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Energy-Transfer Measurements on a Double Fluorescent Labeled Ribonuclease A†

Magali Jullien* and Jean-Renaud Garel

ABSTRACT: Two fluorescent groups have been covalently attached to ribonuclease A: first, the α-amino group is labeled upon reaction with fluorescein isothiocyanate, and second, one of the active site histidine residues is modified by *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid. Among the products of these two successive chemical modifications, a derivative bearing one label on Lys-1 and the other label on His-119 can be isolated and characterized. Because of their spectral properties, these two fluorophores, fluorescein and *N*-[(acetamido)ethyl]-5-naphthylamine-1-sulfonic acid, are suitable for measuring resonance energy transfer within a single protein molecule. The efficiency of the energy transfer is close to 100% in the native state and is reduced to about 50% in the guanidine-unfolded state. This efficiency is further diminished upon reduction of the disulfide bonds in denaturing conditions. The efficiency of energy transfer has been determined independently from both emission and excitation spectra of the double-labeled protein, when unfolded with intact disulfide bonds. The average distance between the two fluorescent groups can be obtained from these measurements: it increases from 20 Å at most in the native state to 46 Å or more in the unfolded state.

The intrinsic sensitivity of fluorescence measurements and the variety of reagents available have increased the use of fluorescent labels as conformational probes of biological structures. Nonradiative energy transfer between a suitable donor–acceptor pair can occur provided that the emission spectrum of the donor overlaps the excitation spectrum of the acceptor and that the distance and relative orientation of the two fluorescent groups be appropriate (Stryer, 1978). This energy transfer can be used to measure the distance between the donor and acceptor: depending on the donor–acceptor pair, distances ranging from 10 to 100 Å can be estimated (Fairclough & Cantor, 1978). In many cases, changes in the distance between donor and acceptor, rather than the distance itself, have been measured, especially for complex formation

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and/or assembly processes: the encounter and/or formation of a complex between two distinct objects can be monitored by energy transfer if these two objects bear each one fluorescent group, donor or acceptor. In this case, the fluorescent labeling can be only partial and/or statistical since any transfer implies an association. Quantitative measurements of distances by resonance energy transfer require that the donor and acceptor groups be at specific sites (Horton & Koshland, 1967) and, hence, is limited by the quality of fluorescent labeling. This is probably one of the reasons why so few studies of *intra*molecular energy transfer have been performed with proteins (Stryer, 1978), despite the fact that this method can yield not only static distances but also dynamic informations on their fluctuations (Haas et al., 1978). This approach is applied here to RNase,[1] one of the best known proteins. It has been shown previously that two specific sites could be labeled with fluorescent groups: the N-terminal amino group (Garel, 1976) and the active site (Jullien & Garel, 1981). This paper reports the preparation of a double-labeled derivative of RNase, bearing both the donor and acceptor covalently attached to specific sites of the protein. In this derivative, energy transfer depends on the conformational state of the protein and is used to determine the average distance between acceptor and donor in an unfolded state, i.e., in a state that is not within the reach of X-ray crystallography.

The three-dimensional structure of RNase is known from X-ray diffraction (Wlodawer et al., 1982), and hence, all the intramolecular distances within the native conformation can be derived; also, analysis of the temperature factors gives an idea of the local mobility of the atoms in this conformation (Frauenfelder & Petsko, 1980). However, the unfolded state(s) is (are) defined only by some overall properties such as viscosity, hydrodynamic volume, etc. (Tanford, 1968). The present work shows that some information on average distances within the unfolded state of RNase (with disulfide bonds intact) can be obtained by energy-transfer measurements, once the chemical aspects of fluorescent labeling have been solved.

Theory

According to Förster (1965), the efficiency of nonradiative energy transfer between a donor D and an acceptor A is

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (1)$$

where R is the distance between D and A, and R_0 is the distance corresponding to 50% transfer efficiency. R_0 (Å) is given by

$$R_0^6 = (8.79 \times 10^{-5}) K^2 n^{-4} \phi_D J_{DA} \quad (2)$$

where K^2 is an orientation factor, ϕ_D is the quantum yield of the donor alone, n is the refractive index, and J_{DA} (M^{-1} cm^{-1} nm^4) is the spectral overlap integral, given by

$$J_{DA} = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda}{\int F_D(\lambda) \, d\lambda} \quad (3)$$

where $F_D(\lambda)$ is the fluorescence intensity emitted by the donor at the wavelength λ and $\epsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor at the same wavelength. The transfer efficiency E is obtained from fluorescence measurements as described below; once R_0 has been determined from the spectral properties of the donor and acceptor, eq 1 yields the distance R between the two fluorescent groups. It must be pointed out that for a given donor–acceptor pair, R_0 has to be determined in given conditions: indeed, n, ϕ_D, and J_{DA} can be affected by the environment of the fluorescent groups.

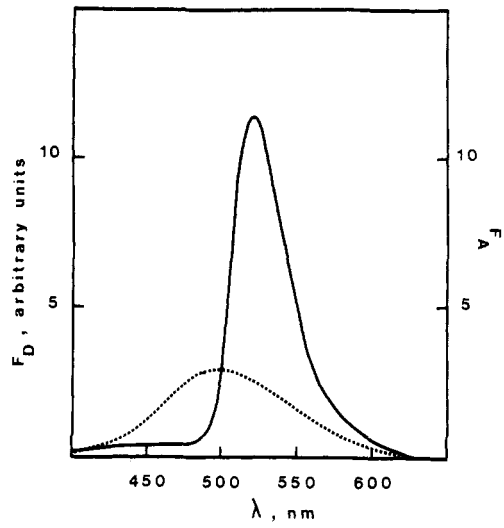

FIGURE 1: Fluorescence emission spectra of D-RNase (---) and A-RNase (—) in 0.1 M cacodylate buffer–6 M Gdn-HCl, pH 6.5. Excitation wavelength 350 nm; protein concentration 0.02 mg/mL.

In the present work, the emission spectra of donor and acceptor overlap each other (Figure 1); since a partial overlap also exists in the excitation spectra, then the fluorescence intensity emitted by the donor–acceptor pair at a wavelength λ' upon excitation at a wavelength λ will contain three contributions: (i) the fluorescence of donor, decreased in case of efficient transfer; (ii) the fluorescence of acceptor if donor were absent; (iii) the fluorescence of acceptor upon transfer from donor. This intensity, $F_{DA}(\lambda,\lambda')$, is given by

$$F_{DA}(\lambda,\lambda') = \alpha I_0(\lambda)[\epsilon_D(\lambda)[(1 - E)\phi_D(\lambda') + E\phi_A(\lambda')] + \epsilon_A(\lambda)\phi_A(\lambda')] \quad (4)$$

where $\epsilon(\lambda)$ is the molar extinction coefficient at excitation wavelength λ, $\phi(\lambda')$ is the fluorescence yield at emission wavelength λ' (assumed to be independent of λ), the subscripts D and A pertain to donor and acceptor, respectively, and $I_0(\lambda)$ is the light source intensity at excitation wavelength λ; E is the transfer efficiency given by eq 1. Equation 4 can be used to analyze the influence of energy transfer on either the excitation or the emission spectra of the donor–acceptor pair. It can be noted that the expression of $F_{DA}(\lambda,\lambda')$ becomes simpler in cases where the donor and acceptor spectra are well separated on the wavelength scale.

Materials and Methods

The sources of materials, the analytical methods, and the preparation of the two single-labeled derivatives have been described previously (Garel, 1976; Jullien & Garel, 1981).

Optical Measurements. A Cary 14 spectrophotometer was used to record absorption spectra and to measure absorbances. Table I gives the molar extinction coefficients obtained for different solvent conditions for the single-labeled derivatives, D-RNase and A-RNase.

Fluorescence spectra were recorded with a Jobin-Yvon JY3 spectrofluorometer. Quantitative comparison of the fluores-

[1] Abbreviations: RNase, bovine pancreatic ribonuclease; A-RNase, RNase labeled with acceptor; D-RNase, RNase labeled with donor; DA-RNase, RNase labeled with donor and acceptor; FITC, fluorescein isothiocyanate; 1,5-IAENS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid; AENS, *N*-[(acetamidoamino)ethyl]-5-naphthylamine-1-sulfonic acid; Gdn-HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane.

Table I: Molar Extinction Coefficients (M^{-1} cm^{-1}) of Fluorophores for Different Conditions

solvent:	0.1 M Tris, pH 8.5		0.1 M Tris–6 M Gdn-HCl, pH 8.5		0.1 M cacodylate, pH 6.5		0.1 M cacodylate–6 M Gdn-HCl, pH 6.5	
wavelength:	350 nm	495 nm	350 nm	495 nm	350 nm	495 nm	350 nm	495 nm
ϵ_A	5.8×10^3	7.2×10^4 [a]	6.6×10^3	6.6×10^4	6.4×10^3	4.7×10^4	6.4×10^3	4.7×10^4
ϵ_D	6.8×10^3	0	6.1×10^3	0	6.8×10^3 [b]	0	6.1×10^3	0

[a] This value of ϵ_A was obtained from amino acid analyses (Garel, 1976); the other values of ϵ_A were determined by comparing the absorbance in given conditions to that at 495 nm in 0.1 M Tris, pH 8.5, for the same protein concentration. [b] This value of ϵ_D was obtained by us (Jullien & Garel, 1981) from direct measurements of protein concentration according to Lowry et al. (1951). It is in excellent agreement with that given by Hudson (1970) for the molar absorbance of the bound AENS; this value of ϵ_D thus corresponds to one AENS group attached to RNase A. The other values of ϵ_D were determined by comparing the absorbance in given conditions to that at 350 nm in 0.1 M cacodylate, pH 6.5, for the same protein concentration.

cence intensities at a given wavelength of different samples is subject to many experimental variables such as the protein concentration and refractive index of each sample, the fluctuations in light source energy from one sample to another, etc. (Brand & Witholt, 1967). These sources of uncertainty were eliminated by systematically using for each sample the ratio of the fluorescence intensities at two selected wavelengths; for a given sample, the value of this ratio depends only upon the molar extinction coefficients and the fluorescence yields at the wavelengths involved.

(*i*) *Analysis of Emission Spectra.* The excitation wavelength was $\lambda = 350$ nm, and the two selected emission wavelengths were $\lambda'_1 = 476$ nm and $\lambda'_2 = 515$ nm, corresponding to the maximum emission of donor and acceptor alone, respectively (Figure 1). The ratio, $Q = F_{DA}(\lambda,\lambda'_2)/F_{DA}(\lambda,\lambda'_1)$ can be determined experimentally. From eq 4 applied to λ'_1 and λ'_2, an expression for Q is obtained, which can be solved for the transfer efficiency, E:

$$E = \frac{\epsilon_D(\lambda)(Qa - ab) + \epsilon_A(\lambda)(Qbc - abc)}{\epsilon_D(\lambda)[(abc - ab) + Q(a - bc)]} \quad (5)$$

where the coefficients a, b, and c correspond to ratios of fluorescence yields:

$$a = \frac{\phi_A(\lambda'_2)}{\phi_A(\lambda'_1)} \qquad b = \frac{\phi_D(\lambda'_2)}{\phi_D(\lambda'_1)} \qquad c = \frac{\phi_A(\lambda'_2)}{\phi_D(\lambda'_2)}$$

These coefficients were determined from the emission spectra of the two single-labeled derivatives (Figure 1). For $\lambda'_1 = 476$ nm, $\lambda'_2 = 515$ nm, and samples of D-RNase and A-RNase with the same absorbance at 350 nm in 0.1 M cacodylate buffer–6 M Gdn-HCl, at pH 6.5, 25 °C, values of $a = 40$, $b = 1.1$, and $c = 4.2$ were obtained and used for subsequent analysis of the double-labeled derivative, DA-RNase. With these coefficients, eq 5 becomes

$$E = \frac{1.27Q - 6.72}{Q + 3.98} \quad (5')$$

(*ii*) *Analysis of Excitation Spectra.* The emission wavelength was $\lambda' = 516$ nm, and the two selected excitation wavelengths were $\lambda_1 = 350$ nm and $\lambda_2 = 495$ nm, corresponding to the maximum excitation (i.e., absorption) of the donor and acceptor alone, respectively (Hudson & Weber, 1973; Lindquist, 1961). The ratio $Q' = F_{DA}(\lambda_2,\lambda')/F_{DA}(\lambda_1,\lambda')$ can be determined experimentally. From eq 4, an expression for Q' is obtained that can be solved for the transfer efficiency, E:

$$E = \frac{A\epsilon_A(\lambda_2) - Q'[\epsilon_A(\lambda_1) + a'\epsilon_D(\lambda_1)]}{Q'(1 - a')\epsilon_D(\lambda_1)} \quad (6)$$

where $a' = \phi_D(\lambda')/\phi_A(\lambda')$ is also a ratio of fluorescence yields and $A = I_0(\lambda_2)/I_0(\lambda_1)$ is an instrumental factor corresponding to the ratio of light source intensities at these two wavelengths. The value of A was determined from the excitation spectrum of A-RNase used as a fluorescent standard with the molar extinction coefficients given in Table I; a value of 2.5 was found for $\lambda_1 = 350$ nm and $\lambda_2 = 495$ nm. A value of $a' = 0.11$ was obtained from a comparison of the excitation spectra of samples of D- and A-RNase with the same absorbance at 350 nm in 0.1 M Tris buffer, at pH 8.5, 25 °C; the same value, $a' = 0.11$, was determined in the presence or absence of 6 M Gdn-HCl. In the presence of 6 M Gdn-HCl, eq 6 becomes

$$E = 30.4/Q' - 1.34 \quad (6')$$

This procedure of using the ratio between two fluorescence intensities rather than the intensities themselves is quite convenient for the analysis of fluorescence excitation; indeed, correction of the excitation spectrum for the light source spectrum is no longer required. In the present work, all fluorescence spectra were analyzed without correction whether for the single-labeled proteins, D- and A-RNase, or the double-labeled derivative DA-RNase. On the other hand, this procedure requires the preliminary determination of some numerical values such as the ratios a, b, c, a', A, ... from measurements on single-labeled proteins (donor alone and/or acceptor alone). This assumes that the optical properties, molar extinction coefficient, and fluorescence quantum yield of one fluorescent group are the same in a single-labeled and a double-labeled protein. This is justified by both the properties of the double-labeled derivative (see below) and the fact that the two fluorescent groups are not in direct contact with each other. With this assumption, one can convert the experimentally measured values of Q and Q' into a transfer efficiency E within the double-labeled protein, DA-RNase, using either eq 5 for emission spectra or eq 6 for excitation spectra. It is important to note that these two determinations of E are independent and that agreement between them supports the present procedure and its implicite assumptions.

Chemical Methods. The double-labeled protein was obtained from A-RNase (Garel, 1976) by reaction with 1,5-IAENS in the following conditions: $(0.5–1) \times 10^{-3}$ M A-RNase and 2×10^{-2} M 1,5-IAENS, in 0.1 M cacodylate buffer, at pH 5.5, 25 °C in the dark (Jullien & Garel, 1981). To minimize the extent of multiple-site labeling, the reaction was stopped when the enzyme activity was still about 20% of its original level, i.e., after about 48 h. The excess of reagent was removed by gel filtration on a 4 × 50 cm column of Sephadex G-25, eluting with 10^{-2} M cacodylate buffer at pH 6.5. The protein fractions, identified by their absorbance at 495 nm, were pooled and fractionated by ion-exchange chromatography on a 1.5 × 50 cm column of CM-cellulose at a flow rate of 12 mL/h, using an ionic strength gradient from 0 to 0.3 M NaCl in 10^{-2} M cacodylate buffer, at pH 6.5, as an eluant. Elution was measured by the protein absorbance at 495, 278, and/or 350 nm; part of the elution diagram is

given in Figure 2. A few milligrams of DA-RNase were routinely obtained from 50 mg of A-RNase.

Cyanogen bromide cleavage of the polypeptide chain was performed on RNase derivatives without reduction of the disulfide bonds in the following conditions: (0.5–1) × 10^{-4} M protein and 3 × 10^{-2} M CNBr, in 70% formic acid, for 24 h, at room temperature, in the dark (Gross & Witkop, 1962). After lyophilization, the products were dissolved in 0.2 N acetic acid and submitted to gel filtration on a 1.5 × 100 cm column of Sephadex G-25, eluting with 0.2 N acetic acid (Gross, 1967). Elution was monitored by absorbance measurements at 350 and 495 nm; because of the pH dependence of the fluorescein absorption spectrum (Lindquist, 1961), quantitative measurements were always performed on samples adjusted to alkaline pH with concentrated ammonia.

Reduction of the disulfide bonds of the RNase derivatives was achieved by a 1-h exposure to 0.1 M dithiothreitol, in 0.1 M Tris buffer–6 M Gdn-HCl, at pH 8.5, 25 °C (Garel, 1977). Carboxymethylation of the thiol groups by 0.25 M iodoacetic acid was done in the same medium.

Results

Preparation of the Double-Labeled Derivative, DA-RNase. Because of their optical properties, AENS and fluorescein can be used as a donor–acceptor pair in fluorescence-transfer measurements (Fairclough & Cantor, 1978). Previously, two chemical derivatives of RNase have been prepared, each being covalently labeled with one of these two fluorescent groups: (i) A-RNase (A for acceptor) bears a fluorescein residue attached to the α-amino group of Lys-1 (Garel, 1976); (ii) D-RNase (D for donor) has a AENS group at the enzyme active site, probably attached to either His-12 or His-119 (Jullien & Garel, 1981).

In order to study intramolecular fluorescence transfer, a double-labeled derivative, DA-RNase, with both donor and acceptor on the same protein molecule, was prepared by introducing the AENS group into the fluorescein-labeled protein, A-RNase. This procedure ensures that the final product is homogeneously labeled in acceptor residue, which is recommended for transfer measurements (Fairclough & Cantor, 1978). A-RNase, prepared as already reported (Garel, 1976), was reacted with IAENS under conditions very similar to those used to prepare D-RNase from RNase (see Materials and Methods). The reaction was followed by the loss of enzymatic activity (Jullien & Garel, 1981) and was stopped when the activity of A-RNase was about 20% of its starting level. After removal of the excess of reagent by gel filtration, the modified protein was fractionated into several components by ion-exchange chromatography (Figure 2). Prior to the reaction, all protein molecules are labeled with one fluorescein residue at their N-terminal end; the number of AENS groups introduced can be determined by absorbance measurements at selected wavelengths. Table I gives the molar extinction coefficients at 350 and 495 nm taken here for one AENS and/or one fluorescein group bound to RNase; these molar extinction coefficients were obtained from the absorption spectra of the monosubstituted derivatives A- and D-RNase, in different solvent conditions. Using the values given in Table I, it is found that the two last fractions to elute are labeled with one AENS residue (Figure 2). The most probably site of attachment of this AENS group is either His-12 or His-119 (Jullien & Garel, 1981), and indeed, these two fractions are enzymatically inactive. Cleavage of the polypeptide chain by cyanogen bromide at Met-13 without reduction of disulfide bonds has been used to discriminate between His-12 and His-119 as the site bearing the AENS residue: this cleavage

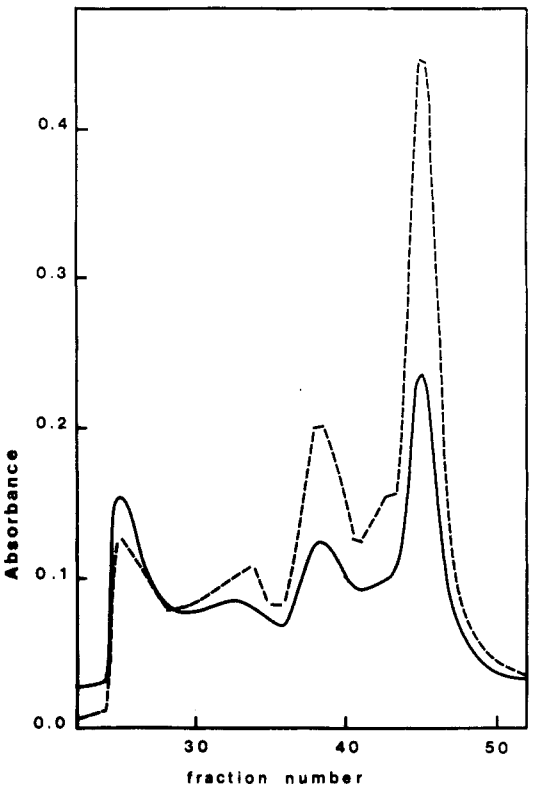

FIGURE 2: Ion-exchange chromatography on CM-cellulose of the products of the reaction of A-RNase with 1,5-IAENS at pH 5.5. Elution was carried out with a 0–0.3 M NaCl gradient in 10^{-2} M cacodylate, pH 6.5, at room temperature. (—) Absorbance at 278 nm; (– –) absorbance at 495 nm.

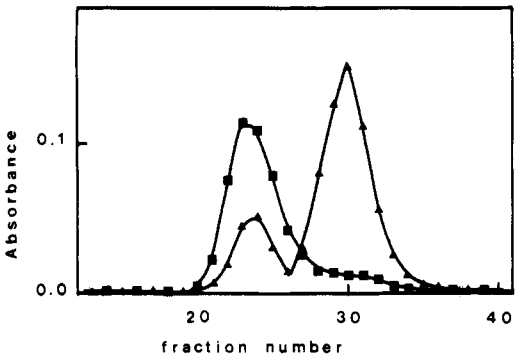

FIGURE 3: Gel filtration on a 120 × 1.5 cm G-25 column of the products of the reaction of DA-RNase with CNBr. Elution was carried out with 0.2 M acetic acid, and volume fractions were of 5 mL. (▲) Absorbance at 495 nm; (■) absorbance at 350 nm; the samples were alkalinized with concentrated NH_4OH.

yields the C-peptide, with residues 1–13, and the C-protein with the rest of the molecule (Gross & Witkop, 1962). Cleavage at Met-13 was performed on the last fraction to elute, and the separation between the products, C-peptide and C-protein, is shown in Figure 3. Only 70–80% of N-terminal fluorescein is found to elute with the C-peptide; the remaining 20–30% elutes with a heavier species. The same result (not shown) is found with single-labeled A-RNase and indicates that cleavage at Met-13 does not occur with 100% yield, at least in these conditions. It can also be seen in Figure 3 that the C-peptide, which bears most of the fluorescein, has no AENS group attached to it, as judged from the lack of absorbance at 350 nm. This shows that His-12 cannot be the dansyl attachment site in this fraction and, thus, that this site is probably His-119. This fraction, which apparently contains a double-labeled RNase, with a donor on residue 119 and an

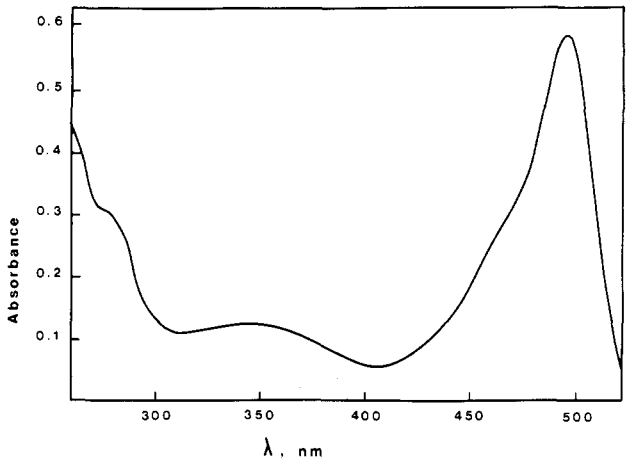

FIGURE 4: Absorption spectrum of DA-RNase in 10^{-2} M cacodylate buffer, pH 6.5. Protein concentration 0.17 mg/mL.

acceptor on residue 1, has been studied in more detail below; its absorption spectrum is given in Figure 4.

Cyanogen bromide cleavage at Met-13, followed by gel filtration to separate the C-peptide from a mixture of C-protein (70–80%) and partially uncleaved protein (20–30%), was also applied to the second peak to elute in Figure 2. In this case, the C-peptide was found to contain the AENS group, together with the fluorescein, which shows that His-12 is modified in this fraction. A similar conclusion, namely, that the donor and acceptor are attached close to one another along the polypeptide chain, comes from fluorescence measurements: in this double-labeled derivative, the efficiency of transfer from AENS to fluorescein is always 100% independent of the state, native or denatured, of the protein. As explained below, this means that the distance between the two fluorescent groups is at most about 20 Å; if it is so in the denatured state, then it also suggests that the donor is on His-12, i.e., much closer to the acceptor on Lys-1 than His-119. Because of this systematic efficient transfer, this derivative was not studied in more detail. The other double-labeled species defined above, with the AENS on His-119, exhibits a marked change in resonance transfer upon unfolding, confirming the conclusion from chemical studies. This derivative, DA-RNase, seems then homogeneously labeled by a donor on residue 119 and an acceptor on residue 1, as desired for resonance transfer.

Determination of Transfer Efficiency E from Emission Spectrum of Double-Labeled DA-RNase. Figure 5 gives the fluorescence emission spectra obtained for the double-labeled derivative DA-RNase in the absence and the presence of 6 M Gdn-HCl, at pH 6.5; the excitation wavelength is 350 nm, i.e., corresponding to the maximum absorption of the donor. In conditions where the protein is native (no Gdn-HCl is present), the emission spectrum is very similar to that of the single-labeled species A-RNase: no contribution from the donor is detected in the 450–480-nm range. In native DA-RNase, the donor fluorescence is completely quenched because of a complete transfer to the acceptor: the transfer efficiency approaches 100%. The emission spectrum obtained for denatured DA-RNAse (in the presence of 6 M Gdn-HCl) is quite different: the acceptor fluorescence, at 515 nm, is lower, and the donor fluorescence, at 450–480 nm, can now be seen (Figure 5). This is exactly what is expected from eq 4 if denaturation causes a decrease in the transfer efficiency. A value of 10 is measured for the ratio Q of the intensities emitted at 515 and 476 nm; using eq 5′ leads to a value of 43% for E, the efficiency of energy transfer from the AENS to the fluorescein in unfolded DA-RNase.

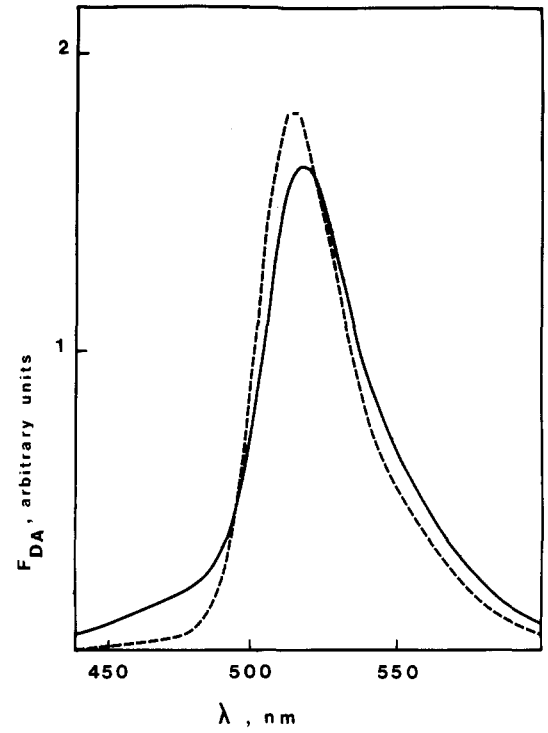

FIGURE 5: Fluorescence emission spectra of DA-RNase in 0.1 M cacodylate buffer in the absence (– –) and in the presence (—) of 6 M Gdn-HCl. Excitation wavelength 350 nm; protein concentration 0.01 mg/mL.

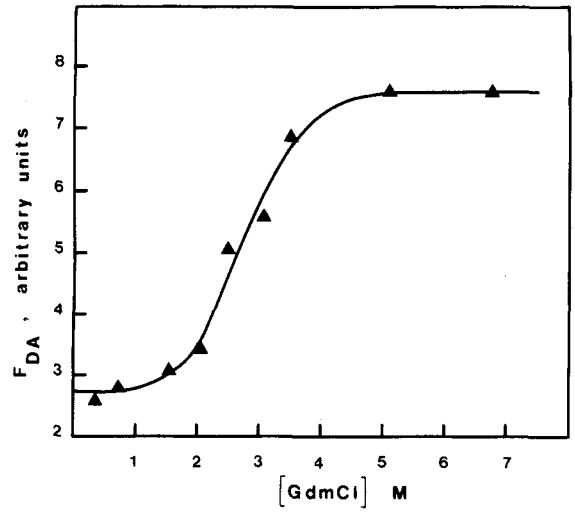

FIGURE 6: Fluorescence intensity of DA-RNase at 476 nm as a function of Gdn-HCl concentration in 0.05 M cacodylate buffer, pH 6.5, at 10 °C. Excitation wavelength 350 nm; protein concentration 0.04 mg/mL.

Unfolding Transition of DA-RNase As Measured by Energy Transfer. Unfolding of DA-RNase by 6 M Gdn-HCl reduces transfer efficiency by a factor of 2, from 100 to 43%. Figure 6 shows the dependence on Gdn-HCl concentration of the intensity emitted at 476 nm by DA-RNase when excited at 350 nm, i.e., of the donor fluorescence. The reappearance of donor fluorescence upon increasing Gdn-HCl concentration has a classical sigmoidal shape; its midpoint of 2.7 M Gdn-HCl agrees with those obtained in exactly the same conditions for D-RNase by fluorescence (Jullien & Garel, 1981) and for A-RNase by absorption (unpublished results). This confirms that the change in transfer efficiency is indeed directly related to the disruption of the protein structure and not to the change in solvent, from 0 to 6 M Gdn-HCl, in itself.

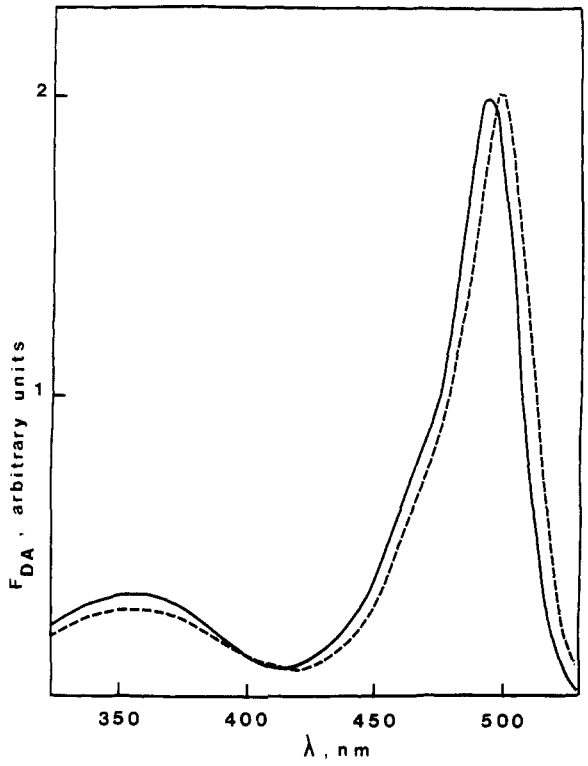

FIGURE 7: Corrected excitation spectra of DA-RNase in the presence (--) and in the absence (—) of 6 M Gdn-HCl in 0.1 M Tris buffer, pH 8.5 at 25 °C. Emission wavelength 516 nm.

Determination of Transfer Efficiency from Excitation Spectrum of DA-RNase. In the wavelength range of donor excitation, there is also some absorption of light by the acceptor; because the absorbance of fluorescein is strongly pH dependent (Lindquist, 1961), this interference of acceptor absorption with the excitation spectrum was minimized by using a pH value of 8.5; Tris buffer was used instead of cacodylate. The excitation spectra obtained for DA-RNase in the absence and presence of 6 M Gdn-HCl, at pH 8.5, are given in Figure 7; the emission wavelength is 516 nm, i.e., corresponding to the maximum emission of the acceptor. The spectra obtained in the presence or the absence of denaturant are very similar; the ratio Q' of the intensities at 495 and 350 nm changes from 14 in the absence of Gdn-HCl to 16 in its presence. Then, the effect of unfolding DA-RNase by 6 M Gdn-HCl is much less obvious on the excitation spectra (Figure 7) than it is on the emission spectra (Figure 5). However, eq 6 shows that the transfer efficiency E depends on the actual values of the molar extinction coefficients of the donor and acceptor at the two wavelengths chosen to determine Q'; these values are different in the presence and the absence of 6 M Gdn-HCl (Table I), and the relationship between E and Q' thus depends on solvent composition. This is shown in Figure 8, where the changes in E as a function of Q' are numerically calculated for both solvents, with and without 6 M Gdn-HCl. It can be seen that the same observed value of Q' will correspond to different values of E, depending on the solvent; for instance, $Q' = 15$ will correspond to $E = 95\%$ in native conditions and to $E = 60\%$ in unfolding conditions. For DA-RNase, the small change from $Q' = 14$ in the native state to $Q' = 16$ in the denatured state indeed corresponds to a decrease in transfer efficiency from $E = 100\%$ to $E = 55\%$ upon unfolding the protein by 6 M Gdn-HCl. Numerical eq 6′ is thus valid only in the presence of 6 M Gdn-HCl.

Determination of Distance between Donor and Acceptor in Unfolded Protein. Equation 1 can be used to determine the distance between donor and acceptor from the transfer efficiency E, provided that R_0 is known and also that the value of E is different from 0 or 100%. The native double-labeled protein shows a value of 100% for E, which means only that the donor–acceptor distance is less than a given value. The distance between donor and acceptor was therefore determined only for the unfolded state, for which E is around 50% (see above). Calculation of R_0 was performed from eq 2 as follows. (a) The orientation factor K^2 can be given the averaged value of $^2/_3$, corresponding to random orientations of rapidly moving donor and acceptor. Indeed, previous measurements have shown that the donor in the unfolded protein is highly mobile, with a rotational relaxation time around 1 ns, i.e., much shorter than the lifetime of the excited state, 11.5 ns (Jullien & Garel, 1981). Also, the acceptor was shown to be a surface label even in the native state (Garel, 1976). (b) A value of 1.43 was found for the refractive index of the unfolding solvent, 6 M Gdn-HCl, independently of the buffer, either cacodylate at pH 6.5 or Tris at pH 8.5. (c) The quantum efficiency of the donor, ϕ_D, was determined from lifetimes. A mean value of 25 ns can be obtained for the radiative lifetime of the AENS group from its fluorescence properties (Hudson & Weber, 1973). The fluorescence lifetime of this group in unfolded protein is 11.5 ns (Jullien & Garel, 1981), corresponding to a quantum efficiency ϕ_D of 0.46. This value was used at both pH 6.5 and 8.5; indeed, the fluorescence properties of the AENS group are pH independent in this range (Hudson & Weber, 1973). (d) The spectral overlap integral J_{DA} was calculated from eq 3, with the emission spectrum of D-RNase and the absorption spectrum of A-RNase (Figure 9). Because these spectra depend on solvent composition, mostly on pH in the present case, the value of J_{DA} has to be determined for each solvent. For unfolding conditions (6 M Gdn-HCl), values of J_{DA} of 1.2×10^{15} and of 1.6×10^{15} M^{-1} cm^{-1} nm^4 are obtained, respectively, for cacodylate buffer, pH 6.5, and Tris buffer, pH 8.5.

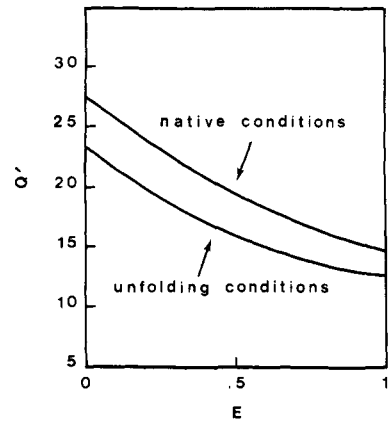

FIGURE 8: Theoretical curves obtained from eq 6 showing effect of solvent on measured transfer efficiency. (Native conditions) 0.1 M Tris buffer, pH 8.5; (unfolding conditions) 0.1 M Tris–6 M Gdn-HCl, pH 8.5.

With the above values, R_0 is found to be 44 Å in cacodylate buffer, pH 6.5, and 47 Å in Tris buffer, pH 8.5, both in the presence of 6 M Gdn-HCl. That the value of R_0 for a given donor–acceptor pair depends on solvent composition is obvious from eq 2 where three terms J_{DA}, n, and ϕ_D may differ according to the solvent and/or the chromophore environment. Indeed, R_0 values from 33 to 48 Å have been determined for the AENS–fluorescein pair in various conditions (Fairclough & Cantor, 1978). The donor–acceptor distance in the unfolded protein can now be calculated: at pH 6.5, $E = 43\%$ and $R_0 = 44$ Å yield a distance of 46 Å, and at pH 8.5, $E = 55\%$ and

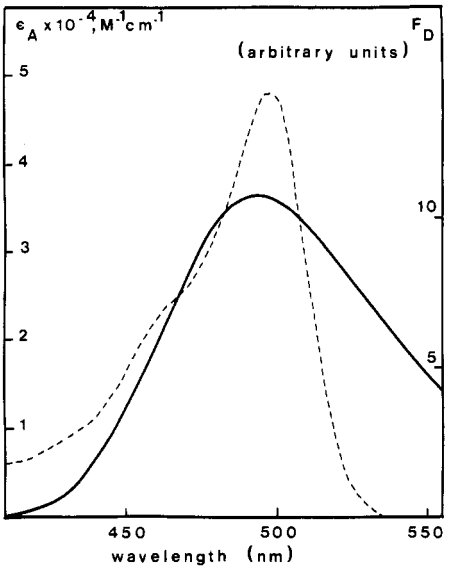

FIGURE 9: Overlap of fluorescence emission spectrum of D-RNase (—) and absorption spectrum of A-RNase (--) in 0.1 M Tris–6 M Gdn-HCl, pH 6.5.

R_0 = 47 Å yield a distance of 45 Å (eq 1). There is a quite good agreement between the two values obtained for the donor–acceptor distance in unfolded double-labeled RNase at pH 6.5 and 8.5, despite the differences in the values of R_0 and E and mostly despite the different procedures used to measure E at these two pH values, from emission spectra at pH 6.5 and excitation spectra at pH 8.5 (see above). The conformation of RNase in 6 M Gdn-HCl, which probably corresponds to a statistical distribution between many different unfolded configurations, is not expected to change appreciably between pH 6.5 and 8.5; only few groups ionize in this pH range, and the high ionic strength provided by Gdn-HCl minimizes all electrostatic interactions (Tanford, 1968). Then, the agreement between the two determinations of the donor–acceptor distance suggests that the value of 45 Å obtained is correct; since the protein is unfolded, the distance obtained corresponds to an average, the physical sense of which is discussed below. Such an average value for the distance between two given residues in an unfolded protein would have been difficult to measure by classical methods.

Reduction of Disulfide Bonds Results in an Increase of the Distance between Donor and Acceptor in Unfolded Protein. Upon reduction of disulfide bonds, the viscosity of unfolded RNase (unfolded by urea or Gdn-HCl) increases markedly, which shows that the presence of cross-links reduces the hydrodynamic volume of the polypeptide chain (Tanford, 1968). The same result is obtained by fluorescence transfer; indeed, upon reduction of disulfide bonds and subsequent carboxymethylation of the thiol groups, the transfer efficiency decreases from around 50 to around 10%, both being measured in 6 M Gdn-HCl. The determination of the actual distance between donor and acceptor from eq 1 with the R_0 values calculated above would require an accurate measure of E. The detailed study of the reduced protein will be reported elsewhere; it is still worth mentioning that reduction of the disulfide bonds increases the average distance between Lys-1 and His-119 from 45 to 60 Å or more.

Discussion

Intramolecular distance measurements by means of energy transfer between two fluorescent groups require that these groups be located at specific sites. In the case of a protein, the preparation of a double-labeled derivative suitable for such measurements represents a difficult step. Previous chemical work on RNase has led to the preparation and characterization of two single-labeled derivatives bearing respectively the donor or acceptor group in a defined site: D-RNase bears a donor AENS group in the active site (Jullien & Garel, 1981), and A-RNase bears an acceptor fluorescein group at the N-terminal end of the chain (Garel, 1976). A two-step chemical modification yields DA-RNase, a double-labeled derivative that probably has the donor attached to His-119 and the acceptor to Lys-1. In the single-labeled derivative D-RNase, unfolding of the protein results in a *decrease* of the fluorescence of the AENS group (Jullien & Garel, 1981). In the double-labeled DA-RNase, unfolding of the protein results in an *increase* of the fluorescence of the AENS group; indeed, the donor fluorescence is totally quenched in native DA-RNase and becomes detectable only after unfolding. This comparison shows that the increase of AENS fluorescence upon unfolding DA-RNase is dominated by the decrease in transfer efficiency and not by exposure to solvent.

There appears to be a qualitative agreement between the efficiency of this energy transfer and what can be expected of the donor–acceptor distance in three "classical" conformational states of the protein: a complete (or almost) transfer agrees with a short distance within the compact native state, a medium transfer agrees with a longer distance in an unfolded state cross-linked by disulfide bonds, and an almost absent transfer agrees with a still longer distance in the random-coiled linear unfolded state. The unfolded state with intact disulfide bonds is well suited for quantitative analysis of energy transfer because the efficiency of this transfer is around 50%. Such analysis implies first the determination of the actual value of transfer efficiency E and second the conversion of E into a distance. The average distance between the donor on His-119 and the acceptor on Lys-1 has thus been determined by using either the emission or the excitation spectra of DA-RNase: these two independent procedures yield the same distance of 46 ± 1 Å between residues 1 and 119 in disulfide-bonded RNase in 6 M Gdn-HCl. The donor–acceptor pair of the present double-labeled derivative is such that DA-RNase is only useful for measuring distances in the *unfolded* protein, because of the rather large value of R_0. This particular derivative is however suitable for studying the residual structure that may exist in the unfolded state and participate in the early part of the folding process (Baldwin, 1980).

The conversion of measured fluorescence intensities into distances involves the prerequisite determination of different numerical factors. The spectroscopic features of the donor and acceptor are usually derived from the properties of the single-labeled derivatives; this assumes that energy transfer is the only interaction existing between the two chromophores and is probably correct here where no ordered structure is present. The calculation of the overlap integral requires the knowledge of the refractive index n of the medium between the donor and acceptor, as well as that of their relative orientation K^2. In the case of an unfolded protein, as is done here, the value of n is simply that of the solvent, and K^2 can be given the value of $^2/_3$, corresponding to an average over all possible orientations (Fairclough & Cantor, 1978). In the case of a native protein, where the two chromophores may not possess a high relative mobility, the choice of $^2/_3$ for K^2 or of the solvent refractive index for that inside the protein may lead to some ambiguities. For a given pair of donor and acceptor, the value of the overlap integral J_{DA} and, hence, of R_0 depends on the solvent: indeed, two slightly different values of R_0, 44 and 47 Å, have been found for unfolded DA-RNase. This

dependence on the solvent, as well as the uncertainties brought by some dubious choice of K^2 and/or n, may explain why the R_0 values found for the pair AENS–fluorescein have been reported to range between 33 and 48 Å (Fairclough & Cantor, 1978). The correct determination of R_0 is crucial for distance measurements: indeed, eq 1 shows that only distances in the range from $0.5R_0$ to $1.9R_0$ can be determined with reasonable accuracy.

In native DA-RNase, the distance between donor and acceptor can be estimated from the crystallographic data (Wlodawer et al., 1982); the center of mass to center of mass distance between Lys-1 and His-119 is about 17 Å, and thus the donor–acceptor distance probably does not exceed 20–25 Å. This is in agreement with the very good transfer observed in this native state. Upon unfolding, this distance increases at least twice and becomes 46 Å. This increase in the donor–acceptor distance is of course related to the larger hydrodynamic volume of the unfolded state (Tanford, 1968).

Estimation of the distance between residues has been proposed for unfolded chains only for the end-to-end distances (Tanford, 1961). RNase has 124 residues, and it can be considered that the end-to-end distance is reasonably approximated by that between residues 1 and 119. Unfolded RNase with intact disulfide bonds has an intrinsic viscosity of 9.4 cm^3/g; this leads to 27 Å for the radius of the equivalent sphere (Tanford, 1961) and to 77 Å for the end-to-end distance of an ideal flexible polymer having the same space occupancy (Tanford, 1961). Brant & Flory (1965) have proposed a method for calculating the end-to-end distance of a polypeptide chain in a random-coil conformation; for 124 residues, this yields a value of 130 Å. However, the topological constrains due to the four disulfide bonds restrict the maximum length of the extended conformation to half of its value (Richards & Wyckoff, 1971); the end-to-end distance of the cross-linked coil would then be 60–70 Å. It seems that the actual value of 46 Å found for the distance between residues 1 and 119 is slightly lower than the 60–80 Å calculated from various models (Tanford, 1968); however, the high degree of approximation of these models in terms of spherical shape, solvation, excluded volume, homogeneity of residues, ideal solvent, etc. preclude any conclusion.

The distance actually measured represents a double average taken in space over all the possible configurations of the chain and taken in time over all the movements occurring during the lifetime of the donor excited state. Indeed, the efficiency of energy transfer is influenced by the dynamics of the chain; for instance, the translational diffusion of the fluorophores may enhance the transfer efficiency by changing the donor–acceptor distance during the lifetime of the donor excited state. In the case of an oligopeptide with a fluorophore at each end, the efficiency of transfer is increased if the solvent viscosity is decreased; at high viscosity, the end-to-end distance is "frozen" during the donor lifetime, whereas at low viscosity the two ends are now free to move toward each other (Haas et al., 1978). An estimation of the bias introduced by the space-averaging procedure can be reached in the limiting situation corresponding to the following assumptions: (1) The donor–acceptor distance in a given molecule does not change during the lifetime of the donor excited state; this would be the case in a very viscous medium. (2) The set of configurations taken by the chain molecules corresponds to a Gaussian distribution of the donor–acceptor distance. (3) The observed overall transfer efficiency is the sum of the individual efficiencies contributed by each of these configurations.

Then, a simple calculation shows that the observed efficiency of 43% corresponds to a root mean square average distance between donor and acceptor of 57 Å instead of 46 Å, as directly determined from eq 1. The contribution of time averaging, i.e., of that related to the relative diffusion of donor and acceptor, can be approached by time-resolved fluorescence measurements (Haas et al., 1978). It is expected that further fluorescence studies will lead to a more accurate representation of the static and dynamic properties of a polypeptide chain.

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