The Nature of the Allosteric Interactions of Ribonuclease and its Ligands

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The allosteric model for ribonuclease activity proposed by Walker, Ralston & Darvey [(1975) *Biochem. J.* 147, 425-433; (1976) *Biochem. J.* 153, 329-337] involves the binding of a large number of molecules of substrate or substrate analogue to a series of allosteric sites on the enzyme. In the present paper, the nature of these allosteric interactions is investigated. The effects of ionic strength, pH, carbamoylation of lysine to homocitrulline and of deamidation of glutamine and asparagine on plots of velocity versus substrate concentration are examined and evidence is presented that the allosteric transition involves an electrostatic interaction between the negatively charged substrate molecules and the cationic groups on the enzyme.

A model for RNAase activity has been presented (Walker *et al.*, 1975) in which the enzyme exists in two catalytically active forms, E and F. The conformation transition, $E \rightleftharpoons F$, is considered to be induced by the binding of a large number of molecules of substrate (or its analogues) to a set of allosteric sites on the enzyme (Walker *et al.*, 1976). The present paper investigates the nature of these allosteric sites.

That anions, phosphates in particular, are able to stabilize RNAase against denaturation, or to promote renaturation, has been frequently demonstrated (Ginsberg & Carroll, 1965; Sherwood & Potts, 1965; Barnard, 1964). Nelson *et al.* (1962), in discussing the different effects of the two substrates, RNA and cyclic nucleotides, on the dissociation constant of the RNAase-2'-CMP complex, conclude that the difference probably reflects some supplementary affinity of RNAase for RNA by non-catalytic cationic binding sites. They continue, 'Regardless of the nature of the cationic groups involved, it seems reasonable to postulate that the native configuration of RNAase is less easily disrupted by denaturing treatment because the polypeptide chain is additionally secured in place by the electrostatic affinity of the polyvalent anions to two or more cationic groups which lie on either side of a critical seam in the molecule which makes up part of the active site'.

RNAase contains four arginine and ten lysine residues, all in positions well exposed to aqueous solvents (Richards & Wyckoff, 1971). Wagner & Arfmann (1974) have demonstrated that polylysine and polyarginine are able to bind large numbers of

nucleotide molecules. Considering, therefore, the possibility that the cationic groups of lysine and arginine may constitute the allosteric sites postulated by Walker *et al.* (1975, 1976), we have examined the effects of a decrease in the electrostatic interaction between substrate and enzyme on plots of velocity versus substrate concentration. This decrease has been brought about by increasing the ionic strength and by decreasing the net positive charge on the enzyme. The positive charge on the enzyme has been decreased by titration with NaOH, by carbamoylation of the positively charged lysine residues to give uncharged homocitrulline, or by deamidation of glutamine or asparagine. In all four cases, a decrease in the electrostatic interaction resulted in a decreased affinity of the allosteric sites for the substrate and a decreased number of substrate molecules involved in the E-to-F transition.

Experimental

Materials

The sodium salt of 2':3'-cyclic CMP was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine pancreatic RNAase was extracted from pancreases obtained from Homebush State Abattoirs and purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and ion-exchange chromatography as described in the following paper (Walker *et al.*, 1978). All other chemicals used were of analytical-reagent grade.

Methods

Enzyme assay. Activity was measured at pH 7.13 and 10.3, unless otherwise specified, by using the pH-stat assay described by Walker *et al.* (1975). For

Abbreviation used: RNAase, ribonuclease.

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the pH and ionic-strength studies, the required conditions were obtained by the addition of NaOH and NaCl respectively.

Carbamoylation of lysine. RNAase (0.03 mM) was incubated with potassium cyanate (1 M; pH 8.2) at 50°C for 2, 7 and 20 min. The reaction was stopped by the removal of potassium cyanate on Sephadex G-25 columns (2 cm × 20 cm), eluted with 0.04 M NaCl.

Deamidation of glutamine and asparagine. Deamidation of RNAase was achieved by incubating the enzyme (9.3 μM) in HCl (2.0 M) for 10 min at 40, 80 and 100°C. The average number of residues deamidated per enzyme molecule was determined from the ammonia released, measured by the method of Kaplan (1965).

Results

Effect of ionic strength on plots of v versus $[S]$

Plots of v versus $[S]$ measured at pH 7.13 and total I 0.3, 1.0 and 2.0 are shown in Fig. 1. As the ionic strength of the system increases, $[S_m]$, the substrate concentration at the midpoint of the transition (Walker *et al.*, 1976) increases. The slope of the transition decreases with increasing ionic strength, until at I 2.0 the plot of v versus $[S]$ approaches a hyperbola.

Effect of pH on plots of v versus $[S]$

Plots of v versus $[S]$ measured at pH 7.13, pH 8.5, pH 9.2, pH 10.0 and pH 10.6 are shown in Fig. 2. As

Fig. 1. Effect of ionic strength on plots of v versus $[S]$ Enzyme assay was as described in the text. Reaction mixture contained 2':3'-cyclic CMP (17–70 mM), RNAase (0.1 μM) and NaCl to final ionic strength of 0.3 mol/l (■), 1.0 mol/l (●) or 2.0 mol/l (▲).

Fig. 2. Effect of pH on plots of v versus $[S]$ Enzyme assay was as described in the text. Reaction mixture contained 2':3'-cyclic CMP (23–60 mM), NaCl to a final ionic strength of 0.3 mol/l and RNAase at pH 7.13 (0.16 μM; ○), pH 8.5 (1.6 μM; ■), pH 9.2 (4 μM; □), pH 10.0 (16 μM; ▲) or pH 10.6 (32 μM; △).

Table 1. Effect of charge modification of RNase by titration and deamidation on [S_m], the substrate concentration at the midpoint of the transition

pH	Titration			Temperature of deamidation (°C)	Deamidation		
	Average charge modification* (relative to pH7.13)	[S _m] (mM)	Δ[S _m] relative to unmodified (mM)		Average charge modification†	[S _m] (mM)	Δ[S _m] relative to unmodified (mM)
7.13	0	33	0	—	0	35	0
				40	0.66	35	0
				80	1.0	40	5
				100	2.0	45	10
8.5	1.8	31	—				
9.2	2.9	47	14				
10.0	5.2	54	21				
10.6	9.0	55	22				

* Calculated from the titration curve (Tanford & Havenstein, 1956).
† Calculated from the ammonia released.

Fig. 3. Effect of lysine carbamoylation on plots of *v* versus [S]

Enzyme assay was as described in the text. Reaction mixture contained 2':3'-cyclic CMP (17–74mM), NaCl to a final ionic strength of 0.3 mol/l and RNAse: Δ, unmodified (0.44 μM); ■, modified for 2 min (0.77 μM); ●, modified for 7 min (1.54 μM); ▲, modified for 20 min (3.08 μM).

Fig. 4. Effect of deamidation on plots of *v* versus [S] Enzyme assay was as described in the text. Reaction mixture contained 2':3'-cyclic CMP (20–55mM), NaCl to a final ionic strength of 0.3 mol/l and RNAse: □, unmodified (0.09 μM); ○, modified at 40°C (0.23 μM); ■, modified at 80°C (0.23 μM); ●, modified at 100°C (0.23 μM).

the pH of the assay system is increased, [S_m] remains constant below pH8.5 and above pH 10, but increases between these values, reflecting the titration curve for lysine side chains (see Table 1). The slope of the

transition region decreases with increasing pH until the transition becomes a plateau at pH10.6. Data were insufficient for reliable estimates of Δ*v*_s, the number of ligand molecules involved in the E-to-F transition (Walker *et al.* 1976), but Δ*v*_s appeared to decrease from pH7.13 to pH10.0.

Effect of lysine carbamoylation on plots of v versus $[S]$

Plots of v versus $[S]$ for unmodified enzyme and enzyme carbamoylated for 2, 7 and 20 min are shown in Fig. 3. All assays were carried out at pH 7.13 and $I\ 0.3$. $[S_m]$ increased with increasing time of modification, which corresponds to decreasing positive charge on the enzyme. The slope of the transition again decreased until, at 20 min modification, the plot of v versus $[S]$ approached a hyperbola.

Effect of deamidation on plots of v versus $[S]$

Plots of v versus $[S]$ for unmodified enzyme and enzyme deamidated in HCl (2.0M) for 10 min at 40, 80 and 100°C are shown in Fig. 4. The number of mol of ammonia released per mol of enzyme as determined by the method of Kaplan (1965) is given as the degree of deamidation shown in Table 1. Again $[S_m]$ increases with decreasing net positive charge on the enzyme, and the slope of the transition decreases.

Discussion

All the studies presented here indicate that, with decreasing net positive charge on RNAase, the transition that occurs in plots of v versus $[S]$ moves to higher substrate concentrations. This corresponds, we postulate, to a decreased affinity of the less positively charged enzyme for the negatively charged nucleotide. At the same time the slope of the transition region decreases. This corresponds, we suggest, to a decrease in the number of negatively charged ligands that have to bind to the enzyme before the E-to-F transition is induced.

The ionic-strength studies indicate that a general decrease in electrostatic interactions decreases the affinity of enzyme for substrate. The pH studies, in which the substrate concentration at the midpoint of the transition reflects the titration curve for lysine, implicates the function of the lysine residues in the allosteric binding.

Lysine-carbamoylation studies, pH studies and deamidation studies all show that, as the net positive charge of RNAase is decreased, the affinity of enzyme for substrate is decreased. Examination of Table 1 shows that a direct comparison of $[S_m]$ as a function of degree of charge modification for the different modification studies cannot be made. This is presumably because, as has been shown in the following

paper (Walker *et al.*, 1978), the degree of spontaneous deamidation increases rapidly in a few days and this itself changes the value of $[S_m]$ before further modification of the enzyme is carried out. Comparison of titration and deamidation studies (see Table 1), where the degree of charge modification could be calculated or measured, shows an approximately linear increase of $[S_m]$ with decreasing charge up to a charge loss of about 4. With further charge modification, diminished changes in $[S_m]$ are seen.

Examination of curves from all four studies show that, for a given substrate concentration, the lower the electrostatic interaction, the further the equilibrium between the E and F conformations is shifted towards conformation E. In each study, when the electrostatic interaction has been sufficiently decreased, the E→F transition fails to occur in the substrate-concentration range examined.

Previous studies (Walker *et al.*, 1975, 1976) have demonstrated that conditions that favour the binding of ligands to the allosteric sites (e.g. increased concentrations of the substrate or its analogues) favour a shift in the equilibrium towards form F of the enzyme. The evidence presented here suggests that factors that hinder binding at the allosteric sites appear to favour form E.

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