The Active Site and Mechanism of Action of Bovine Pancreatic Ribonuclease

6. KINETIC AND SPECTROPHOTOMETRIC INVESTIGATION OF THE INTERACTION OF THE ENZYME WITH INHIBITORS AND *p*-NITROPHENYL ACETATE

By C. A. ROSS, A. P. MATHIAS AND B. R. RABIN

Department of Biochemistry, University College London, Gower Street, London, W.C. 1

(Received 15 March 1962)

The evidence presented by Herries, Mathias & Rabin (1962) and Findlay, Mathias & Rabin (1962) indicates that two imidazole residues are involved at the catalytic site of ribonuclease. Since ribonuclease contains no thiol groups, imidazole groups are the most likely to act as ligands for metal ions at pH values near neutrality. No detailed study of the inhibition of ribonuclease by metal ions, in which well-characterized substrates of low molecular weight are used, has been reported. Davis & Allen (1955) reinvestigated many of the reported inhibitors and confirmed that Cu^{2+} ions, and to a less extent Zn^{2+} ions, inhibited the enzyme. A summary of some of the salient points is given by Anfinsen & White (1961). With a view to confirming the existence of imidazole residues at the catalytic site, we investigated the inhibition by Zn^{2+} ions alone and in the presence of cytidine nucleotides.

Hartley & Kilby (1954) investigated the hydrolysis of *p*-nitrophenyl acetate catalysed by chymotrypsin for which Gutfreund & Sturtevant (1956) postulated a mechanism involving general base catalysis by an imidazole group. The hydrolysis of *p*-nitrophenyl acetate catalysed by imidazole and its derivatives has been extensively investigated (Bruce & Schmir, 1957; Bender & Turnquest, 1957; Koltun, Dexter, Clark & Gurd, 1958). We have investigated the hydrolysis of *p*-nitrophenyl acetate by ribonuclease and the effect of Zn^{2+} ions on this.

Cytidine 2', 3'- and 5'-phosphate are competitive inhibitors of ribonuclease (Herries, 1961; Davis & Allen, 1955) and they are closely related to the substrate, cytidine 2',3'-phosphate. We have investigated the interaction of these inhibitors with ribonuclease and with the enzymically inactive derivative, carboxymethylribonuclease. This material has been prepared by the reaction of ribonuclease at pH 5.5 with bromoacetic acid (Barnard & Stein, 1959) or iodoacetic acid (Gundlach, Stein & Moore, 1959). The reaction occurs mainly with residue 119 though a small amount of a derivative in which the sole histidine alkylated is 12 is formed (S. Moore, personal communication).

A preliminary communication of the spectrophotometric work has appeared (Mathias, Rabin &

Ross, 1960) and related investigations have been reported by other workers (Hummel, Verploes & Nelson, 1961).

EXPERIMENTAL

Materials

Buffers. Except for the investigation of the pH curve of inhibition by Zn^{2+} ions, *N*-ethylmorpholine buffers (0.1 M with respect to total *N*-ethylmorpholine) of pH 6.90 or 7.21 and 1.0–2.0 were used throughout. For investigation of the pH curve of inhibition by Zn^{2+} ions the following buffers, all of 1.0–2.0, were used: acetate, pH 4.5 and 5.0; malonate, pH 6.25; pentane-3,3-dicarboxylate, pH 6.56 (see Herries *et al.* 1962).

Ribonuclease. The preparation used was that described by Herries *et al.* (1962).

Cytidine 2',3'-phosphate, cytidine 2'-phosphate and cytidine 3'-phosphate. These were as described by Herries *et al.* (1962).

Cytidine 5'-phosphate. This was obtained as the disodium salt (lot no. CMP. 5905, Schwartz Laboratories, Mount Vernon, N.Y., U.S.A.).

***p*-Nitrophenyl acetate.** Highly purified material, recrystallized several times from 50% (v/v) ethanol, (m.p. 80–85°), was available in this Laboratory.

Zinc chloride. A standard 0.1 M solution was prepared by dissolving the stoichiometric amount of ignited zinc oxide in constant-boiling hydrochloric acid.

Bromoacetic acid. Except where stated to the contrary the crude material (L. Light and Co. Ltd., Colnbrook, Bucks.) was recrystallized from light petroleum (b.p. 40–60°).

Preparation of carboxymethylribonuclease. Under conditions essentially as described by Barnard & Stein (1959) the rate of carboxymethylation, as measured by loss of catalytic activity, is considerably enhanced by the purification of the bromoacetic acid. The enzyme activity at various times was measured by removing 0.1 ml. portions and assaying these by the simplified procedure of Crook, Mathias & Rabin (1960).

The bulk preparation of carboxymethylribonuclease was made by using purified bromoacetic acid at pH 5.5 and 40°. The concentrations of reactants were those of Barnard & Stein (1959), but the isolation procedure of Gundlach *et al.* (1959) was used. After 45 min. no detectable activity remained. The reaction mixture was then poured on a column of ion-exchange resin as described by Gundlach *et al.* (1959) and desalted with Amberlite Monobed Resin. From 30 mg. of ribonuclease, 17 mg. of carboxymethylribonuclease, as a freeze-dried powder, was obtained.

Methods

Hydrolysis of cytidine 2',3'-phosphate. This was determined spectrophotometrically as described by Crook *et al.* (1960).

Hydrolysis of *p*-nitrophenyl acetate. This was determined at 400 μ in a stoppered cell of 1 cm. light-path at 25° in a Hilger Uvispek model H.700.307. The value of $\epsilon_{400\mu}$ for *p*-nitrophenol at the pH values and temperatures used was found to be 12840. A freshly prepared stock solution of *p*-nitrophenyl acetate in acetone was used. The final acetone concentration was 4% (v/v) except in experiments on the variation of initial velocity with substrate concentration. Controls for the aqueous hydrolysis of *p*-nitrophenyl acetate were carried out. All reaction mixtures were made up with *N*-ethylmorpholine buffer, pH 7.21.

Spectrophotometry. A Hilger Uvispek model H.700.307 with a thermostatically-controlled cell holder and matched stoppered quartz cells of 1 cm. light-path were employed. The temperature was maintained at $25 \pm 0.1^\circ$. Since the work depends on the observation of small differences, elaborate precautions are necessary. All volumetric apparatus was grade 'A' or equivalent. First the absorption spectrum of the protein in 2.5 ml. of buffer was measured against a blank of buffer. The nucleotide, dissolved in 0.05 ml., was added to the cell containing protein and the spectrum again measured and corrected for the small volume change. The absorption spectrum of the nucleotide was measured in a separate experiment under identical conditions, the same pipette being used to add the nucleotide from the same stock solution. Zinc chloride solution was added from a 0.01 ml. constriction pipette and the absorption readings were corrected for the dilution. The concentrations of proteins and nucleotides, $54.4 \mu\text{M}$, were such that Beer's law was obeyed over the entire spectral range. The slit width was kept constant at 0.6 mm. The buffer used was *N*-ethylmorpholine, pH 6.90, I 0.2.

RESULTS

Inhibition by zinc ions and cytidine nucleotides. Typical progress curves for the hydrolysis of cytidine 2',3'-phosphate are shown in Fig. 1. The remarkable feature of these curves is that the initial velocities are almost independent of Zn^{2+} ions over the concentration range illustrated, and the main effect of Zn^{2+} ions is to cause marked deviation from first-order behaviour with respect to substrate concentration. At higher concentrations of Zn^{2+} ions the initial velocity is lowered as shown by the progress curves in Fig. 2. Thus Zn^{2+} ions have a dual effect on the activity. At low concentrations, inhibition only occurs during the course of the reaction, as the product concentration builds up, and is probably due to a protein-zinc-product complex. At high concentrations, there is apparently an interaction between the protein and Zn^{2+} ions causing inhibition which does not involve the product.

It was of interest to see if the inhibition at high concentrations of Zn^{2+} ions could be interpreted on the basis of simple mass law relationships.

Let

$$K_{\text{Zn}^{2+}} = \frac{[\text{ribonuclease-Zn}^{2+}]}{[\text{ribonuclease}][\text{Zn}^{2+}]}$$

assuming that binding of Zn^{2+} ions by buffer and substrate can be neglected. Since all experiments are conducted in the first-order region:

$$[\text{substrate}] \ll K_m \quad \text{and} \quad V = \frac{k_{+2}[\text{ribonuclease}][\text{substrate}]}{K_m}$$

where [ribonuclease] is the concentration of uncomplexed ribonuclease. Hence

$$\frac{V_0}{V_0 - V} = 1 + \frac{1}{K_{\text{Zn}^{2+}}[\text{Zn}^{2+}]} \quad (1)$$

where V_0 and V are the initial velocities in the absence and presence of Zn^{2+} ions respectively. From Fig. 3, $\log K_{\text{Zn}^{2+}}$ was found to be 3.9. This is somewhat higher than expected for a single imidazole group (Edsall, Felsenfeld, Goodman & Gurd, 1954). Thus if the Zn^{2+} ions are bound to an imidazole group, as suggested by Tanford (1952) for other proteins, then an additional co-ordinating group of the protein is probably also involved in binding the metal.

Fig. 1. Progress curves of hydrolysis of cytidine 2',3'-phosphate in *N*-ethylmorpholine buffer (0.1 M), pH 7.2 and I 0.20, at 25°. The concn. of ribonuclease was $1.6 \mu\text{M}$, and that of cytidine 2',3'-phosphate was 0.22 mM. ○, No ZnCl_2 , curve displaced upwards by 0.06; ▲, in the presence of ZnCl_2 ($10 \mu\text{M}$), curve displaced upwards by 0.04; ■, in the presence of ZnCl_2 ($20 \mu\text{M}$), curve displaced upwards by 0.02; ●, in the presence of ZnCl_2 ($40 \mu\text{M}$).

The inhibition by Zn^{2+} ions varies markedly with pH in the pH range 6.0–7.0 at concentrations of 4–8 mM-zinc chloride. We have been unable to compute $K_{\text{Zn}^{2+}}$ as a function of pH from these

results owing to buffer- Zn^{2+} ion interactions, but the pK value of the group which interacts with Zn^{2+} ions to cause loss of enzyme activity is certainly less than 6.5.

To gain further understanding of the inhibition at low concentrations of Zn^{2+} ions, which appeared to involve ternary-complex formation between product, enzyme and Zn^{2+} ions, progress curves for the hydrolysis of cytidine 2',3'-phosphate in the presence of other cytidine nucleotides, with and without Zn^{2+} ions, have been investigated. Some representative examples are shown in Figs. 4 and 5. In the presence of cytidine 2'-phosphate or cytidine 5'-phosphate (Fig. 5), Zn^{2+} ions have a negligible effect on the initial velocity, and a reduction in the reaction rate is observed only after a considerable proportion of the substrate has been hydrolysed, i.e. when cytidine 3'-phosphate is present. In the presence of cytidine 3'-phosphate Zn^{2+} ions have a considerable effect on the initial velocity (Fig. 4). It can be concluded that, of the cytidine nucleotides examined, only cytidine 3'-phosphate forms a ternary complex with Zn^{2+} ions and ribonuclease. Thus the inhibition of ribonuclease by Zn^{2+} ions is due to two separable phenomena: at concentrations of Zn^{2+} ions less than 0.1 mM the dominant effect is due to the formation of a ternary product-zinc-protein complex; at higher concentrations of

Fig. 2. Progress curves of hydrolysis of cytidine 2',3'-phosphate (conditions as in Fig. 1). ○, No ZnCl_2 , curve displaced upwards by 0.04; ■, in the presence of ZnCl_2 ($60 \mu\text{M}$), curve displaced upwards by 0.02; ▲, in the presence of ZnCl_2 ($80 \mu\text{M}$).

Fig. 3. Effect of concentration of Zn^{2+} ions on the initial velocity of hydrolysis of cytidine 2',3'-phosphate in *N*-ethylmorpholine buffer (0.1M), pH 7.2 and *I* 0.2, at 25°. The concn. of ribonuclease was $1.6 \mu\text{M}$ and that of cytidine 2',3'-phosphate was 0.22 mM.

Fig. 4. Hydrolysis of cytidine 2',3'-phosphate in the presence of cytidine nucleotides (conditions as in Fig. 1). ○, No additions; □, in the presence of cytidine 3'-phosphate ($10 \mu\text{M}$); ■, in the presence of cytidine 3'-phosphate ($10 \mu\text{M}$) and ZnCl_2 ($40 \mu\text{M}$). Some experimental points have been omitted for clarity.

Fig. 5. Hydrolysis of cytidine 2',3'-phosphate in the presence of cytidine nucleotides (conditions as in Fig. 1). ○, No additions; □, in the presence of cytidine 5'-phosphate (0.11 mM); ■, in the presence of cytidine 5'-phosphate (0.11 mM) and ZnCl_2 (40 μM); △, in the presence of cytidine 2'-phosphate (0.11 mM); ▲, in the presence of cytidine 2'-phosphate (0.11 mM) and ZnCl_2 (40 μM).

Zn^{2+} ions the formation of a zinc-protein complex becomes kinetically important.

Reaction of ribonuclease with *p*-nitrophenyl acetate. Over the concentration range 0.01–0.1 mM, the initial rate of hydrolysis of *p*-nitrophenyl acetate is proportional to the ribonuclease concentration. The rates cannot strictly be compared with those obtained by Hartley & Kilby (1954) for insulin, because of the slightly different pH and *I*; nevertheless, they are of the same order of magnitude. No inactivation of ribonuclease by *p*-nitrophenyl acetate, as measured by the hydrolysis of cytidine 2',3'-phosphate, has been observed.

At concentrations less than 0.02 mM, Zn^{2+} ions had no detectable effect on the ribonuclease-catalysed hydrolysis of *p*-nitrophenyl acetate. At 0.1 and 2 mM concentrations of Zn^{2+} ions the rate was 75% of its original value. No further decrease of rate was obtained by increasing the concentration of Zn^{2+} ions to 2 mM. Caution is required in interpreting these results quantitatively. However, reduction of the rate to 75% of its original value strongly suggests that only one of the four histidine residues in ribonuclease binds Zn^{2+} ions.

Spectrophotometric investigations. The absorption spectrum of a mixture of cytosine and ribonuclease was identical with that computed by assuming

Fig. 6. Effect of Zn^{2+} ions on the spectrum of ribonuclease. The spectra are of ribonuclease (54.4 μM) in *N*-ethylmorpholine buffer (0.1 M), pH 6.9 and *I* 0.2, at 25°, before (full line) and after (broken line) the addition of ZnCl_2 (160 μM).

additivity of the extinctions of the individual components. This is to be expected, since cytosine is not an inhibitor of ribonuclease. The experiment provides a useful additional check both on the reliability of the volumetric procedures and the applicability of Beer's law, on which the assumptions of additivity depend. Walaas (1958) has shown that the absorption spectra of nucleotides are unaffected by the presence of several metal ions despite complex formation. We have shown that this is also true of the isomeric cytidine nucleotides and Zn^{2+} ions at the concentrations employed in these investigations.

The effect of added zinc chloride on the spectrum of ribonuclease is shown in Fig. 6. The zinc chloride stock solution was 20 mM and was added in increments of 0.01 ml. The absorption spectra were corrected for the small volume change. The first two increments produced a decrease in the extinction coefficient over the range 240–300 $\text{m}\mu$. Further increments caused the extinction to increase over this wavelength range, probably due to incipient precipitations.

For mixtures of ribonuclease and cytidine 2'-phosphate the extinctions in the range 240–280 $\text{m}\mu$ were less than the computed values, and the maxima of the measured spectra were shifted to longer wavelengths (Fig. 7). A similar but less marked effect was observed for cytidine 3'-phosphate (Fig. 8). Similar, but smaller effects were observed when carboxymethylribonuclease replaced ribonuclease (Figs. 9 and 10).

Fig. 7. Spectrum of ribonuclease (54.4 μM) plus cytidine 2'-phosphate (54.4 μM) (conditions as for Fig. 6). Full line, observed spectrum; broken line, computed spectrum.

Fig. 9. Spectrum of carboxymethylribonuclease plus cytidine 2'-phosphate (conditions as for Fig. 7).

Fig. 8. Spectrum of ribonuclease plus cytidine 3'-phosphate (conditions as for Fig. 7).

Fig. 10. Spectrum of carboxymethylribonuclease plus cytidine 3'-phosphate (conditions as for Fig. 7).

Fig. 11. Difference spectra of ribonuclease plus cytidine 3'-phosphate in the presence of increasing concentrations of zinc chloride: A, 0.04 mM; B, 0.08 mM; C, 0.12 mM; D, 0.16 mM (omitted at wavelengths longer than 280 mμ); E, 0.20 mM (omitted at wavelengths longer than 280 mμ).

The effect of zinc chloride on the spectra of nucleotide-protein mixtures was measured. For combinations of ribonuclease with cytidine 2'-phosphate or cytidine 5'-phosphate, the addition of the first two increments of zinc chloride produced a slight lowering of the extinctions; further increments of zinc chloride increased the extinctions. This behaviour is identical to that observed for ribonuclease alone. For carboxymethylribonuclease no lowering of the extinctions occurred on the addition of the first increment of zinc chloride. Nor did any lowering of the extinctions occur on the addition of zinc chloride to combinations of carboxymethylribonuclease and cytidine 2', 3' or 5'-phosphate. A different effect of zinc chloride was observed on the spectrum of an equimolar combination of ribonuclease and cytidine 3'-phosphate, as shown in the difference spectra in Fig. 11. Zinc chloride depressed the extinction up to 282 mμ and enhanced it above this wavelength.

To assist in the interpretation of these phenomena the effect of the isomeric cytidine nucleotides on the kinetics of the reaction of ribonuclease with bromoacetic acid was investigated (Fig. 12). Cytidine 2'-phosphate gave almost complete protection of the enzyme, and the protection given by the nucleotides in descending order is 2'-phosphate, 3'-phosphate, 5'-phosphate; this is the same order as their effectiveness as inhibitors of the ribonuclease-catalysed hydrolysis of the cyclic phosphate.

Fig. 12. Effect of cytidine nucleotides on the reaction of ribonuclease with bromoacetic acid in acetate buffer (0.1M), pH 5.5, at 40°. The concn. of ribonuclease was 70 μM, and that of bromoacetic acid was 40 mg./ml. ▼, No addition; ▲, in the presence of cytidine 5'-phosphate (5.5 mM); ■, in the presence of cytidine 3'-phosphate (5.5 mM); ●, in the presence of cytidine 2'-phosphate (5.5 mM).

DISCUSSION

The spectrophotometric results given above supply information about the nature of the binding of the inhibitors to the enzyme.

There is an apparent contradiction between the observations that cytidine nucleotides protect the enzyme from reaction with alkylating reagents, and yet these same nucleotides combine with the alkylated derivative. The protection of the protein against alkylation may be due to the masking of a group which promotes the alkylation reaction. Stein (1960) has commented on the remarkable nature of the reaction of iodoacetate with ribonuclease at pH 5.5 and quotes evidence that the alkylation depends on electrostatic interaction between the carboxyl group and a positive locus, which is probably an imidazolium ion from the pH profile of the reaction. Further study of the effect of pH on the rate of reaction of ribonuclease with iodoacetic acid (M. P. Laiden, A. P. Mathias & B. R. Rabin; unpublished work) substantiates the

identification of the groups involved as histidine residues.

The almost complete protection of ribonuclease against alkylation by cytidine 2'-phosphate, as measured by activity loss, is evidence that the protein-nucleotide complexes do not react with alkylating reagents. We conclude that the binding site for iodo- or bromo-acetic acid can also be occupied by nucleotides, but the identification of this as part of the active site remains to be substantiated since this site can also be occupied by sulphate (Gundlach *et al.* 1959), which, however, does not inhibit hydrolysis of cytidine 2',3'-phosphate by the enzyme (Ross, 1961) at $I\ 0.2$.

The slight depression of the spectrum of ribonuclease by low concentrations of Zn^{2+} ions might be taken as evidence for complex formation. The absence of this effect with carboxymethylribonuclease indicates that the alkylated histidine residue is involved in binding the metal. Of the nucleotides studied spectrophotometrically, only cytidine 3'-phosphate forms a ternary complex with Zn^{2+} ions and ribonuclease. This conclusion is substantiated by the kinetic experiments. The analogous complex with carboxymethylribonuclease is not formed. This is further evidence that Zn^{2+} ions and the halogenated acids interact at the same site. Presumably, in the ternary complex, the oxygen atoms of the phosphate residue in cytidine 3'-phosphate are in a stereochemically suitable position to co-ordinate with Zn^{2+} ions bound to the histidine residue.

SUMMARY

1. The interaction of ribonuclease or carboxymethylribonuclease with cytidine 2'- and 3'-phosphate has been demonstrated spectrophotometrically.

2. At pH 7.2, $I\ 0.1$, 25° and at a concentration of zinc chloride of 0.04 mM , the inhibition of ribonuclease by Zn^{2+} ions is due to the formation of a ternary complex of protein, Zn^{2+} ions and cytidine 3'-phosphate, which can also be demonstrated spectrophotometrically. At higher concentrations of zinc chloride, inhibition occurs in the absence of

product. No evidence for formation of a ternary complex with either cytidine 5'-phosphate or cytidine 2'-phosphate has been obtained.

3. The isomeric cytidine nucleotides protect ribonuclease against alkylation by iodoacetic acid and their effectiveness in descending order is: 2'-phosphate, 3'-phosphate, 5'-phosphate.

4. The inhibition of ribonuclease by Zn^{2+} ions varies with pH in the region 6–7, suggesting the involvement of a histidine residue.

5. Ribonuclease catalyses the hydrolysis of *p*-nitrophenyl acetate and the reaction is inhibited by Zn^{2+} ions.

REFERENCES

- Anfinsen, C. B. & White, F. H., jun. (1961). *The Enzymes*, vol. 5, p. 95. Ed. by Boyer, P. O., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Barnard, E. A. & Stein, W. D. (1959). *J. molec. Biol.* **1**, 339.
- Bender, M. L. & Turnquest, B. W. (1957). *J. Amer. chem. Soc.* **79**, 1652.
- Bruice, T. C. & Schmir, G. L. (1957). *J. Amer. chem. Soc.* **79**, 1663.
- Crook, E. M., Mathias, A. P. & Rabin, B. R. (1960). *Biochem. J.* **74**, 230.
- Davis, F. F. & Allen, F. W. (1955). *J. biol. Chem.* **217**, 13.
- Edsall, J. T., Felsenfeld, G., Goodman, D. S. & Gurd, F. R. N. (1954). *J. Amer. chem. Soc.* **76**, 3054.
- Findlay, D., Mathias, A. P. & Rabin, B. R. (1962). *Biochem. J.* **85**, 139.
- Gundlach, H. G., Stein, W. H. & Moore, S. (1959). *J. biol. Chem.* **234**, 1754.
- Gutfreund, H. & Sturtevant, J. M. (1956). *Biochem. J.* **63**, 656.
- Hartley, E. S. & Kilby, B. A. (1954). *Biochem. J.* **56**, 288.
- Herries, D. G. (1961). Ph.D. Thesis: University of London.
- Herries, D. G., Mathias, A. P. & Rabin, B. R. (1962). *Biochem. J.* **85**, 127.
- Hummel, J. P., Verploes, D. A. & Nelson, C. A. (1961). *J. biol. Chem.* **236**, 3168.
- Koltun, W. L., Dexter, R. N., Clark, R. W. & Gurd, F. R. N. (1958). *J. Amer. chem. Soc.* **80**, 4188.
- Mathias, A. P., Rabin, B. R. & Ross, C. A. (1960). *Biochem. biophys. Res. Commun.* **3**, 625.
- Ross, C. A. (1961). Ph.D. Thesis: University of London.
- Stein, W. H. (1960). *Brookhaven Symp. Biol.: Protein Structure and Function*, **13**, 104.
- Tanford, C. (1952). *J. Amer. chem. Soc.* **74**, 211.
- Walaas, E. (1958). *Acta chem. scand.* **12**, 528.