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Quenching by acrylamide and temperature of a fluorescent probe attached to the active site of Ribonuclease

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Abstract. The fluorescence properties of ribonuclease labelled at its active site with N-(iodoacetylamino)-ethyl-5-naphthylamine-1-sulfonic acid have been studied at different temperatures and in the presence of acrylamide. The rate constant for the quenching of the fluorescence of labelled ribonuclease by acrylamide is apparently not limited by the "accessibility" of the probe: similar values are obtained for the native and denatured states of the protein. Instead, acrylamide seems to be a rather inefficient quencher of this fluorescent group ((acetamidoamino)ethyl-5-naphthylamine-1-sulfonic acid), as shown by non-linear Stern-Volmer representations, biphasic decay kinetics, and a low value of the rate constant.

The fluorescence intensity of the native state of the labelled protein is highly sensitive to temperature and exhibits a 20% decrease for an increase of temperature of from 10 °C to 30 °C, independent of solvent viscosity. This thermal quenching is specific for the native conformation and disappears when the protein is unfolded. When the fluorescence lifetime of the label is shortened by addition of acrylamide, the effect of temperature becomes identical for native and unfolded structures. This suggests that the cause of the thermal quenching is the presence of conformational fluctuations within the native protein which apparently take place in the time range from 35 to 200 ns.

Key words: Fluorescence quenching, protein conformation, ribonuclease A

Introduction

Fluorescence efficiency depends on the relative rates of radiative and non-radiative deactivation processes. In the case of a fluorophore attached to a protein, the non-radiative processes can be related to (Cooper 1981):

(i) the intrinsic properties of the fluorophore, such as intersystem crossing and internal conversion, which depend on its chemical nature and on its environment (polarity, dielectric constant, etc.)

(ii) the transient events which occur during the lifetime of its excited state and are due to transient changes of its environment as provided by the protein and/or the solvent, such as collisions, forming or breaking non-covalent interactions, charge transfer, etc.

Fluorescent groups have radiative lifetimes in the ns time range, and are thus convenient to monitor the rapid conformational changes of protein structure.

AEDANS possesses a large radiative lifetime, around 30 ns (Hudson and Weber 1973), and therefore its fluorescence will be affected by processes occurring in less than 100 ns and perturbing the excited state. AEDANS can be covalently attached to the active site of ribonuclease (RNase) A (Jullien and Garel 1981), and its fluorescence may report movements of the protein structure in this fast time-range which are not otherwise easily accessible.

Abbreviations used: 1,5-IAEDANS, N-(iodoacetylamino)ethyl-5-naphthylamine-1-sulfonic acid; AEDANS, (acetamidoamino)-ethyl-5-naphthylamine-1-sulfonic acid; RNase, bovine pancreatic ribonuclease; AEDANS-RNase, RNase labelled with AEDANS; ME-AEDANS, (hydroxyethylthioacetamido)ethyl-5-naphthylamine-1-sulfonic acid: the product of the reaction between 1,5-IAEDANS and β-mercaptoethanol (Hudson and Weber 1973); Gu-HCl, guanidine hydrochloride

Theory

The fluorescence yield of a fluorophore A is given by (Parker 1968)

$$F = \frac{k_r}{k_r + k_{nr} + k_q[Q]},$$

where k_r and k_{nr} are the rate constants for the radiative and non-radiative deactivation processes, and k_q is the bimolecular quenching rate constant of an added quencher Q, if any. This fluorescence yield can be written as the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{sv}[Q],$$

where F_0 and τ_0 are the fluorescence yield and lifetime of A in the absence of Q, and K_{sv} is the Stern-Volmer constant.

The simplest scheme for external quenching, which involves a diffusional encounter followed by deactivation, leads to a biphasic fluorescence decay (Gratton et al. 1984)

$$F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$$

but the Stern-Volmer equation can still hold for a mean lifetime defined as:

$$\langle \tau \rangle = \frac{A_1}{A_1 + A_2} \tau_1 + \frac{A_2}{A_1 + A_2} \tau_2 .$$

Materials and methods

The sources of materials and the preparation of the labelled derivative have been described previously (Jullien and Garel 1981); acrylamide was from BDH. A fluorescent model compound was obtained by labelling β-mercaptoethanol on its SH group with AEDANS, according to the Hudson and Weber (1973) procedure. The reagent 1,5-IAEDANS itself cannot be used to measure the properties of the isolated fluorophore because it decomposes spontaneously to release iodine, which perturbs the fluorescence.

Fluorescence intensities were measured with a Jobin-Yvon JY3 spectrofluorometer. Fluorescence decays and fluorescence anisotropy decays were measured by the single photoelectron counting method (Yguerabide 1972) with an apparatus previously described (Brochon 1980). The excitation light pulse (1.8 ns measured fwhm) was synchrotron radiation generated by the electron storage ring ACO working at a frequency of 13.6 MHz. The instrumental response function was measured with a scattering solution (LUDOX solution) at the emission wavelength. The numerical values of relaxation times and amplitude factors were determined by non-linear least-squares procedures (Grinvald and Steinberg 1974; Wahl 1979) taking into account the measured instrumental response function.

Results and discussion

Fluorescence properties of the probe itself

The intrinsic properties of the fluorescent probe alone markedly depend on the solvent polarity; its fluorescence lifetime decreases from 18.3 ns in ethanol to 9.4 ns in water (Hudson and Weber 1973). Indeed, it was also found that the quantum yield of ME-AEDANS in ethanol is 1.8 times that in an aqueous solution of guanidine, after correction for the absorption coefficients and refractive indexes.

The fluorescence intensity of ME-AEDANS does not change with temperature between 10 °C and 30 °C when in aqueous guanidine solution, and it decreases slightly when in ethanol, by 5% over the same temperature range. In both cases, the presence of an external quencher, acrylamide, reduces the fluorescence intensity according to the Stern-Volmer equation (Fig. 1); the constant K_{sv} has a value of $5.6\,M^{-1}$ in ethanol and of $2.1\,M^{-1}$ in $7.6\,M$ guanidine. Using values of τ_0 of 18.3 ns in ethanol and 9.4 ns in aqueous solution, values of $3.1 \times 10^8\,M^{-1}\,s^{-1}$ in ethanol and $2.2 \times 10^8\,M^{-1}\,s^{-1}$ in aqueous solution are found for k_q, the rate constant for acrylamide quenching (one could have used only one lifetime

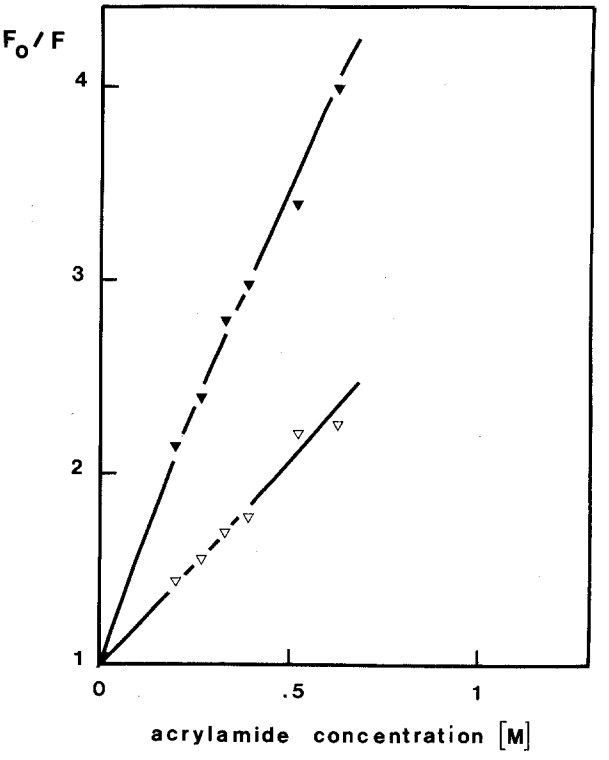

Fig. 1. Stern-Volmer representation of the quenching of the fluorescence of ME-AEDANS by acrylamide in ethanol (▼) and in $7.5\,M$ guanidine (▽). Excitation wavelength: 350 nm, emission wavelength: 500 nm. Temperature: 10 °C

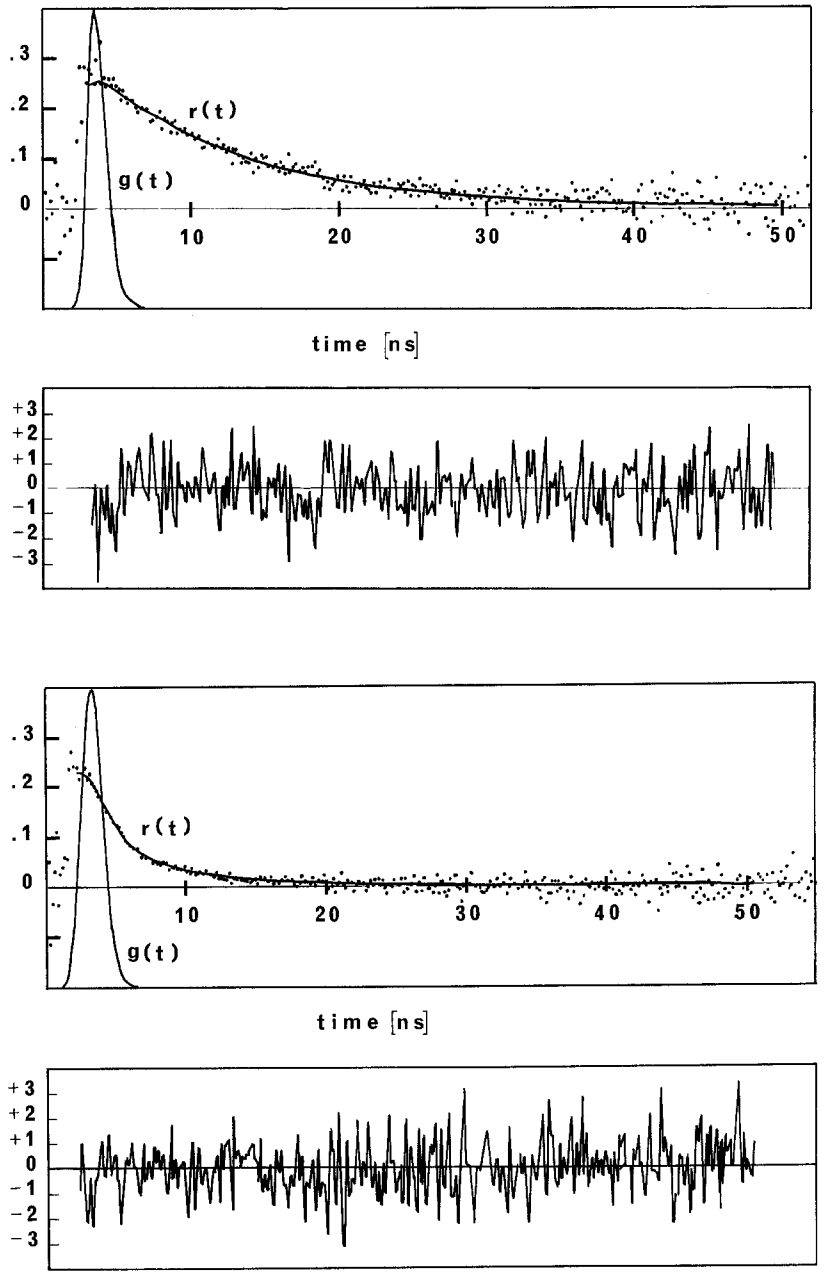

Fig. 2. Fluorescence anisotropy decay of native AEDANS-RNase at 10 °C. Upper curves: experimental points (····) and calculated convolution (——); a single exponential with a time constant of 10 ns and an amplitude of 0.30 is used; $g(t)$ is the apparatus response function. *Lower curve:* deviation function, *Buffer*: 50 m*M* cacodylate pH 6.5, Excitation wavelength: 380 nm, Emission wavelength: 550 nm

Fig. 3. Fluorescence anisotropy decay of AEDANS-RNase unfolded by 6 *M* guanidine hydrochloride. Upper curves: experimental points (····) and calculated convolution (——); the sum of 2 exponentials with time constants of 0.7 ns and 5.4 ns and amplitudes of 0.20 and 0.10 respectively is used; $g(t)$ is the apparatus response function. *Lower curve:* deviation function. Other conditions as given in the legend of Fig. 2

value, and assumed proportionality between quantum yields and lifetimes to derive the same values for k_q). The ratio between the values of k_q found in the two solvents is the inverse of that between the viscosities of these solvents, suggesting that diffusion of acrylamide is involved in the quenching process. However, the values of k_q are smaller than the ones expected for a free diffusion process, by at least one order of magnitude. Acrylamide can indeed quench the fluorescent probe AEDANS by a collisional process, albeit not very efficiently; in the case of indole derivatives, the acrylamide quenching constant is about $7 \times 10^9\, M^{-1}\, s^{-1}$ (Eftink and Ghiron 1984), which approaches the limit of diffusion controlled reactions.

Fluorescence properties of AEDANS-RNase

The unique fluorescent probe of AEDANS-RNase is attached to the enzyme active site and its fluorescence properties depend upon the conformational state of the protein. In the native state, the fluorescence shows a larger lifetime, a higher quantum yield, a lower maximum emission wavelength, and a larger polarization than in the unfolded state; the AEDANS group appears to be rigidly held in a hydrophobic environment by the folded conformation (Jullien and Garel 1981).

Anisotropy decay measurements using native AEDANS-RNase show a single correlation time of 10 ns (Fig. 2); extrapolation to zero time yields a

value of 0.30 for the limiting anisotropy, identical to that found for the model compound ME-AEDANS in glycerol. Therefore, the fluorescent group of native AEDANS-RNase has no movements other than those involving the whole protein. In the denatured state however, fluorescence anisotropy decays much more rapidly, with a correlation time of 0.7 ns for the major portion (Fig. 3). This indicates a marked increase in the freedom of the AEDANS group to reorientation. Nothing similar is observed with native AEDANS-RNase in the presence of up to 1 *M* acrylamide: there is a single correlation time which increases slightly from 10 to 12 ns (Fig. 4).

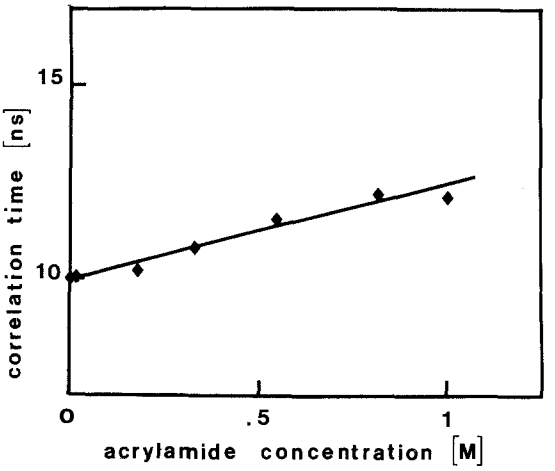

Fig. 4. The correlation time, τ_c, of native AEDANS-RNase as a function of acrylamide concentration. Temperature: 10 °C. The slight increase in τ_c is due to the increase in solvent viscosity

Thermal quenching of the fluorescence of AEDANS-RNase

Increasing the temperature from 10 °C to 30 °C leads to a decrease in the fluorescence intensity emitted by native AEDANS-RNase without significantly changing the maximum emission wavelength; this decrease is almost linear with temperature with a relative change of around 1% per °C (Fig. 5). This thermal quenching is unaffected by the presence of 50% sucrose i.e. by an increase in the solvent viscosity.

Under the same conditions of pH and temperature the fluorescence of AEDANS-RNase unfolded by concentrated guanidine does not appreciably change (Fig. 5).

The fluorescent probe by itself shows no significant thermal quenching whether in ethanol or aqueous solution (see above), therefore thermal quenching is not related to environment polarity alone. It is rather the presence of a folded protein structure around it which makes the fluorescence of the AEDANS group so sensitive to temperature. The origin of this thermal quenching could thus be due to rapid movements of the protein structure around the fluorescent probe occurring during the lifetime of its excited state, the rate of these movements being temperature dependent (Cooper 1981).

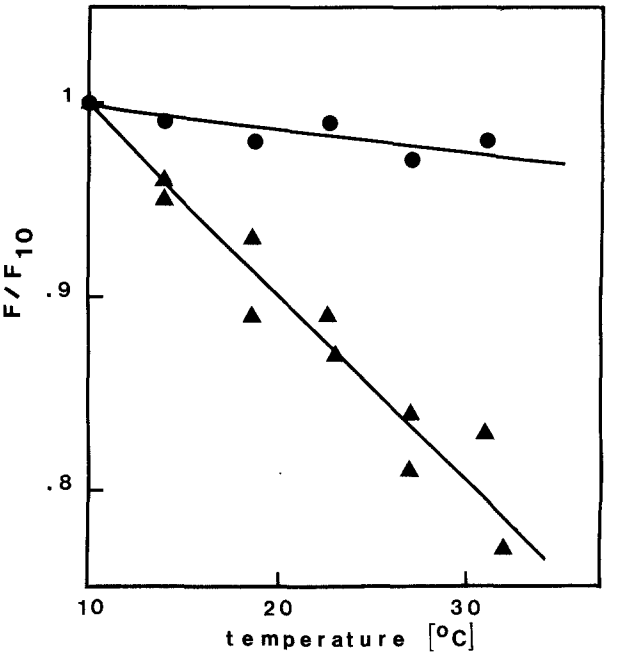

Fig. 5. Effect of temperature on fluorescence intensity of native (▲) and guanidine unfolded (●) AEDANS-RNase. Intensities are expressed relative to that measured at 10 °C for the same sample (native or unfolded). Buffer is 50 m*M* cacodylate, pH 6.5, in the absence or presence of 6 *M* guanidine hydrochloride. Protein concentration is 10^{-5} *M*. Excitation wavelength: 350 nm, emission wavelength: 476 nm

Quenching of AEDANS-RNase by acrylamide

The addition of acrylamide to AEDANS-RNase decreases its fluorescence intensity without changing its maximum emission wavelength. In order to avoid the influence of static (non-collisional) processes, acrylamide quenching has been measured by fluorescence lifetimes instead of fluorescence intensity; the emission wavelength was 550 nm, at the red edge of the spectrum to favour homogeneous emission (Hudson and Weber 1973).

The fluorescence decay of native AEDANS-RNase follows a single exponential, with a lifetime of 18 ns. Upon addition of acrylamide, this decay becomes biphasic (Fig. 6), and the relative amplitude associated with the faster phase increases with the quencher concentration (Table 1). Several arguments suggest that AEDANS-RNase remains native in the presence of acrylamide:

(i) its maximum emission wavelength remains unchanged;

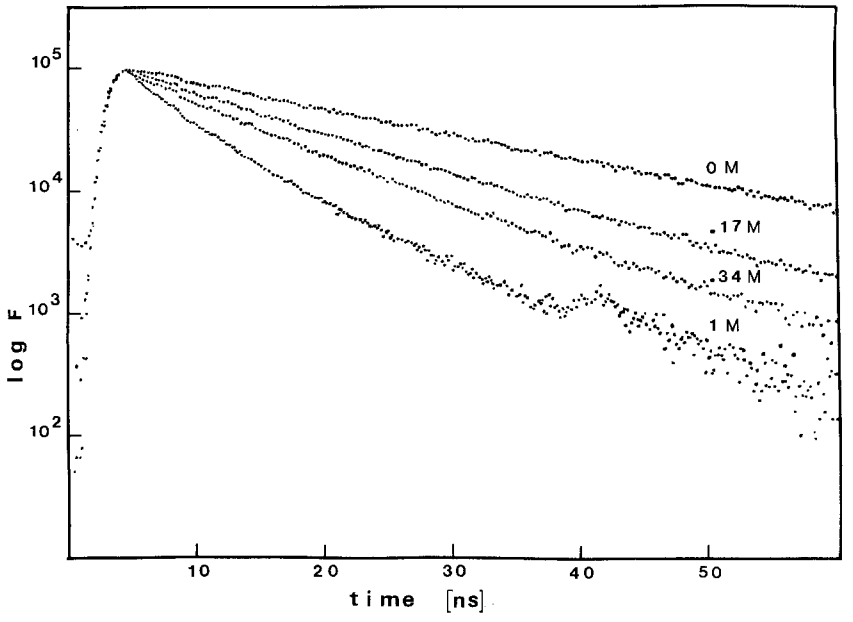

Fig. 6. Fluorescence decays of native AEDANS-RNase in the presence of different concentrations of acrylamide. Buffer is 50 m*M* cacodylate, pH 6.5, 10 °C. Protein concentration is $5 \cdot 10^{-6}$ *M*. Excitation wavelength: 380 nm, emission wavelength: 550 nm

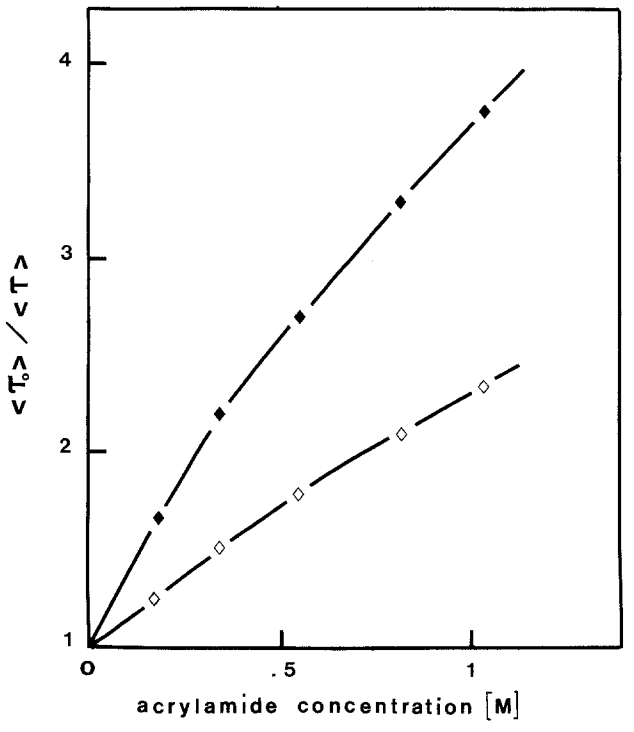

Fig. 7. Stern-Volmer representations of the quenching of the fluorescence of AEDANS-RNase by acrylamide; (◆) native protein, (◇) protein unfolded by 6.2 *M* GuHCl. Conditions as given in the legend of Fig. 4

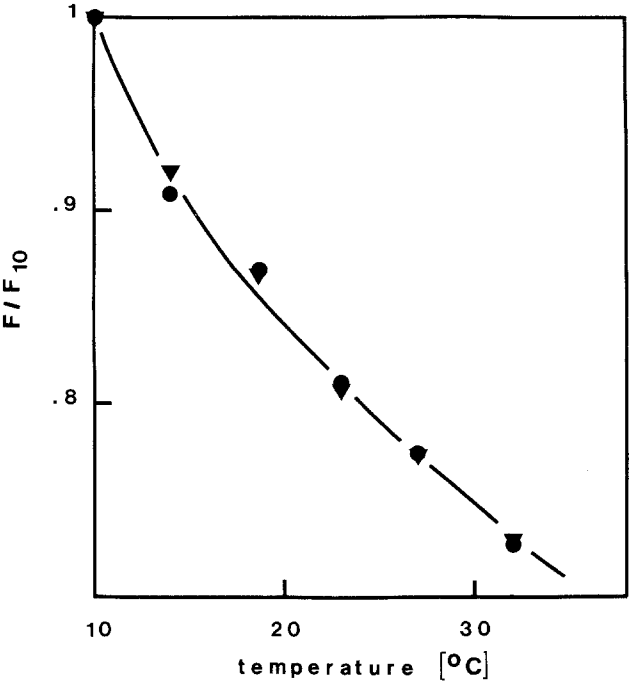

Fig. 8. Effect of temperature on fluorescence intensity, in the presence of 0.9 *M* acrylamide, of native (▼) and unfolded (●) AEDANS-RNase. Conditions as given in the legend of Fig. 5, except for protein concentration which is $4 \cdot 10^{-6}$ *M*

(ii) the fluorescence anisotropy decay shows no increase in the mobility of the AEDANS group (Fig. 4);

(iii) unmodified RNase is enzymatically active in the presence of 0.8 *M* acrylamide.

The biphasic fluorescence decay is apparently not due to a partial acrylamide-induced denaturation of AEDANS-RNase; rather, such a pattern is expected in the case of an inefficient quencher (Gratton et al. 1984). Also expected is the downward curvature of the Stern-Volmer plot of the mean lifetime $\langle \tau \rangle$ (Eftink and Ghiron 1981) (Fig. 7).

The reciprocal of the quenching rate constant, k_q, varies linearly as a function of quencher concen-

Table 1. Fluorescence decay parameters of AEDANS-RNase in the presence of acrylamide

Acrylamide concentration [M]	τ_1 [ns]	τ_2 [ns]	A_1/A_1+A_2	$\langle\tau\rangle$ [ns]	χ^a
0.17	6.0	12.3	0.24	10.8	2.7
0.34	5.2	10.6	0.45	8.2	2.4
0.55	4.7	9.0	0.52	6.8	3.4
0.82	3.4	7.6	0.49	5.6	2.8
1.04	3.2	7.9	0.64	4.9	2.3

[a] χ is the classical criterion for fitting experimental curves (Grinvald and Steinberg 1974)

tration between $0.2\,M$ and $1\,M$; extrapolation of k_q^{-1} to $0\,M$ acrylamide yields a value of $2.4\times10^8\,M^{-1}\,s^{-1}$ for the initial slope of the Stern-Volmer plot (Gratton et al. 1984).

Acrylamide also quenches the fluorescence of AEDANS-RNase unfolded by concentrated guanidine; the Stern-Volmer plot is almost linear in this case (Fig. 7), and corresponds to a quenching rate constant of $1.3\times10^8\,M^{-1}\,s^{-1}$. Measurements performed at $4.5\,M$ and $6.9\,M$ guanidine show that k_q depends on the viscosity of the solvent; after correcting for the viscosity of the concentrated guanidine, the quenching rate constant k_q becomes $2.3\times10^8\,M^{-1}\,s^{-1}$. The rate constants of acrylamide quenching are thus quite similar for the native and unfolded states of AEDANS-RNase.

Quenching of the fluorescence of AEDANS-RNase by temperature and acrylamide: internal and external quenching

In the presence of $0.9\,M$ acrylamide, the fluorescence intensities emitted by both native and unfolded AEDANS-RNase are temperature dependent (Fig. 8). The extent of quenching is expected to depend on temperature via k_q, which reflects the collisional step of acrylamide quenching; processes which are rate-limited by the diffusion through the solvent usually have activation energies of 4 to 5 kcal/mole (Fenichel and Horowitz 1965). Such a value is indeed sufficient to account for the temperature-dependence of AEDANS-RNase fluorescence given in Fig. 8, as if acrylamide diffusion through the solvent were the temperature sensitive step. In the presence of $0.9\,M$ acrylamide, the mean fluorescence lifetimes of native and unfolded AEDANS-RNase are similar, 5.4 ns and 5.2 ns respectively; these two states also show the same temperature dependence, as if the particular thermal quenching of the native protein (Fig. 5) was suppressed by the presence of acrylamide. Assuming that the protein structure is not perturbed by acrylamide (see above), this loss of thermal quenching can be explained by the shorter lifetime: in the presence of an external quencher, the activated state does not last long enough to be sensitive to the fluctuations of its environment.

The radiative lifetime of AEDANS is about 30 ns (Hudson and Weber 1973) and is not expected to vary with temperature. The fluorescence lifetime of native AEDANS-RNase being 18.3 ns, in the absence of quencher, the global rate constant k_{nr} of all the non radiative processes (see Theory) is about $2.5\times10^7\,s^{-1}$. Assuming that the observed fluorescence intensity decrease with temperature (Fig. 5) represents a decrease in fluorescence lifetime, there is therefore a significant effect of temperature on one of the processes included in k_{nr}. The rate constant of this process is thus a non-negligible part of k_{nr}. The presence of $0.9\,M$ acrylamide results in an additional quenching with a rate $k_q[Q]=13\times10^7\,s^{-1}$, i.e. larger than k_{nr}. The rate of the temperature-dependent non-radiative process has now become a negligible part of the sum, $k_{nr}+k_q[Q]=15.5\times10^7\,s^{-1}$; with an arbitrary limit of "negligibility" of 20%, this rate is smaller than $3\times10^7\,s^{-1}$ and larger than $0.5\times10^7\,s^{-1}$, i.e. it corresponds to a time constant between 35 ns and 200 ns (these limits are only indicative). It is possible that this peculiar temperature-dependent process is due to conformational fluctuations of the immediate environment of the fluorophore (Cooper 1981). These fluctuations appear to be one order of magnitude slower than the process involved in the ability of acrylamide to quench the fluorescence of the protein ($1/k_q[Q]=8$ ns at $0.9\,M$ acrylamide); further resolution of these effects will depend upon a more detailed knowledge of the molecular mechanism of the quenching.

Conclusion

Acrylamide is not an efficient quencher of the fluorescence of the AEDANS group; this can be seen from the non-linear Stern-Volmer plots (Fig. 7), the biphasic decay curves (Fig. 6), and the low values of k_q, the quenching rate constant, ca. $2\times10^8\,M^{-1}\,s^{-1}$. Thus the rate of quenching does not only reflect the rate of collision between the quencher and its target, but also the low probability that such a collision leads to actual quenching; k_q does not strictly measure the "accessibility" of the fluorophore as defined by the probability of encounter, and therefore finding similar values of k_q for the

native and unfolded states of AEDANS-RNase does not show a similar degree of exposure to solvent.

The native conformation of AEDANS-RNase shows a peculiar thermal quenching (Fig. 5) which disappears either upon shortening its fluorescence lifetime with acrylamide (Fig. 8) or unfolding its conformation with guanidine (Fig. 5). A possible explanation is that the vicinity of the AEDANS group in the native state fluctuates and/or moves during the lifetime of the excited state and influences its deactivation; the efficiency of this quenching would depend on the relative rates of deactivation of the fluorophore and fluctuations of its protein environment. From the values of the lifetimes in the absence, 18.3 ns, and the presence, 5.4 ns, of external quencher, one can estimate that these fluctuations in the active site of RNase are in the time range from 35 to 200 ns.

References

Brochon JC (1980) Protein structure and dynamics by polarized pulse fluorometry. In: Ishiwata S (ed) Protein dynamics and energy transduction. Proceedings of the Sixth Taniguchi International Symposium, Biophysics Division, pp 163–189

Cooper A (1981) Spurious conformational transitions in proteins? Proc Natl Acad Sci USA 78:3551–3553

Eftink MR, Ghiron CA (1981) Fluorescence quenching studies with proteins. Anal Biochem 114:199–227

Eftink MR, Ghiron CA (1984) Indole fluorescence quenching studies on proteins and model systems: use of the inefficient quencher succinimide. Biochemistry 23:3891–3899

Fenichel IR, Horowitz SB (1965) Diffusional specificity in water. Ann NY Acad Sci 125:290–297

Gratton E, Jameson DM, Weber G, Alpert B (1984) A model of dynamic quenching of fluorescence in globular proteins. Biophys J 45:789–803

Grinvald A, Steinberg IZ (1974) On the analysis of fluorescence decay kinetics by the method of least-squares. Anal Biochem 59:583–598

Hudson EN, Weber G (1973) Synthesis and characterization of two fluorescent sulfhydryl reagents. Biochemistry 12: 4154–4161

Jullien M, Garel J-R (1981) Fluorescent probe of ribonuclease A conformation. Biochemistry 20:7021–7026

Parker CA (1968) Photoluminescence of solutions. Elsevier, Amsterdam

Wahl P (1979) Time-resolved fluorometry. In: Chen RF, Edelhoch H (eds) Biochemical fluorescence concepts. Marcel Dekker, New York, pp 1–41

Yguerabide J (1972) Nanosecond fluorescence spectroscopy of macromolecules. In: Hirs CHW, Timasheff SN (eds) Methods in enzymology, Vol 26. Academic Press, New York, pp 498–578