Optical Rotatory Dispersion of Ribonuclease and Ribonuclease–Nucleotide Complexes*

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ABSTRACT: The optical rotatory dispersion of bovine pancreatic ribonuclease and that of ribonuclease complexed with cytidine 3′-phosphate, cytidine 2′-phosphate, and pyrophosphate has been studied in aqueous solution over the wavelength region 200–600 mμ. In the pH range 5–8 ribonuclease displays a small Cotton effect centered around 278 mμ and a trough at 228 mμ; no dependence of the rotatory dispersion on pH was found.

The optical rotation of the ribonuclease–cytidine 3′-phosphate complex at pH 5.5 was slightly more negative at 228 mμ than calculated by addition of the rotation of the free enzyme and nucleotide; this effect was more marked for the ribonuclease–cytidine 2′-phosphate complex. In addition the enzyme Cotton effect centered about 278 mμ was not apparent in either enzyme–nucleotide complex. A slight decrease was also observed in the ultraviolet rotation of the ribonuclease–pyrophosphate complex relative to that of ribonuclease, but the difference was within the estimated limit of experimental error. Even in the visible portion of the spectrum the rotation was somewhat more negative for the ribonuclease–nucleotide complexes. The observed changes are ascribed to a slight alteration in protein structure and/or to interaction of the nucleotide–protein chromophores.

The use of optical rotatory dispersion for providing information about the structure of macromolecules and the interaction of macromolecules with small molecules is now well established (Urnes and Doty, 1961; Ridgeway, 1963; Ulmer and Vallee, 1965). Although a detailed interpretation of the optical rotation of proteins is still lacking, empirical correlations between the rotation and protein structure or between changes in rotation related to small molecule–macromolecule interactions is of considerable interest. When bovine pancreatic ribonuclease complexes with cytidine 3′-phosphate, the product of the enzymatic hydrolysis of cytidine 2′,3′-cyclic phosphate, or with cytidine 2′-phosphate, an inhibitor of the reaction, an ultraviolet difference spectrum is observed (Hummel *et al.*, 1961, Hammes and Schimmel, 1965). Optical rotatory dispersion measurements provide a further means for investigating this interaction and detecting possible configurational changes in the enzyme related to the binding process.

We present here the results of measurements of the optical rotatory dispersion of ribonuclease at various pH values and of ribonuclease complexed with pyrophosphate, cytidine 3′-phosphate, and cytidine 2′-phosphate.

Experimental Section

Optical rotation measurements were performed on a Cary 60 recording spectropolarimeter over the wavelength region 200–600 mμ as previously described (Fasella and Hammes, 1965). The temperature was maintained at 27 ± 1°. Cells of 0.1-, 1.0-, and 10.0-mm path lengths were employed. Average values of the molar rotations (see below) were calculated from a minimum of two experiments. The molar rotations for the enzyme–cytidine 2′-phosphate and enzyme–cytidine 3′-phosphate complexes were obtained from measurements performed under conditions where the enzyme essentially is saturated (Hummel *et al.*, 1961; Hammes and Schimmel, 1965). The error in the molar rotations of the enzyme and the complexes is estimated to be about ±3%; the error is considerably larger (±20%) in the case of the nucleotides because the experimentally observed rotation was quite small.

Ribonuclease A (Worthington), lyophilized and phosphate free, was assayed by the method of Crook *et al.* (1960). Cytidine 3′-phosphate and cytidine 2′-phosphate were prepared by previously described methods (Cathou and Hammes, 1964; French and Hammes, 1965). Concentrations of ribonuclease and the cytidylic acids were determined spectrophotometrically (Cathou and Hammes, 1964; Harris *et al.*, 1953). The range of concentrations employed was as follows: ribonuclease, 0.83–2.2 × 10^{-4} M; cytidine 3′-phosphate, 1.2–10.5 × 10^{-4} M; cytidine 2′-phosphate, 1.5–7.9 × 10^{-4} M. All solutions contained 0.05 M NaCl

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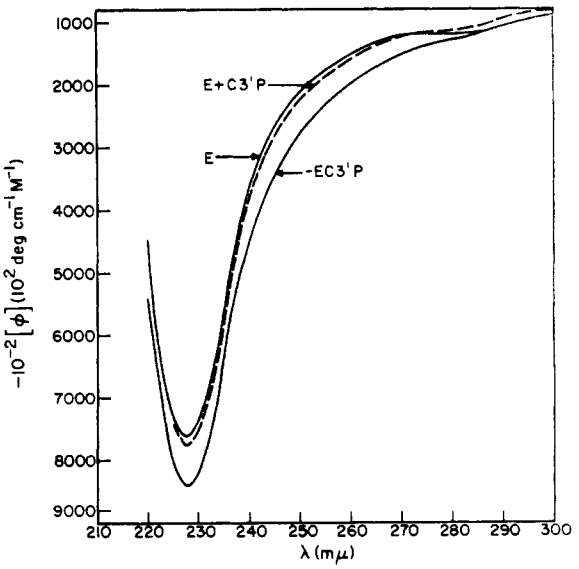

FIGURE 1: Ultraviolet rotatory dispersion of ribonuclease (E) and the ribonuclease–cytidine 3′-phosphate complex (EC3′P). The dashed curve is the sum of the molar rotations of E and C3′P.

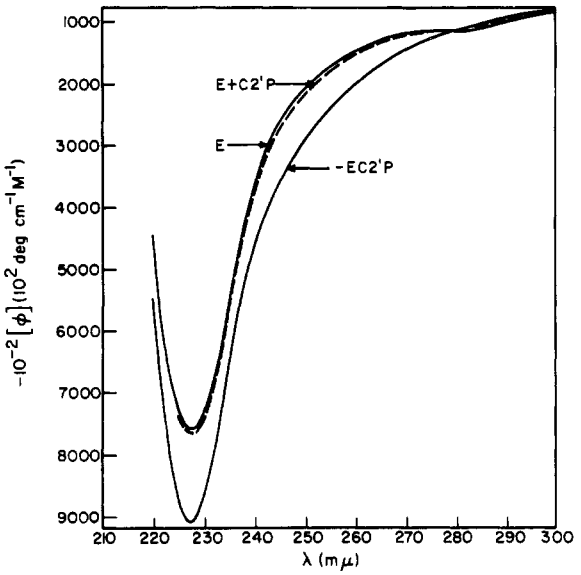

FIGURE 2: Ultraviolet rotatory dispersion of ribonuclease (E) and the ribonuclease–cytidine 2′-phosphate complex (EC2′P). The dashed curve is the sum of the molar rotations of E and C2′P.

and 0.1 M Tris–acetate buffer. The resultant ionic strength was 0.15 M.

Results and Treatment of Data

Since we wish to compare rotations of optically active substances of different molecular weight, the specific rotation is not a useful quantity. Accordingly, the data will be analyzed in terms of the molar rotation, $[\phi]$, which is defined as

$$[\phi] = 10^2 \frac{\alpha}{lc} \qquad (1)$$

where α is the observed rotation in degrees, l is the path length in centimeters, and c is the concentration of optically active solute in moles/liter. (A considerably more logical definition would replace the factor of 10^2 by unity, but this is contrary to current practice.) The molar rotation is simply related to the specific rotation, $[\alpha]$, by

$$[\phi] = \frac{MW}{10^2}[\alpha] \qquad (2)$$

where MW is the molecular weight of the optically active species. The molecular weight of ribonuclease was taken as 13,680 (Hirs *et al.*, 1956); a molecular weight of 14,000 was assumed for the enzyme–nucleotide complexes.

The ultraviolet rotatory dispersion at pH 5.5 of ribonuclease (E),[1] the ribonuclease–cytidine 3′-phosphate complex (EC3′P), and the ribonuclease–cytidine 2′-phosphate complex (EC2′P) is shown in Figures 1 and 2. The ultraviolet rotatory dispersion of the nucleotides at pH 5.5 is shown in Figure 3. Both nucleotides exhibit characteristic Cotton effects centered about their absorption maxima. The rotatory dispersion curves of EC3′P and EC2′P were computed by subtracting the rotation of a solution containing nucleotide only (at an appropriate concentration) from that of enzyme–nucleotide mixtures in which the nucleotide was in considerable excess.

Experiments with pyrophosphate, a known competitive inhibitor of ribonuclease (Nelson *et al.*, 1962), consistently showed a slight decrease (about 2%) in the rotation of the enzyme–pyrophosphate complex, EPP, relative to that of E in the wavelength region 220–280 mμ. Since the experimental error is about ±3%, the significance of the observed difference is questionable.

The visible portion of the rotatory dispersion of E and EC3′P is reproduced in Figure 4. The curve for EC2′P corresponds closely to that of EC3′P. Values of the specific rotation of ribonuclease at pH 5.4, 0.1 M KCl, and 20° have been tabulated at various wavelengths by Schellman and Schellman (1956); the values reported here are in quantitative agreement with these values over the visible portion of the spectrum (within 2%), although a small discrepancy (10–15%) is found in the near-ultraviolet. Our results are not in quantitative agreement with those of Zimmerman and Schellman (1962), particularly with regard to the wavelength at which the minimum of the ultraviolet trough is located.

[1] Abbreviations used: E, ribonuclease; EC3′P, ribonuclease–cytidine 3′-phosphate complex; EC2′P, ribonuclease–cytidine 2′-phosphate complex; EPP, enzyme pyrophosphate complex.

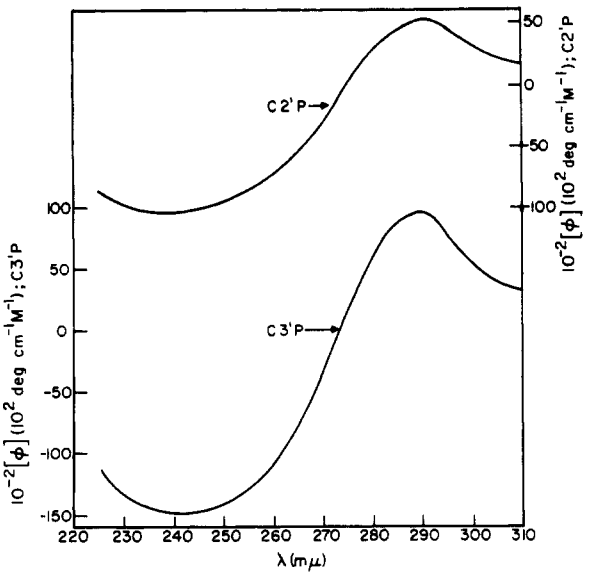

FIGURE 3: Ultraviolet rotatory dispersion of cytidine 2′-phosphate (C2′P) and cytidine 3′-phosphate (C3′P).

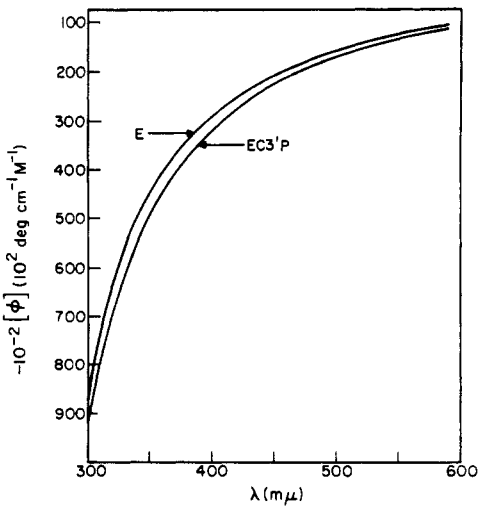

FIGURE 4: Visible rotatory dispersion of ribonuclease (E) and the ribonuclease–cytidine 3′-phosphate complex (EC3′P). The curve for the sum of the molar rotations of E and C3′P is essentially the same as that for E.

This discrepancy is probably due to the relatively poorer instrumentation used in the earlier work. More recent results from Dr. Schellman's laboratory (Dr. J. Schellman, private communication) are in accord with the data reported here.

A study of the pH dependence of the rotatory dispersion (220–600 mμ) of the free enzyme in the pH range 5–8 showed that there was no significant pH dependence of the optical rotation. This agrees with the findings of Jirgensons (1958) who reported no significant change in $[\alpha]^{25}_{404.7}$ in the pH range 4.4–9.

Discussion

All enzyme species show the characteristic protein trough at 228 mμ although the depth of this trough varies. The location of the minimum at 228 mμ is a characteristic which ribonuclease shares with some other proteins of apparently low helical content such as pepsin and trypsin (Jirgensons, 1964). In addition, the shape of the dispersion curves varies considerably in the region 275–285 mμ. Ribonuclease has a Cotton effect in this region which appears to be centered near its absorption maximum at 278 mμ and is probably associated with the tyrosine residues [*cf.* Hooker and Tanford (1964), who observed a similar Cotton effect with solutions of free tyrosine]. This Cotton effect is not evident in the rotatory dispersion of the species EC3′P and EC2′P. The absence of the characteristic enzyme Cotton effect in these species may be due to an altered structure of the enzyme or may be due to alterations in the environment of the tyrosine or bound-nucleotide chromophores, without the occurrence of any significant structural changes in the enzyme. On the basis of studies of the difference spectra caused by interaction of ribonuclease with the cytidine nucleotides, it has been suggested that an alteration in the environment of the tyrosine residues accompanies the binding process (Hummel *et al.*, 1961; Hammes and Schimmel, 1965). Pyrophosphate, which is optically inactive, binds to the same site on the enzyme as the cytidylic acids and does not alter the Cotton effect of the enzyme centered about 278 mμ, within the limits of experimental error (the rotation at this wavelength is consistently slightly more negative for EPP).

The rotatory dispersion of the species E, EC2′P, and EC3′P differs most significantly at 228 mμ; although both cytidylic acids have negative rotations at this wavelength, the molar rotation of each complex is more negative than the sum of the molar rotations of the enzyme and the respective nucleotide, the difference being greatest for EC2′P (*cf.* Figures 1 and 2). The differences are small, but definitely real. The values of the molar rotations at 228 mμ are given in Table I.

TABLE I: Values of the Molar Rotation at 228 mμ.

Species	$10^{-5}[\phi]_{228}$ (10^2 deg cm^{-1} M^{-1})[a]
E	−7.65
EC3′P	−8.42 (−7.78)
EC2′P	−9.11 (−7.75)

[a] The value in parenthesis is the sum of the molar rotation of E and the nucleotide. The estimated experimental error for the results presented in this table is ±3%.

As previously mentioned, only a very slight decrease (if any) is observed in the rotation of EPP relative to that of E. Again the observed differences may be due to changes in the protein structure and/or to changes in the environment of the nucleotide and protein chromophores. The magnitude of the optical rotation changes is roughly correlated with the binding constants for the pyrophosphate and nucleotide–ribonuclease interactions; pyrophosphate binds less strongly than cytidine 3′-phosphate which binds less strongly than cytidine 2′-phosphate (Nelson *et al.*, 1962; Hammes and Schimmel, 1965). The visible rotatory dispersion of EC2′P and EC3′P differs very slightly from that of the enzyme. Again, the observed decrease in rotation of the complexes may be due to structural alterations and/or chromophore interactions.

French and Hammes (1965) have reported that a pH-dependent isomerization of ribonuclease occurs around neutral pH values. Since the rotatory dispersion of ribonuclease is independent of pH over the range 5–8, it seems unlikely that gross structural changes accompany isomerization. This is further indication that the optical rotation of proteins is often not a very sensitive measure of small configurational changes (Sloane, 1964; Kägi, 1964; Fasella and Hammes, 1965).

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