A STUDY ON THE INTERACTION OF RIBONUCLEASE ST WITH GUANOSINE 3'-MONOPHOSPHATE BY CIRCULAR DICHROISM

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SUMMARY

The interaction of ribonuclease St with guanosine 2'(3')-monophosphate, a competitive inhibitor for the enzyme, has been studied by means of their circular dichroism spectra. Ribonuclease St exhibits a strong positive band at 234 mμ and a negative band at 280 mμ. Upon addition of the nucleotide to the enzyme, a significant change in the circular dichroism spectrum occurs in the 280 mμ region alone, and this has been used to characterize the interaction of nucleotide and enzyme. Mixing enzyme which had been inactivated by modification with iodoacetate with the nucleotide did not produce any change in the spectrum, though the circular dichroism spectra of the native and the modified enzyms, are virtually identical. The results are discussed together with the results obtained using bovine pancreatic ribonuclease and cytidine 2'(3')-monophosphate.

The extracellular ribonuclease of Streptomyces erythreus (RNase St) is specific for guanosine 3'-phosphodiester bonds (2) and consists of 107 amino acid residues with no tryptophanyl group (3). Analysis of changes in enzyme conformation by means of circular dichroism (CD) measurement should be simpler using this enzyme rather than ribonuclease T_1 (RNase T_1), which has similar substrate specificity but contains a tryptophan residue (4).

We wished to see if the formation of the complex between RNase St and the specific substrate would be reflected in any change in the CD spectra. However, since the lifetime of the complex of RNase St with substrate, i.e., guanosine 2',3'-cyclic phosphate (5), is far shorter than the time required for CD measurement, we

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This work was presented in part at the 21st Symposium of Protein Structure in October 1970 at Chiba, Japan (1).

have examined complexing with guanosine 2'(3')-monophosphate (Gp) which is a reversible inhibitor for the enzyme and a structural analogue of true substrate. We also report here preliminary results on the CD of bovine pancreatic ribonuclease (RNase A) complex with cytidine 2'(3')-monophosphate (Cp).

MATERIALS AND METHODS

RNase St was prepared as described in a previous paper (3). RNase A was purchased from Mann Research Laboratories. Gp and Cp were purchased from Kokoku Rayon and Pulp Co. (Tokyo). The inhibitor constants (K_i) of the nuclotides were determined from a Dixon plot (6), where ribonuclease activity was measured by the rate of acid liberation from guanosine 2',3'-cyclic phosphate for RNase St and from cytidine 2',3'-cyclic phosphate for RNase A (5). The CD spectra were recorded at room temperature (25°) on a JASCO ORD/UV6.

RESULTS AND DISCUSSION

The CD spectrum of RNase St at pH 7.4, the pH at which the enzyme shows optimum ribonuclease activity (2), reveals two bands, at 280 and 234 mμ, in the 220-330 mμ region (Fig. 1). The CD extremum at 234 mμ shows a biphasic dependence upon temperature, a linear change between 20° and 50° being followed by a sharp one above 55°. On cooling from 80° to 25°, the ellipticity at 234 mμ was reversibly recovered only in very dilute solutions (below 10^{-6} M), in which enzymatic activity was also fully recovered (1). Therefore, the positive band at 234 mμ may be assigned the B_{1u} band of tyrosine influenced by the peptide chromophores. However, it is not yet clear what regular conformation is responsible for the positive band. The negative band centered near 280 mμ is correlated with the B_{2u} band of tyrosine. On the other hand, the CD spectrum of Gp, which is shown by kinetic data to possess the ability to combine with the active site of RNase St in a competitive manner, exhibits two weak bands at 215 and 250 mμ which do not overlap those of RNase St. Thus, it should be possible to characterize the conformation

of RNase St-Gp complex (E·S complex-like intermediate) by CD spectrum if there is any difference between the CD of the native enzyme and that of the complex.

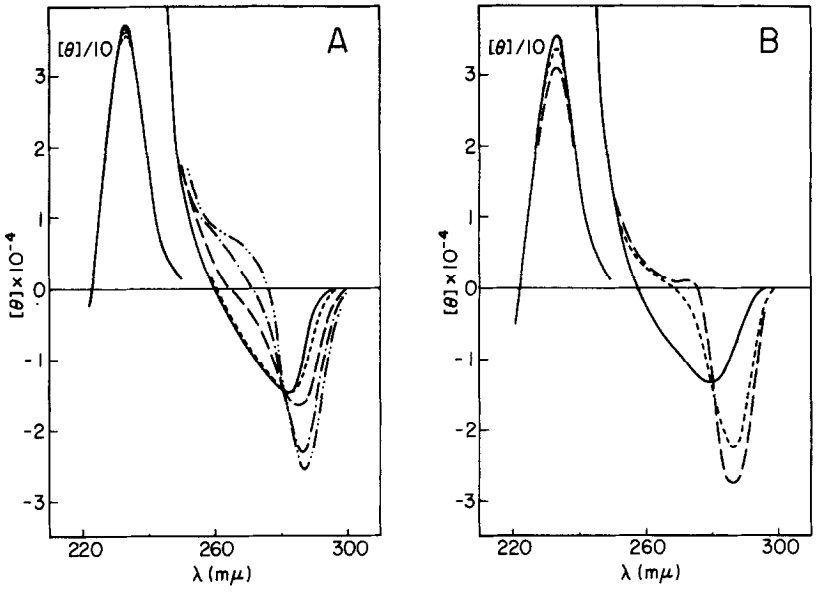

Fig. 1. Effect of Gp on the CD spectra of (A) 0.88 mM RNase St at pH 7.4. Molar ratio of Gp to RNase St: 0 : 1 (———), 0.14 : 1 (-----), 0.7 : 1 (– – –), 1.4 : 1 (—·—·), 2.3 : 1 (—·· –); (B) 0.087 mM RNase St at pH 5.0. Molar ratio of Gp to RNase St; 0 : 1 (———), 1 : 1 (-----), 1: 5 (– – –).

When RNase St is complexed with Gp, its negative peak at 280 mμ shifts to 288 mμ, the intensity of the latter peak increasing with the amount of Gp complexed; there is no change during complexing, however, in the band at 234 mμ. This behavior is shown in Fig. 1A, where the concentration of the enzyme was 0.88 mM and a ratio of Gp to RNase St was varied from 0.14 to 2.3 at pH 7.4. Higher ratios could not be used because of the absorbancy of the nucleotide. Fig. 1B shows the profile of CD change for the complex at pH 5.0 and 0.087 mM enzyme concentration. Assuming 1 : 1 stoichiometry of binding, the dissociation constant, K_D, can be estimated according to the following equation:

$$K_D = \frac{[E_0 - E{\cdot}I][I_0 - E{\cdot}I]}{[E{\cdot}I]} \quad (1)$$

$$\Delta[\theta] \equiv [\theta]_0 - [\theta]_E - a[\theta]_I = \{[\theta]_{E\cdot I} - [\theta]_E - [\theta]_I\}x \quad (2)$$

$$x = \tfrac{1}{2}[(1 + a + k_d) - \{(1 + a + k_d)^2 - 4a\}^{1/2}] \quad (3)$$

where $[E_0]$ and $[I_0]$ are initial concentration of the enzyme and the inhibitor, respectively; $[\theta]_0$, $[\theta]_E$, $[\theta]_I$ and $[\theta]_{E\cdot I}$ are the molecular ellipticity of the mixture, the enzyme, the nucleotide and the E·I complex, respectively; a is the molar ratio of nucleotide to enzyme, $[I_0]/[E_0]$; and k_d is the K_D value divided by the initial enzyme molar concentration, $K_D/[E_0]$. According to eqs 2 and 3, a plot of the difference in ellipticity at a given wavelength ($\Delta[\theta]_{288}$) as a function of the molar ratio (x) of E·I complex to enzyme should yield a straight line for the proper value of the dissociation constant, K_D. Substitution of various trial values for K_D into eqs 2 and 3 yields a family of curves, only one of which (that for the proper K_D) is a straight line. From the slope of this line one can obtain the molecular ellipticity of the E·I complex, $[\theta]_{E\cdot I}$. The K_D values given by this process are in fair agreement with the values of the corresponding inhibitor constant, K_i, estimated from a Dixon plot. The results are summarized in Table I.

Table I. Dissociation Constant and Optical Parameters Estimated from CD Spectra and Inhibitor Constant Determined Kinetically

Enzyme	Inhibitor	From CD data				From kinetics data	
		pH	K_D (M)	$\lambda_{max}^{E\cdot I}$ (mμ)	$[\theta]_{E\cdot I}^{max} \times 10^{-4}$ (deg. cm²/decimole)	pH	K_i (M)
RNase St	Gp	7.4	1×10^{-4}	288	-2.7	7.4	1.2×10^{-4}
		5.0	0.8×10^{-5}	288	-2.7	5.7*	3.2×10^{-5}
RNase St	Cp	7.4	0.8×10^{-5}	272	+1.9	7.4	3.3×10^{-3}
RNase A	Cp	7.0	1.5×10^{-4}	273	+6.1	7.0	3.2×10^{-4}
		5.8	4×10^{-5}	273	+6.1	5.8	4.2×10^{-5}

* K_i value at pH 5.0 could not be estimated because of the limitation of the assay method at lower than pH 5.4.

Since the competative binding of Gp was not accompanied by a distinguishable change in the molar ellipticity at 235 mμ, it is possible that the conformation of the enzyme remains unaltered during complex formation. The marked change in the CD band above 280 mμ should, therefore, be due to the attachment of Gp at the active site of the enzyme which is involved in combination with the specific substrate. This shift in the CD band, and the considerably large $[\theta]_{E\cdot I}$, suggest that there is appreciable electronic interaction between the guanine base and an amino acid residue(s), presumably the tyrosyl residue, of the enzyme; such interaction possibly being a specific binding of the base to a side chain, e.g., a phenol group, to form a stable hydrogen bond and/or salt bridge. Exactly what chemical role is played by which amino acid seems difficult to ascertain unless an X-ray analysis of the complex can be made.

Iodoacetate has been shown to be an effective alkylating agent for the amino acid residue at the active site of RNase T_1 (7). We reacted iodoacetate with RNase St under the same conditions as those used for RNase T_1 (pH 5.5, 37°). Inactivation of RNase St was much slower than that of RNase T_1, 42 hours reaction being required for complete inactivation compared with only 5 hours for RNase T_1. RNase St inactivated by modification with iodoacetate (CM-RNase St) shows the same CD spectrum as the native enzyme. Also, in the presence of Gp at either pH 5.0 or 7.4, CM-RNase St exhibits a spectrum indistiguishable from that of native RNase St. These observations lead us to propose that CM-RNase St has lost capacity for binding both to specific substrate and to the competitive inhibitor, though the conformations of the native and the modified RNase St are virtually identical. An identification of modified amino acid(s) is in progress with CM-RNase St.

The CD spectra of the complexes formed from RNase A and Cp at pH 7.0 and pH 5.8 are quite different from the superimposed spectra of both the components (see Fig. 2). This difference would seem to be attributable to phenomena similar to

those operative in the RNase St-Gp complex. Assuming the sugar-base conformation is in the anti-range (8), the maximum molecular ellipticity of the Cotton effect at 275 mμ would be expected to be 22,000. Therefore, the very large ellipticity of the RNase A-Cp complex, $[\theta]_{273} = 61{,}000$ (Table I), may reflect not only the perturbation caused by the fixing of the cytosine base at the active site of the enzyme but by the interaction with amino acid side chain(s) near the base. As in the case of RNase St-Gp complex, K_D values of Cp to RNase A are obtained from the $\Delta[\theta]_{273}$ and appear to be in good agreement with the K_i values estimated from the kinetics for the inhibition of RNase activity by Cp (Table I).

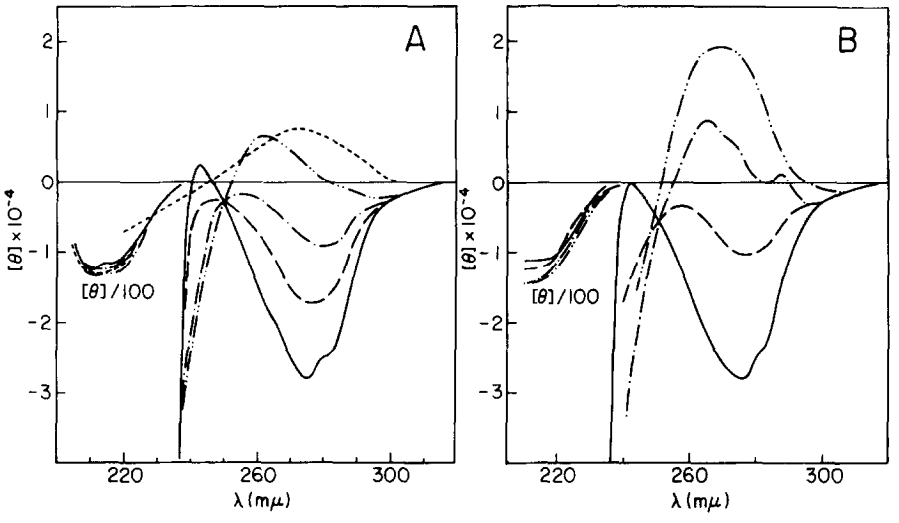

Fig. 2. CD spectra of (A) 0.085 mM RNase A at pH 7.0 (——), and on addition of Cp in molar ratios: 0.5 : 1 (– – –), 1 : 1 (–·–·–), 1 : 2 (–·· –), 1 : 0 (·····); (B) 0.084 mM RNase A at pH 5.8 (——), and on addition of Cp in molar ratios: 0.5 : 1 (– – –), 1 : 1 (–·–), 1 : 2 (–··–).

On addition of Gp (2×10^{-4}M) to RNase A (1×10^{-4}M) at pH 7.4, the CD spectrum is similar to the superimposed spectra of the separate components. In contrast to this the CD of RNase St-Cp system is quite different from the sum of the spectra of the components (Fig. 3). The CD change for this system, $[\theta]^{272}_{E.I}$ +19,000 can be accounted for by the fixing of the anti conformation (8) and by an absence of electronic inter-

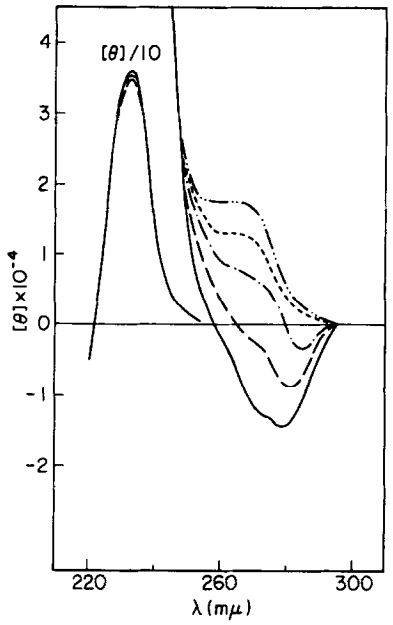

Fig. 3. Change in the CD spectra of RNase St on addition of increasing amounts of Cp to 0.076 mM RNase St at pH 7.4. Molar ratios of Cp to RNase St: 0 : 1 (——), 0.5 : 1 (– – –), 1 : 1 (–·–·), 1.5 : 1 (·····), 2 : 1 (–··–).

action between the cytosine base and RNase St. Therefore, this CD change does not presumably correspond to that for the RNase A-Cp complex which is accompanied by the formation of a Michaelis-Menten type complex. The fact that the K_D value for the RNase St-Cp complex near 10^{-5} M is very much smaller than K_i (3×10^{-3}M), as shown in Table I, is consistent with the above result. A discussion and more examples of the ribonucleases and nucleotides will be given later.

REFERENCES

1. N. Yoshida, K. Kuriyama, T. Iwata and H. Otsuka, Abstracts, 21st Symposium of Protein Structure (1970) p.28.
2. K. Tanaka, J. Biochem. (Tokyo), 50, 62 (1961).
3. N. Yoshida, H. Inoue, A. Sasaki and H. Otsuka, Biochim. Biophys. Acta, 228, 636 (1971).
4. F. Egami, K. Takahashi and T. Uchida, Progress in Nucleic Acid Research and Molecular Biology, Vol. III, p. 59, Academic Press (1964).

5. N. Yoshida and H. Otsuka, Biochim. Biophys. Acta, 228, 648 (1971).

6. M. Dixon, Biochem. J., 55, 170 (1953).

7. K. Takahashi, W. H. Stein and S. Moore, J. Biol. Chem., 242, 4682 (1967).

8. D. W. Miles, M. J. Robins, R. K. Robins, M. W. Winkley and H. Eyring, J. Amer. Chem. Soc., 91, 831 (1969).