Further Evidence for an Allosteric Model for Ribonuclease

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

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Evidence is presented from three experimental systems to support the allosteric model of Walker *et al.* (1975) (*Biochem. J.* 147, 425–433) which explains the substrate-concentration-dependent transition observed in the RNAase (ribonuclease)-catalysed hydrolysis of 2':3'-cyclic CMP (cytidine 2':3'-cyclic monophosphate). 1. Kinetic studies of the initial rate of hydrolysis of 2':3'-cyclic CMP show that the midpoint of the transition shifts to lower concentrations of 2':3'-cyclic CMP in the presence of the substrate analogues 3'-CMP, 5'-CMP, 3'-AMP, 3'-UMP and P_i; 2'-CMP and 2'-UMP do not cause such a shift. 2. Trypsin-digestion studies show that a conformational change in RNAase to a form less susceptible to tryptic inactivation is induced in the presence of the substrate analogues 3'-CMP, 5'-CMP, 3'-AMP and 3'-UMP. 2'-CMP, 2'-AMP and 2'-UMP do not induce this conformational change. 3. Equilibrium-dialysis experiments demonstrate the multiple binding of molecules of 3'-CMP, 3'-AMP and 5'-AMP to a molecule of RNAase. 2'-CMP binds in the ratio 1:1 over the analogue concentration range studied.

Measurements of the initial rate (v) of the RNAase*-catalysed hydrolysis of 2':3'-cyclic CMP show a substrate-concentration-dependent transition during which v decreases abruptly over a narrow range of substrate concentration ($[S]$) (Walker *et al.*, 1975). To explain this transition, Walker *et al.* (1975) proposed a model for RNAase in which the enzyme exists in two catalytically active forms E and F (see Scheme 1). These two forms of enzyme have different affinities for substrate at the catalytic site. They also bind substrate molecules with different affinities at sites other than the catalytic site; these sites are called allosteric sites and the conformational change $E \rightleftharpoons F$ is induced by the binding of a large number of substrate molecules to these allosteric sites.

In addition to the kinetic data, further evidence to support the model was presented from a study of the effect of the product of the reaction, 3'-CMP, on the digestion of RNAase by trypsin (Walker *et al.*, 1975). 3'-CMP protects RNAase against tryptic digestion and the initial rate of RNAase digestion decreases sigmoidally as the 3'-CMP concentration increases (Walker *et al.*, 1975). The shape of the curve of the initial rate of trypsin digestion versus $[3'\text{-CMP}]$ suggests that a large number of molecules of 3'-CMP are bound to RNAase inducing a conformational change in the enzyme.

One might expect that analogues of the substrate other than product (e.g. competitive inhibitors) would also bind to the allosteric sites of RNAase and induce the $E \rightleftharpoons F$ conformational change. In this paper we examine the effects of such substrate

analogues (A) on the kinetics of the RNAase-catalysed hydrolysis of 2':3'-cyclic CMP and on the digestion of the enzyme by trypsin. In addition, equilibrium dialysis is used to obtain direct information on the number of molecules of substrate analogues bound to RNAase.

Theory

The model of Walker *et al.* (1975) is extended to account for the presence of various substrate analogues (product P or competitive inhibitor I).

Scheme 1. A mechanism for the action of RNAase on cyclic CMP in the presence of an inhibitor

E and F denote two different conformations of the enzyme with various numbers of molecules of S, P and I bound to the allosteric sites.

* RNAase, ribonuclease.

We have restricted our study to the $E \rightleftharpoons F$ transition and do not consider here the second transition which occurs at values of $[S]$ greater than 80mm in the absence of substrate analogue (Walker *et al.*, 1975). In the presence of P and I the model may be represented by Scheme 1. The model assumes that all steps other than the conversions $ES \rightarrow EP$ and $FS \rightarrow FP$ are in rapid equilibrium.

The rate equation

The apparent equilibrium constants for the transitions $E \rightleftharpoons F$, $ES \rightleftharpoons FS$ and $EA \rightleftharpoons FA$ are defined by:

$$K_{F/E,app} = \frac{[F]}{[E]} \quad (1)$$

$$K_{FS/ES,app} = \frac{[FS]}{[ES]} \quad (2)$$

$$K_{FA/EA,app} = \frac{[FA]}{[EA]} \quad (3)$$

where A denotes either P or I and E and F denote $\sum_{i,j} S_i A_j E$ and $\sum_{i,j} S_i A_j F$ respectively, where $S_i A_j$ written to the left of an enzyme species denotes i molecules of S and j molecules of A binding to the allosteric sites ($i, j = 0, 1, 2, \dots$).

The Michaelis constants for S ($K_{S,E}$ and $K_{S,F}$) and the dissociation constants for A ($K_{A,E}$ and $K_{A,F}$) are defined by:

$$K_{S,E} = \frac{[E][S]}{[ES]} \quad (4)$$

$$K_{S,F} = \frac{[F][S]}{[FS]} \quad (5)$$

$$K_{A,E} = \frac{[E][A]}{[EA]} \quad (6)$$

$$K_{A,F} = \frac{[F][A]}{[FA]} \quad (7)$$

The initial velocity (v) of the reaction $S \rightarrow P$ is given by:

$$v = k_E[ES] + k_F[FS] \quad (8)$$

and eqns. (1)–(8) can be shown to yield:

$$v = \frac{(k_E + k_F K_{FS/ES,app})[S][E_t]}{K'_{S,E} + [S] + (K'_{S,F} + [S])K_{FS/ES,app}} \quad (9)$$

where

$$K'_{S,E} = K_{S,E} \left(1 + \frac{[A]}{K_{A,E}} \right) \quad (10)$$

$$K'_{S,F} = K_{S,F} \left(1 + \frac{[A]}{K_{A,F}} \right) \quad (11)$$

and $[E_t]$ is the total concentration of all species of enzyme.

Determination of the apparent equilibrium constant

From eqn. (9):

$$K_{FS/ES,app} = \frac{(v_1 - v)(K'_{S,E} + [S])}{(v - v_2)(K'_{S,F} + [S])} \quad (12)$$

where:

$$v_1 = \frac{k_E[S][E_t]}{K'_{S,E} + [S]} \quad (13)$$

$$v_2 = \frac{k_F[S][E_t]}{K'_{S,F} + [S]} \quad (14)$$

v_1 is the value of v over the $[S]$ range below the $E \rightleftharpoons F$ transition, and v_2 is the value of v obtained for $[S]$ values above the transition. Values of $K_{FS/ES,app}$ may be obtained by using the procedure described by Walker *et al.* (1975).

Model for the binding of ligand at the allosteric sites

So far, we have not incorporated into our model the binding of S or A to the allosteric sites. From the theory of linked functions (Wyman, 1964) it follows that, irrespective of the model chosen to describe the binding of S or A to the allosteric sites:

$$\left(\frac{\partial \ln(K_{FS/ES,app})}{\partial \ln[S]} \right)_{[A]} = \Delta \bar{\nu}_S \quad (15)$$

$$\left(\frac{\partial \ln(K_{FS/ES,app})}{\partial \ln[A]} \right)_{[S]} = \Delta \bar{\nu}_A \quad (16)$$

where $\Delta \bar{\nu}_S$ and $\Delta \bar{\nu}_A$ denote the average increase in the number of molecules of S and A respectively bound to the allosteric sites when an enzyme molecule in conformation E is converted into form F. Thus for a fixed value of $[A]$, $\Delta \bar{\nu}_S$ can be estimated, and for a fixed value of $[S]$, $\Delta \bar{\nu}_A$ can be estimated from plots of $\ln(K_{FS/ES,app})$ against $\ln[S]$ and $\ln[A]$ respectively in the transition region. In the latter case several sets of data for v versus $[S]$, each obtained at a different value of $[A]$, are required.

To avoid complex algebraic expressions and to keep the number of parameters to a minimum, we consider that no molecules of S or A are bound to the allosteric sites of the enzyme in the E conformation. We further assume that, in the F conformation, there are n equivalent and independent allosteric sites and that S and A compete for these sites. If K_S and K_A denote the association constants for the binding of S and A to an allosteric site when the enzyme exists in the F conformation, then $\Delta \bar{\nu}_S$ and $\Delta \bar{\nu}_A$ are given by (Edsall & Wyman, 1958):

$$\Delta \bar{\nu}_S = \frac{nK_S[S]}{1 + K_S[S] + K_A[A]} \quad (17)$$

$$\Delta \bar{\nu}_A = \frac{nK_A[A]}{1 + K_S[S] + K_A[A]} \quad (18)$$

For this particular model of binding of S and A at the allosteric sites, $K_{FS/ES,app.}$ becomes:

$$K_{FS/ES,app.} = K_{FS/ES,int.}(1 + K_S[S] + K_A[A])^n \quad (19)$$

where $K_{FS/ES,int.}$ is the intrinsic constant for the $FS \rightleftharpoons ES$ equilibrium when no molecules of S or A are bound to the allosteric sites of either conformation of the enzyme.

It should be noted that in our previous paper (Walker *et al.*, 1975), when we considered a system in which $[A] = 0$, eqn. (19) was written as:

$$K_{FS/ES,app.} = K_{FS/ES,int.}[S]^n \quad (20)$$

i.e. the $K_{FS/ES,int.}$ used previously is equivalent to the present $K_{FS/ES,int.}$ multiplied by K_S^n , since we assumed previously that $K_S[S] \gg 1$ for values of $[S]$ in the transition region.

Analysis of the model for binding of ligands at the allosteric sites

The values of $\Delta\bar{v}_S$ and $\Delta\bar{v}_A$ are determined at the midpoint of the transition because this is an easily characterized point and also because errors in the determination of the ratio $(v_1 - v)/(v - v_2)$ are least when the ratio is approx. 1.

At the midpoint of the transition, when $K_{FS/ES,app.} = 1$, from eqn. (19) the value of $[S]$ ($[S_m]$) is given by:

$$[S_m] = -\frac{K_A}{K_S}[A] + \frac{(K_{FS/ES,int.})^{-1/n} - 1}{K_S} \quad (21)$$

At $[S] = [S_m]$, let $\Delta\bar{v}_S = \Delta\bar{v}_{S,m}$ and $\Delta\bar{v}_A = \Delta\bar{v}_{A,m}$; substitution of eqn. (21) into eqns. (17) and (18) gives:

$$\Delta\bar{v}_{S,m} = -nK_A(K_{FS/ES,int.})^{1/n}[A] + n[1 - (K_{FS/ES,int.})^{1/n}] \quad (22)$$

$$\Delta\bar{v}_{A,m} = nK_A(K_{FS/ES,int.})^{1/n}[A] \quad (23)$$

That is, $[S_m]$ and $\Delta\bar{v}_{S,m}$ decrease linearly with $[A]$, $\Delta\bar{v}_{A,m}$ increases; with $[A]$ and $(\Delta\bar{v}_{S,m} + \Delta\bar{v}_{A,m})$ is independent of $[S]$, $[A]$, K_S and K_A .

Experimental

Materials

Bovine pancreatic RNAase (EC 3.1.4.22; type XA, lot nos. 52C 8018 and 94C 8050), pancreatic trypsin type 1 (EC 3.4.21.4), cytidine 2':3'-cyclic monophosphate (sodium salt), 3'-CMP (free acid), 5'-CMP (free acid), 2'-CMP (lithium salt), 3'-AMP, 5'-AMP and 2'-AMP (free acids), and 3'-UMP, 5'-UMP and 2'-UMP (sodium, disodium and lithium salts respectively) were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All chemicals used were of analytical grade.

Methods

Spectrophotometric assay. The spectrophotometric enzyme assay, used in equilibrium-dialysis and trypsin-inactivation experiments, was carried out as described by Walker *et al.* (1975). Substrate, enzyme and substrate-analogue concentrations were determined spectrophotometrically by using a Cary 14 or a Unicam SP.500 spectrophotometer and extinction coefficients given by Dunn & Hall (1968) for nucleotides and by Tanford *et al.* (1955) for RNAase. Protein concentration was determined by the Folin-Ciocalteu reaction (Layne, 1957).

Analogue-inhibition studies. The effects of the 2'-monophosphates of cytidine and uridine, the 3'-monophosphates of cytidine, uridine and adenosine, the 5'-monophosphate of cytidine, and P_i , on the velocity of the hydrolysis of 2':3'-cyclic CMP by RNAase were determined by the polarimetric assay of Walker *et al.* (1975), with the following modifications. A Perkin-Elmer 141 polarimeter was used to measure rotation at 313 nm in a water-jacketed 10 cm micro-cell at 24°C. The reaction mixture contained RNAase (1.9, 4.0 or 5.4 μ M), 2':3'-cyclic CMP (0–65 mM) and different substrate analogues. The change in rotation as 1 mmol of substrate/l is converted into product was measured as 0.05 degree/10 cm cell.

Trypsin inactivation. The ability of 2'- and 3'-monophosphates of cytidine, uridine and adenosine and the 5'-monophosphates of cytidine and adenosine to protect RNAase against digestion by trypsin was measured as previously described (Walker *et al.*, 1975).

Equilibrium dialysis. Equilibrium dialysis was carried out in the apparatus constructed according to the design of Furlong *et al.* (1972). The wells held approx. 0.1 ml on each side of the membrane. The membrane was prepared by heating Visking dialysis tubing to 80°C in 3% (v/v) acetic acid and then washing with copious quantities of water. Dialysis was carried out for 40 h at 23°C with stirring accomplished at a rotation speed of approx. 50 rev./min. Enzyme and ligand were dissolved in Tris/NaCl buffer (0.1 M-Tris, pH 7.13, 1.02 mol/l) containing NaN_3 (0.02%) to inhibit bacterial growth. Initial checks were made to demonstrate that no enzyme leaked through the membrane. The protein concentration decreased by approx. 10% during dialysis, presumably owing both to diffusion of water into the more concentrated solution and to some adsorption of the protein on the membrane and chamber. The enzyme activity, as determined spectrophotometrically, had decreased by approx. 20% at the end of the dialysis. The loss of activity was dependent on the size of the air bubble in the well and the speed and duration of rotation. It was assumed that denatured enzyme did not bind ligand and so percentage activity

was used as a measure of protein concentration. The ligand concentration was determined from the E_{260} , taking into account the contribution of total protein to the absorbancy.

Analysis of data

Kinetic data. Plots of v versus $[S]$ before the transition region were fitted to an unweighted least-squares regression of:

$$\frac{1}{v_1} = \frac{1}{k_E[E_t]} + \frac{K_{S,E}}{k_E[E_t]} \cdot \frac{1}{[S]} + \frac{K_{S,E}}{k_E[E_t]K_{A,E}} \cdot \frac{[A]}{[S]} \quad (24)$$

Data for which $[S] < 10 \text{ mM}$ were excluded, since a slight sigmoidicity occurs in the plots of v versus $[S]$ when $[S]$ is approx. 6 mM (Rübsamen *et al.*, 1974).

Data for 3'-AMP and P_i beyond the transition were similarly fitted to:

$$\frac{1}{v_2} = \frac{1}{k_F[E_t]} + \frac{K_{S,F}}{k_F[E_t]} \cdot \frac{1}{[S]} + \frac{K_{S,F}}{k_F[E_t]K_{A,F}} \cdot \frac{[A]}{[S]} \quad (25)$$

For the other analogues, where data in this region were insufficient to fit to an unweighted regression, the data were fitted to hyperbolae by eye. It was shown theoretically, and experimentally justified, that the choice of hyperbola had little effect on the slopes of plots of $\log K_{FS/ES,app.}$ versus $\log[S]$ (at constant $[A]$) and versus $\log[A]$ (at constant $[S]$) since, within the range of values of $[S]$ and $[A]$ used in the log-log plots,

$$\log \frac{[S] + K_{S,E}(1 + [A]/K_{A,E})}{[S] + K_{S,F}(1 + [A]/K_{A,F})}$$

remains essentially constant. $[S_m]$ was estimated directly from each v versus $[S]$ plot as $[S]$ when v was the average of the values of v at the extremes of the transition.

Trypsin-protection studies. For curves showing a sigmoidal dependence of the initial rate of trypsin digestion (u) on $[A]$, one can determine the degree of co-operativity by plotting $\log[(u_1 - u)/(u - u_2)]$ against $\log[A]$, where u_1 is the value that u approaches as $[A] \rightarrow 0$ and u_2 is the value that u approaches as $[A] \rightarrow \infty$. However, we stress that this 'degree of co-operativity' cannot be given a simple well-defined theoretical significance.

Results

Kinetic assays

Sets of plots of v versus $[S]$ for reaction mixtures containing various concentrations of 3'-CMP, 3'-AMP and P_i are shown in Figs. 1(a), 1(b) and 1(c) respectively. In each case, the value of $[S]$ at the midpoint of the transition ($[S_m]$) decreased and the velocity at each $[S]$ decreased as $[A]$ increased. There is also some evidence of the sigmoidicity at low $[S]$

which Rübsamen *et al.* (1974) propose as indicating that the enzyme is undergoing a conformational change. Similar plots were obtained for 3'-UMP and 5'-CMP.

The effects of 2'-CMP on plots of v against $[S]$ are shown in Fig. 1(d). In this case, although v decreased at all values of $[S]$ as the 2'-CMP concentration was increased, there was no change in the value of $[S_m]$. A similar set of plots was obtained for 2'-UMP.

Figs. 2(a)–2(e) show the effect of $[A]$ on the value of $[S_m]$ determined as described in the Experimental section. The substrate analogues shown are 3'-CMP, 3'-AMP, P_i , 5'-CMP and 3'-UMP respectively. The value of $[S_m]$ decreased approximately linearly with increasing $[A]$, except for 3'-AMP, where some deviation from linearity was observed. Values of K_A/K_S were obtained from the slopes of the plots in Fig. 2 (see eqn. 21) and are listed in Table 1.

For each set of v versus $[S]$ data at fixed $[A]$, plots of $\log K_{FS/ES,app.}$ versus $\log[S]$ were linear over the transition region. The slopes of these log-log plots, i.e. $\Delta \bar{v}_{s,m}$, are plotted as a function of $[A]$ in Fig. 3. Figs. 3(a)–3(d) show plots of $\Delta \bar{v}_{s,m}$ versus $[A]$ for the analogues 3'-CMP, 3'-AMP, P_i and 5'-CMP respectively. The plots all show a linear decrease in $\Delta \bar{v}_{s,m}$ with increasing $[A]$ up to $[A] \approx 4 \text{ mM}$.

Plots of $\log K_{FS/ES,app.}$ versus $\log[A]$ obtained from analogue-inhibition studies with 3'-AMP are shown in Fig. 4. For each value of $[S]$, the slope of the plots ($\Delta \bar{v}_A$) increases with increasing $[A]$ and $\Delta \bar{v}_A \rightarrow 0$ as $[A] \rightarrow 0$. At fixed $[A]$, the slopes of the plots decrease with increasing $[S]$. Similar plots were obtained for 3'-CMP and P_i . For each value of $[A]$ used in the analogue-inhibition studies, the value of $\Delta \bar{v}_A$ obtained from the curve where $[S] = [S_m]$ is defined as $\Delta \bar{v}_{A,m}$.

Plots of $\Delta \bar{v}_{A,m}$ versus $[A]$ for the analogues 3'-CMP, 3'-AMP and P_i are shown in Figs. 5(a)–5(c). The plots show that there are multiple binding sites for the analogues on the enzyme, the binding affinity for P_i being the strongest. The plots also show a difference in the dependence of $\Delta \bar{v}_{A,m}$ on $[A]$ for 3'-CMP and the other two analogues, reflected in the different shaped curves.

Equilibrium dialysis

Plots of the total number of molecules of analogue bound per enzyme molecule as determined from equilibrium-dialysis studies with 3'-CMP, 3'-AMP, 5'-AMP and 2'-CMP are shown in Figs. 6(a)–6(d) respectively. The plots show multiple binding sites, except for 2'-CMP, where the binding is probably only at the active site. The total number of analogue molecules bound per enzyme molecule shows a similar dependence on $[A]$ to that shown by $\Delta \bar{v}_{A,m}$ estimated from the analogue-inhibition studies

Fig. 1. Effect of four substrate analogues on the initial rate of hydrolysis of 2':3'-cyclic CMP by RNAase

Data were obtained at 24°C by using the polarimeter assay described in the text. The reaction mixture contained Tris/NaCl buffer (pH 7.13, 0.1M; I 0.2mol/l), 2':3'-cyclic CMP (0–65mM) and: (a) RNAase (3.9μM), 3'-CMP (▼, 0mM; ▽, 0.5mM; ■, 1.0mM; □, 1.5mM; ▲, 2.0mM; △, 2.5mM; ●, 3.1mM; ○, 4.95mM); (b) RNAase (5.4μM), 3'-AMP (▼, 0mM; ○, 0.9mM; ●, 1.8mM; △, 2.7mM; ▲, 4.1mM; □, 5.0mM; ■, 6.1mM; ▽, 8.0mM); (c) RNAase (1.9μM), P_i (▼, 0.5mM; ○, 1.0mM; ●, 1.6mM; △, 2.1mM; ▲, 3.1mM; □, 6.0mM; ■, 8.0mM); (d) RNAase (4.2μM), 2'-CMP (□, 0mM; ●, 0.8mM; ■, 1.6mM; ▲, 2.3mM).

(compare Figs. 5 and 6); again 3'-CMP shows a different dependence from that shown by the other analogues.

Trypsin inactivation

Figs. 7(a)–7(f) show the effect of [A] on the inactivation of RNAase by trypsin for 3'-CMP,

3'-AMP, 5'-CMP, 3'-UMP, 2'-CMP and 2'-AMP respectively. For those curves showing a sigmoidal dependence of u on [A], plots of $\log[(u_1 - u)/(u - u_2)]$ against $\log[A]$ (see the Experimental section) were linear over the transition region with gradients ranging from 5 to 7. No sigmoidal dependence on [A] was found for the 2'-nucleotides.

Fig. 2. Effect of substrate-analogue concentrations [A] on the value of [S_m] for various substrate analogues

The substrate analogues are (a) 3'-CMP, (b) 3'-AMP, (c) P_i, (d) 5'-CMP, (e) 3'-UMP. The reaction mixture contained Tris/NaCl buffer (pH 7.13, 0.1M, 1.02 mol/l), 2':3'-cyclic CMP (0–65 mM), RNAase (1.9–5.4 μM) and various substrate analogues as shown. [S_m] was estimated as the value of [S] when *v* was the average of the values of *v* at the extremes of the transition.

Table 1. Estimates of the value of K_A/K_S from the slopes of Fig. 2

Substrate analogue	K_A/K_S
3'-CMP	3
3'-AMP	3*
P _i	5
5'-CMP	3
3'-UMP	6

* Determined as the gradient of the curve between [3'-AMP] = 2 mM and [3'-AMP] = 6 mM.

Discussion

The Model

In our previous paper (Walker *et al.*, 1975) we proposed an allosteric model for RNAase activity in which the enzyme existed in three conformations

E, F and G. Of these E and F are catalytically active and G is an inactive form induced at very high [S]. In the present paper we have limited our study to the range of [S] over which the E ⇌ F transition occurs.

In the Theory section we extended this model to allow for the presence of various substrate analogues and proposed that there are, in the F conformation, *n* equivalent and independent allosteric sites for which S and A compete. This model predicts that [S_m] decreases linearly with [A] (see eqn. 21). As shown in Fig. 2, the results at low [A] are consistent with such a model. The magnitude of K_A/K_S (Table 1) shows that all five analogues bind more tightly than does the substrate. At [A] ≥ 4 mM deviations from linearity are observed, probably owing to the oversimplifications we have made in our model. For 2'-CMP and 2'-UMP, [S_m] does not

Fig. 3. Effect of substrate-analogue concentrations [A] on $\Delta \bar{v}_{s,m}$ as determined from kinetic studies

Substrate analogues are: (a) 3'-CMP; (b) 3'-AMP; (c) P₁; (d) 5'-CMP. Methods of assay and reaction mixtures are as given in Fig. 2.

Fig. 4. Determination of $\Delta \bar{v}_A$ for 3'-AMP

$\ln [K_{FS/ES, app.}]$ is plotted against $\ln [A]$ for the data in the transition region of Fig. 1(b), where [S] is: ▼, 20 mM; ▽, 26 mM; ■, 28 mM; □, 30 mM; ▲, 32 mM; △, 34 mM; ○, 36 mM; ●, 38 mM.

decrease with increasing [A], indicating that $K_A = 0$ for the 2'-nucleotides.

The model also predicts that $\Delta \bar{v}_{s,m}$ decreases linearly with increasing [A] (see eqn. 22). Examination of Fig. 3 shows that the data are consistent with the model at low [A]. Deviations again appear at $[A] \geq 4$ mM. Further, the model predicts that $\Delta \bar{v}_{A,m}$ increases linearly with [A] (see eqn. 23). Examination of Fig. 5 shows a non-linear increase in $\Delta \bar{v}_{A,m}$ with [A], perhaps arising from the existence of classes of allosteric binding sites with different affinities, again representing an oversimplification in the original assumptions used to derive eqn. (23).

It should be noted, however, that the total number of analogue molecules binding per enzyme molecule, determined by equilibrium dialysis and shown in Fig. 6, shows the same type of concentration dependence as that shown in Fig. 5 for each of the analogues 3'-CMP and 3'-AMP. The fact that at a given [A] the total number binding determined from equilibrium dialysis is less than $\Delta \bar{v}_{A,m}$ derived from kinetics is probably due to the different experimental conditions in the kinetic and dialysis experiments, particularly the higher protein concentration in the dialysis experiments.

Fig. 5. Effect of substrate-analogue concentration $[A]$ on $\Delta\bar{v}_{A,m}$ as determined from kinetic studies

Substrate analogues are: (a) 3'-CMP; (b) 3'-AMP; (c) P_i . Methods of assay and reaction mixture are as given in Fig. 1. For a given $[A]$, $\Delta\bar{v}_{A,m}$ is determined from the gradient at $[A]$ of that curve in Fig. 4 where $[S] = [S_m]$.

Trypsin-digestion studies present further evidence that the binding of 3'- and 5'-analogues to the enzyme induces a co-operative conformational change, since the plots of $\log[(u_1 - u)/(u - u_2)]$ against $\log[A]$ demonstrate a high degree of co-operativity.

That nucleotides bind to amino acids has been clearly shown. For example, Wagner & Arfmann (1974) have demonstrated that large numbers of nucleotide molecules bind to poly(amino acids) such as polylysine and polyarginine. Since RNAase contains four arginine and ten lysine residues, all in positions well exposed to aqueous solvents (Richards & Wyckoff, 1971), weak binding sites may exist on RNAase for a large number of nucleotides, particularly since models based on the X-ray-diffraction data of Wyckoff *et al.* (1967) for RNAase S show that 11 of these residues occur in a cluster centred around lysine-31.

Our results, particularly those with P_i , suggest that the phosphate group on the nucleotide is involved in the binding and that its affinity for RNAase depends both on the nucleotide to which it is attached and on the position of its attachment. The inability of the 2'-nucleotides to induce the conformational change in the enzyme caused by 3'- and 5'-nucleotides may reflect the fact that the terminal phosphate of an RNA segment produced by RNAase activity always occurs in the 3' or 5' position rather than the 2' position.

Ferguson *et al.* (1974) have reported evidence for the self-aggregation of nucleotides. Therefore as an alternative to the strongly co-operative binding of substrate to enzyme it might be suggested that, at high substrate concentrations, molecules of sub-

Fig. 6. Effect of substrate-analogue concentration $[A]$ on the total number of ligand molecules bound per enzyme molecule as determined from equilibrium dialysis

Dialysis was carried out for 40h at 23°C with stirring accomplished by rotation at approx. 50rev./min in an apparatus built according to the design of Furlong *et al.* (1972). Reaction mixtures contained, on one side of the membrane, RNAase (0.25mm) and substrate analogue (0–22mm) dissolved in Tris/NaCl buffer (0.1M-Tris; pH7.13; I 0.2mol/l) containing NaN_3 (0.02%). On the other side of the membrane, the reaction mixture contained all components except RNAase. Substrate analogues used were: (a) 3'-CMP; (b) 3'-AMP; (c) 5'-AMP; (d) 2'-CMP.

Fig. 7. Effect of substrate-analogue concentration [A] on the digestion of RNAase by trypsin

The reaction mixture contained RNAase ($100\mu\text{M}$), trypsin ($1.2\mu\text{M}$), substrate analogue ($0\text{--}10\text{mM}$) and $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (pH 7.2). At each analogue concentration the remaining ribonuclease activity after incubation at 60°C for 0, 0.5, 1.0 and 1.5 min was used to determine the initial rate of trypsin digestion as μmol of RNA digested/min. Substrate analogues used were: (a) 3'-CMP; (b) 3'-AMP; (c) 5'-CMP; (d) 3'-UMP; (e) 2'-CMP; (f) 2'-AMP.

strate may associate before addition to the enzyme. The rate equation, however, would be unchanged by this variation of the model. The model would still involve the binding of a large number of ligand molecules to induce a conformational change in RNAase.

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