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Thermal Unfolding Transition of Ribonuclease A Measured by 2′-CMP Binding†

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ABSTRACT: We report an approach to the problem of detecting and characterizing intermediates in the unfolding of ribonuclease A. Two distinct properties of the protein are compared at equilibrium within the unfolding transition zone: (1) a physical property of the protein, the absorbance of buried tyrosine residues, and (2) a functional property, the ability to bind the specific ligand, 2′-CMP. A direct comparison of these two properties is made within the pH 5.8 transition zone, and an indirect comparison is made by using the stopped-flow instrument to sample rapidly the equilibrium properties of the pH 2.0 transition. At both pH 2.0 and pH 5.8, the results indicate that there are no intermediates in folding which have the physical properties of the native enzyme but which have lost the ability to bind a specific ligand.

When the heat-induced unfolding transition of RNase A[1] is measured by different physical properties (Harrington and Schellman, 1956; Ginsburg and Carroll, 1965; Brandts and Hunt, 1967; Tsong et al., 1970; Tiktopulo and Privalov, 1974; Chen and Lord, 1976), the transition is found to be highly cooperative. Transition curves for unfolding at pH 2.0 are found to be superimposable when measured by viscosity, optical rotation, or tyrosine absorbance (Ginsburg and Carroll, 1965), and the two-state approximation ($N \rightleftharpoons U$) has been used to describe the unfolding transition curves (Brandts and Hunt, 1967). Laser Raman measurements suggest that noncoincident unfolding curves may be observable by this technique, but greater accuracy is needed to be certain (Chen and Lord, 1976). NMR measurements of thermal unfolding curves of individual protons for the four histidine residues show a single unfolding curve at pH 4.0 (Matthews and Westmoreland, 1973). At the acidic pH 1.3, the unfolding curve of His-12 precedes by 1° the common curve of the other three residues (Westmoreland and Matthews, 1973), probably because His-12 is involved in a local unfolding reaction (see also Benz and Roberts, 1975).

There are two possible explanations as to why it is difficult to detect intermediates in unfolding at equilibrium above pH 1.3: (1) either intermediates are populated at levels too low to be detectable or (2) the intermediates have physical properties close to those of native RNase A or of its unfolded form. The second explanation may appear unlikely: however, it has been shown to be correct in the case of an intermediate detected in kinetic experiments. There are two forms of unfolded RNase A that have very different rates of refolding (Garel and Baldwin, 1973, 1975a,b; Brandts et al., 1975; Hagerman and Baldwin, 1976) and the fast-refolding species U_2 is an intermediate in the folding of the slow-refolding species U_1 (Hagerman and Baldwin, 1976). Most physical properties of U_1 and U_2 are indistinguishable, but a small pK difference has been detected (Garel and Baldwin, 1975b). Moreover, the equilibrium ratio $(U_2)/(U_1)$ does not depend significantly on temperature (Garel and Baldwin, 1973, 1975a), so that, even if a thermal unfolding curve could be measured by a property that is very different for U_2 and U_1, this curve would still coincide with the unfolding curves measured by tyrosine absorbance or viscosity.

Consequently, it is necessary to scrutinize carefully the experiments which have failed to detect any intermediates with properties close to N. Such intermediates would be expected to retain the compact structure of N but to show some local unfolding. A "quasi-native" intermediate has been reported recently in the guanidine-induced unfolding of α-lactalbumin (Kuwajima et al., 1976). In the case of RNase A, if a comparable quasi-native intermediate exists it should have been detected in kinetic studies of unfolding (cf. Hagerman and Bal-

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[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A, disulfide bonds intact; NMR, nuclear magnetic resonance; N, native; U, unfolded.

dwin, 1976) but it could have been missed if it is in rapid equilibrium with N or if it does not differ from N in tyrosine absorbance.

To search for quasi-native intermediates in RNase A unfolding, we designed an experiment based on these considerations. (1) The measurements are made at equilibrium, so that a quasi-native intermediate is detectable even if it is rapidly interconvertible with N. (2) The binding of a specific ligand (2′-CMP) is measured so that any partial unfolding which weakens the 2′-CMP binding site can be detected. (3) The measurements of 2′-CMP binding are compared with a standard physical measurement of unfolding, based on the absorbance changes of the three buried tyrosine groups. In order to study the unfolding transition as it exists in the absence of 2′-CMP, the experiment has also been made in another way: a stopped-flow apparatus has been used for rapid sampling of the thermal unfolding transition at pH 2.0.

Materials and Methods

Materials

RNase A (lot No. RAF 53H372 and 54J355) were obtained from Worthington, and 2′-CMP (lot No. 273-10) from P-L Biochemicals. Concentrations were determined from absorbance measurements using molar absorbances of 9.8×10^3 for RNase A at 278 nm (Sela and Anfinsen, 1957) and of 7.6×10^3 for 2′-CMP at 260 nm, neutral pH (Beaven et al., 1955). Protein solutions were heated for 10 min, at 65 °C, neutral pH, to remove aggregates (Crestfield et al., 1963). The following buffers were used: (a) 0.05 M cacodylate–0.1 M $NaClO_4$, pH 5.8, and (b) 0.1 M $NaClO_4$, pH 2.0.

Methods

(a) *Equilibrium Measurements.* Absorbance equilibrium measurements were carried out using either a Cary 14 or a Gilford spectrophotometer. The temperature of the cell was controlled by water circulation and directly measured by a thermistor Digitec thermometer. The cuvettes were sealed by Teflon tape stretched over the top of the cuvettes and held in place by tight-fitting rubber caps. All samples were degassed before use, and the reversibility of thermal unfolding was checked by comparing heating and cooling curves. The RNase A unfolding transition was monitored at 287 nm using 1-cm cells. The binding of 2′-CMP to RNase A was measured at 250 nm using tandem cells of three different path lengths (2 mm, 4 mm, and 9 mm) so as to keep the background absorbance to a comparable value in all experiments.

(b) *Kinetic Experiments.* Changes in absorbance upon pH jumps from pH 2.0 to pH 5.8 were measured with a modified Gibson-Durrum stopped-flow coupled to a Biomation 802 transient recorder. The signal change upon mixing was stored in the transient recorder (Figure 1), and data were accepted only in cases where the signals before and after mixing were the same within 0.5% (see the legend of Figure 1). Signal changes upon mixing were corrected for the dead-time of the instrument, 3 ms. Signal changes were converted into absorbance changes after calibration of the stopped-flow with solutions of known absorbance. In order to eliminate the thermal artifact observed by Gibson (1964), the drive syringes and the observation chamber were thermostated by separate Haake water baths. The test used for proper thermostating was to mix RNase A at pH 5.8 with 2′-CMP at pH 5.8, a control mixing experiment which should give no kinetic changes in the stopped-flow time range. In order to obtain long-term signal stability, a 100-W tungsten lamp (Osram or Norelco) was used as a light source. This made it necessary to use wide slit settings

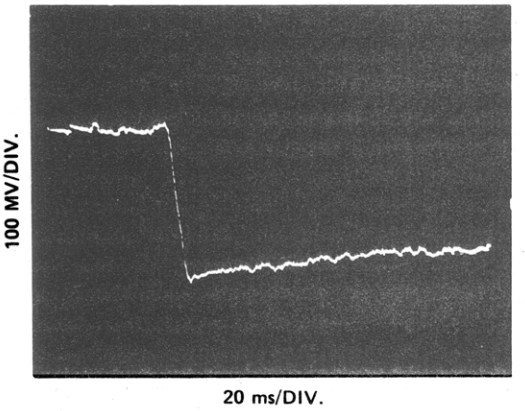

FIGURE 1: Measurement of the signal change upon mixing RNase A at pH 2.0 and 2′-CMP in buffer at pH 5.8. Final conditions = 4.5×10^{-5} M RNase A, 7.5×10^{-5} M 2′-CMP, 0.05 M cacodylate buffer, 0.1 M $NaClO_4$, pH 5.8, 47 °C. Wavelength: 250 nm. A photograph of an oscilloscope display of the transient signal stored in a transient recorder set in a pretrigger record mode. The horizontal line on the left of the display is the baseline before mixing, i.e., corresponds to the signal due to the completely mixed solutions of the previous run. The rapid fall in signal in the center corresponds to the replacement of the old solution by the newly mixed reagents in the observation chamber of the stopped-flow. The slightly sloping line on the right corresponds to the beginning of the refolding reaction after flow has stopped. In all cases, this refolding reaction is followed to completion, using a digital panel meter to monitor signal changes and to check that the final signal value at infinite time is within 0.5% of the corresponding value of the previous run. A setting of 5.00 V is usually used for these infinite time signal values. It can be seen that a fluctuation of 20 mV (i.e., 0.4% of 5.00 V) represents only a few percent of the total measured signal change (of the order of 350 mV in the present case).

(a band pass of about 20 nm) and interference filters to eliminate stray light. The optical path length was calibrated using solutions whose absorbances had been measured in a Cary 14 spectrophotometer. Once calibrated, the same combination of lamp, slit settings, and filters was used in all stopped-flow experiments. To determine the absorbance of unfolded RNase A as a function of temperature at pH 5.8, before refolding after a stopped-flow pH jump from pH 2.0, the $NaClO_4$ was omitted from the initial pH 2 buffer and added during mixing. This depresses the T_m in the initial conditions yielding completely unfolded protein at $t = 0$ after the pH jump.

(c) *Calculations.* In order to determine 2′-CMP binding spectrophotometrically, and to compute the fraction of native protein that is present and capable of binding 2′-CMP, the following equations are used:

$$f_B = (PC)/(P_T) \tag{1a}$$

$$f_N = [(P) + (PC)]/(P_T) \tag{1b}$$

where P = native RNase A, not complexed to 2′-CMP, C = unbound 2′-CMP, PC = complex, P_T = total RNase A (including unfolded protein), and C_T = total 2′-CMP. The molar difference extinction coefficient of the complex (Hammes and Schimmel, 1965) is:

$$\Delta\epsilon_{PC} = \epsilon_{PC} - \epsilon_P - \epsilon_C \tag{2}$$

and, provided that a wavelength can be found where the extinction coefficient is the same for the native and unfolded forms of the protein (this is approximately true for RNase A at 250 nm; Garel and Baldwin, 1973), then the measured difference in absorbance is:

$$\Delta A = l\Delta\epsilon_{PC}(PC) \tag{3a}$$

$$\Delta A_{max} = l\Delta\epsilon_{PC}(P_T) \tag{3b}$$

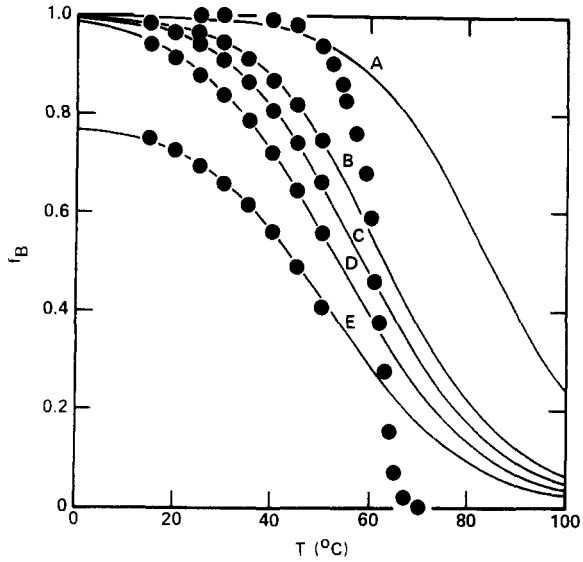

FIGURE 2: Temperature dependence of the fraction of RNase A which has bound 2′-CMP. Conditions: RNase A, 2.95×10^{-5} M (curve A) and 7.2×10^{-5} M (curves B to E), 2′-CMP, 7×10^{-4} M (curve A), 1.5×10^{-4} M (curve B), 1.12×10^{-4} M (curve C), 8.4×10^{-5} M (curve D), and 5.6×10^{-5} M (curve E), 0.05 M cacodylate, 0.1 M $NaClO_4$, pH 5.8. The solid lines are calculated using the parameters given in the text for the binding equilibrium, and assuming that all RNase A molecules can bind 2′-CMP at saturation. This assumption is valid below 45 °C, but not at higher temperatures because unfolding of RNase A occurs.

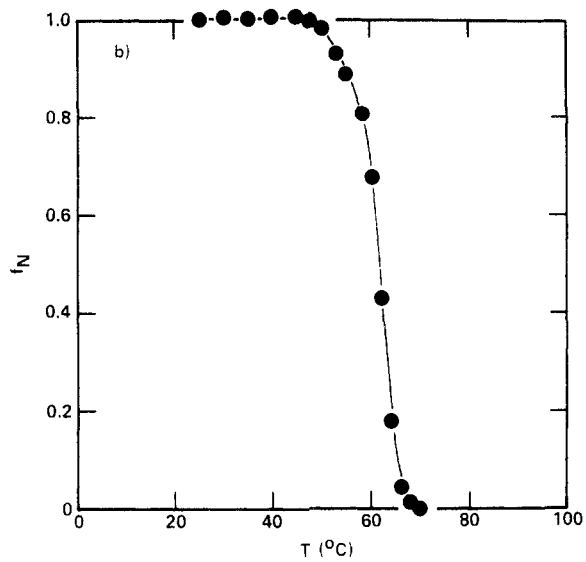

FIGURE 3: Comparison between the temperature dependence of the fraction of native RNase A measured by its ability to bind 2′-CMP (●) and by changes in A_{287} (—) at pH 5.8. Conditions: 2.95×10^{-5} M RNase A, 7×10^{-5} M 2′-CMP, 0.05 M cacodylate, 0.1 M $NaClO_4$, pH 5.8.

where l is the path length, A is the absorbance at 250 nm, and ΔA_{max} is measured in experiments at saturating ligand concentrations. Then:

$$f_B = \Delta A / \Delta A_{max} \quad (4a)$$

$$f_N = f_B + f_B / K_a[(C_T) - f_B(P_T)] \quad (4b)$$

where K_a is the association constant for 2′-CMP binding. Equations 4a and 4b are correct, even if the extinction coefficients of the native and unfolded forms of the protein are different, for the case that binding of the ligand does not change the equilibrium between native and unfolded forms. In the unfolding measurements based on changes in tyrosine absorbance, f_N is measured at 287 nm by use of the two-state approximation, after extrapolating the absorbances of the native and unfolded forms into the transition zone for unfolding (cf. Brandts and Hunt, 1967). Because of the wide slits used in the stopped-flow experiments, it is not possible to use the equilibrium data for the dependences on temperature of the native and unfolded forms; instead, these have been determined separately in stopped-flow experiments. In equilibrium experiments, the value we determine for $\Delta\epsilon_{PC}$ is 2280 ± 80 cm^{-1} M^{-1} at 250 nm in 0.05 M sodium cacodylate (pH 5.8)–0.1 M $NaClO_4$; it is taken to be independent of temperature. Our value agrees closely with the difference spectra determined by Anderson et al. (1968) in 0.10 M KNO_3, and is about 10% lower than their data for 0.10 M KCl.

Results and Discussion

Equilibrium Measurements of the Binding of 2′-CMP to Native RNase A. The binding of 2′-CMP to native RNase A is a 1:1 stoichiometric reaction which produces a substantial change in A_{250} (Hummel et al., 1961); this change in A_{250} arises from a perturbation of the nucleotide spectrum, and not from the protein (Irie and Sawada, 1967). The fraction of RNase A capable of binding 2′-CMP is obtained by comparing the change in A_{250} observed at given protein and 2′-CMP concentrations to that observed at saturation in 2′-CMP for the same protein concentration. Figure 2 gives the temperature dependence of the fraction of RNase A capable of binding 2′-CMP obtained in this way at different 2′-CMP concentrations. Up to 45 °C, i.e., in conditions where RNase A is fully native, these data can be satisfactorily fitted by a single binding constant characterized by the apparent thermodynamic parameters $\Delta H = -20.4$ kcal/mol and $\Delta S = -42.5$ eu/mol, and by a temperature-independent value of 2.28×10^3 for the molar absorbance change at 250 nm due to the binding of 2′-CMP to the protein. These values are in satisfactory agreement with comparable published results for the apparent enthalpy change (Hummel et al., 1961) and the molar absorbance change (Hummel et al., 1961; Anderson et al., 1968) accompanying 2′-CMP binding to RNase A in similar conditions. Comparison with calorimetric data for the binding of NAD to two glyceraldehyde phosphate dehydrogenases (Velick et al., 1971) suggests that the actual values of ΔH and ΔS are likely to be strongly temperature dependent, and the above values should be regarded as empirical constants. Isothermal calorimetric measurements of ΔH for 2′-CMP binding at 25 °C give pH-dependent values lower than the one above (Flogel et al., 1975).

Comparison between the Unfolding Transition of RNase A at pH 5.8 Measured Either by 2′-CMP Binding or by Tyrosine Absorbance. The binding of 2′-CMP to native RNase A can be described by a simple binding equilibrium characterized by the parameters given above. This is no longer true above 45 °C, where RNase A is partly unfolded: the fraction of protein which binds 2′-CMP is lower than that predicted from the data obtained below 45 °C, i.e., in the absence of unfolding (curve A in Figure 2). At a given 2′-CMP concentration and temperature, the fraction of RNase A capable of binding 2′-CMP is obtained by comparing the observed amount of bound 2′-CMP to that predicted in the absence of any unfolding. This procedure is justified by the fact that unfolded RNase A does not bind 2′-CMP. Figure 3 gives the temperature dependence of the fraction of RNase A capable of binding 2′-CMP in the transition region, i.e., the unfolding curve measured by 2′-CMP binding.

The unfolding of RNase A has also been measured by changes in tyrosine absorbance at 287 nm. At this wavelength,

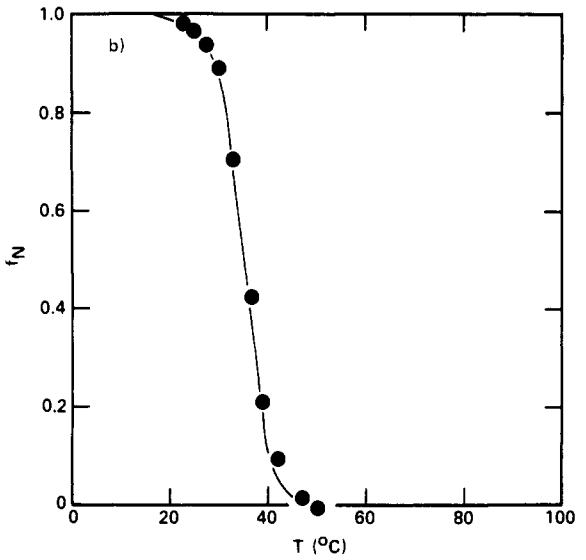

FIGURE 4: Comparison between the unfolding curves of RNase A at pH 2.0 measured either at equilibrium at 287 nm (—) or by rapid sampling experiments after a jump to pH 5.8 (●). Results are expressed as the fraction of native RNase A vs. temperature by normalizing the observed absorbance change with respect to the absorbance difference between fully native and fully unfolded RNase A.

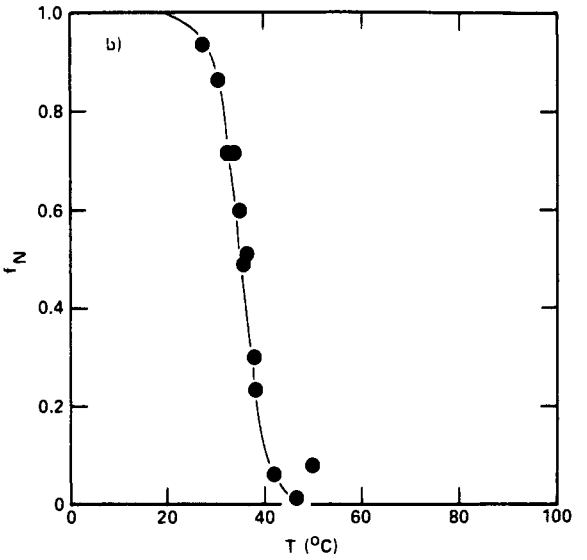

FIGURE 5: Comparison between the unfolding curves of RNase A at pH 2.0, expressed as fraction of native protein vs. temperature, measured either at equilibrium at 287 nm (—) or by rapid sampling experiments of the ability to "instantaneously" bind to 2′-CMP upon a pH jump to pH 5.8.

the binding of 2′-CMP to native RNase A causes only a negligible change in absorbance (Hummel et al., 1961; Anderson et al., 1968), so that unfolding of the protein can be observed independently of 2′-CMP binding, even in the presence of inhibitor. The temperature dependence of the fraction of native RNase A, as judged from the absorbance of its tyrosine residues, is also given in Figure 3.

The unfolding transition curves obtained for RNase A at pH 5.8 either from the ability to bind 2′-CMP or from the protein absorbance are identical within experimental errors in the same conditions (Figure 3). The two definitions of native RNase A by a functional or a physical property are therefore equivalent. Species which would have a nonfunctional binding site for 2′-CMP, and still retain three buried tyrosine residues, do not appear to be populated to a detectable level.

Rapid Sampling of the Unfolding Transition of RNase A at pH 2.0. Temperature-jump measurements of RNase A unfolding based on tyrosine absorbance do not detect any very fast reactions that take place in a time range prohibited to stopped-flow experiments, i.e., below a few milliseconds (Tsong et al., 1971). Consequently, the fraction of unfolded RNase A present at equilibrium inside the transition region at pH 2.0 can be measured from the amount of protein which gives rise to a refolding reaction after a pH jump to pH 5.8, at temperatures where RNase A at pH 5.8 is entirely native. The total amplitude of the refolding reactions is measured by extrapolating the kinetic curves back to zero time, and using the final transmittance measured at infinite time as described in the Methods section (see Figure 1). Figure 4 gives the results of these rapid sampling experiments, together with the equilibrium unfolding curve at pH 2.0, and shows that both types of measurement give identical results within experimental errors.

Rapid Sampling of the Unfolding Transition of RNase A at pH 2.0 Using 2′-CMP Binding. The binding of 2′-CMP to RNase A is a fast process (Hammes, 1968) and has already been used as a probe for the reappearance of native protein in refolding experiments (Garel and Baldwin, 1973; Garel et al., 1976). Since binding of 2′-CMP to RNase A occurs within the dead-time of the stopped-flow apparatus (Garel and Baldwin, 1973) and since no major refolding reaction occurs within this time (see above), we can use 2′-CMP binding to measure the amount of RNase A which is native at equilibrium at pH 2.0 from its ability to bind 2′-CMP "instantaneously" upon jumping to pH 5.8 in the presence of inhibitor. Note that in the above experiment, we have used a rapid sampling technique to measure the amount of unfolded RNase A present at equilibrium in the pH 2.0 unfolding transition, whereas using 2′-CMP binding measures the amount of *native* protein present at equilibrium in the same initial conditions. Figure 5 gives the temperature dependence of the fraction of RNase A which is native at equilibrium at pH 2.0, as monitored by the rapid sampling measurement of "instantaneous" 2′-CMP binding; Figure 5 also gives the unfolding curve obtained at equilibrium at pH 2.0 from changes in A_{287}. Again the two types of measurements agree within experimental error. This result shows that all RNase A species which appear as native at equilibrium at pH 2.0, as judged from their absorbance at 287 nm, also appear as native by their ability to bind 2′-CMP instantaneously after pH jump to pH 5.8. The same conclusion, about the absence of any detectable species that is unable to bind 2′-CMP but has the absorbance at 287 nm of native RNase A, has also been reached from equilibrium measurements at pH 5.8 (see above). The rapid sampling of the pH 2.0 unfolding transition using 2′-CMP binding yields the further conclusion that such putative "quasi-native" species are not formed as transient intermediates during the refolding reaction after stopped-flow mixing is complete. The equilibrium measurements at pH 5.8 are made in the presence of 2′-CMP (Figures 2 and 3), whereas the rapid sampling experiments correspond to a pH 2.0 unfolding transition in the absence of 2′-CMP, and therefore the two experiments probe the RNase A unfolding transition both in the presence and in the absence of 2′-CMP. However, the pH 2.0 results would not detect a quasi-native intermediate which is converted to N at pH 5.8 in a time short compared with the stopped-flow mixing time.

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Partial Purification and Characterization of an N^2-Guanine RNA Methyltransferase from Chicken Embryos†

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ABSTRACT: An N^2-guanine RNA methyltransferase has been purified 1000-fold from chick embryo homogenates by phosphocellulose chromatography followed by chromatography on *S*-adenosylhomocysteine-Sepharose. The enzyme was shown to methylate the G_{10} position of *Escherichia coli* B $tRNA^{Phe}$ and has a K_m of 3×10^{-7} M for $tRNA^{Phe}$ and 1.38×10^{-6} M for *S*-adenosylmethionine. The molecular weight was estimated to be 77 000 by gel filtration and the pH optimum was 8.0 to 8.5. Magnesium ion was not required for activity but it stimulated the rate of methylation 1.5-fold with an optimum at 12 mM. Ammonium ion stimulated activity about twofold with an optimum at about 83 mM. Sodium and potassium ions above 0.1 M were inhibitory.

Many enzymes in cells are involved in nucleic acid modification. The methylation of nucleosides in various positions and combinations is the most common modification and in the transfer RNA of some eukaryotes 5 to 10% of the bases may be modified by enzymatic methylation. The other major fractions of nucleic acids are also methylated including DNA, messenger RNA, 18S and 28S ribosomal RNA, and small molecular weight nuclear RNA (for reviews, see Borek and Srinivasan, 1966; Starr and Sells, 1969; Weinberg, 1973). The biologic function(s) of these diverse reactions are generally unknown, though a few have been discovered. For example, 6-methylaminopurine and 5-methylcytosine in bacterial DNA have been shown to prevent cleavage of DNA at specific sites by restriction enzymes (Arbor, 1974), and the 7-methylguanine at the 5′-phosphate end of messenger RNA has been shown (Muthukrishnan et al., 1975) to be a requirement for translation in some cases though not all (Rose and Lodish, 1976).

Two technical problems have hindered the study of this group of enzymes. First, the high specificity of the enzymes for a particular base, position on the base, and the structure around

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