Lee, J. J., & Berns, D. S. (1968) *Biochem. J. 110*, 465–470.
MacColl, R., Berns, D. S., & Koven, N. L. (1971a) *Arch. Biochem. Biophys. 146*, 477–482.
MacColl, R., Lee, J. J., & Berns, D. S. (1971b) *Biochem. J. 122*, 421–426.
MacColl, R., Csatorday, K., Berns, D. S., & Traeger, E. (1980) *Biochemistry 19*, 2817–2820.
Saito, T., Iso, M. N., & Mizuno, H. (1974) *Bull. Chem. Soc. Jpn. 47*, 1375–1379.
Saito, T., Iso, N., Mizuno, H., & Kitamura, I. (1978) *Bull. Chem. Soc. Jpn. 51*, 3471–3474.
Scott, E. (1965) Ph.D. Thesis, Albany Medical College, Union University, Albany, NY.
Scott, E., & Berns, D. S. (1965) *Biochemistry 4*, 2597–2606.
Steiner, R. F. (1952) *Arch. Biochem. Biophys. 39*, 333–354.
Viograd, J., Bruner, R., Kent, R., & Weigle, J. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49*, 902–910.
Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Fluorescent Probe of Ribonuclease A Conformation†

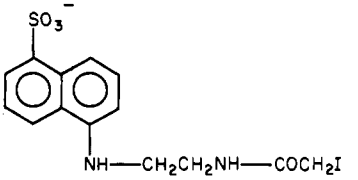
Magali Jullien and Jean-Renaud Garel*

ABSTRACT: The reaction of ribonuclease (RNase) with *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (1,5-IAENS) yields a derivative in which one fluorescent group is covalently attached to the protein. Several arguments suggest that the chemical modification has occurred at the enzyme active site: (i) 1,5-IAENS should have the same specificity as iodoacetamide, i.e., carboxymethylate one histidine of the active site; (ii) the derivatized protein is enzymatically inactive; (iii) in the native state of the protein, the fluorescent group is (almost) completely protected from the aqueous solvent; (iv) this group has no motions other than those of the protein. The fluorescence properties of the derivatized RNase change markedly upon unfolding induced by guanidine hydrochloride (Gdn·HCl), as seen from fluorescence intensity, maximum emission wavelength, and polarization measurements. Upon Gdn·HCl-induced unfolding, the fluorescent group is transferred from a nonpolar to a highly polar environment. Dynamic fluorescence measurements show also that unfolding results in a markedly increased mobility of the fluorescent label with respect to its proteic environment. These results are compared to those of Young & Potts (1963) [Young, D. M., & Potts, J. T. (1963) *J. Biol. Chem. 238*, 1995–2002], who studied the fluorescence properties of a surface-labeled derivative of RNase.

The use of reporter groups has proved to be a useful technique for studying conformational changes in proteins. The changes observed in a given property of the reporter group(s) are assumed to reflect changes in its environment as provided by the protein. Such reporter groups can be either built in the protein structure (e.g., aromatic residues, prosthetic groups, etc.) or introduced into the protein by a suitable chemical modification. A most desirable situation is when a single reporter group exists, because the origin of the observed changes can be unambiguously assigned to a defined region of the protein. Among all the conformational changes which can take place within a polypeptide chain, the major one, at least in amplitude, is its folding, i.e., the process by which this chain acquires its unique, compact, native structure. Ribonuclease (RNase)[1] is one of the small globular proteins the folding process of which has been, and still is, extensively studied, and the present paper shows that a fluorescent reporter group can be introduced into RNase and that it can be used to monitor folding of the protein.

The reason for choosing fluorescence as the observed parameter is that different information can be obtained depending on the type of measurement (Stryer, 1968). Fluorescence intensity, maximum emission wavelength, and lifetime are influenced by the properties of the close environment of the probe, such as polarity, solvation, etc. Fluorescence polarization and anisotropy decay depend on the mobility of the probe and can report on the relative motions of the probe and its environment. Therefore, a fluorescent probe can be characterized both by its static and by its dynamic behavior in different conformational states of the protein. RNase has no tryptophan; its intrinsic fluorescence is only due to its six tyrosines. Tyrosine fluorescence has been used to measure RNase folding, but only as an overall parameter, through fluorescence intensity (Schmid, 1981).

The strategy for introducing a covalent fluorescent probe into RNase rests on the particular reactivity of the active site toward halo acids and their amide derivatives. At slightly acidic pH, these reagents modify almost specifically one histidine in native RNase, either His-12 or His-119, but not both of them (Richards & Wyckoff, 1971). RNase was therefore reacted with *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (1,5-IAENS), a reagent which combines a fluorescent group similar to dansyl and a reactive end resembling iodoacetamide, separated by free-rotating bonds (Hudson & Weber, 1973):



The reaction of 1,5-IAENS with RNase yields a chemical derivative, AENS-RNase, which has one fluorescent group

† From the Unité de Biochemie Cellulaire, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 75724 Paris Cedex 15, France. *Received May 15, 1981.* This work was supported by the Centre National de la Recherche Scientifique (Grant No. 30) and the Institut Pasteur.

[1] Abbreviations used: RNase, bovine pancreatic ribonuclease; 1,5-IAENS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid; AENS, (acetamidoamino)ethyl-5-naphthylamine-1-sulfonic acid; Gdn·HCl, guanidine hydrochloride; 2′,3′-CMP, cytidine cyclic 2′,3′-phosphate; 2′-CMP, cytidine 2′-phosphate.

0006-2960/81/0420-7021$01.25/0

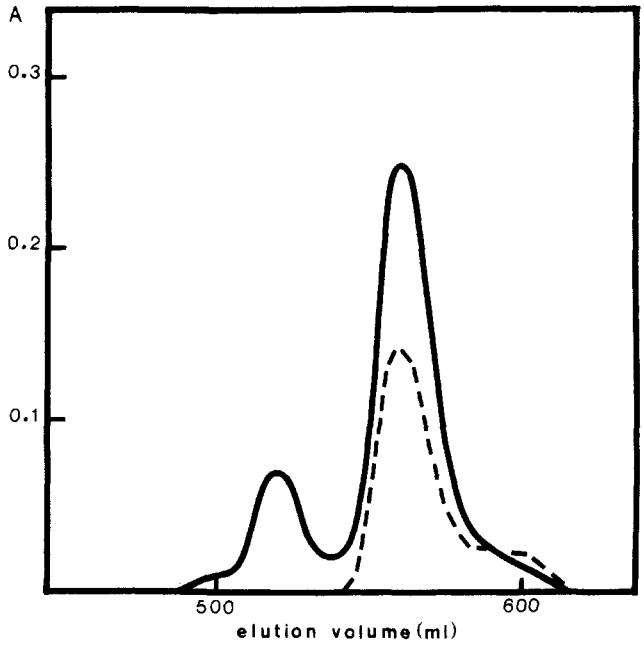


FIGURE 1: Ion-exchange chromatography on a 1.3 cm × 50 cm column of CM-cellulose of the products from the reaction of RNase A with 1,5-IAENS at pH 5.5. Elution was carried out with 1000 mL of a linear gradient of 0–0.3 M NaCl in 10 mM sodium cacodylate, pH 6.5, at room temperature. (—) Absorbance at 278 nm; (---) absorbance at 340 nm.

per protein molecule, and which is suitable for a fluorescence study of protein folding.

Experimental Procedures

Materials. RNase A was from Sigma (lot 95 C 8147) and was purified by ion-exchange chromatography (Garel, 1976). 2′,3′-CMP and 1,5-IAENS were from Sigma. Cacodylate (sodium salt) was from Merck; guanidine hydrochloride (Gdn·HCl) and sucrose were Schwarz/Mann ultrapure. Sephadex G-25 (medium) was from Pharmacia and CM 52 cellulose from Whatman.

Methods. Enzymatic activity toward 2′,3′-CMP was determined at 25 °C from the change in absorbance at 292 nm as a function of time, using a Cary 14 spectrophotometer (Crook et al., 1960). Fluorescence spectra were obtained with a Jobin-Yvon JY3 spectrofluorometer. Fluorescence polarizations and anisotropy decays were measured in Dr. Le Pecq's laboratory, using photon counting techniques. Concentrations of sucrose and Gdn·HCl solutions were measured by refractometry.

Preparation of the Conjugate AENS-RNase. RNase was reacted with 1,5-IAENS under the following conditions: (0.5–1) × 10^{-3} M RNase, (1–2) × 10^{-2} M, 1,5-IAENS, in 200 mM cacodylate, pH 5.5. The reaction was allowed to proceed in the dark at room temperature. The extent of alkylation of amino acid residues at the active site was determined by the loss of catalytic activity against 2′,3′-CMP. A 70% conversion of the enzyme to an inactive product was obtained in 48 h. The excess of unreacted dye was then removed by gel filtration on Sephadex G-25. The labeled protein was further fractionated by ion-exchange chromatography on CM-cellulose at pH 6.5; three species were resolved (Figure 1). The fractions corresponding to the major peak were pooled, dialyzed against water, and lyophilized. The yield in labeled protein was about 30%.

Results and Discussion

Reaction of RNase with 1,5-IAENS and Characterization of the Derivative AENS-RNase. A disappearance of enzymatic activity is observed during the reaction of RNase with 1,5-IAENS (Figure 2). This disappearance is not observed in the presence of an excess of the competitive inhibitor 2′-CMP (Figure 2). The protection by 2′-CMP against RNase inactivation suggests that 1,5-IAENS abolishes activity by reacting with the enzyme active site. With 10^{-2} M 1,5-IAENS, the half-life for RNase inactivation is 15–20 h (Figure 2). Fruchter & Crestfield (1967) have found that, in similar conditions, iodoacetamide inactivates RNase with a rate constant of about 10^{-4} M^{-1} s^{-1}; with 10^{-2} M iodoacetamide, this corresponds to an inactivation half-life of 200 h or so. Thus, 1,5-IAENS reacts with the RNase active site at least 1 order of magnitude faster than does iodoacetamide. This enhanced reactivity could arise from the weak binding of the aromatic rings and negative charge of the AENS group to the RNase active site.

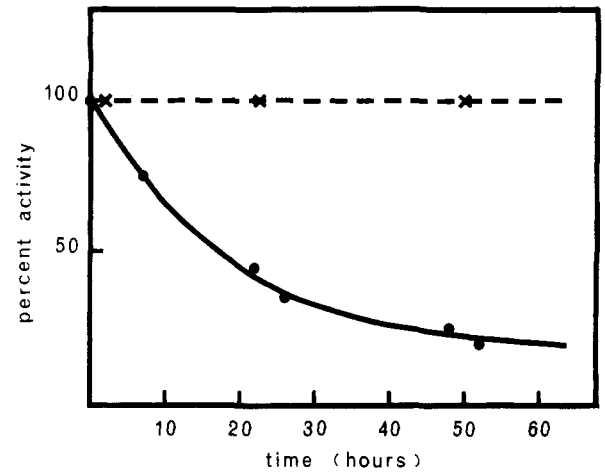


FIGURE 2: Time dependence of the enzymatic activity toward 2′,3′-CMP as a substrate during the reaction of RNase with 1,5-IAENS in the absence (●) or in th presence (×) of 2 × 10^{-3} M 2′-CMP. Experimental conditions: 6 × 10^{-4} M RNase, 10^{-2} M 1,5-IAENS, 0.2 M cacodylate buffer, pH 5.5, room temperature, in the dark.

Ion-exchange chromatography of the products of the reaction of RNase with 1,5-IAENS on CM-cellulose shows the presence of a major labeled protein fraction (Figure 1). This fraction, termed AENS-RNase from here on, has less than 1% of the enzymatic activity of unmodified RNase, using 2′,3′-CMP as a substrate. The stoichiometry of labeling of RNase by 1,5-IAENS was determined by spectrophotometric measurements. Figure 3 shows the absorption spectrum of AENS-RNase, which is composed of three main bands, at 340, 278, and 255 nm. The absorbance at 340 nm of AENS-RNase is due only to the bound AENS groups and can thus be used to measure the total concentration of bound fluorescent groups, using a molar absorbance of 6.8 × 10^3 M^{-1} cm^{-1} (Hudson, 1970). The absorbance at 278 nm of AENS-RNase is mainly due to the protein moiety; at this wavelength, the RNase molar absorbance is 9.8 × 10^3 M^{-1} cm^{-1} (Richards & Wyckoff, 1971), whereas that of the AENS group is only about 10^3 M^{-1} cm^{-1} (Hudson, 1970; Hudson & Weber, 1973). An estimate of the number of AENS groups bound per RNase molecule can therefore be obtained by (i) determining the concentration of AENS groups from A_{340nm} and (ii) determining the concentration of protein from A_{278nm} after correction for the absorption of he bound dye. In addition, the actual molar absorbance of AENS-RNase at 278 nm was measured by direct determination of protein concentration according to Lowry et al. (1951), using RNase as a standard; a value of (1.1 ± 0.05) × 10^4 M^{-1} cm^{-1} was obtained. Not only were the two methods of measuring protein concentration in very good agreement

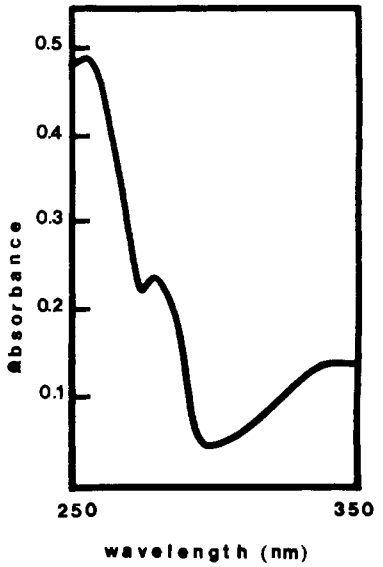


FIGURE 3: Absorption spectrum of AENS-RNase in 10 mM sodium cacodylate, pH 6.5, 25 °C.

but also they both gave consistently a ratio of AENS group per RNase molecule of 1 ± 0.1; AENS-RNase is an inactive derivative which bears one covalently attached fluorescent group per protein molecule. Because the AENS and protein moieties can be measured almost independently of each other at 340 and 278 nm, respectively, spectrophotometric determination of the labeling stoichiometry is rather precise, and no other method had to be used to confirm the 1:1 labeling ratio.

The covalent binding of one AENS group abolishes RNase activity; this binding does not take place when 2′-CMP is present (Figure 2), suggesting that 1,5-IAENS has reacted with the active site. Fruchter & Crestfield (1967) found that two residues of RNase react preferentially with iodoacetamide: His-12, with loss of activity, and a methionine, without such a loss. They could separate these two mono-substituted derivatives by chromatography on a cation exchanger and showed that they were formed at about the same rate. It is very unlikely that AENS-RNase contains a significant fraction of protein molecules alkylated on a methionine because these molecules should (i) show enzymatic activity and (ii) have been removed by chromatography on a cation exchanger like CM-cellulose. At pH 5.5, Lys-41 does not react with either iodoacetamide or halo acids (Heinrikson, 1966; Fruchter & Crestfield, 1967). The most probable site of reaction with 1,5-IAENS seems to be one of the two histidines in the active site, His-12 and His-119. If 1,5-IAENS has the same chemical specificity as iodoacetamide, it may have alkylated mostly His-12. If, on the other hand, 1,5-IAENS has the same specificity as halo acids (as its increased rate of reaction might suggest), it may have alkylated either His-12 or His-119, but not both in the same protein molecule. It is not known whether AENS-RNase is chemically homogeneous or corresponds to a mixture; no evidence of heterogeneity was observed in the physical behavior of AENS-RNase (see below), which indicates that the AENS group is located in a single environment, independently of a possible heterogeneity of its attachment site. In conclusion, it seems as if AENS-RNase is a physically homogeneous derivative bearing a single fluorescent group, most likely at the protein active site on one of the histidines.

When excited in the highest wavelength absorption band, that at 340 nm, AENS-RNase is strongly fluorescent, with a maximum emission wavelength at 481 nm (Figure 4). Using polarization spectra, we could verify that this 340-nm band

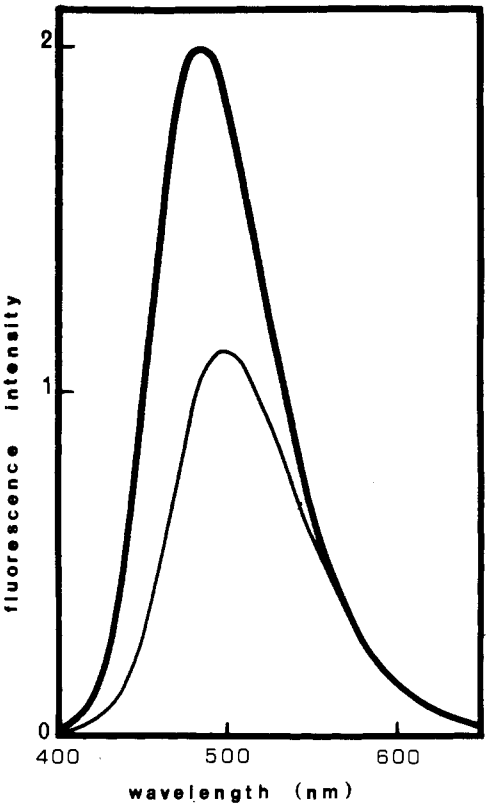


FIGURE 4: Uncorrected fluorescence emission spectra of native (thick solid line) and unfolded (thin solid line) AENS-RNase in 100 mM sodium cacodylate, pH 6.5, at 25 °C. Excitation wavelength, 352 nm; protein concentration, 5 μM.

is actually composed of two different electronic transitions, as initially shown by Stryer (1965).

Equilibrium Fluorescence Measurements of the Folding–Unfolding Transition of AENS-RNase. The fluorescence properties of AENS-RNase depend upon the conformational state of the protein. Upon the transition from the native state to the Gdn·HCl unfolded state, the fluorescence intensity decreases markedly, and the maximum emission wavelength increases from 481 to 498 nm (Figure 4). Both parameters can be used to monitor the Gdn·dHCl-induced transition of AENS-RNase, and both give the same transition curves, with a midpoint at 2.5 M Gdn·HCl (Figure 5). The shape and midpoint of the transition curve of AENS-RNase are similar to those obtained for unmodified RNase (Salahuddin & Tanford, 1970), which indicates that the introduction of the AENS group into RNase has not markedly altered the stability of the protein toward Gdn·HCl-induced unfolding. This Gdn·HCl-induced transition shows complete reversibility.

The detailed interpretation of the changes in either fluorescence intensity or maximum emission wavelength is difficult, but it is, however, admitted that these two parameters are sensitive to different features of the environment of the probe: the maximum emission wavelength reflects the overall dipolar character of this environment, whereas the intensity is related to the quantum yield and is rather influenced by particular deactivating processes (Stryer, 1968). Hudson & Weber (1973) have carried out a study of the fluorescence properties of the AENS group in its free state, using solvents of different polarities. A decrease in solvent polarity results in an increase in fluorescence intensity and in a decrease in the maximum emission wavelength. These results show that when AENS-RNase is unfolded, the fluorescent probe is transferred from a highly hydrophobic environment in the native state to a much more polar environment in the unfolded

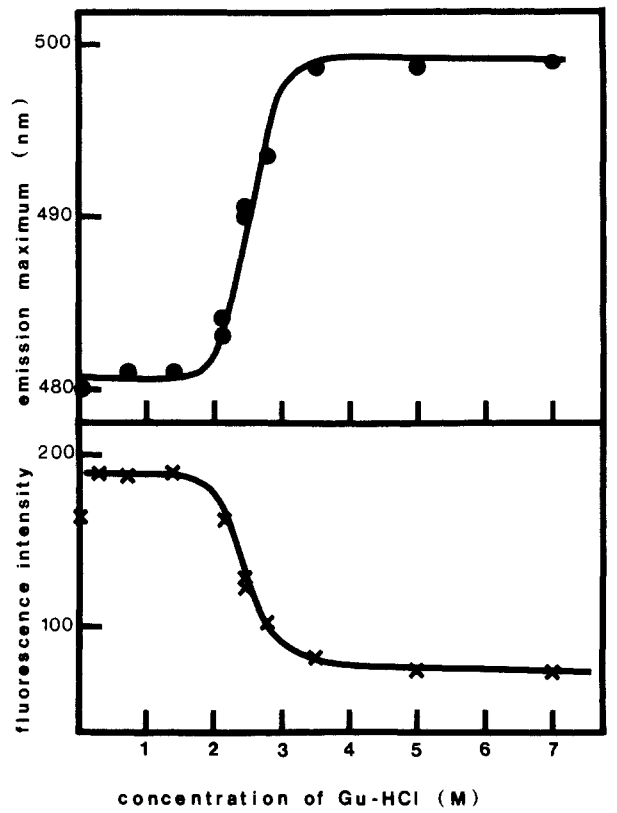


FIGURE 5: Effect of Gdn·HCl concentration on the fluorescence of AENS-RNase. Folding transition curve at 10 °C measured by the shift of emission maximum (●) or by the change of fluorescence intensity at 483 nm (×). Excitation wavelength, 353 nm; protein concentration, 0.2 mg/mL.

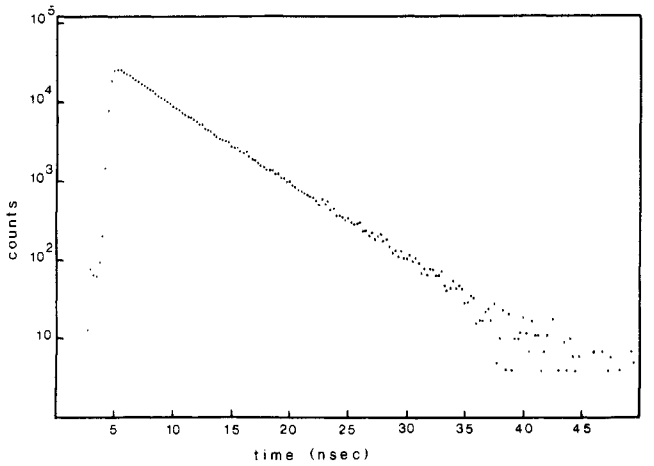


FIGURE 6: Time dependence of the total fluorescence intensity emitted by AENS bound to RNase A, in 10 mM sodium cacodylate at 25 °C. Excitation wavelength, 330 nm; protein concentration, 0.3 mg/mL.

state. The fluorescence properties of native AENS-RNase are those of the free fluorophore in 80% ethanol, which suggests that the AENS group is almost completely shielded from the aqueous solvent by the protein structure. Since RNase has no hole or cleft large enough to accommodate a bulky AENS group except its active site (Richards & Wyckoff, 1971), this also indicates that this active site is the place where modification by 1,5-IAENS has taken place.

This difference in environment of the AENS group between the native and unfolded states of AENS-RNase is also shown by fluorescence lifetime measurements. A value of 20.5 ns was obtained for the lifetime in the native state (Figure 6), while a value of only 11.5 ns was found for the unfolded state.

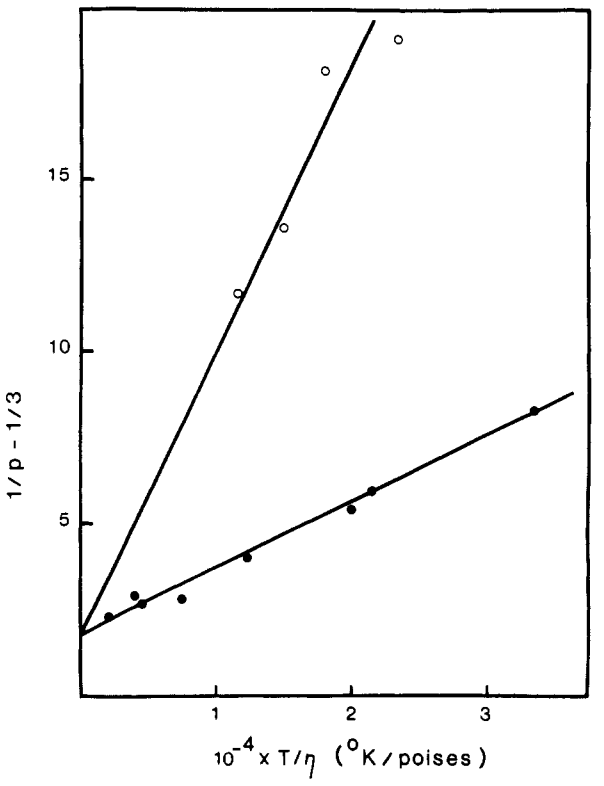


FIGURE 7: Perrin plots for fluorescence polarization of native (●) and unfolded (○) AENS-RNase. Viscosity was increased by adding sucrose up to 50%; temperature varied in the range 10–25 °C. Excitation wavelength, 380 nm; emission wavelength, 500 nm; protein concentration, 0.1 mg/mL.

Comparison with the results of Hudson & Weber (1973) again shows that the AENS group is not in contact with aqueous solvent in the native state, whereas it is in the unfolded state. Finding that the fluorescent group is exposed to solvent in the unfolded state is not unexpected. It is, however, more surprising to monitor the almost complete burial of the AENS group in the native state: results of fluorescence intensity, maximum emission wavelength, and lifetime are in agreement to show little contact between the aqueous solvent and the probe when AENS-RNase is native. This suggests that a strong interaction exists between the probe and the protein, which could be due to the resemblance, aromatic rings and negative charge, between the AENS group and the usual ligands of RNase. This resemblance has been already invoked above as a possible explanation for the enhanced reactivity of 1,5-IAENS as compared to iodoacetamide.

Dynamic Fluorescence Measurements on AENS-RNase. The motions of the AENS group in AENS-RNase were studied by steady-state fluorescence polarization and by fluorescence anisotropy decay measurements. Figure 7 shows a Perrin plot of the fluorescence polarization of the probe in native AENS-RNase; this plot is a straight line, which indicates that the derivative behaves as a homogeneous species with respect to the motions of the AENS group. Extrapolation to infinite viscosity gives a value for the fundamental polarization, $p_0 = 0.45$. A close value was found for the free fluorophore in glycerol, suggesting that the electronic properties of the probe are the same whether free or bound to RNase. Using the value of 20.5 ns found above for the lifetime of the excited state, we could calculate a mean relaxation time of 6 ns for the AENS group. This value of 6 ns is in good agreement with the rotational relaxation time of RNase, whether derived from electric birefringence measurements of the rotational diffusion coefficient or calculated for a hydrated spherical particle

(Richards & Wyckoff, 1971). This agreement suggests that the AENS group has no faster motion than that imposed on it by the rotational relaxation of the protein.

The value of the mean relaxation time of the AENS group in native AENS-RNase was also measured directly by anisotropy decay; the time course of fluorescence anisotropy decay could be reasonably well fitted by a single exponential, which again is a good indication of the physical homogeneity of AENS-RNase. The time constant for this decay, corrected to standard conditions (in water at 25 °C), is $\theta_w^{25} = 6.1$ ns, in close agreement with the value of 6 ns obtained from steady-state measurements. This confirms that the AENS group has overall motions which are severely restricted by the protein. Taking this result together with the tight interaction between the probe and the protein in native AENS-RNase (see above), it seems as if the reporter group is in a frozen position with respect to its proteic environment. RNase behaves almost as a rigid sphere as regards its rotational relaxation, and the AENS group is so tightly bound to it that its only motions are the protein overall motions.

The same measurements were carried out on Gdn·HCl-unfolded AENS-RNase. When the protein is unfolded, a marked decrease in steady-state polarization was observed, which made all measurements less accurate. The data are consistent however with the same value of the fundamental polarization, $p_0 = 0.45$, as that found above (Figure 7). Using a fluorescence lifetime for the excited state of 11.5 ns, we could estimate a mean relaxation time of around 2 ns. In the same conditions, the decay of anisotropy is also more difficult to measure, but a value around 1 ns could be evaluated, after correction to standard conditions. These values of 1 and 2 ns, although approximate, are significantly lower than that of 6 ns obtained for native AENS-RNase. The fluorescent probe in unfolded AENS-RNase then appears much more mobile; it possesses rapid motions which result in a fast reorientation with respect to the polarized light. Unfolded RNase no longer behaves as a rigid body, and each segment of the polypeptide chain has its own motions. The same is true of the fluorescent probe for which unfolding results in the loss of the tight interaction with the protein and in a markedly increased rate of motions.

That this change in mobility of the AENS group actually parallels the protein unfolding is shown in Figure 8; indeed, the dependence on Gdn·HCl concentration of the steady-state fluorescence polarization gives a transition curve which is the same as those obtained above (Figure 5). Therefore, unfolding AENS-RNase results in measurable changes of all the fluorescence properties of the reporter group; this group is solvent shielded and rigidly attached to the protein in the native state, whereas it is exposed to solvent and mobile in the unfolded state.

Conclusions

The reaction of RNase with 1,5-IAENS yields a derivative, AENS-RNase, bearing one fluorescent group per protein molecule. That labeling has taken place at the enzyme active site is suggested by the following arguments: (i) AENS-RNase has no enzymatic activity; (ii) the presence of 2′-CMP prevents inactivation of RNase by 1,5-IAENS; (iii) 1,5-IAENS probably inactivates RNase as iodoacetamide or halo acids do, i.e., by alkylating one of the two histidines of the active site, but not both of them (Heinrikson, 1966; Fruchter & Crestfield, 1967); (iv) the fluorescent probe of AENS-RNase is almost completely shielded from solvent in the native state, and the only cleft in RNase is the active site (Richards & Wyckoff, 1971); (v) there is a strong enough interaction between the

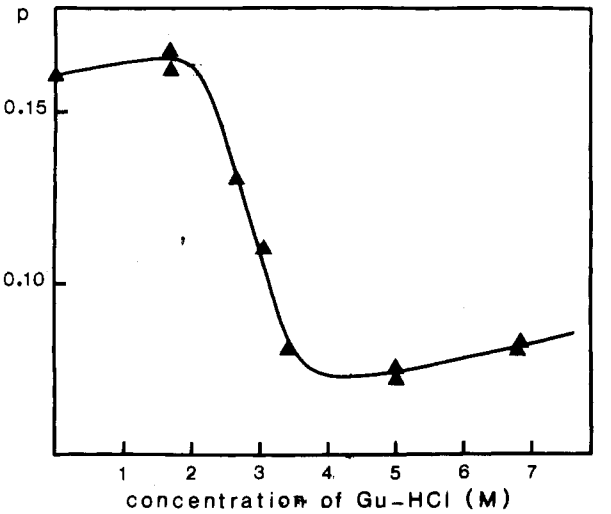


FIGURE 8: Degree of fluorescence polarization of AENS-RNase as a function of Gdn·HCl concentration at 10 °C; degrees of polarization are not corrected for viscosity of the solvent. Excitation wavelength, 380 nm; emission wavelength, 500 nm. Buffer, 50 mM sodium cacodylate, pH 6.5. Protein concentration, 0.1 mg/mL.

AENS group and the protein so that the probe has no motions other than those of AENS-RNase.

This assignment of the AENS group to the active site is consistent with both the known properties (chemical and structural) of RNase and our experimental results. Even if the actual site of covalent attachment of the AENS group might be questioned, this probe behaves as if it is in a unique environment; i.e., AENS-RNase appears as a physically homogeneous species. In the native state of the protein, the fluorescent probe seems to be a solid part of AENS-RNase and is probably embedded in the active site.

All the fluorescence properties of the AENS group examined so far are sensitive to the conformational state of the protein, as seen from the Gdn·HCl-induced transition. In the unfolded state, the fluorescent probe is exposed to aqueous solvent and possesses rapid motions. In the native state, the probe is buried (or almost) inside the protein; the tight interaction between the probe and the protein restricts the probe mobility to the extent that the AENS group has no motions other than those due to the overall motions of AENS-RNase. These differences in both exposure to solvent and mobility of the AENS group indeed reflect the folding–unfolding transition of the protein: the same transition curves are obtained from different fluorescence measurements, and they resemble the corresponding curve for unmodified RNase. Therefore, the AENS group represents a valuable probe to monitor the folding of the protein. A similar approach has been used earlier, also on RNase, by Young & Potts (1963) with dansyl chloride as a reagent. This reagent was chosen most likely because it reacts primarily with exposed amino groups and thus yields a statistical labeling of the protein surface. Such a procedure has the advantage of minimizing the possible perturbations due to a chemical modification, and the disadvantage of an external heterogeneous label. And indeed, with labeling ratios ranging between 0.5 and 3 fluorescent groups per RNase molecule, Young & Potts (1963) did not abolish enzymatic activity but were unable to measure a defined unfolding transition curve. Also, the motions of the probe(s) did not seem to be significantly different in the native and the unfolded states of the protein, even though the unfolded state was reduced and carboxymethylated. These differences in their and our results, using the same protein and closely related fluorescent probes, illustrate the importance of the labeling

procedure in terms of the reagent used and of the chemical and structural features of its target site. The derivative described here, AENS-RNase, with its single probe located in a specific site which makes it sensitive to changes in the environment, can obviously be further utilized to investigate various equilibrium or kinetic aspects of protein folding through measurements of its different fluorescence properties.

Acknowledgments

We are indebted to Drs. J. B. Le Pecq and M. Le Bret for their help in dynamic fluorescence measurements.

References

Crook, E. M., Mathias, A. P., & Rabin, B. R. (1960) *Biochem. J. 74*, 234-238.
Fruchter, R. G., & Crestfield, A. M. (1967) *J. Biol. Chem. 242*, 5807-5812.
Garel, J. R. (1976) *Eur. J. Biochem. 70*, 179-189.
Heinrikson, R. L. (1966) *J. Biol. Chem. 241*, 1393-1405.
Hudson, E. N. (1970) Ph.D. Dissertation, Unversity of Illinois.
Hudson, E. N., & Weber, G. (1973) *Biochemistry 12*, 4154-4161.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647-806.
Salahuddin, A., & Tanford, C. (1970) *Biochemistry 9*, 1342-1347.
Schmid, F. X. (1981) *Eur. J. Biochem. 114*, 105-109.
Stryer, L. (1965) *J. Mol. Biol. 13*, 482-495.
Stryer, L. (1968) *Science (Washington, D.C.) 162*, 526-533.
Young, D. M., & Potts, J. T. (1963) *J. Biol. Chem. 238*, 1995-2002.

Hydroxylamine Oxidoreductase: A 20-Heme, 200 000 Molecular Weight Cytochrome *c* with Unusual Denaturation Properties Which Forms a 63 000 Molecular Weight Monomer after Heme Removal†

Kathleen R. Terry and Alan B. Hooper*

ABSTRACT: Hydroxylamine oxidoreductase catalyzes the successive dehydrogenation and oxygenation of NH_2OH to HNO_2. Molecular weight (M_r) values of 180 000–200 000 have been reported for the enzyme. Per M_r 200 000, the enzyme contains 18 *c*-type and approximately 2 P-460 hemes [Hooper, A. B., Maxwell, P. C., & Terry, K. R. (1978) *Biochemistry 17*, 2984]. Boiling the enzyme in the presence of 2% sodium dodecyl sulfate ($NaDodSO_4$) and 5% 2-mercaptoethanol followed by polyacrylamide gel electrophoresis in 0.1% $NaDodSO_4$ resulted in five bands with the following designations and molecular weights: I, 225 000; II, 195 000; III, 125 000; IV, 56 500; V, 11 000. Bands I–III and V contained heme. Partial proteolysis of bands I–III produced identical fragments. The ratio of bands I:II:III depended on the presence or absence of 2-mercaptoethanol and the length of heating in $NaDodSO_4$. Production of band I required prolonged treatment and occurred in two discrete steps; bands II and III were apparently intermediates in a sequential denaturation or polymerization. Band IV, which was always present in less than one molecule per molecule of large subunit, was either a contaminant or a hemeless fragment of the large polypeptide. Band V, which was released from the enzyme in $NaDodSO_4$ without 2-mercaptoethanol, was present in a ratio of three or four molecules per molecule of large polypeptide. Some samples of purified enzyme contained heme P-460, 20 molecules of *c*-type heme, and full enzymatic activity, though lacking band IV or V. Further, bands I–III exhibited some enzyme activity on $NaDodSO_4$ gels. Thus the large polypeptide contains all factors necessary for catalysis. Treatment of the enzyme or band I with 2-nitrophenylsulfenyl chloride, which removes thioether-linked heme, resulted in the formation of a M_r 63 000 polypeptide in approximately a 1:1 molar ratio with the band V cytochrome. We conclude that, as isolated, hydroxylamine oxidoreductase probably consists of three molecules of a monoheme *c*-type cytochrome, M_r 11 000, and three tightly complexed molecules of a catalytically active M_r 63 000 protein containing six *c*-type hemes and one P-460 heme.

In nature, essentially all ammonia in aerobic soils or waters is rapidly oxidized to nitrite by the nitrifying bacteria. The nitrifying bacterium *Nitrosomonas europaea* grows autotrophically, utilizing the oxidation of ammonia to nitrite as the sole energy source. Hydroxylamine, or a closely related chemical species which is enzyme bound, is an intermediate in the process. The enzyme hydroxylamine oxidoreductase from *Nitrosomonas* catalyzes the rapid aerobic oxidation of hydroxylamine to nitrite in the presence of phenazine methosulfate (Hooper, 1978). Molecular weight (M_r)[1] values in the range 180 000–200 000 have been reported for the enzyme (Rees, 1968; Maxwell, 1976; Hooper et al., 1978; Yamanaka et al., 1979). On the basis of a particle weight of 200 000, Hooper et al. (1978) estimated that the enzyme contains 18 *c*-type hemes and 2 hemes of a unique CO-binding P-460. The subunit composition of the enzyme is unknown. The present paper describes the dissociation and denaturation pattern and subunit composition of hydroxylamine oxidoreductase based on analysis by polyacrylamide gel electrophoresis in NaDod-

† From the Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108. *Received January 26, 1981.* Supported by a grant from the National Science Foundation (PCM80-8710).

[1] Abbreviations used: M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate (SDS in figures); sulfenyl chloride, 2-nitrophenylsulfenyl chloride; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/81/0420-7026$01.25/0