Penetration of Small Molecules into Proteins Studied by Quenching of Phosphorescence and Fluorescence†

D. B. Calhoun, J. M. Vanderkooi, and S. W. Englander*

ABSTRACT: Experiments were done to test the ability of a variety of small molecules to quench the phosphorescence and fluorescence of the well-buried and the more accessible tryptophans of horse liver alcohol dehydrogenase (LADH) and alkaline phosphatase (AP). Quenchers studied include acrylamide derivatives, saturated and unsaturated ketones, and inorganic anions and cations. Room temperature phosphorescence of the buried tryptophan in LADH is quenched by many of these agents, with rate constants grouped in a relatively narrow range 5–6 decades below the diffusional limit. The phosphorescent residue in AP is reached several decades more slowly still. Evidently, facile penetration through the proteins by these small molecules does not occur. The ability to quench LADH phosphorescence is independent of the size and charge of the small molecule quenchers, indicating that the tryptophan–quencher contact is governed by some protein opening reaction that is at least several amino acid residues in size. Thus, even the 5-decades-reduced quenching that is observed in LADH is not due to simple protein penetration. Dioxygen quenches the phosphorescence in both proteins at close to the diffusion-limited rate, so that unusual protein rigidity can be ruled out. The fluorescence (but not the phosphorescence) of these same buried tryptophans in LADH and AP could be quenched with rate constants near the diffusional limit by some of the quenchers, namely, those that display a small degree of Forster overlap, pointing to through-space resonance energy transfer rather than physical contact. Evidently, the buried tryptophans project very close to the protein surface. Fluorescence of the more accessible (nonphosphorescent) tryptophans is quenched by many of these agents with the same relatively low rate constant (7×10^8 M^{-1} s^{-1}), independently of quencher size, indicating that these tryptophans are still partially solvent exposed; most protein tryptophans so far studied have even higher rate constants. This sets the scale of solvent exposure vs. quencher accessibility higher than has previously been appreciated. These results cast doubt on the ability of small molecules (except dioxygen) to move into and through native proteins.

Following the work of Lehrer (1971) on the quenching of lysozyme fluorescence by iodide, Lakowicz & Weber (1973a,b) examined the ability of oxygen to quench the fluorescence of a number of proteins, found surprisingly large quenching rate constants varying between 2×10^9 and 7×10^9 M^{-1} s^{-1}, and concluded that oxygen must be able to penetrate into proteins on a nanosecond time scale. Eftink & Ghiron (1975–1977) found that acrylamide could also quench many proteins and that this agent was more sensitive than oxygen to details of protein structure, displaying quenching rate constants spread out over a wider range, and thereupon proposed acrylamide as a "kinetic ruler" that could report the degree of exposure of particular tryptophan residues to solvent. Their results further suggested that acrylamide might provide in its measured quenching rate an index of the actual depth of tryptophan burial, since it appeared to quench even fully buried tryptophans. If so, then acrylamide must be able to penetrate into and move about rapidly within the matrix of proteins. The Eftink–Ghiron approach will clearly have utility for studying tryptophan placement in proteins. In addition, the latter conclusion extends the similar ideas of Lakowicz & Weber (1973a,b) concerning oxygen penetrability into proteins and carries major implications for the structural character and internal dynamical behavior of proteins. However, the reality of acrylamide penetration into proteins is as yet based on inferential arguments, such as the expected dependence of protein penetration rates on solvent viscosity and ambient temperature. Here we reexamine this issue.

Our approach is to study protein tryptophans that are surely buried, namely, the residues in liver alcohol dehydrogenase (LADH)[1] and alkaline phosphatase (AP) that display long-lived phosphorescence at room temperature (Purkey & Galley, 1970; Saviotti & Galley, 1974), and to observe the ability of a variety of potential quenching agents, for example, homologues of acrylamide with increasing size, to quench the fluorescence and the phosphorescence of these residues. If luminescence quenching requires protein penetration, then it can be expected that the ability to move about inside proteins will be a sharp function of quencher size (Richards, 1979). In these studies, we have used as quenchers a number of saturated and unsaturated amides and ketones, some inorganic ions, and oxygen. LADH and AP also have other tryptophans that are not buried, and parallel observations on these proved enlightening.

Materials and Methods

Sample preparation and instrumentation were as described in the preceding paper (Calhoun et al., 1983). The proteins used, alcohol dehydrogenase from horse liver (LADH) and *Escherichia coli* alkaline phosphatase (AP), were also previously described.

Phosphorescence lifetimes and the effects thereon of various quenching agents were measured in a Perkin-Elmer MPF2A fluorometer by closing a mechanical shutter (ca. 1-ms closing time) and recording the subsequent decay of luminescence intensity in time. Fluorescence intensity was measured in the steady-state mode. Quenching data were plotted according to the Stern–Volmer equation as described in the preceding paper. With some of the quenching agents studied here, fluorescence of the different tryptophans in a given protein

† From the Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received August 30, 1982.* This research was supported by National Institutes of Health Research Grants GM 12202 to J.M.V. and AM 11295 to S.W.E.

[1] Abbreviations: LADH, horse liver alcohol dehydrogenase; AP, alkaline phosphatase from *Escherichia coli*; NATA, *N*-acetyltryptophanamide; cinnamamide, 3-phenylacrylamide; mesityl oxide, 4-methyl-3-penten-2-one.

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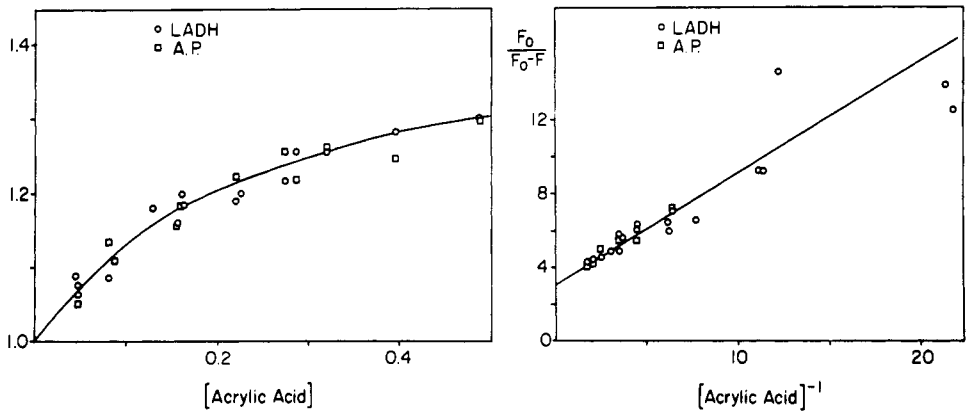

FIGURE 1: Quenching of LADH fluorescence by acrylic acid. Stern–Volmer plot and Lehrer inverse plot displaying fraction of fluorescence that is quenchable.

proved differentially accessible, and this led to grossly nonlinear Stern–Volmer plots. The data were then also plotted according to the Lehrer (1971) inverse equation:

$$F_0/(F_0 - F) = 1/(fK_q\tau_0 Q) + 1/f \quad (1)$$

Here, F_0 and F represent fluorescence intensity in the absence and presence, respectively, of quenching agent at concentration Q, and t_0 is the unquenched lifetime. The fraction of fluorescence that is quenchable, indicated by f, was obtained from the ordinate intercept by extrapolating the straight-line part of the plot to infinite Q, and the quenching rate constant, k_q, was computed from the slope of the line (eq 1).

Eftink & Selvidge (1982) have discussed nonlinear Stern–Volmer plots before. When the exciting wavelength is moved toward the red edge of the tryptophan absorbance, an increasing fraction of the observed fluorescence becomes insensitive to quenching. This is undoubtedly due to the red absorbance shift displayed by the most effectively buried tryptophan, namely, the same tryptophan that displays room temperature phosphorescence [see Calhoun et al. (1983)]. In these experiments, we excited at 300 nm in order to maximize the contribution to overall fluorescence of the most protected tryptophan so that the quenching of its fluorescence and its phosphorescence might be effectively compared. The same experiments also clearly display the fluorescence quenching of the more exposed tryptophans.

A number of the quenching agents studied have significant spectral absorbance at the excitation and emission wavelengths we used. The fluorescence intensity data must be corrected accordingly. Small corrections were also applied for background fluorescence due to some of the quenchers and for sample dilution caused by adding sequential aliquots of quenching agent. The fluorescence cuvette we used had a 4-mm path length in the excitation direction, and the narrow exciting beam was centered at the 5-mm position of the 10-mm length viewed by the emission slit [like Figure 78b in Parker (1968)]. To correct for absorbance attenuation of the exciting beam by added quenchers, we multiplied the measured fluorescence intensity by the factor given in eq 2, obtainable by integration of the decreasing beam intensity through the sample cell.

$$f_{exc} = 2.3(OD)/(1 - 10^{-OD}) \quad (2)$$

Here, OD is the measured optical density at the exciting wavelength (per 4-mm light path). The factor indicated in eq 3 was applied to correct for attenuation of the emitted luminescence,

$$f_{em} = 10^{OD/2} \quad (3)$$

Table I: Quenching Constants for Fluorescence[a]

quencher	NATA[b]	LADH[c,d]	AP[c,d]
O_2	15	3 (exposed only)	0.8 (exposed only)
ionic			
$NaNO_2$	13	2.5[e]	1.3[e]
$NaNO_3$	8	0.4	0.4
NaCl	0	0	0
KI	2	0.2	0.07
CsCl	0.6	0.07	0.04
unsaturated amides			
acrylamide	9	0.8	0.8
bis(acrylamide)	8	1.3	1.3
N-isopropylacrylamide	5	0.14	0.14
acrylic acid	3	0.7	0.7
ketones			
acetone	3	0.6	0.6
methyl ethyl ketone	5	0.6	
methyl vinyl ketone	10	4[e]	4[e]
mesityl oxide	10	4[e]	4[e]

[a] All values are quenching constants (k_Q) in units of 10^9 M^{-1} s^{-1}. [b] τ_0 = 2.8 ns. Stern–Volmer plots show positive deviation from linearity at high concentrations of quenchers. [c] τ_0 = 7.0 ns for exposed Trp in LADH [see Calhoun et al. (1983)], and taken as 5 ns in other cases. [d] Stern–Volmer plots leveled off at high concentrations. k_Q, determined from Lehrer plots, relates to exposed Trp (except as indicated by *e*). [e] Stern–Volmer plots linear; k_q averages in all Trp.

Here, OD is the measured optical density at the emission wavelength on a per centimeter basis since the emitted fluorescence traverses 5 mm of solution. The total correction factor, obtained by multiplying these two and the dilution factor, was applied after background fluorescence contributions were subtracted from the measured intensity. These correction factors permitted measurements over 1 OD with good accuracy, as indicated by the quality of the corrected data in the plateau region and when straight-line Stern–Volmer plots were obtained. No corrections were needed in the phosphorescence experiments which measured decay time rather than intensity.

Results

Fluorescence Quenching. The ability of a number of agents to quench the fluorescence of LADH and AP was studied. These display fluorescence quenching rate constants for fully accessible NATA (*N*-acetyltryptophanamide) close to the diffusion-limited rate (Table I).

With the proteins, most quenchers generated curved Stern–Volmer plots that tend to plateau at an F_0/F value of about 1.4 (e.g., see left panel of Figure 1 for acrylic acid), indicating that some of the tryptophans in these proteins are effectively quenched and others are not. These data when

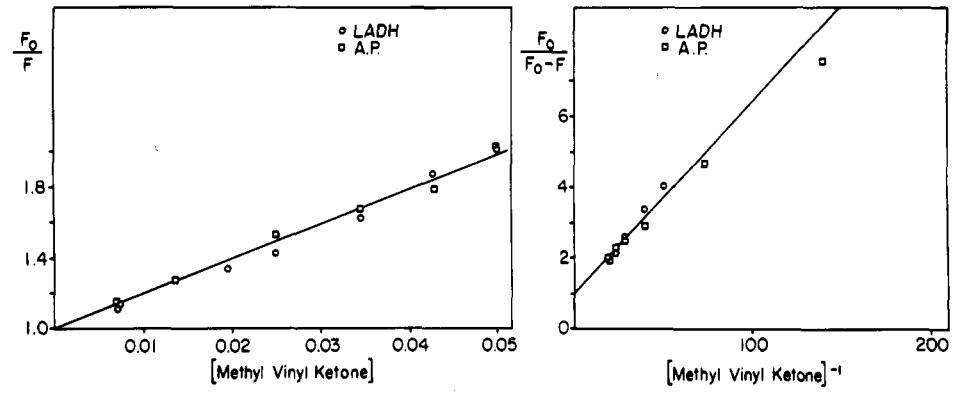

FIGURE 2: Quenching of LADH fluorescence by methyl vinyl ketone. Stern–Volmer plot and Lehrer inverse plot indicating that all the fluorescence is quenchable.

replotted according to eq 1 (right panel of Figure 1) show that the quenchable fluorescence is more or less uniformly sensitive to any given quencher (straight line in Lehrer plot) and accounts for about 30% of the total fluorescence [extrapolated $F_0/(F_0 - F) = 3.3$; unquenched $F/F_0 = 70\%$]. The remaining two-thirds of fluorescence is quenched far less if at all.

Quenchers in this category are the unsaturated amides (electronic analogues of acrylic acid), the saturated ketones (acetone and methyl ethyl ketone), and the nitrate anion, all of which display rate constants for reaching the relatively exposed tryptophans close to 7×10^8 M^{-1} s^{-1} (Table I). Bis(acrylamide), which has two active groups per molecule, is twice as effective on a molar basis but equally effective per amide moiety. The heavy ions I^- and Cs^+ are less efficient but approximately in proportion to their lesser efficiency with NATA. *N*-Isopropylacrylamide appears somewhat blocked for both fluorescence and phosphorescence quenching.

In most cases, fluorescence quenching data for LADH and AP plotted in this way were hardly distinguishable. Evidently their accessible tryptophans have approximately equal degrees of exposure. LADH is a little more sensitive to the ionic quenchers, probably owing to a nonspecific salt effect (see Table II). LADH also displays some unusual sensitivity to acrylamide at high concentration; rather than reaching a plateau level of quenching at $F_0/F = 1.4$, quenching continues to rise slowly past this level. Correspondingly, the Lehrer plot for acrylamide at low concentration is linear as usual, but it turns down toward an ordinate value of 1 as the concentration exceeds 1 M. It appears that acrylamide binds weakly to LADH (Eftink & Selvidge, 1982), and this labilizes the buried tryptophan to quenching.

In summary, a number of quenching agents, though they vary in size and in charge, display similar capability for quenching the more exposed tryptophans of LADH and AP. Their rate constants are only a little lower than that observed for quenching by oxygen (3×10^9 M^{-1} s^{-1}). In contrast, the buried tryptophans of these proteins, though they are reached easily by oxygen (1×10^9 M^{-1} s^{-1}), are less accessible by far to the small molecule quenchers tested.

Spectral Overlap. Some of the agents listed in Table II display an enhanced quenching capability. Nitrite, methyl vinyl ketone, and mesityl oxide are especially effective against NATA and also against the more exposed protein tryptophans. Most strikingly, these agents quench even the protected tryptophans of LADH and AP; their Stern–Volmer plots rise smoothly through the usual limiting value of $F_0/F = 1.4$ (left panel of Figure 2 and Figure 4), and Lehrer plots extrapolate to f values of 1 (right panel of Figure 2), indicating that all the fluorescence is being quenched. These results suggest a

Table II: Quenching Constants for Phosphorescence[a]

quencher	LADH[b]	alkaline phosphatase[c]
O_2	0.6×10^9	1×10^9
ionic		
$NaNO_2$	5×10^5	30
$NaNO_3$	<2	
NaCl	7	<1
KI	1	<1
CsCl	2	<1
unsaturated amides		
acrylamide	2×10^4	<1
cinnamamide[d]	2×10^5	
bis(acrylamide)	9×10^3	<1
N-isopropylacrylamide	2×10^3	<2
ketones		
acetone	<1	<1
methyl ethyl ketone	10	<1
methyl vinyl ketone	1×10^5	300
mesityl oxide[d]	8×10^4	<2

[a] Values shown are quenching constants (k_q) in units of M^{-1} s^{-1}. [b] $\tau_0 = 0.3 \pm 0.05$ s. [c] $\tau_0 = 1.4 \pm 0.1$ s. [d] Cinnamamide is 3-phenylacrylamide; mesityl oxide is 4-methyl-3-penten-2-one.

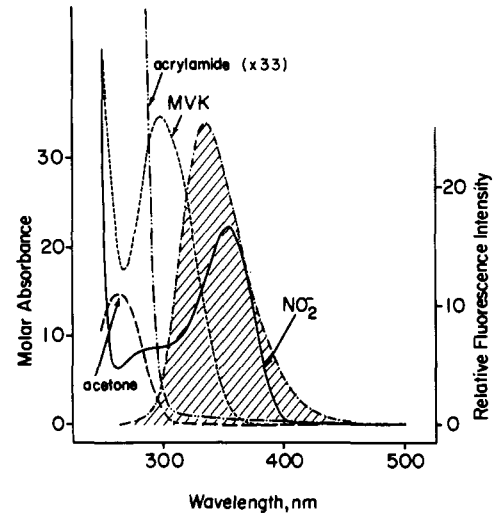

FIGURE 3: Spectral overlap. Fluorescence emission spectrum of LADH (hatched area) and absorption spectra of some quenching agents.

role for resonance energy transfer. Figure 3 compares the fluorescence emission spectrum of LADH with the absorption spectra of some of the quenching agents studied. The good quenchers display significant spectral overlap and when processed through the Forster (1948) energy transfer equations indicate a characteristic transfer distance (R_0) for these agents

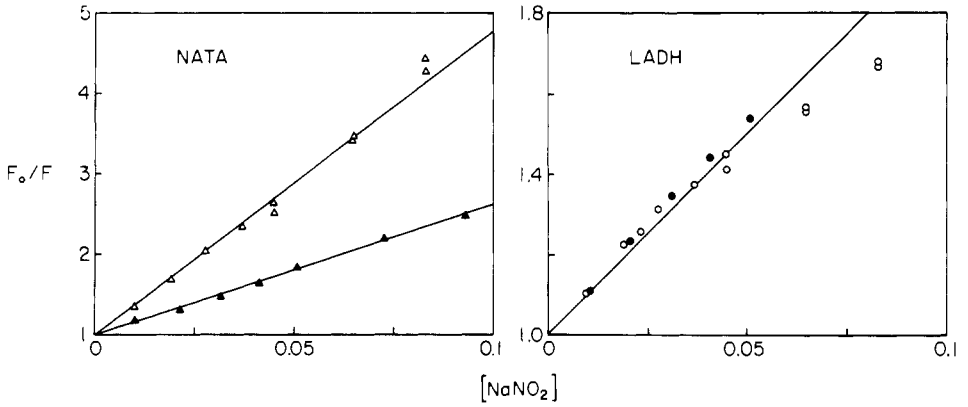

FIGURE 4: Quenching of fluorescence by $NaNO_2$. Stern–Volmer and Lehrer plots indicating fluorescence quenching of NATA and LADH by $NaNO_2$ without glycerol (open symbols) and in 50% glycerol (closed symbols).

of about 10 Å (see Discussion).

In summary, a list of small molecule quenchers reaches the more exposed tryptophans of these two proteins with a common relatively low rate constant and does not detectably quench the tryptophans known to be buried. Some quenchers that do affect the buried tryptophans do so not by collision but by through-space energy transfer. The buried tryptophans in LADH and AP must be close to the protein surface since they are quenched by resonance energy transfer agents having minimal R_0 values; nevertheless, they are wholly inaccessible to the small molecule quenchers on the fluorescence time scale.

Viscosity Dependence. Figure 4 shows some data for the dependence of quenching by nitrite on solvent viscosity, adjusted by making the solvent 50% (w/w) in glycerol. An increase in macroviscosity by 5× [perhaps 2× in microviscosity; compare effect on oxygen found by Calhoun et al. (1983)] has no effect on quenching rate. This suggests that nitrite, which appears to act largely through resonance energy transfer, is in these experiments close to the fast diffusion limit (Thomas et al., 1978). From the equation for diffusional displacement vs. time ($s^2 = 6Dt$), one can compute that the average displacement of solvent nitrite during the tryptophan fluorescence lifetime is about 50 Å, about the same as the average distance between quenching molecules at the nitrite concentrations used here (Figure 4). Thus, the fluorescent tryptophans see essentially the time-averaged concentration distribution of nitrite, and the viscosity-dependent slowing of diffusion has little effect on the quenching due to energy transfer.

Phosphorescence Quenching. The rareness of long-lived phosphorescence in proteins at room temperature and the quality of the phosphorescence emission spectra and single exponential decay observed for LADH and AP (Calhoun et al., 1983) indicate that only one tryptophan in each protein enjoys the special structural environment that can protect the long-lived triplet excited state [see also Purkey & Galley (1970)]. The phosphorescent tryptophan in AP displays a lifetime of 1.4 s at room temperature; the lifetime in LADH is 0.3 s. These are longer than the fluorescence lifetime by 8 decades and immensely extend the possible range of quencher accessibility measurements.

In spite of their special structural protection, these residues can be easily reached and quenched by oxygen in the medium. Both fluorescence and phosphorescence measurements show that oxygen quenches the buried tryptophans with a rate constant of about 1×10^9 M^{-1} s^{-1} (Calhoun et al., 1983; Eftink & Jameson, 1982). Evidently the structure protecting these residues is in no sense totally rigid. Nevertheless, other small molecule quenchers are unable to reach these residues on the fluorescence time scale.

Table II lists a number of (ineffective) fluorescence quenchers that were tested for their ability to quench phosphorescence. Some show measurable quenching ability on the phosphorescence time scale; others are totally ineffective. Phosphorescence quenching involves a contact-dependent excited-state electron exchange in which triplet-state energy levels play a determining role (Turro, 1978). Though exact triplet-state energy levels for the quenchers we studied are not available, differences therein appear to account for the major difference between the better quenchers and the ineffective ones. For example, methyl ethyl ketone is less effective than methyl vinyl ketone by 3–4 decades. These are close structural though not electronic analogues. The smaller homologue acetone is even less effective.

Against LADH, the better quenchers are slower than the diffusional rate by about 5 orders of magnitude, in comparison to which they all fall within a narrow range. There is no correlation between quencher efficiency and molecular size; cinnamamide, one of the best quenchers we found, is nearly the largest, and acrylamide and bis(acrylamide) show similar quenching constants. The common level of efficiency displayed by the better quenchers against LADH therefore appears to reflect some characteristic of the protein itself, or rather of the dynamical distortion of the protein that allows quenchers to contact the buried indoles. Against AP, the two best quenchers we found show some activity (Table II), but this is at a level lower than in LADH by 3 decades or more, and slower than the diffusion-limited rate by about 8 decades. Again, this is in contrast to the easy accessibility of these same tryptophans to dioxygen (Calhoun et al., 1983).

Discussion

The phosphorescence quenching data presented here relate to a single, well-buried tryptophan (per identical subunit) in LADH (Trp-314; see preceding paper) and a similar although as yet unidentified residue in AP. In both proteins, the fluorescent tryptophans divide into two distinct categories, with greater and lesser sensitivity to quenching. The phosphorescent residues undoubtedly represent the ones that are also more protected against fluorescence quenching.

These results bear on several different mechanistic issues. We consider first the more buried tryptophans, studied specifically in the phosphorescence experiments and somewhat selectively, by preferential red-edge excitation, in the fluorescence experiments.

Buried Tryptophans and Fluorescence Quenching. Phosphorescence of the protected tryptophan in LADH is quenched by a variety of agents with rate constants around 10^5 M^{-1} s^{-1}. Some of these agents (nitrite, methyl vinyl ketone, and mesityl

oxide) quench the fluorescence of this very same tryptophan in LADH, and an analogous residue in alkaline phosphatase, on a nanosecond time scale with rate constants of about 1×10^9 M^{-1} s^{-1}.

How can this be? There is an obvious difference between those agents that quench the fluorescence of the buried tryptophans and those that do not. The molecules that quench the buried fluorescence are just those that display significant Forster overlap (Figure 3). Concurrently, these same agents show enhanced quenching activity even against NATA in free solution and also unusually effective quenching of the exposed tryptophans in the two proteins studied (Table I). The overlap data in Figure 3 indicate for the good quenchers a characteristic Forster energy transfer distance (R_0) of about 10 Å. Evidently it is this quality that allows these agents to so effectively quench fluorescence in NATA and in the proteins, including tryptophans that they can only contact far more slowly (the phosphorescent residues) than is otherwise necessary for significant fluorescence quenching.

The same considerations provide an explanation for the data shown in Figure 4, which indicate the absence of any dependence on solvent viscosity for the quenching by nitrite of LADH fluorescence. Under these conditions, nitrite can diffuse farther during the fluorescence lifetime than the average distance between nitrite ions in solution. In respect to resonance energy transfer, therefore, nitrite is in the fast diffusion limit (Thomas et al., 1978), and slowing diffusion by a factor of 2, as with added glycerol, has little effect.

It is most interesting that the characteristic energy transfer distance of 10 Å is sufficient to allow facile quenching of even the most buried tryptophans. Evidently the protected tryptophans are not very deeply buried. In qualitative terms, this may not appear too surprising. A spherical protein the size of an LADH or AP subunit (40 000 daltons) would be ca. 20 Å in radius; half the mass of such a particle would be within 5 Å of the surface, and 80% within 10 Å. Real protein subunits are by no means spherical, so that their mass is even closer to the surface. The rate of resonance energy transfer can be quantified by use of equations given by Thomas et al. (1978), in terms of quencher R_0 and concentration and the depth of tryptophan burial (distance of closest approach). Given the parameters of the present experiments, the "buried" tryptophan must be very close to the protein surface.

Buried Tryptophans and Phosphorescence Quenching. Many small molecules were found to quench the phosphorescence of the protected tryptophans in LADH and were less effective in AP (Table II). Some of these small molecules are the good quenchers just discussed (nitrite, methyl vinyl ketone, and mesityl oxide), but other analogues (unsaturated amides) which cannot quench the buried fluorescence are about as effective against LADH phosphorescence as the good quenchers are. Oxygen quenches the phosphorescent tryptophans with a rate constant of 1×10^9 M^{-1} s^{-1}, only 1 decade less well than the diffusion-limited rate it displays against NATA. The other small molecule quenchers are less effective in LADH by another 4–5 decades. In spite of their disparate character, ranging from the nitrite anion through a series of unsaturated amides and ketones, and in contrast with their grossly reduced effectiveness relative to oxygen, these fall within a remarkably narrow range (Table I). Nitrite, cinnamamide, methyl vinyl ketone, and mesityl oxide all display the same order of magnitude of quenching capability. Acrylamide and bis(acrylamide) begin to trail off to lower values, and *N*-isopropylacrylamide appears, in comparison, significantly blocked, just as it is for fluorescence quenching.

In searching for the interpretation of these results, we assume that the set of quenchers with similar rate constants can quench triplet-state indole on contact. This has been found true of a number of analogous systems (Turro, 1978) in which the acceptor triplet-state energy level is lower than that of the phosphorescent donor. When this condition is not met, quenching efficiency falls off extremely rapidly with small changes in the acceptor energy level. Such differences in the electronic energy level presumably explain the great differences in quenching ability between structurally similar agents, for example, between methyl vinyl ketone and methyl ethyl ketone and between nitrite and nitrate. The fact that a number of the disparate quenchers studied here show such similar rate constants in spite of their varying structure and charge indicates that they are all very near the contact-limited plateau.

How do all these quenching agents reach the buried tryptophan in LADH? Evidently some aspect of the fluctuational behavior of the protein provides the pathway utilized by the various quenchers, and that pathway is nonselective with respect to quencher size and charge. Protein penetration mechanisms that have been either discussed to explain fluorescence quenching (Lakowicz & Weber, 1973a,b; Eftink & Ghiron, 1981) or discussed in other contexts (Ellis et al., 1975; Lumry & Rosenberg, 1975; Nakanishi et al., 1972; Richards, 1979; Case & Karplus, 1979) visualize the transient opening of holes or channels and the sequential stepping of small molecules through the protein, analogous to Eyring diffusion through water (Glasstone et al., 1941). When channel size is only about as large as the penetrating molecule, the penetration rate generated by such a mechanism is expected to be exceedingly sensitive to molecule size (Richards, 1979) and probably also to charge. But that is not what we observe.

Since the opening process that exposes the well-buried tryptophans to quenchers does not distinguish among them on the basis of molecular size, the effective opening must be large relative to the quenchers studied. Under these circumstances, one has to invoke an opening process that provides "channels" larger than the quenching molecules, that is, larger than a single amino acid residue. No protein penetration model put forward to date deals with such large openings.

Openings of this size could be provided by the local unfolding mechanism suggested to account for protein hydrogen exchange behavior (Englander, 1975), which considers concerted denaturation-like reactions involving the collapse of segments of structure several amino acid residues in size. The reaction scheme can be written as in eq 4.

$$\text{closed} \underset{}{\overset{K_{op}}{\rightleftharpoons}} \text{open} \xrightarrow{k_{ch}} \text{quenched} \qquad (4)$$

When the structural reclosing rate is faster than the quenching reaction in the opened form, this kinetic scheme makes the measured quenching rate proportional to quencher concentration, as was observed; in the simplest case, the proportionality constant is $K_{op}k_{ch}$, the equilibrium opening constant times the quenching rate constant in the open form. Since the quenching reaction proceeds in LADH at about 10^{-4} of its free solution rate, one might then conclude that the local structure masking the phosphorescent tryptophan is open 10^{-4} of the time. The analogous equilibrium opening constant in AP would be even smaller.

In the hydrogen exchange literature, the value of k_{ch} in eq 4 is usually assumed equal to the rate for the fully exposed group in free solution. In the present case, it seems likely that in a locally collapsed structural form (the "open" form in eq 4), the indole residue will be surrounded by other hydrophobic

side chains and spend most of its time still partly protected from solvent contact. Thus, k_{ch} in eq 4 is probably significantly less than the diffusion-limited quenching constant, so that K_{op} may well be greater than 10^{-4}. Some part of the (small) range of quenching constants found for the effective phosphorescence quenchers may be due to their varying ability to penetrate into the remaining "oil drop" milieu about the uncovered tryptophan.

One can conjecture that the constraints discussed here provide the criteria for room temperature protein phosphorescence. The indole should be inaccessible to solvent in the stable protein form (though not necessarily deeply buried), and when transient breathing reactions deform the main chain structure protecting them (eq 4), they must still remain covered by a sheath of apolar side chains. These seem to be necessary conditions; whether they are sufficient remains to be seen.

Fluorescence Quenching of the More Exposed Tryptophans. Though excitation and emission wavelengths used in the fluorescence quenching experiments were chosen to maximize the contribution made by the buried (phosphorescent) tryptophans, the relatively exposed tryptophans continued to make a substantial contribution, and could be independently measured. All the quenching agents listed in Table I, though they have varying size and character, reached the more exposed tryptophans, and rather remarkably reached them with nearly the same efficiency, namely, with a rate constant close to 7×10^8 M^{-1} s^{-1}. (Minor exceptions were described under Results.)

In prior work, the quenching effectiveness of agents like acrylamide has been used as an index of the degree of accessibility of particular protein tryptophans (Eftink & Ghiron, 1981). This has sometimes been viewed in terms of the depth of indole burial, though in most cases it has not been clear whether the operative degree of accessibility relates to actual burial or to a greater or lesser degree of exposure at the protein surface (Eftink & Ghiron, 1977). Evidently the nonphosphorescent tryptophans in LADH and AP are by no means totally buried. The fact that quenchers of varying size and character reach them equally well indicates that some fraction of the indole ring is freely exposed to solvent.

The superficial similarity of these results to the phosphorescence quenching results should not be a cause for confusion. In the phosphorescence case, the successful quenchers display similar rate constants that are lower than the diffusion limited value by 4 decades or so. The phosphorescent residues are clearly buried, and their quenching behavior requires an explanation in terms of protein fluctuations that expose them. The tryptophans being discussed here are all reached by all the quenchers with rate constants within about 1 decade of the diffusional limit. Their quenching behavior depends on their normal exposure in the native protein form.

Penetration of Proteins by Small Molecules. The results obtained in this work do not support the view that small molecules can penetrate readily into proteins. The phosphorescence results seem most indicative. The phosphorescent tryptophans in both LADH and AP appear to project very close to the protein surface (see discussion of energy transfer), yet they are not reached at all easily by small molecules like acrylamide. The rate constant for even this minimal penetration into AP is less than the diffusion-limited rate by 8 decades or more. Penetration is slowed by a least 4 decades in LADH and very probably more. In view of the rapid accessibility of these same tryptophans to incoming oxygen (Calhoun et al., 1983), their inaccessibility to other small molecule quenchers does not seem to depend upon some special degree of protein structural rigidity.

It appears that oxygen, owing to its small and apolar character, may be exceptional in its protein penetrating ability. On the other hand, it might be argued that since the indoles are so near to the surface of the proteins tested here, even oxygen may reach the indoles by way of a minimal, surface penetration rather than by its ability to move about freely and deeply within the protein. Oxygen does of course penetrate deeply into myoglobin and hemoglobin; however, the globins may not provide a suitable general model for other proteins in this regard, since their structure must be designed to allow easy access of oxygen to the deeply buried heme iron. The analysis presented in the previous paper (Calhoun et al., 1983) suggests that deep internal penetration rather than superficial entry does provide the operative mechanism for oxygen quenching. Further evidence on this issue would be valuable.

The results with the more exposed tryptophans studied here are also pertinent to the general question of penetration. These residues are reached by acrylamide and a variety of other quenchers with a rate constant close to 7×10^8 M^{-1} s^{-1}. Evidently, on the "kinetic ruler" scale of quenching rates, this value still refers to solvent-exposed residues and non-penetration-dependent quenching. Tryptophans with higher rate constants are presumably even more exposed, so that the ability of acrylamide to quench these provides no information concerning protein penetrability. Among all the proteins studied in this way, only a small number display lower quenching constants. Do any of these provide evidence for penetration by acrylamide?

The single tryptophan in human serum albumin (at pH 5.5) is quenched by acrylamide with a rate constant of 5×10^8 M^{-1} s^{-1}. Results presented by Eftink & Ghiron (1976, 1977) suggest that this residue may still be solvent exposed; its fluorescence is quite red (λ_{max} = 342 nm), its lifetime is solvent sensitive (increased by 15% in 50% glycerol), and its static quenching parameter, which in some sense may indicate unblocked volume about the indole, is in the range characteristic of highly exposed residues ($V = 0.8$ M^{-1}). Thus, even this level of quenching constant may still refer to nonburied indoles.

Few other proteins have acrylamide quenching constants lower than this. The buried tryptophans in LADH and AP, studied here, are lower and do not display fluorescence quenching by acrylamide; the same is true of azurin (Eftink & Ghiron, 1976, 1977). The remaining test population includes only aldolase and ribonuclease T1.

Aldolase displays marginally measurable quenching by acrylamide ($k_q = 0.1 \times 10^9$ M^{-1} s^{-1}). This may or may not indicate penetration. Eftink & Ghiron (1977) report a solvent perturbation of the fluorescence lifetime of aldolase (up by 25% in 50% glycerol). Also, acrylamide at the high concentrations used in the quenching experiments (over 1 M) shows evidence of binding to the protein: this is enhanced with increasing temperature and perhaps with addition of concentrated glycerol. In this protein, tests for static quenching (due to binding) as opposed to dynamic (collisional) quenching were not done.

The positive evidence put forward as favoring penetration involves the temperature dependence of acrylamide quenching rates, and their viscosity dependence (Eftink & Ghiron, 1977). The acrylamide quenching of aldolase and ribonuclease T1 shows activation energies of 11 and 9 kcal/mol, compared to a range between 2.1 and 5 kcal for five other proteins and free indole (Eftink & Ghiron, 1977). This kind of information can be discussed in terms of *possible* models but perhaps does not

guarantee the kind of mechanistic determination one wishes. The effect on quenching rates of 50% glycerol added to the solvent has also been reported. The degree of glycerol-induced slowing decreases monotonically with decreasing quenching rate constant, even for proteins with clearly exposed tryptophans. The reason for this is unclear. Added glycerol has very little effect on the acrylamide quenching rate in those proteins with the smallest rate constants (human serum albumin, aldolase, and ribonuclease T1). This was taken to indicate the dominance of a penetration-dependent pathway (Eftink & Ghiron, 1977). Perhaps it does. However, in view of all the considerations just discussed, it appears to us premature to conclude that proteins in general are easily penetrated by molecules like acrylamide. On the contrary, the results reported in these papers seem to show that, with the probable exception of oxygen, small molecules in general cannot enter native protein structures on any significant time scale.

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Registry No. LADH, 9031-72-5; AP, 9001-78-9; NATA, 2382-79-8; tryptophan, 73-22-3; nitrate, 14797-55-8; nitrite, 14797-65-0; iodide, 20461-54-5; cesium, 7440-46-2; acrylamide, 79-06-1; bis(acrylamide), 20602-80-6; *N*-isopropylacrylamide, 2210-25-5; acrylic acid, 79-10-7; acetone, 67-64-1; methyl ethyl ketone, 78-93-3; methyl vinyl ketone, 78-94-4; mesityl oxide, 141-79-7; cinnamamide, 621-79-4; oxygen, 7782-44-7.

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