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STEADY-STATE ENZYME KINETICS OF THE PANCREATIC RIBONUCLEASES FROM FIVE MAMMALIAN SPECIES

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Summary

The kinetic parameters K_m, k_{+2} and k_{+2}/K_m of the pancreatic ribonucleases (EC 3.1.4.22) from cow, giraffe, horse, rat and lesser rorqual have been determined, using 2′,3′-cyclic cytidine monophosphate and 2′,3′-cyclic uridine monophosphate as substrates. No large differences were found between the activities of the five enzymes. The relative differences between the activities of the five enzymes are mainly due to differences in the rates of hydrolysis and not to differences in the affinities for the substrates.

Introduction

Unlike bovine pancreatic ribonuclease A (EC 3.1.4.22) [1—7], little attention has been paid to the kinetics of the pancreatic ribonucleases from other animals [8—10]. Here we present a comparative study of the kinetics of the ribonucleases from cow (*Bos taurus*), giraffe (*Giraffa camelopardalis*), horse (*Equus caballus*), rat (*Rattus rattus*) and lesser rorqual (*Balaenoptera acutorostrata*). The measurements are made under identical conditions so that the results obtained for these five ribonucleases can be compared. The kinetic parameters for the hydrolysis of 2′,3′-cyclic cytidine monophosphate are measured at 25°C for a number of pH values, those for the hydrolysis of 2′,3′-cyclic uridine monophosphate at 20 and 30°C at pH 6.5.

Since the ribonucleases used in this study have also been characterized as to their primary structure and carbohydrate content [11—15] and are assumed to have a three-dimensional structure comparable to that of bovine ribonuclease [16] an attempt is made to explain some of the observed differences in activity by difference in structure.

Materials and Methods

Bovine ribonuclease A was purchased from Miles-Seravac (Maidenhead, U.K.). Rat ribonuclease was isolated as described by Beintema et al. [8] and giraffe, horse and lesser rorqual ribonucleases were isolated as described by Wierenga et al. [17]. The sodium salt of 2′,3′-cyclic cytidine monophosphate was from Sigma Chemical Co. (St. Louis, U.S.A.). 2′,3′-Cyclic uridine monophosphate (sodium salt) was from Boehringer (Mannheim, G.F.R.). All other chemicals were analytical grade products from Merck (Darmstadt, G.F.R.).

The hydrolysis of 2′,3′-cyclic cytidine monophosphate by ribonuclease was followed by monitoring the change in absorbance at 284 nm ($\Delta\epsilon$ = 1500) in a Zeiss PMQ II spectrophotometer at 25°C for 16 min as described by Crook et al. [1]. The hydrolysis of 2′,3′-cyclic uridine monophosphate was measured in the same way as the hydrolysis of 2′,3′-cyclic cytidine monophosphate at 274 nm at 20 and 30°C at pH 6.5. Since the difference spectrum of 2′,3′-cyclic uridine monophosphate and 3′-uridine monophosphate was found to be pH dependent, we had to use a value for $\Delta\epsilon$ of 1700 instead of the value of 1800, used at pH 7.5 [8].

A 0.1 M Tris · HCl buffer was used for the pH range 7.0—8.5 and a 0.1 M acetate buffer for the pH range 5.5—6.5. All buffer systems were of ionic strength 0.2, the inert electrolyte being NaCl. A stock solution of about 1.5 mg/ml ribonuclease in 0.2 M NaCl was used. Exact enzyme concentrations were calculated from amino acid analyses performed on samples of the stock solutions on a Technicon TSM 1 amino acid analyzer.

The concentrations of the cyclic nucleotides varied from 0.1 to 4.0 mM. Exact nucleotide concentrations were determined spectrophotometrically, using molar absorbances published in refs. 18—20. Depending on the nucleotide concentration cuvettes with an optical pathlength of 1.0, 0.5 or 0.1 cm were used. Under the conditions used the change in absorbance was linear with time. The parameters K_m and k_{+2} were derived from plots of the initial velocity against the quotient of initial velocity and substrate concentration. The estimated uncertainty in the kinetic parameters obtained from the plots are ±15% for K_m and ±10% for k_{+2}. Most of the experiments were performed in triplicate, a few in duplicate.

Results

2′,3′-cyclic cytidine monophosphate

In Table I the kinetic parameters for the hydrolysis of 2′,3′-cyclic cytidine monophosphate at 25°C by the five different ribonucleases are given. Comparison of these data shows that the differences in activity are caused by different rates of hydrolysis rather than by different affinities of the enzymes for the substrate. In Fig. 1 the k_{+2}/K_m values for the five enzymes are plotted versus pH. As can be seen from this figure, the pH optimum is not the same for all ribonucleases. Giraffe ribonuclease has its optimum at pH 6.4, cow, horse and rat ribonucleases at pH 6.1, and lesser rorqual ribonuclease at pH 5.9. Obviously, the five ribonucleases may be ordered according to their relative activities as follows: horse > cow > lesser rorqual > giraffe > rat.

TABLE I

REACTION CONSTANTS FOR THE HYDROLYSIS OF 2',3'-CYCLIC CYTIDINE MONOPHOSPHATE BY FIVE MAMMALIAN PANCREATIC RIBONUCLEASES

In this table data from the literature are included. In those cases where K_m is significantly higher than the substrate concentration only the quotient of k_{+2}/K_m is measurable. No discrimination was made here between the two whale species. See text for experimental conditions.

pH	K_m (mM)					k_{+2} (s^{-1})					k_{+2}/K_m (s^{-1}, mM^{-1})				
	Cow	Giraffe	Horse	Rat	Whale	Cow	Giraffe	Horse	Rat	Whale	Cow	Giraffe	Horse	Rat	Whale
8.5											0.086	0.051	0.10	0.030	0.044
8.0											0.20	0.16	0.30	0.097	0.14
7.5											0.48	0.43	0.66	0.18	0.29
7.0	6.6	2.8	4.0	1.8	3.2	9.7	3.0	7.8	1.2	3.9	1.5	1.1	1.9	0.67	1.2
6.5	0.58	0.83	0.48	0.62	0.73	2.5	1.4	2.5	0.83	2.7	4.3	1.7	5.2	1.3	3.8
6.0	0.30	0.61	0.33	0.29	0.41	1.7	0.98	2.3	0.49	1.8	5.6	1.6	6.9	1.7	4.4
5.5	0.35	0.39	0.24	0.45	0.30	1.4	0.54	1.1	0.53	1.3	3.9	1.4	4.7	1.2	4.3

Literature															Ref.
8.5	36					5.0					0.14				4
8.0	41					10.2					0.25				4
7.5	17					14.5					0.85				4
	5.2—7.5			5.1—5.9		2.7—3.7			1.6—2.5		0.50—1.12			0.27—0.63	8
7.0	11.1					18.1					1.64				4
	5.4	1.3									1.60	0.92			10
	3.3					5.5					1.7				2
	0.28		0.49		0.49										9
			0.24		0.37										9
6.5	3.3					12.1					3.57				4
6.0	1.7					7.63					4.59				4
5.8	0.4					2.0					5.0				2
5.0	3.8					1.27					2.65				4
4.5	0.49					0.42					0.85				4
4.0	1.1					0.15					0.13				4

TABLE II

REACTION CONSTANTS FOR THE HYDROLYSIS OF 2',3'-CYCLIC URIDINE MONOPHOSPHATE BY FIVE MAMMALIAN PANCREATIC RIBONUCLEASES

See legend Table I and text for experimental conditions.

pH	K_m (mM)					k_{+2} (s^{-1})					k_{+2}/K_m (s^{-1}, mM^{-1})				
	Cow	Giraffe	Horse	Rat	Whale	Cow	Giraffe	Horse	Rat	Whale	Cow	Giraffe	Horse	Rat	Whale
6.5 (20° C)	0.78	1.24	0.51	0.50	0.64	0.86	1.48	1.18	0.51	0.71	1.10	1.19	2.32	1.02	0.93
6.5 (30° C)	0.70	0.49	0.31	0.34	0.58	0.54	1.07	0.56	0.28	0.59	0.77	2.17	1.80	0.81	1.02
Literature															Ref.
8.0	15.8					1.14					0.08				6
7.5	8.65					1.88					0.21				6
	2.4—3.5			3.0—3.1		0.6—1.1			1.5—2.3		0.26—0.38			0.46—0.75	8
7.0	7.3					4.16					0.57				6
	0.45		0.49		0.30										9
			0.49		0.24										9
	8.2	8.0									0.56	0.38			10
	5.0					2.2					0.40				2
	3.6					1.6					0.40				5
6.5	3.87					4.20					1.09				6
6.0	1.40					2.26					1.61				6
5.8	0.5					1.0					2.0				2
5.5	0.85					1.37					1.61				6
5.0	0.93					0.812					0.87				6
	0.6					0.5					0.8				2
4.5	1.29					0.435					0.34				6
4.0	3.23					0.254					0.079				6
	2.0					0.16					0.08				3

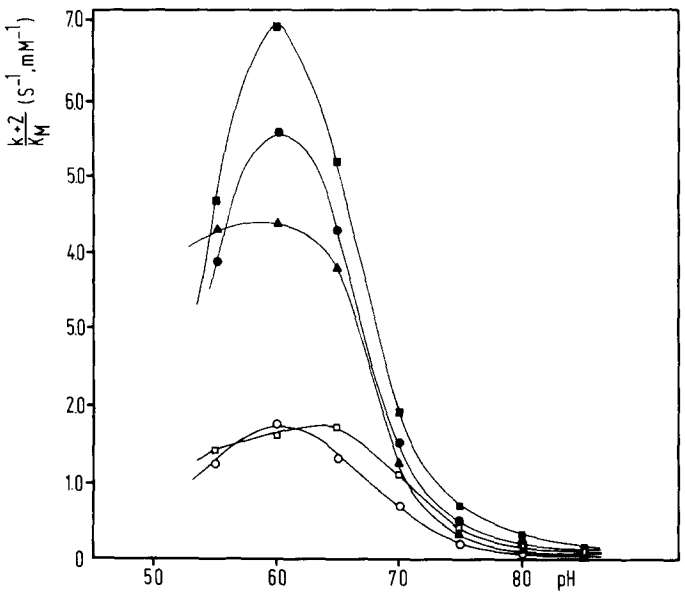

Fig. 1. The pH dependence of the activity on 2',3'-cyclic cytidine monophosphate of the five pancreatic ribonucleases. ●———●, bovine ribonuclease; □———□, giraffe ribonuclease; ■———■, horse ribonuclease; ▲———▲, lesser rorqual ribonuclease; ○———○, rat ribonuclease.

2',3'-Cyclic uridine monophosphate

The kinetic parameters for the hydrolysis of 2',3'-cyclic uridine monophosphate at pH 6.5 at 20 and 30°C by the five ribonucleases are listed in Table II. With this substrate too the differences in activity are mainly due to differences in the rate of hydrolysis and not to differences in affinity for the substrate. As can be seen from these data, horse ribonuclease is still the most active of the five ribonucleases but there are remarkably smaller differences in the activity of the four other ribonucleases. Thus, giraffe and rat ribonucleases are relatively more active on 2',3'-cyclic uridine monophosphate than on 2',3'-cyclic cytidine monophosphate.

TABLE III

DIFFERENCE MATRIX OF THE FIVE PANCREATIC RIBONUCLEASES USED IN THIS STUDY

Both the number and the percentage of the differences in the amino acid sequences for a chain length of 124 residues are included in this table. A deletion was treated as an amino acid.

	Cow	Giraffe	Horse	Whale	Rat
Cow	—	9%	27%	24%	33%
Giraffe	11	—	25%	26%	36%
Horse	33	31	—	23%	32%
Whale	30	32	29	—	31%
Rat	41	45	40	39	—

Discussion

As can be seen from the data in Tables I and II, the activities of the five ribonucleases do not differ by more than a factor four, although their primary structures differ in 11—45 of the 124 positions (Table III). Most of these differences occur in regions of the molecule remote from the active centre. Giraffe ribonuclease which has only eleven differences compared with bovine ribonuclease is interesting, since two of them occur in positions next to the two histidine residues in the active centre of the enzyme [12].

The relatively small differences in activity of the five ribonucleases are not surprising, since by comparing the proton magnetic resonance titration curves of the two active centre histidine residues of rat and bovine ribonucleases [21], it could already be concluded that there are more constraints on the structure of the active site in the enzyme-substrate complex than on that of the substrate-free enzyme.

In our study, we confirmed the preference of bovine ribonuclease for cytidine derivatives over uridine derivatives. The ribonucleases from the lesser rorqual and the horse have the same preference, whereas no distinct preference for one of the two substrates is shown by the ribonucleases from giraffe and rat. The kinetics of rat ribonuclease were already studied previously [8]. In this study rat ribonuclease was found to be twice as active on 2',3'-cyclic uridine monophosphate as bovine ribonuclease at pH 7.5. In contrast to these results we found rat ribonuclease to be only slightly more active on 2',3'-cyclic uridine monophosphate than bovine ribonuclease. A possible explanation for this discrepancy is the difference in ionic strength of the reaction mixture and the pH.

On both substrates studied, horse ribonuclease was the most active of the five ribonucleases. Like bovine ribonuclease, the enzyme from horse showed a preference for 2',3'-cyclic cytidine monophosphate, this in contrast to the results obtained by Irie [9], who observed the opposite preference.

Irie [9] also studied the kinetics of the ribonuclease from the common rorqual (*Balaenoptera physalus*). The amino acid sequence of this enzyme has not yet been completely determined [22], but is very similar to that of the lesser rorqual [15]. Therefore, we have not discriminated between both whale species in Tables I and II. As was found by Irie [9] for the common rorqual ribonuclease, we also found that the activity of the lesser rorqual ribonuclease on both substrates differed not significantly from that of bovine ribonuclease, with a preference for 2',3'-cyclic cytidine monophosphate over 2',3'-cyclic uridine monophosphate. This in contrast to the activity on RNA which is about 4—8 times less for the ribonucleases from the common rorqual [9] and lesser rorqual (Emmens, M., unpublished results), compared to that of bovine ribonuclease. Another interesting feature of lesser rorqual ribonuclease is its high activity on double-stranded RNA (Libonati, M., personal communication).

The values for k_{+2}/K_m show that the specific activity of giraffe ribonuclease on 2',3'-cyclic cytidine monophosphate is lower than the specific activity of bovine ribonuclease. The specific activity of giraffe ribonuclease on 2',3'-cyclic uridine monophosphate is higher than that of bovine ribonuclease. The enhanced specific activity of giraffe ribonuclease on 2',3'-cyclic uridine monophosphate in comparison with bovine ribonuclease was predicted by Merrifield and Hodges

[23] on the basis of their recombination experiments of bovine ribonuclease shortened at the C-terminal end with synthetic C-terminal peptides. In this way these authors could exchange phenylalanine at position 120 in the sequence of bovine ribonuclease for tyrosine, the amino acid residue found at this position in giraffe ribonuclease. This partially synthetic enzyme had a larger specific activity on 2',3'-cyclic uridine monophosphate than bovine ribonuclease. Their prediction was verified with a sample of giraffe ribonuclease [10].

The explanation for this enhanced activity may be that a tyrosine residue at position 120 forms an additional hydrogen bond with the hydroxyl group of serine 123 [24]. This serine hydroxyl group serves as the acceptor in a hydrogen bond formed upon binding of 2',3'-cyclic cytidine monophosphate and as the donor in a hydrogen bond formed upon the binding of 2',3'-cyclic uridine monophosphate. By rotation around the C-O bond, the hydroxyl group may function either as an acceptor or as a donor without serious disruption of the protein conformation.

The possible correlation between the orientation of the hydroxyl group of the serine residue at position 123 and the relative preference for uridine nucleotides shown by giraffe ribonuclease is also indicated by other experiments of Hodges and Merrifield [25]. It was found that substitution of serine at position 123 by alanine had no effect on the activity on 2',3'-cyclic cytidine monophosphate, but it lowered the activity on 2',3'-cyclic uridine monophosphate by 75%.

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