Preparation of Allosteric Ribonuclease

By ELAINE J. WALKER, GREGORY B. RALSTON* and IVAN G. DARVEY
Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006, Australia

(Received 24 August 1977)

A method for the preparation of allosteric ribonuclease from bovine pancreas is described. The effects of freeze-drying ribonuclease from acid and alkaline solutions on plots of velocity versus substrate concentration for the hydrolysis of 2':3'-cyclic CMP are examined. Comparison of these plots with the plots obtained with several commercial enzyme preparations indicates that the conformation of the enzyme is dependent on the method of preparation. Aging experiments demonstrate that further conformational changes occur at different rates, depending on the methods of storage. Results suggest that the allosteric behaviour of ribonuclease has not always been observed with commercial preparations, owing to variations in methods of preparation and storage of the enzyme.

Bovine pancreatic RNAase has frequently been reported to show Michaelis–Menten kinetics [see, for example, Hummel *et al.* (1958), Litt (1961), Nelson & Hummel (1961), Herries *et al.* (1962), Ramsden & Laidler (1966), Irie (1968), Wigler (1968), Muller & Talsky (1970)].

Rübsamen *et al.* (1974) reported sigmoidicity in plots of velocity versus substrate concentration for the RNAase-catalysed hydrolysis of 2':3'-cyclic CMP in the substrate concentration range 0–12 mM. We have previously reported (Walker *et al.*, 1975, 1976) that the velocity increases as [S], substrate concentration, increases from zero to 30 mM and between 40 and 70 mM, but that the velocity decreases sharply in the region 30–40 mM-substrate. To explain this unusual behaviour, we have proposed a model involving two conformations of the enzyme. Kinetic data to support such a model have been obtained with six different batches of commercial RNAase purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), but we have since received two batches of RNAase from the same source, one showing typical Michaelis–Menten kinetics and the other displaying kinetics intermediate between the two types.

We describe here a modification of the preparative methods of Hirs *et al.* (1953) and of Kunitz (1940) which gives consistently allosteric preparations of RNAase. We also consider some of the conditions of preparation and storage that result in apparently non-allosteric preparations.

Experimental

Materials

Bovine pancreatic ribonuclease was obtained: (1) from Sigma in both the form freeze-dried from acid

(R 5375) and the non-freeze-dried form (R 5250); (2) from Worthington (Croydon CR9 3QL, Surrey, U.K.) in the form freeze-dried from alkali (a gift from Professor J. H. Bradbury, Australian National University, Canberra); or (3) by preparation from bovine pancreas as described below.

CM-Sephadex CM-50 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. 2':3'-Cyclic CMP was obtained from Sigma. All other chemicals used were of analytical-reagent grade.

Methods

Enzyme preparation. Bovine pancreatic ribonuclease was extracted from pancreas obtained within a few minutes of the death of the animal. The gland was cut into small pieces and embedded in CO₂ snow. It could be stored in this form for several days without noticeable effect on the enzyme, but storage for extended periods gave rise to enzyme preparations with increasingly altered kinetics. Freeze-drying the tissue at this stage also gave rise to enzyme preparations with apparently non-allosteric kinetics.

The pancreas was pulverized in a Waring blender while still frozen, then homogenized with ice-cold H₂SO₄ (0.125 M) (100 g of tissue/200 ml of acid). It was then stirred, filtered and centrifuged by the method of Hirs *et al.* (1953). The appropriate fraction was adjusted to pH 5.5 by the gradual addition of cold NaOH (1 M) and then re-centrifuged as described by Hirs *et al.* (1953).

Elution of this fraction from Sephadex CM-50 with Na₂HPO₄/NaH₂PO₄ buffer (0.025 M, pH 7.2) containing NaN₃ (0.02 %) and a gradient of NaCl (0–0.2 M) gave incomplete separation of RNAase activity from a protein with molecular weight as determined by disc gel electrophoresis (Fairbanks *et al.*, 1971) of approx. 20000 and with an isoelectric

Abbreviation used: RNAase, ribonuclease.

* To whom reprint requests should be addressed.

point as determined by isoelectric focusing (Wrigley, 1971) of pH 9.5.

Fractionation with $(\text{NH}_4)_2\text{SO}_4$ by the method of Kunitz (1940) was therefore used to remove this contaminating protein. The supernatant from the second centrifugation as above was adjusted to 45 g of $(\text{NH}_4)_2\text{SO}_4$ /100 ml of the original supernatant by the gradual addition of solid $(\text{NH}_4)_2\text{SO}_4$. The precipitated protein was removed by centrifugation as above three times during this addition. The supernatant of the third centrifugation [45 g of $(\text{NH}_4)_2\text{SO}_4$ /100 ml of original supernatant] was then adjusted to 66 g/100 ml of original supernatant by further addition of solid $(\text{NH}_4)_2\text{SO}_4$. This solution was centrifuged as above and the pellet dissolved in a minimum amount of NaCl (0.04 M). The resulting solution contained 60–70 % of the total RNAase activity.

The remaining $(\text{NH}_4)_2\text{SO}_4$ was removed from the solution by elution from a Sephadex G-25 column (2 cm $\times$ 30 cm) with NaCl (0.04 M). Fractions showing RNAase activity were pooled and added to a column (2 cm $\times$ 20 cm) of Sephadex CM-50. It was eluted with Na_2HPO_4 / NaH_2PO_4 buffer (0.025 M, pH 7.2) with a gradient of NaCl (0.04–0.16 M). The time of elution is critical. Elution times of more than 24 h resulted in failure to elute RNAase from the column. Concentrations of phosphate of less than 0.025 M had a similar effect. Phosphate presumably aids in the elution of RNAase by lowering the apparent isoelectric point of RNAase (Crestfield & Allan, 1954).

Freeze-drying from acid solution

RNAase prepared as described above was desalted by elution with acetic acid (0.01 M) from a Sephadex G-25 column (2 cm $\times$ 20 cm) which had been equilibrated with acetic acid (0.01 M). Fractions showing A_{278} were pooled. The concentration of enzyme was determined by using an absorption coefficient of $9800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Tanford & Havenstein, 1956), and an equimolar concentration of phosphate (Na_2HPO_4 / NaH_2PO_4 , pH 7.2) was added back. The enzyme was freeze-dried from the acetic acid/phosphate solution and redissolved in the minimum volume of NaCl (0.1 M), giving a final pH of approx. 4.0.

Freeze-drying from alkaline solution

Most of the phosphate was removed from a solution of RNAase in phosphate buffer (0.025 M) by elution with water from a Sephadex G-25 column (2 cm $\times$ 30 cm). The one phosphate molecule that binds tightly to the active site of RNAase (Nelson *et al.*, 1962) was then removed by elution with distilled water from a Dowex 2 column (1 cm $\times$ 20 cm) pre-equilibrated with acetic acid (0.1 M). Fractions showing A_{278} were pooled and had pH 4.8. The solution was freeze-dried until almost dry, then water was added and the freeze-drying procedure was

repeated twice more until the solution had pH approx. 8.5.

Enzyme activity

Enzyme assays were carried out by the pH-stat assay of Walker *et al.* (1976) at pH 7.13, I 0.3, by using substrate concentrations below the allosteric transition.

Enzyme storage

RNAase was stored at 4°C in sealed sterilized vials in either Na_2HPO_4 / NaH_2PO_4 buffer (0.025 M, pH 7.2) containing 0.02 % NaN_3 , or in NaCl (0.04 M) after the removal of phosphate as described above.

Results

Enzyme preparation

The elution profile from Sephadex CM-50 is shown in Fig. 1. It shows peaks similar to those obtained by Hirs *et al.* (1953) using Amberlite IRC 50 resin. Although Taborsky (1959) has reported resolution of commercial RNAase on CM-cellulose into four active components, chromatography of our preparation on CM-Sephadex shows only one active component. It is possible that the four components reported by Taborsky (1959) arise from the heat-treatment steps used in the commercial preparation of RNAase (Shapira & Parker, 1960).

The purified enzyme after $(\text{NH}_4)_2\text{SO}_4$ fractionation and rechromatography had an apparent molecular weight as determined by disc gel electrophoresis in

Fig. 1. Ion-exchange chromatography of pancreas extract. Pancreas extract prepared as described in the text was eluted from a Sephadex CM-50 column (2 cm $\times$ 20 cm) with Na_2HPO_4 / NaH_2PO_4 buffer (0.025 M, pH 7.2) with a NaCl gradient (0–0.2 M) at 4°C. ----, A_{278} ; —, RNAase-catalysed hydrolysis of 2':3'-cyclic CMP.

Fig. 2. Curves of velocity versus substrate concentration for the hydrolysis of 2':3'-cyclic CMP by RNAase prepared as described in text, and for three batches of commercial enzyme

The reaction mixture contained 2':3'-cyclic CMP (0–50 mM), RNAase (0.5 μM) and NaCl to a final ionic strength of 0.3 mol/l. ○, RNAase prepared as described in the text. ●, ■, ▲, Three separate batches of non-freeze-dried RNAase obtained from the Sigma Chemical Co., arbitrarily labelled (a), (b) and (c).

Fig. 3. Effect of freeze-drying RNAase from acid solution on plots of velocity versus substrate concentration

Reaction mixture contained 2':3'-cyclic CMP (0–50 mM), NaCl to a final ionic strength of 0.3 mol/l and RNAase prepared as described in the text, (a) non-freeze-dried (0.5 μM), or (b) acid freeze-dried (0.9 μM), or (c) crystalline RNAase from Sigma Chemical Co. (0.5 μM).

Fig. 4. Effect of freeze-drying RNAase from alkaline solution on plots of velocity versus substrate concentration. Reaction mixture contained 2':3'-cyclic CMP (0–50 mM), NaCl to a final ionic strength of 0.3 mol/l and RNAase prepared as described in text (a) non-freeze-dried (0.5 μM), (b) alkaline freeze-dried (0.26 μM) immediately after being dissolved or (c) alkaline freeze-dried (0.26 μM) 24 h after being dissolved, or RNAase freeze-dried from alkaline solution by Worthington, (d) immediately after dissolving (0.44 μM), (e) 24 h after dissolving (0.44 μM), or (f) 10 days after dissolving (0.44 μM).

sodium dodecyl sulphate of 12000–13000 and an isoelectric point, as determined by isoelectric focusing, of 9.6 [cf. mol.wt. 13680 calculated from amino acid composition (Richards & Wyckoff, 1971) and pI 9.60 (Tanford & Havenstein, 1956)].

A plot of velocity (v) versus substrate concentration $[S]$ for the enzyme preparation is shown in Fig. 2 and may be compared with plots of v versus $[S]$ obtained with three different batches of non-freeze-dried commercial enzyme from Sigma (Fig. 2, curves a , b , c). Early batches of non-freeze-dried RNAase obtained from Sigma show plots of v versus $[S]$ (curve a) similar to that obtained with prepared RNAase. A more recent batch of Sigma enzyme shows much decreased allostercity (Fig. 2, curve b), and a third batch of enzyme from Sigma has a plot of v versus $[S]$ which approaches a hyperbola (curve c).

Freeze-drying of RNAase

Fig. 3 shows plots of v versus $[S]$ for enzyme prepared as described above (curve *a*) and freeze-dried from acid (curve *b*). After freeze-drying from acid once, the enzyme shows approximately the same specific activity at substrate concentrations below the allosteric transition, but a marked decrease in allostericity. $[S_m]$, the substrate concentration at the midpoint of the transition, is slightly less than before freeze-drying. The shape of the curve is very similar to that obtained with a specimen of crystalline RNAase obtained from Sigma which also had an acid pH when dissolved in NaCl (0.1 M) (see Fig. 3, curve *c*).

Fig. 4 shows plots of v versus $[S]$ for RNAase that had been freeze-dried at pH 8.5 in the absence of phosphate. Activities were measured both immediately after redissolving the enzyme (curve *a*) and after the enzyme had remained in solution for 24 h at 4°C (curve *b*). Fig. 4 shows that immediately after solution the enzyme showed apparently Michaelis-Menten kinetics. During 24 h at 4°C, a conformational change appeared to produce a form partially able to undergo the allosteric transition. Even after 2 weeks at 4°C, however, full allostericity was not restored (curve *c*), though specific activity was approximately the same as that before freeze-drying. However, $[S_m]$ was greater after freeze-drying than was the case with freshly prepared enzyme.

These results are very similar to those obtained with a sample of enzyme from Worthington, which

Fig. 5. Effect of storage in the absence of phosphate on prepared RNAase

Prepared RNAase was stored at 4°C in NaCl (0.04 M) containing 0.02% azide for (a) 5 days (b) 12 days. Conditions for assay as given in Fig. 2.

Table 1. Effect of phosphate on the rate of deamidation of RNAase during storage

Time of storage (days)	Deamidation (residues/enzyme molecule)	
	No phosphate	+25 mM-phosphate
5	0.5	0.4
12	2.7	0.7
19	8.1	0.7

gave an alkaline solution when dissolved in NaCl (see Fig. 4, curves *d*, *e*, *f*).

Storage of RNAase

Storage of RNAase, purified by the method described in the present paper, resulted in a gradual loss of activity, an increase in the value of $[S_m]$, and a spontaneous loss of amide groups from the protein. Fig. 5 shows the effect of storage of the purified enzyme at 4°C for (a) 5 and (b) 12 days in the absence of phosphate. Storage of the enzyme in the presence of phosphate (25 mM) considerably slowed the rate at which these changes occurred. The degree of deamidation occurring during the storage of the enzyme in the presence and absence of phosphate was measured by the determination of free ammonia in the solution by the method of Kaplan (1965). The results, shown in Table 1, indicate that storage of the enzyme in the absence of phosphate resulted in rapid spontaneous deamidation, and that the presence of 25 mM-phosphate largely prevented this change.

Storage of the frozen pancreas at 0°C for up to 19 days before extraction of the RNAase also resulted in slow changes in the enzyme. However, these changes were much slower than those observed in the purified enzyme in the absence of phosphate. Thus it appears that storage of the enzyme either in the tissue or in the presence of phosphate minimizes the degree of spontaneous deamidation and subsequent changes in enzyme activity.

Discussion

The data presented here suggest a number of reasons why the allosteric kinetics of RNAase have not been previously reported with commercial batches of enzyme. It is clear that different methods of preparation can result in conformations of the enzyme different from that found in the native state. Shapira & Parker (1960) have suggested that the microheterogeneity reported by Taborsky (1959) and also by Aqvist & Anfinsen (1959) may be an artifact introduced by a heat-treatment step in the preparation.

We have noticed the variability of the commercial preparations available from Sigma with respect to

their conformational adaptability to ligands. With the freeze-dried Sigma preparations, it appears to be the freeze-drying process itself that decreases the enzyme's ability to undergo conformational change. Freeze-drying of our preparations from acid solutions results in a similar loss of conformational adaptability.

With the non-freeze-dried preparations, the variability may be due to a spontaneous deamidation process. Robinson *et al.* (1970) have pointed out the marked correlation between the percentage of glutamine and asparagine residues in a protein and the rapidity of turnover of that protein. They suggest that the deamidation of a protein is possibly a 'planned obsolescence mechanism' by which the protein pool can be kept at optimal metabolic activity. They report that RNAase, with an unusually high content of glutamine and asparagine residues (13.7%), has a half-life of less than 1 day in the animal.

We have demonstrated that up to eight residues per enzyme molecule are deamidated during storage for 3 weeks at 4°C in the absence of phosphate. We have shown that storage in the presence of phosphate (25mM) or in the frozen tissue results in a more gradual deamidation. In this case, the activity of the preparation is little affected at low substrate concentrations, consistent with reports that several commercial preparations are stable for several weeks when kept under refrigeration (see, e.g., the Worthington catalogue).

This deamidation results in an increase in $[S_m]$, the substrate concentration at the midpoint of the transition, in the manner predicted by the allosteric model. In the preceding paper (Walker *et al.*, 1978) we provide evidence that the decrease in the net positive charge of the enzyme, which arises from deamidation, results in a decreased affinity of the allosteric sites of the negatively charged nucleotides and hence an increase in $[S_m]$. This shift in $[S_m]$ results in a change in the shape of the plots of v versus $[S]$ and an increasing difficulty in detecting deviations from Michaelis-Menten behaviour.

In addition to the variability that may be introduced by freeze-drying or aging of the enzyme, it is possible that the variations in the commercial preparations obtained from Sigma may be due to variations in the phosphate concentrations of the preparations. J. H. Bradbury (personal communication) has observed marked variation in the phosphate concentration in the Sigma preparations, and we have previously reported (Walker *et al.*, 1976) that the allostericity of RNAase is affected by phosphate.

Studies of velocity as a function of $[S]$ after freeze-drying from acid and alkaline solution lends further support to the allosteric model that we have proposed (Walker *et al.*, 1975, 1976). In the preceding paper (Walker *et al.*, 1978) we present evidence that altering the pH used during assay of the enzyme results in a

shift in equilibrium between the two enzyme conformations, E and F (Walker *et al.*, 1975). A decrease in pH favours form F and results in a decrease in $[S_m]$. An increase in pH favours form E and results in an increase in $[S_m]$.

With freeze-drying from acid solution in the presence of phosphate there is a slight decrease in $[S_m]$, indicating a shift in equilibrium towards form F. Freeze-drying from alkaline solution results in an enzyme conformation with apparently Michaelis-Menten kinetics. After 24h at 4°C this form of the enzyme undergoes a slow conformational change to a form of the enzyme that is able to undergo the $E \rightleftharpoons F$ transition. In this case the value of $[S_m]$ is slightly higher than that of the non-freeze-dried enzyme, indicating, perhaps, a shift in the $E \rightleftharpoons F$ equilibrium favouring form E.

These observations of the conformational changes induced by acid and alkaline freeze-drying are consistent both with the allosteric model that we have proposed above and with evidence for conformational changes induced by freeze-drying reported by Craig *et al.* (1963). They examined the rate of diffusion of RNAase through dialysis tubing and report that the enzyme exists in at least two different conformations, a fast-diffusing and a slow diffusing form. They report that, in the Armour preparations which are freeze-dried from acid, and in their own samples freeze-dried from acid, the fast-moving form predominates. The Worthington preparations, freeze-dried from alkaline solution, exist as the slow-moving form. After freeze-drying, a slow conformational change occurs (3h at 40°C, slower at 25°C), re-establishing equilibrium. In addition, they report that sulphate increases the rate of diffusion, whereas increasing ionic strength decreases the rate.

Such observations are consistent with the model that we propose. We have postulated that form E is favoured by alkaline pH and high ionic strength (see Walker *et al.*, 1978). We have shown that form F is favoured by increasing phosphate concentrations (Walker *et al.*, 1976). In addition, we have demonstrated that form E (that favoured by high pH) is more susceptible to tryptic digestion (Walker *et al.*, 1976), indicating, perhaps, a more open structure. Similarly, Crompton & Bradbury (1976) have reported that deuterium exchange is more rapid in the conformation favoured by freeze-drying from alkaline solution.

All of these data are consistent with a model in which alkaline conditions favour form E, a more open form of the enzyme which would be more susceptible to tryptic digestion, deuterium exchange and slowed diffusion.

Data from all of these systems indicate that the predominant conformation and its measurable kinetics are therefore very dependent on the method of isolation, storage and assay of the enzyme.

References

- Aqvist, S. E. G. & Anfinsen, C. B. (1959) *J. Biol. Chem.* **234**, 1112-1117
- Craig, L. C., King, T. P. & Crestfield, A. M. (1963) *Biopolymers* **1**, 231-238
- Crestfield, A. M. & Allan, F. W. (1954) *J. Biol. Chem.* **211**, 363-366
- Crompton, M. & Bradbury, J. H. (1976) *Abstr. Aust. Biochem. Soc. 3rd Spec. Conf. Enzymes*
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606-2617
- Herries, D. G., Mathias, A. P. & Rabin, B. R. (1962) *Biochem. J.* **85**, 127-134
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1953) *J. Biol. Chem.* **200**, 493-506
- Hummel, J. P., Flores, M. & Nelson, G. (1958) *J. Biol. Chem.* **233**, 717-721
- Irie, M. (1968) *J. Biochem. (Tokyo)* **63**, 501-508
- Kaplan, A. (1965) *Stand. Meth. Clin. Chem.* **5**, 245-256
- Kunitz, M. (1940) *J. Gen. Physiol.* **24**, 15-32
- Litt, M. (1961) *J. Biol. Chem.* **236**, 1786-1790
- Muller, W. & Talsky, G. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1564-1570
- Nelson, C. A. & Hummel, J. P. (1961) *J. Biol. Chem.* **236**, 3173-3176
- Nelson, C. A., Hummel, J. P., Svenson, C. A. & Friedman, L. (1962) *J. Biol. Chem.* **237**, 1575-1580
- Ramsden, E. N. & Laidler, K. J. (1966) *Can. J. Chem.* **44**, 2597-2610
- Richards, F. M. & Wyckoff, H. W. (1971) *Enzymes 3rd Ed.* **4**, 647-806
- Robinson, A. B., McKerrow, J. H. & Cary, P. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 753-757
- Rübsamen, H., Kandler, R. & Witzel, H. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 687-708
- Shapira, R. & Parker, S. (1960) *Biochem. Biophys. Res. Commun.* **3**, 200-204
- Taborsky, G. (1959) *J. Biol. Chem.* **234**, 2652-2656
- Tanford, C. & Havenstein, J. D. (1956) *J. Am. Chem. Soc.* **77**, 6409-6413
- Walker, E. J., Ralston, G. B. & Darvey, I. G. (1975) *Biochem. J.* **147**, 425-433
- Walker, E. J., Ralston, G. B. & Darvey, I. G. (1976) *Biochem. J.* **153**, 329-337
- Walker, E. J., Ralston, G. B. & Darvey, I. G. (1978) *Biochem. J.* **173**, 1-4
- Wigler, P. W. (1968) *J. Biol. Chem.* **243**, 3466-3472
- Wrigley, C. W. (1971) *Methods Enzymol.* **22**, 559-564