Exposure of Tryptophanyl Residues and Protein Dynamics†

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ABSTRACT: The acrylamide quenching reaction is shown to be very discriminating in sensing the exposure of fluorescing tryptophanyl residues in globular proteins. The quenching rate constants for some proteins, such as aldolase and human serum albumin, are reported to be independent of the solvent viscosity, indicating that the reaction is limited by penetration of the quencher through the protein matrix. Temperature-dependent studies are performed to determine the activation energy and entropy for the penetration of acrylamide into these proteins. The tryptophanyl residues in aldolase are shown to be shielded by a large activation energy barrier, while the single residue in human serum albumin is shielded by a large activation entropy barrier. These parameters characterize the nature of the protein matrix enveloping the fluorophors.

In a globular protein, the degree to which an amino acid side chain is exposed to the solvent will depend on just how well it is shielded by the surrounding protein segments. The exposure of a residue can be judged from x-ray crystallographic information (Lee & Richards, 1971). In this manner, however, one obtains only a static view of the positioning of various groups. In solution, protein molecules will be subject to a wide range of conformational fluctuations (Linderstrom-lang & Schellman, 1959; Weber, 1975). As a result of this mobility, a residue that appears buried in the crystal may become periodically exposed in the solution state. The frequency at which such residues become "dynamically exposed" will depend on the nature of the surrounding protein fabric. If several layers of protein fabric blanket a residue, or if the layers are very resistant to disruption, encounters between the buried group and solvent molecules may be very infrequent.

An excellent way to experimentally determine the degree of exposure of tryptophanyl residues in solution is by fluorescence quenching (Lehrer, 1971). Using a neutral probe, acrylamide, the degree of exposure of this residue in a number of proteins has been measured in terms of a bimolecular rate constant for collision between the probe and the indole side chains (Eftink & Ghiron, 1976a). A study of particular interest is that with RNase T_1 (Eftink & Ghiron, 1975). The fluorescence of the supposedly buried tryptophanyl residue in this protein was quenched by acrylamide with a surprisingly large rate constant. The argument was made that in order for collision between acrylamide and the buried indole ring to occur, fluctuations in the protein matrix must facilitate the inward movement of the quencher. The activation energy for the quenching process was very large and characterized the dynamics of the protein. The purpose of the present work was to study the manner by which tryptophanyl residues in other proteins become dynamically exposed to acrylamide.

Experimental Section

Materials. The proteins used in this study are the same as those previously described (Eftink & Ghiron, 1976a). The proteins were dissolved in the buffer solutions listed in Table I. *N*-Acetyl-L-tryptophanamide (*N*-AcTrpNH)[1] was obtained from Fox Chemical Co. Acrylamide was recrystallized from ethyl acetate and an 8 M stock solution prepared (both in water and in an equivolume glycerol–water mixture). Glycerol was spectoquality from Matheson Coleman Bell. Fructose 1,6-bisphosphate, hydrazine sulfate, and 2,4-dinitrophenylhydrazine were obtained from Sigma Chemical Co.

Quenching Analysis. Acrylamide fluorescence quenching data can be analyzed according to the modified Stern–Volmer equation (Eftink and Ghiron, 1976b, and references therein):

$$\frac{F_0}{F \exp(V[Q])} = 1 + K_{sv}[Q] = \frac{\tau_0}{\tau} \qquad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, V is the static quenching constant, and K_{sv} is the dynamic quenching constant. K_{sv} is equal to $k_q\tau_0$, where k_q is the rate constant for the quenching reaction (and is an index of exposure of fluorophors in proteins) and τ_0 and τ are the fluorescence lifetimes of the fluorophor being studied in the absence and presence of quencher. V is related to the probability of finding a quencher molecule in the immediate vicinity of the fluorophor at any time (Eftink & Ghiron, 1976b).

For proteins containing more than one tryptophanyl residue, analysis of quenching data with the above equation is generally not feasible due to the heterogeneity of the emission. In such cases the initial slope of plots of F_0/F vs. [Q] is taken as an effective dynamic quenching constant (Eftink & Ghiron, 1976a).

Methods. Fluorescence intensity measurements were performed on a Farrand Mark I spectrofluorometer. For temperature-dependence studies, a Perkin-Elmer MPS-3 or a Baird-Atomic SF-1 spectrofluorometer was used. The K_{sv} for acrylamide quenching of several proteins was determined from 5 to 40 °C. The procedure for studying the temperature dependence of K_{sv} ($K_{sv}(T)$) is described in Eftink & Ghiron (1975, 1976b).

It was previously demonstrated that acrylamide does not seriously perturb the native conformation of proteins by noting the full retention of enzymic activity of a number of proteins in the presence of this compound. In the present study, this

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[1] Abbreviations used are: ACTH, adrenocorticotropin; LADH, horse liver alcohol dehydrogenase; NAcTrpNH, *N*-acetyl-L-tryptophanamide; HSA, human serum albumin; RNase T_1, ribonuclease T_1; Tris, tris(hydroxymethyl)aminomethane.

point requires further consideration. One must be concerned as to whether acrylamide denatures the proteins at the higher temperatures. If denaturation were to occur, a mistakenly large temperature dependence of k_q (and possibly a nonlinear Arrhenius plot) would be observed.

As will become clear in the Results section, the proteins for which these concerns are most relavant are RNase T_1, aldolase, and HSA. For RNase T_1, the Stern–Volmer plot is linear up to 0.8 M acrylamide at 40 °C. If the denaturation were to occur at the higher acrylamide concentrations, an abrupt upward curving plot would have been expected (Eftink et al., 1977). The Stern–Volmer plots for HSA, although not linear due to static quenching, have a similar shape at all temperatures as seen in Figure 2.

The results for aldolase, however, show an upward curvature at higher temperatures suggesting denaturation. Aldolase is the only protein for which we have observed signs of denaturation by acrylamide. This is not surprising since aldolase is a multisubunit protein and because very high acrylamide concentrations were used due to the low K_{sv}. In auxiliary studies it was found that full activity of aldolase was retained even when assayed in the presence of 1 M acrylamide at 40 °C according to the procedure of Sibley and Lehninger (1949). However, difference spectroscopy studies at 40 °C show a decrease in absorbance at 294 nm at acrylamide concentrations above 0.5 M, suggesting an increase in tryptophanyl exposure. The Stern–Volmer plot for aldolase at 40 °C also shows a deviation from linearity above 0.5 M acrylamide (see Figure 2). Only the linear portion of the data was used to obtain a K_{sv} value. The above information suggests that activity measurements may not be the best criterion for the retention of a native conformation. The presence of substrate may stabilize the protein in the active state.

Acrylamide is also known to react with sulfhydryl groups in proteins (Cavins & Friedman, 1968), but the reaction is so slow (half-time $\simeq$ 1 h in denatured, reduced serum albumin) that it is of no consequence in the present studies.

For most of the proteins, the fluorescence in the absence and presence of a given amount of quencher was used to find $K_{sv}(T)$ as given by $[(F_0/F) - 1]/[Q]$. For cases in which V is significant (HSA, *N*-AcTrpNH, and, to a lesser extent, the other single tryptophan containing proteins) it was included in the analysis. Complete Stern–Volmer plots were obtained at each temperature for HSA because of its large V. No temperature dependence of V was found for the quenching of HSA (or *N*-AcTrpNH; Eftink & Ghiron, 1976b).

For the K_{sv} values, the temperature dependence of k_q can be found provided the fluorescence lifetime, τ_0, can be estimated as a function of temperature. This can be done by assuming the proportionality of τ_0 to the fluorescence yield, Φ (Badley & Teale, 1969; Weinryb & Steiner, 1968). Values of Φ for the proteins at 25 °C were taken from Longworth (1971). The temperature dependence of Φ was obtained by warming a protein solution in a cuvette to 40–45 °C and observing the change in the fluorescence intensity as the temperature was allowed to slowly drop. From a plot of $(\Phi^{-1} - 1)$ vs. $1/T$, values of the Φ (and hence τ_0) could be determined at any given temperature.

Fluorescence lifetime measurements at 25 °C were made as previously described (Eftink & Ghiron, 1976a) in the laboratory of J. W. Longworth. When noted in the text, literature values for the lifetime of certain proteins are used.

For the viscosity studies, the proteins were dissolved in buffers containing 50% glycerol–water. Quenching was performed by adding acrylamide from a glycerol-containing stock solution. To correct for the possible change in fluorescence lifetime, τ_0, in the presence of glycerol, the relative fluorescence yield, Φ(50% glycerol)/Φ(water) (measured as the ratio of the fluorescence intensity of a protein solution including glycerol to that in the absence of glycerol), was determined. The ratio of the collisional quenching constants (K_{sv}(water)/K_{sv}(50% glycerol)) was then divided by the relative fluorescence yield to obtain the ratio of the quenching rate constants.

TABLE I: Acrylamide Quenching Parameters for Proteins.[a]

	K_{sv} (M^{-1})	V (M^{-1})	τ_0 (ns)	$10^{-9}k_q$ (M^{-1} s^{-1})
N-AcTrpNH	17.5	2.0	2.8	6.5
ACTH[b]	13.5	1.0	3.1	4.2
Glucagon[c]	10.5	1.0	2.8	3.7
Monellin[b]	5.2	0.3	2.6	2.0
β-Trypsin[d]	2.4		2.2	1.1
HSA, pH 2.5[e]	3.3	0.6	3.3	1.0
Nuclease[f]	5.2	0.25	5.0	1.0
HSA–0.2 M KCl[g]	5.4	0.5	(6.0)[i]	0.9
HSA, pH 5.5[g]	3.3	0.8	6.0	0.55
RNase T_1[b]	1.0		3.5	0.25
Aldolase[g]	0.2		2.3[j]	0.10
Auzurin[h]	~0		4.0[k]	<0.05

[a] Studies at room temperature (~25 °C); excited at 295 nm; fluorescence monitored at the emission λ_{max}. The estimated limits of error for the assigned V values are about ±25%; this leads to a variability of the resulting K_{sv} of only about 10%. [b] pH 7.0, 0.01 M Tris buffer. [c] pH 6, 0.1 M NaCl. [d] pH 3, HCl. [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 5.5, 0.01 M acetate buffer. [h] pH 8, 0.01 M phosphate buffer. [i] Value based on the similarity of Φ to that of HSA, pH 5.5. [j] Value taken from Lakowicz & Weber (1973). [k] Value taken from Grinvald et al. (1975).

Results and Interpretation

Table I lists previously reported acrylamide quenching constants and k_q values for a number of proteins (see Eftink & Ghiron (1976a) for added discussion of these studies).[2] Many of the proteins included in Table I possess a single tryptophanyl residue. These proteins have been emphasized in the present work, since, having a single emitting center, their analysis is simplified. Although aldolase, β-trypsin, and pepsin are multi-tryptophan-containing proteins, their Stern–Volmer plots are linear and therefore can be considered to have a number of fluorophors that have a similar degree of exposure. Aldolase is of particular significance; its low K_{sv} indicates that all of its fluorophors must be buried.

The measurable k_q values range from 4×10^9 to $\sim 5 \times 10^7$ M^{-1} s^{-1}. The large values found for the polypeptides ACTH and glucagon are the magnitude expected for collision with a fully exposed residue (Eftink & Ghiron, 1976a). The lower

[2] The argument identifying k_q as a collisional rate constant was made by Eftink & Ghiron (1976a) based on the observation that the fluorescence lifetime, τ, of a number of proteins dropped in the presence of acrylamide. The fluorescence decay rate of HSA was also observed to increase in the presence of acrylamide, indicating that dynamic quenching occurs. For HSA, however, the analysis of lifetime data is complicated by the nonexponentiality of the decay (deLauder & Wahl, 1971). Nevertheless, the decrease in the average (single component fit) lifetime for HSA produced by a given concentration of acrylamide was less than expected for the F_0/F ratio. This is in agreement with our assignment of a large static quenching component for HSA. (A similar discrepancy of τ_0/τ and F_0/F data for HSA has also been noted by Lakowicz & Weber (1973) in their O_2 quenching studies.) Due to the small quenching constant of aldolase, fluorescence decay measurements of this protein in the presence of quencher were not attempted. At acrylamide concentrations high enough to produce a measurable drop in τ, the absorbance at the excitation wavelength would have been so large as to make such measurements impractical.

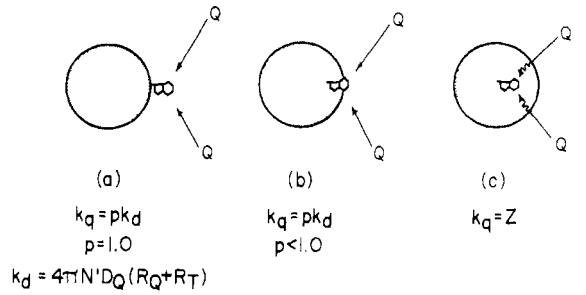

FIGURE 1: Models for acrylamide quenching of tryptophanyl residues in proteins: (a and b) static model; (c) dynamic model.

TABLE II: Viscosity Dependence of Acrylamide Quenching of Proteins.[a]

	K_{sv} (M^{-1})			
	Water	50% glycerol	Φ(g)/Φ(w)	k_q(w)/k_q(g)
N-AcTrpNH	17.3	5.4	1.35	4.5
ACTH	13.0	6.0	1.7	3.6
Glucagon	10.5	5.0	1.7	3.5
Pepsin	9.5	3.5	1.1	3.0
Monellin	4.2	2.2	1.35	2.6
Nuclease	5.2	2.4	1.2	2.6
HSA, pH 2.5	3.4	2.1	1.2	2.0
β-Trypsin	2.4	1.9	1.25	1.6
HSA-0.2 M KCl	5.1	4.5	1.2	1.4
HSA, pH 5.5	3.2	2.9	1.15	1.3
RNase T_1	1.1	0.9	1.0	1.2
Aldolase	0.5	0.55	1.25	1.1

[a] Quenching studies at room temperature. The proteins were studied under the same conditions described in Table I.

values for the other proteins reflect the varying degree to which the indole rings are folded into the globular structure of the proteins. The manner in which shielding by the protein might lead to a reduction in k_q can be explained according to two models as depicted in Figure 1.

Static Model. The reduction of k_q could simply result from the lowering of the collision cross-section of the reaction due to partial steric shielding of the indole ring. In this model quenching occurs only by approach of the quencher through the solution to strike the ring. The protein is considered to be a rigid sphere. The extent of the steric burial is described by the factor p, such that $k_q = pk_d$. The diffusion limited rate constant k_d is equal to $4\pi N' D_Q(R_F + R_Q)$, where N' is Avagodro's number per millimole, R_Q and R_F are the encounter radii of the quencher and indole ring, respectively, and D_Q is the diffusion coefficient of the quencher. The diffusion coefficient of the fluorophor attached to the macromolecule is assumed to be negligibly small.

Dynamic Model. According to this interpretation, the protein matrix is considered to have fluid characteristics. The quenching of surface residues would proceed by the diffusion of quencher through the solution as before. For partially buried residues, however, the path taken by the quencher might be through the solution or through parts of the protein itself. In the extreme case of an indole ring that is completely surrounded by other segments of the protein, quenching could occur only by penetration of the probe into the globular structure. Movement of the probe through this matrix would be facilitated by fluctuations in the structure of the protein. Fluctuations leading to the formation of free volume elements (holes) would allow for the stepwise inward movement of the

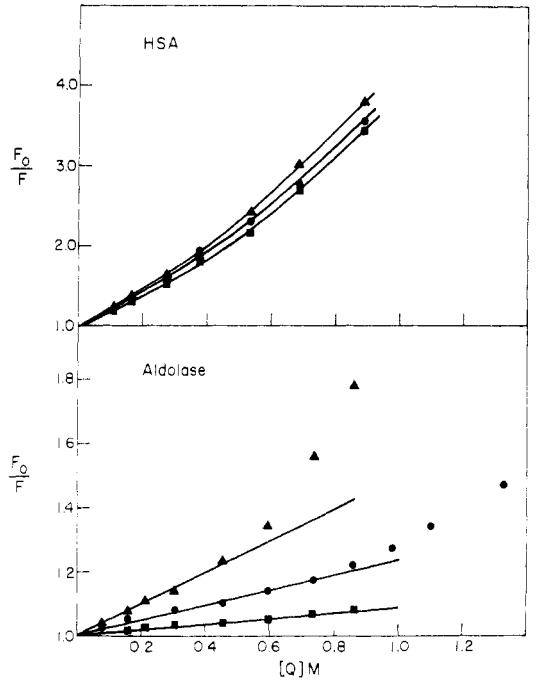

FIGURE 2: Acrylamide quenching of HSA and aldolase as a function of temperature. Top: HSA (0.01 M NaOAc buffer, pH 5.5), (▲) 40 °C; (●) 25 °C; (■) 7 °C. Bottom: aldolase, (▲) 40 °C; (●) 25 °C; (■) 16 °C.

quencher. The quenching rate constant, k_q, would then represent the frequency, Z, at which holes or channels the size of the probe become available (Weber, 1975).

Viscosity Dependence. The low values of k_q found for certain of the proteins might, therefore, be explained in two ways. To decide which interpretation is most appropriate, an experiment was performed in which the viscosity, η, of the solution was increased. According to the first model, the rate constant for quenching should vary inversely with the bulk viscosity (since $k_q \propto D_Q$). If, however, diffusion through the protein limits the quenching rate, an increase in η should not alter k_q to any extent. Table II lists the quenching constants obtained for aqueous and 50% glycerol-water solutions. At this percentage of glycerol, the viscosity is about 5 times that of water. According to the static model, a corresponding drop in k_q would be expected. For the exposed residues in ACTH and glucagon a large drop in k_q is observed (k_q(water) = 3.5k_q(glycerol)). Continuing down the list one finds that the ratio k_q(water)/k_q(glycerol) becomes progressively smaller, approaching a value of 1.0 for RNase T_1, aldolase, and HSA. The lack of η dependence found in these cases can only be explained in terms of the dynamic model for proteins. For these proteins, k_q must be limited not by diffusion through the solvent (k_d), but by penetration of the protein matrix.

Temperature Dependence. Although a protein matrix is not a homogeneous fluid, one might still attempt to characterize its structural/dynamic properties in terms of an activation energy, E_a, for the inward diffusion of the probe. The temperature dependence for the quenching of a number of proteins was studied as previously described for RNase T_1 (Eftink & Ghiron, 1975). As examples, the Stern-Volmer plots for the quenching of HSA and aldolase at various temperatures are shown in Figure 2. Arrhenius plots of k_q vs. $1/T$ were constructed (examples shown in Figure 3) and activation energies, E_a, and activation entropies, $\Delta S^{\ddagger}$, were obtained according to:

$$k_q = \frac{kT}{h} \exp(\Delta S^{\ddagger}/R) \exp(-E_a/RT) \qquad (2)$$

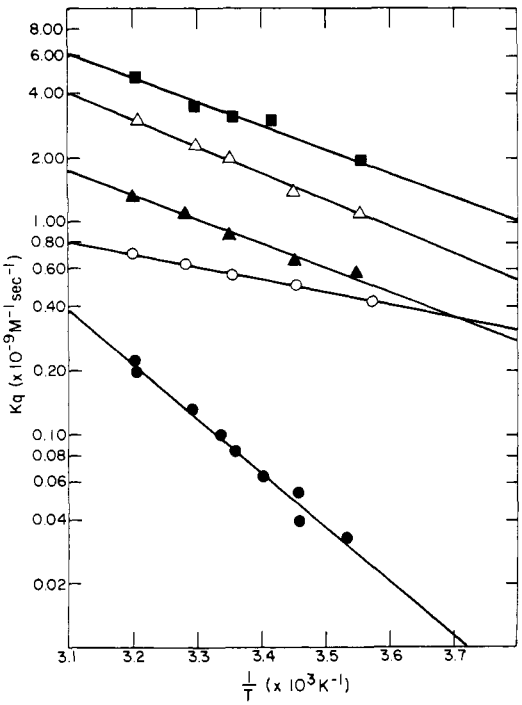

FIGURE 3: Arrhenius plots for the acrylamide quenching of aldolase (●), HSA, pH 5.5 (○), nuclease (▲), monellin (△), and glucagon (■). Conditions are given in Table I.

$$E_a - T\Delta S^{\ddagger} = \Delta G^{\ddagger} = -RT \ln (k_q h/kT) \qquad (3)$$

Values of E_a, $\Delta S^{\ddagger}$, k_q, and the activation free energy, $\Delta G^{\ddagger}$, are listed in Table III. A wide range of E_a and $\Delta S^{\ddagger}$ values are found.[3]

To aid in interpreting these results, the data are plotted as E_a vs. $\Delta S^{\ddagger}$ for each protein in Figure 4. The lower dotted line is drawn through the combinations of E_a and $\Delta S^{\ddagger}$ that gives a $\Delta G^{\ddagger}$ of 4 kcal/mol at 300 K. This $\Delta G^{\ddagger}$ is associated with the upper limit for the quenching rate constant obtainable in aqueous solution ($k_q = 8 \times 10^9$) at room temperature (according to eq 3). The higher dotted line represents $\Delta G^{\ddagger} = 7$ kcal/mol ($k_q = 5 \times 10^7$) which sets the approximate lower limit that can be experimentally measured by this particular method. The solid line represents $\Delta G^{\ddagger} = 5$ kcal/mol for which $k_q = 1.5 \times 10^9$ (slope of line is 300 °C). The greater the displacement of the experimental points above the solid line, the greater the degree of burial of the residue (lower k_q); the further below the line, the more it is exposed. The points for most of the proteins are found to cluster around $E_a = 5$ kcal/mol and $\Delta S^{\ddagger} = 0$ eu. These entries happen to be those that show the largest viscosity dependence for acrylamide quenching (and are deemphasized in the figure). Those proteins showing the least dependence and greatest degree of tryptophan burial also happen to show the most extreme combinations of E_a and $\Delta S^{\ddagger}$ values (and are emphasized in the figure). For these proteins, quenching occurs by dynamic penetration of the quencher through the structure of the protein to reach the protected indole rings. Thus, the E_a and $\Delta S^{\ddagger}$ values are characteristic of the fluidity of the respective protein matrices. RNase T_1 and aldolase have very large E_a and $\Delta S^{\ddagger}$ values, while HSA (pH 5.5 and with KCl) have a much smaller E_a and a large negative $\Delta S^{\ddagger}$.

[3] The data have been expressed in terms of activation energies, instead of activation enthalpies, since the former is more commonly used in biochemical discussion. The latter can be obtained by subtracting 0.6 kcal/mol from the former.

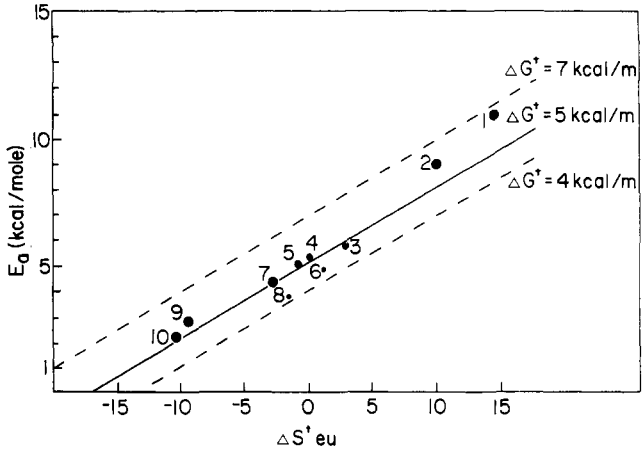

FIGURE 4: Activation energies and entropies for acrylamide quenching of proteins: (1) aldolase; (2) RNase T_1; (3) monellin; (4) nuclease; (5) HSA, pH 2.5; (6) glucagon; (7) β-trypsin; (8) *N*-AcTrpNH; (9) HSA, pH 5; and (10) HSA–0.2 M KCl. The proteins showing the least viscosity dependence are emphasized in the above plot by representation with larger symbols: (●) $k_q(w)/k_q(g)$ between 1.0 and 2.0; (•) $k_q(w)/k_q(g)$ between 2.0 and 3.0; and (·) $k_q(w)/k_q(g)$ greater than 3.0.

TABLE III: Activation Energy, Entropy, and Free Energy for Acrylamide Quenching of Proteins.[a]

	E_a (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	$\Delta G^{\ddagger}_{300\,°C}$ (kcal/mol)	$10^{-9}k_q^{300\,°C}$ ($M^{-1}s^{-1}$)
Aldolase	11.0	14.5	6.65	0.10
RNase T_1	9.0	10.0	6.0	0.25
HSA, pH 5.5	2.7	−9.5	5.55	0.55
HSA–0.2 M KCl	2.1	−11.0	5.3	0.9
Nuclease	5.3	0.5	5.15	1.0
HSA, pH 2.5	5.0	−0.5	5.15	1.0
β-Trypsin	4.3	−2.3	5.0	1.1
Monellin	5.7	2.8	4.85	2.0
Glucagon	4.8	1.0	4.5	3.7
N-AcTrpNH	3.7	−1.4	4.1	6.5

[a] The proteins were studied under the same conditions described in Table I.

The tryptophan(s) in RNase T_1 and aldolase are *energetically* buried by the protein. For an acrylamide molecule to collide with the indole ring(s) a large energy barrier must be surmounted. In order to form and propagate the holes required to facilitate the rapid migration of the probe, intramolecular interactions in the protein must be broken or distorted. The cohesive interactions resisting the fluctuations are rather strong, amounting to 9–11 kcal/mol. An energy barrier of this size might be attributed to the breaking of several hydrogen bonds or a large number of weaker van der Waals contacts. With such a large E_a barrier one is surprised to find the frequency of the structural fluctuations ($Z = k_q$) to be so large. However, quenching is observed due to the compensatingly large $\Delta S^{\ddagger}$ value (Lumry & Rajender, 1970). The formation of the holes must decrease the order of the protein matrix. Upon breaking the contacts to liberate the free volume, the entropy of the portions of the protein involved must greatly increase. The process might be visualized as a local relaxation or expansion of the matrix. However, the structural fluctuations must be very subtle, since they are proceeding on a nanosecond time scale. Large scale unfolding of a protein normally proceeds more slowly and with a much larger E_a. A picture arises in which the protein is normally held together by strong intramolecular interactions in a very tightly wound

fashion. Occasionally the interactions are broken and the protein becomes loosened, thus forming the paths leading to the interior tryptophan.

The tryptophan in HSA is buried in an entirely different manner. At pH 5.5 (with or without KCl) the residue is *entropically* buried. The E_a barrier for diffusion of acrylamide through the protein is very low (comparable to the E_a for diffusion through a weakly interacting hydrocarbon solvent). This suggests that only weak van der Waals interactions need to be broken to allow the penetration of acrylamide. Despite the fact that the matrix of HSA is not very resistant to the inward flux of the probe, the k_q value is not extremely large because of the negative $\Delta S^{\ddagger}$. The decrease in entropy for the reaction indicates that in order to form the diffusion paths, segments of the protein (or surrounding water molecules) must actually become more ordered. The frequency at which the groups on the protein can become properly aligned so as to form a "channel" thus limits the encounter rate. The longer the required tunnel, the more negative the $\Delta S^{\ddagger}$ would be. This study suggests that the tryptophanyl residue in HSA is surrounded by layers of a very amorphous, sloppy protein matrix. This characterization of HSA is not surprising in light of the difficulty in crystallizing this protein and its "configurational adaptability" in being able to assemble a binding site for a wide variety of ligands (Foster, 1960).

Returning to the points clustered in the center of the graph, note that the E_a for quenching does not differ much from that for diffusion through water (for which E_a = 4 kcal/mol). For this group of proteins it appears that the predominant quenching path is limited by the diffusion of the quencher through the aqueous solvent before colliding with the indole rings. This is in line with the greater η dependence found for these proteins. The fact that the E_a values for the proteins are slightly larger than that found for *N*-AcTrpNH suggests that "structured water" surrounding the macromolecules might hinder the movement of the probe.

Discussion

With the advent of the fluorescence quenching experiment, the concept of the degree of burial of an amino acid side chain in a protein takes on a new meaning. Quenching reactions sense the exposure of a target residue in a random, kinetic fashion. Besides quenching surface residues, the probe may also occasionally quench a residue located beneath the surface by penetrating the somewhat fluid protein matrix. The question then becomes how deeply the residue is buried.

The inward movement of a probe will be facilitated by certain fluctuations in the structure of protein leading to the formation of lattice vacancies. The frequency of these fluctuations will be regulated by the intramolecular interactions which stabilize the protein's globular structure. The breaking of hydrogen bonds, the separation of oily groups, or the expansion of existing packing defects may be involved. As demonstrated by the present study, a residue may be guarded either energetically or entropically by the surrounding protein matrix.

For these quenching studies, the amplitudes of the protein fluctuations are such so as to accommodate a molecule the size of acrylamide. If a smaller quencher were used, smaller holes would need to be formed and one would expect the free-energy barrier for diffusion into the protein to be less prohibitive. This could be the case for the study by Lakowicz and Weber (1973) using O_2 as a quencher of protein fluorescence. These workers found O_2 to be able to quench even buried tryptophanyl residues at a very large rate. In analogous studies on the diffusion of gases through polymers, it is commonly found that as the size of the diffusate increases, the diffusion coefficient drops and the activation energy for diffusion increases (Kumins, 1965; Meares, 1965). Transport through polymers has also been explained in terms of the movement of "holes" as a result of polymer segmental mobility.

Much evidence suggests that lattice vacancies exist, or can be readily formed, in globular proteins (Kuntz, 1972; Richards, 1974; Wishnia, 1969; Lumry & Rosenberg, 1975). A number of spectroscopic studies have detected molecular events occurring within proteins on the nanosecond time scale (Hull & Sykes, 1975; Visscher & Gurd, 1975; Likhtenshtein et al., 1972) (see also Gellin & Karplus, 1975). As discussed by Cooper (1976), the occurrence of rapid structural fluctuations is consistent with our present understanding of the thermodynamics (i.e., heat capacity and compressibility) of proteins in solution.

However, proteins may be found that have a restricted conformational mobility. In such cases one would expect the inward diffusion of a quenching probe to be hindered. It was reported earlier that no acrylamide quenching of the fluorescence of the single tryptophan in azurin could be detected (Eftink & Ghiron, 1976a). The k_q was estimated to be less than 5×10^7 M^{-1} s^{-1}, placing it above the top dotted line in Figure 4. One can only speculate as to the reason for the extensive burial of this residue. It could be energetically fortified inside a very rigid protein, as in the case of RNase T_1, or it could be entropically insulated as in HSA (due to the small size of azurin, the former argument is favored). In any case, the combination of E_a and $\Delta S^{\ddagger}$ for azurin must be such to place it above the $\Delta G^{\ddagger}$ = 7 kcal/mol line, thus deeply burying the fluorophor.

There is also evidence that certain tryptophanyl residues in the protein horse liver alcohol dehydrogenase (LADH) are extremely well buried. From room temperature phosphorescence quenching studies, Saviotti and Galley (1974) concluded that in the native state, LADH possesses a class of residues that could not be quenched by O_2. This then could be an example of an indole side chain that is buried much more than those in the proteins surveyed by Lakowicz and Weber (1973) in their O_2 fluorescence quenching study. Acrylamide quenching studies are also consistent with the proposed burial of a class of tryptophanyl residues in LADH. A downward curving Stern–Volmer plot is obtained indicating that some of the fluorescence from this protein is very difficult to quench (Zajicek & Ghiron, unpublished work). Saviotti and Galley have interpreted their phosphorescence studies in terms of a model in which the buried residues in the native protein are completely shielded, with quenching of the long-lived triplet state of these indole rings occurring only as a result of a large scale unfolding of the protein (exposing the residues). If this is the case, the fluctuations in the structure of the protein needed to facilitate the phosphorescence quenching process would be very drastic, large E_a ones, much larger and slower than the small amplitude fluctuations discussed for the present work. Thus, for LADH (and possibly azurin) the segments of the protein may be packed so well that a rapid stepwise inward diffusion of the quencher is prohibited ($\Delta G^{\ddagger}$ for the small fluctuations is too large).

In light of the present studies it is interesting to consider the significance of the static quenching constant, V. (See Table I for a list of values.) This parameter is a measure of the ability of a quencher molecule to exist next to an indole ring in solution, and thus provides a second means of assessing the exposure of a residue in a protein. It was noted for most single tryptophanyl containing proteins that there was an agreement between the V and k_q values (Eftink & Ghiron, 1976a).

However, for HSA the V value was much larger than that expected from its low k_q. The large V indicates that the probability of finding an acrylamide molecule adjacent to the residue in this protein is very favorable, even though the fluorophor is buried within the protein (as indicated by its low k_q). For another protein having a buried tryptophanyl residue, RNase T_1, the V value is very small (essentially zero). Although acrylamide can penetrate the matrix of RNase T_1, it must not be able to remain in contact with the buried residue very long. The probe must be rapidly ejected from the interior of this protein in order to restore the stable conformation. For HSA, on the other hand, the quencher apparently is able to remain in the vicinity of the buried residue for a much longer time without greatly upsetting the protein. Acrylamide must be accommodated into "holes" inside this protein. In other studies, it was observed that the hydrophobic probe, trichloroethanol, was very effective at quenching the fluorescence of HSA (Eftink et al., 1977). To account for the high degree of quenching, it was again proposed that the probe was accumulated into "holes" in this protein. It thus appears that HSA possesses domains that are very poorly packed and that can be readily filled by small molecules (such as acrylamide or, to a greater extent, trichloroethanol, due to its hydrophobicity). The low E_a values found in the present studies with HSA are consistent with this view. If HSA possesses regions that are poorly packed and are lacking in strong, directional intramolecular interaction, the matrix would be very flexible and easy to penetrate.

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