins is not as definite as that for proteins possessing only a single tryptophan, the fluorescence can still be described by the classification system given above, depending on the shape of the Stern–Volmer plot, and the average location of the fluorescing centers in the protein can also be judged.

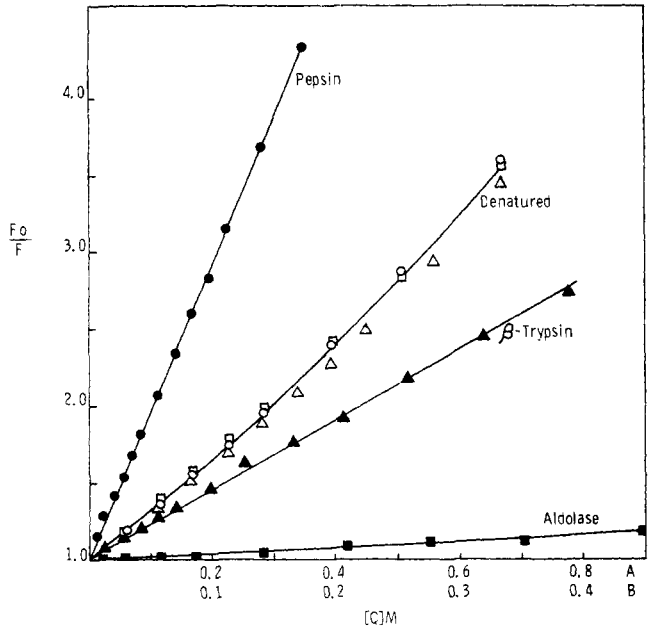

FIGURE 5: Acrylamide quenching of the milti-tryptophan containing proteins: pepsin (●,○), trypsin (▲,△), and aldolase (■,□). Darkened symbols (scale A) are for native proteins; experimental conditions given in Table II. Open symbols (scale B) are for denatured proteins in 6.7 M Gdn·HCl.

Investigation of Protein Conformation Changes. The sensitivity of acrylamide quenching to exposure suggests that it should be very useful for monitoring protein conformation changes. Besides being able to merely detect a conformational rearrangement, the technique should also report the direction of the change in terms of an increase or decrease in the exposure of tryptophanyl residues. If there is only a single fluorophor, the probe will sense a perturbation of the structure of the protein in the proximity of that group. If there are a large number of fluorescing centers, conformational changes occurring over a larger domain will be reported. An example of such an application (see Table II) is the quenching aldolase at neutral and low pH values. Aldolase is a tetrameric protein that is known to dissociate into subunits as the pH is lowered (Stellwagen and Schachman, 1962). With this dissociation, a marked increase in quenching is observed, indicating that tryptophanyl residues throughout the protein are becoming greatly exposed to acrylamide. Also, the Stern–Volmer plot at pH 2.2 curves upward, suggesting that the dissociated subunits are unfolded proteins.

This structural change in aldolase is very drastic. A more limited one is given by the N ⇌ F transition for HSA (Foster, 1960). As the pH of a solution is lowered below 4, the protein is believed to expand, as contacts between the individual globular "subunits" of the macromolecule are broken. By comparison of the k_q values in Table I, we find that the single tryptophan becomes more exposed by this transconformational change. Apparently, the dissociation of the "subunits" uncovers the otherwise buried residue.

Besides these rather extensive structural changes, the quenching technique can also detect very subtle conformational rearrangements which accompany the binding of a competitive inhibitor to an enzyme, such as β-trypsin. When 0.02 M cyclohexylcarboxamidine hydrochloride (99.9% saturation) is added to a β-trypsin solution (pH 7.5,

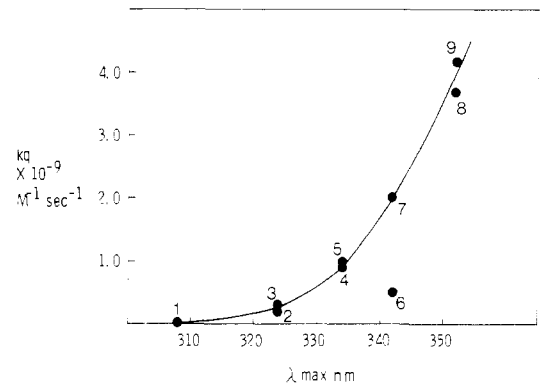

FIGURE 6: Relationship between k_q and λ_{max} for single tryptophan proteins. 1, azurin; 2, HSA-SDS; 3, RNase T_1; 4, nuclease; 5, HSA, pH 2.5; 6, HSA, pH 5.5; 7, monellin; 8, glucagon; 9, ACTH.

0.01 M phosphate buffer), the K_{sv}(eff) drops from 2.8 to 2.0 M^{-1} (Ramachandran, 1973). (A similar reduction in quenching constant is found when *N*-acetylglucosamine is bound to lysozyme.) There are two general explanations for this decrease in tryptophanyl exposure upon inhibitor binding. (It is assumed that the K_{sv}(eff) drop is not a result of a decrease in the lifetime, since the relative fluorescence yield is not significantly altered when the inhibitor binds.) First, the inhibitor itself might act to sterically shield a fluorescing tryptophanyl residue from collision with acrylamide. Trp-215 lies close enough to the active site of trypsin that it might possibly be shielded by a bound inhibitor (Krieger et al., 1974). The other explanation is that inhibitor binding induces a propagated conformational change in the enzyme, resulting in a slight decrease in the exposure of most of the fluorescing residues. To explore these possibilities, studies were performed with two other trypsin inhibitors of greatly different molecular sizes: Gdn·HCl (mol wt 95.5, at a concentration of ~1 M, see Russin et al., 1974) and LBTI (mol wt 9900, twofold molar excess). Both of these inhibitors elicited a similar drop in the quenching constant for trypsin (K_{sv}(eff) equal to 2.0 and 1.9 M^{-1}, respectively). If this drop is a result of the shielding of an active site residue, a much smaller change would have been expected with guanidine and a larger drop with LBTI. The more reasonable interpretation is that a structural change throughout the protein occurs upon inhibitor binding.

Relationship of Emission Spectra to Exposure. The fluorescence maxima (λ_{max}) of tryptophanyl residues in native proteins commonly ranges from about 325 to 350 nm, with azurin having an unusually blue emission at 308 nm. It has often been speculated that there may be some relationship between the λ_{max} and the exposure of tryptophan in a protein (Teale, 1960; Burstein et al., 1973). This is based on the fact that denatured and unfolded proteins have a red (~350 nm) emission, and that in solvents of low dielectric constant, such as dioxane, the λ_{max} of indole is blue shifted (~320 nm). The theory is that surface residues that are exposed to water would fluoresce red, and those buried in a relatively apolar interior region of a protein would appear blue. Alternatively, a tryptophan might fluoresce blue if it were sandwiched in a rigid portion of a protein matrix, even if its microenvironment were not particularly apolar. The true, kinetic exposure of a tryptophan, as measured by k_q for acrylamide quenching, is plotted vs. the λ_{max} for the single tryptophan proteins in Figure 6. A crude relationship exists, as can be seen by the smooth curve drawn through most of the data points. The relationship is not linear. For example, a relatively large change in the λ_{max} occurs between RNase T_1 and nuclease as compared to the k_q change. In this range, a pronounced red shift in the emission accompanies a rather small, incremental deshielding of the indole ring (Longworth and Battista, 1970). HSA at pH 5.5 falls conspicuously off the curve. It is also of interest to note that the exposure (k_q) of the tryptophan in HSA in the F form (pH 2.5) is greater than the exposure in the N form (pH 5.5) despite the fact that the protein fluoresces bluer in the F form. This is evidence that the λ_{max} cannot be relied upon to predict the exposure of a residue. Other factors, such as specific interactions between the indole ring and polar groups on the protein, or the presence of water molecules in the protein's interior may play a part in determining the position of the fluorescence spectrum.

For most of the proteins, a small blue shift in the fluorescence spectrum was observed with quenching. Some extreme examples are lysozyme and pepsin. For these proteins a blue shift of approximately 5 nm was found at about 50% quenching. The most probable explanation for this shift lies in the heterogeneity of the fluorescence of these multi-tryptophan proteins (Lehrer, 1971; Ostashevskii et al., 1973). Those residues that fluoresce at longer wavelengths would be preferentially quenched since they are the most exposed. A small, but discernible blue shift (1-2 nm) has also been observed for certain of the single tryptophan proteins, HSA, and nuclease. Heterogeneity of the fluorescence due to sample impurity or multiple forms of the protein might also explain the blue shift for these proteins. However, a more attractive explanation is that newly formed excited indole rings in proteins undergo relaxation with solvent molecules and other polar groups in their immediate environment at a rate competitive with the collisional quenching reaction. The relaxation processes lead to a red shift in the fluorescence of a tryptophan residue (Grinvald and Steinberg, 1974; Brand and Gohlke, 1971; Bakshiev et al., 1966). Therefore, as the fluorescence is quenched, emission from the bluer, less relaxed fluorophors becomes dominant.

Comparison to Other Techniques. In many ways, fluorescence quenching is an ideal probing technique from both an interpretive and experimental point of view. Because exposure can be reported by mere collision with the excited state of a tryptophan before it fluoresces, the quencher does not have to be in constant contact with the fluorophor, as is the case with solvent perturbation and related techniques (Steiner et al., 1964; Hinman et al., 1974). (Quenching by the static mechanism is more analogous to the solvent perturbation type methods in that a steady-state concentration of quencher in the vicinity of the fluorophor is required.) Consequently, a much lower total concentration of the probing agent is needed. Since the technique is basically a fluorescence experiment, only a very small amount of protein is required (less than a milligram), and use of 295 nm as the excitation wavelength avoids any interference from tyrosine residues in the protein. In contrast to chemical modification studies (Kronman et al., 1967), quenching with acrylamide leads to ground-state molecules, so that the protein is not permanently damaged, and the reaction itself does not induce a structural change in the protein. The only matter of concern is whether or not the protein is denatured by the presence of acrylamide in the solution. In our experiments, we have observed no indications of denaturation (i.e., red shift of emission spectrum, anomalous inflections

in the Stern–Volmer plot, or loss of enzymatic activity) even on occasions when 1 M acrylamide was employed. Previously, full activity of trypsin and RNase T_1 in the presence of acrylamide has been reported (Sellers and Ghiron, 1973; Eftink and Ghiron, 1975). For pepsin and chymotrypsin we also have observed only a 15% or less alteration in the activity when assayed in the presence of about 0.5 M acrylamide.

The solvent perturbation technique developed by Herskovitz and Laskowski (Herskovits, 1967) has heretofore been perhaps the most valuable method for determining the exposure of aromatic amino acids. With this technique the total fractional exposure of tryptophanyl residues can be evaluated. One might expect this method and fluorescence quenching to yield similar estimates of exposure for single tryptophan proteins. Of the two methods, acrylamide quenching appears to be the more sensitive probe, especially for buried residues. Solvent perturbation type studies perceive the residue in nuclease to be completely buried (Cuatrecasas et al., 1968). Similar studies with RNase T_1 and azurin would not be expected to be able to distinguish between the burial of the tryptophans in these proteins and that in nuclease. On the other hand, our quenching studies can readily sense the structural differences in these three cases.

A major problem faced in solvent perturbation studies with multi-tryptophan proteins is the inability to clearly distinguish between a few completely exposed groups and the partial exposure of a large number of groups (Kronman and Robbins, 1970). Acrylamide quenching studies can partly overcome this problem, through the categorization discussed above. For lysozyme and chymotrypsin, some residues are probably much more exposed than others; for proteins like trypsin, on the other hand, there does not appear to be a great difference in the exposure of the various residues. The average degree of exposure of the fluorescing residues in a multi-tryptophan proteins is revealed directly by the magnitude of k_q(eff). From our quenching studies it is clear that the groups in pepsin are more exposed than those in trypsin, which, in turn, are more exposed than those in aldolase. However, it must be realized that the quenching technique does not necessarily weigh all the residues in a multi-tryptophan protein equally. Those with the largest fluorescence yield dominate the study. Nonfluorescent residues do not contribute at all. In some cases this might be misleading. If the fluorescence of a given protein comes predominately from a single fluorophor, the effective exposure (k_q(eff)) that would be reported would be biased toward that particular residue. Quenching studies *cannot* be used to assign the number of "exposed" residues in a protein.

In light of the above discussion, it is interesting to consider the data for pepsin. From the magnitude of k_q(eff) we see that the fluorophors in pepsin are fairly exposed, as compared to those in other proteins. Since the Stern–Volmer plot is linear, all the fluorophors must have a similar accessibility to the quencher. But this does not mean that all five of the tryptophans are exposed. Solvent perturbation studies, on the other hand, reveal only about half of the five tryptophans to be exposed (Herskovits and Sorensen, 1968). By considering both of these studies one might conclude that some of the residues in pepsin are buried and nonfluorescent. Those residues that do fluoresce must be physically exposed to the solvent to a degree that is about 40% that of a fully exposed residue, such as in ACTH.

Concluding Remarks

Previously, biochemists have been able to experimentally describe the positioning of tryptophanyl residues in proteins only with very qualitative terms, such as "exposed", "buried", or "partially exposed". Fluorescence quenching studies with acrylamide provide a kinetic yardstick for surveying the topography of these residues in proteins in a much more quantitative fashion. The degree of exposure is directly measured by the magnitude of k_q, which is the frequency at which a probing molecule the size of acrylamide encounters the fluorophor. The static parameter, V, is an estimate of the steady-state population of quencher molecules adjacent to the residue, and thus provides a second description of its exposure. The location of an indole ring in a globular protein can be visualized by comparison of the k_q and V values to those for the extreme cases for azurin and ACTH.

The familiar portrayal of the structure of a protein that is provided by x-ray crystallography is a static one. Most biochemists are inclined to visualize the exposure of a group in a static sense as well (Lee and Richards, 1971). However, it must be realized that proteins in solution are not perfectly rigid structures. Certain regions of a protein may have quite a bit of flexibility. A residue that is buried on the time average basis may occasionally become exposed as a result of local or large scale conformational fluctuations of the protein. As discussed in the case of RNase T_1 (Eftink and Ghiron, 1975), a probing molecule, such as acrylamide, might be able to diffuse stepwise through a protein matrix, facilitated by thermal fluctuations in the macromolecule, to meet a buried tryptophanyl residue. We need, therefore, to consider not only the time averaged exposure of a residue, but its dynamic exposure as well. Fluorescence quenching studies with acrylamide do just this. Not only does the probe sense the existence of an indole ring on the surface of a protein, but it can report how deeply a residue is buried within a protein as well.

Acknowledgments

We express our thanks to Dr. J. W. Longworth, Oak Ridge National Laboratory, Oak Ridge, Tenn., not only for allowing us to use his fluorescence lifetime instrument, but for the assistance that he and S. Stevens, Oak Ridge National Laboratory, provided in analyzing our lifetime data. We thank Dr. J. Franz, Department of Biochemistry, University of Missouri, for the use of his spectrofluorometer.

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