An Allosteric Model for Ribonuclease

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Data from two assay systems show that the kinetics of the hydrolysis of cytidine 2':3'-cyclic monophosphate by bovine pancreatic RNAase (ribonuclease) is not consistent with conventional models. An allosteric model involving a substrate-dependent change in the equilibrium between two enzyme conformations is proposed. Such a model gives rise to a calculated curve of velocity versus substrate concentration which fits the experimental data. The model is also consistent with the results of an examination of the tryptic digestion of RNAase. Substrate analogues are able to protect RNAase against hydrolysis by trypsin and the percentage of RNAase activity which remains after digestion increases sigmoidally as the analogue concentration is increased. The model also explains the pattern seen in the K_m values quoted in the literature and is consistent with strong physical evidence for a ligand-induced conformational change for RNAase reported in the literature.

It is generally accepted that globular proteins may exhibit a number of different conformational states which are in dynamic equilibrium with each other. Citri (1973) has reviewed over nine hundred papers which present evidence of the 'conformational adaptability of enzymes to their specific ligands'. However, the concept of conformational adaptability is not often incorporated into models of enzyme behaviour.

There is much physical evidence from studies using X-ray diffraction (Wyckoff *et al.*, 1967), optical rotatory dispersion (Simons, 1971), difference spectroscopy (Deavin *et al.*, 1968), fluorescence quenching (Pongs, 1970), temperature-jump studies (French & Hammes, 1965) and other methods to suggest that RNAase* exists in at least two conformational states and that a transition between these two states may be induced by the binding of such substrate analogues as cytidine 3'-monophosphate and cytidine 2'-monophosphate.

The kinetics of the RNAase-catalysed hydrolysis of cyclic CMP (cytidine 2':3'-cyclic monophosphate) has been extensively studied. An examination of nine such studies at pH 7 and an ionic strength of 0.2 (see references in Table 1) shows that, although the data in each case have been fitted to a Michaelis-Menten type model, the K_m values thus obtained varied with the substrate concentration used. The division of these papers into three groups on the basis of the substrate concentration used showed that, with one exception (Pollard & Nagyvary, 1973), studies with substrate concentrations less than 10 mM resulted in K_m values less than 1 mM; studies with substrate concentrations not exceeding 60 mM resulted in K_m values of 3.4 and 2.29 mM, whereas a

study which used a substrate concentration range of 50–250 mM (Ramsden & Laidler, 1966) resulted in a K_m value of 7.2 mM (see Table 1). If the plots of velocity versus substrate concentration were hyperbolic no such trend would result from examining different regions of the curve.

In this paper we show that (a) plots of velocity versus substrate concentration for the RNAase-catalysed hydrolysis of cyclic CMP over a wide range of substrate concentrations are not consistent with conventional kinetic models; (b) substrate analogues protect RNAase against tryptic digestion and the residual RNAase activity after digestion increases sigmoidally as the analogue concentration is increased.

All the above observations may be explained by an allosteric model for RNAase activity based on a substrate-induced conformational change of the enzyme.

Experimental

Materials

Cytidine 3'-monophosphate (free acid), cyclic CMP (sodium salt), bovine pancreatic RNAase (EC 3.1.4.22) and pancreatic trypsin type II (EC 3.4.21.4) were obtained from Sigma (St. Louis, Mo., U.S.A.). Cellulose CC41 was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K. The cyclic CMP was shown by t.l.c. on cellulose developed in propan-2-ol–conc. NH_3 –water (14:5:1, by vol.) to contain approx. 1% hydrolysis products. Further purification was not attempted since repeated chromatography leads to the formation of a small amount of the hydrolysis products (Szer & Shugar, 1963).

* Abbreviation: RNAase, ribonuclease.

CO₂-free NaOH was prepared from boiling glass-distilled water and was flushed with and stored under N₂ in a vessel with a soda lime trap. It was checked for the presence of CO₂ with BaCl₂ and standardized with potassium hydrogen phthalate (0.05 M) (Kolthoff & Sandel, 1958). All chemicals used were of analytical grade.

Methods

Substrate and product concentrations were determined spectrophotometrically in a Cary 14 or a Perkin-Elmer 402 UV-Vis spectrophotometer and the extinction coefficients determined by Crook *et al.* (1960) (substrate: $\epsilon_{268} = 0.795 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; $\epsilon_{284} = 0.335 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; $\epsilon_{290} = 0.094 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; product: $\epsilon_{270} = 0.84 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; $\epsilon_{284} = 0.463 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; $\epsilon_{290} = 0.193 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The enzyme was assumed to have a molar extinction coefficient of $9800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm (Tanford *et al.*, 1955).

(1) *Spectrophotometric assay.* The spectrophotometric assay measured the change in u.v. absorption as the ring of cyclic CMP is broken (Crook *et al.*, 1960). The assay was carried out in a Cary 14 spectrophotometer and quartz cells of 2 or 5 mm pathlength in a thermostatically controlled cell compartment maintained at 23°C. The reaction was measured at 284 nm or 290 nm. The reaction mixture consisted of Tris-NaCl buffer (0.1 M-Tris; pH 7.13; *I* 0.2), RNAase (5.0 or 6.7 μM) and cyclic CMP (0.175–9 mm). Substrate and product were both shown to obey Beer-Lambert's Law over the concentration range studied.

(2) *Polarimetric assay.* The polarimetric assay measured the change in optical rotation at 320 nm which accompanies the hydrolysis of cyclic CMP (the change in rotation was found to be 18.0° for each mol/litre of substrate hydrolysed). The assay was carried out by using a Jasco model ORD/UV5 ORD recorder and quartz cells of 5–50 mm pathlength in a cell compartment maintained at 17°C. The reaction mixture consisted of Tris-NaCl buffer (0.1 M-Tris; pH 7.13, *I* 0.2), RNAase (6.5–13 μM) and cyclic CMP (4–139 mm). Rotation was shown to be proportional to concentration for both substrate and product over the concentration range used.

(3) *pH-stat assay.* The pH-stat assay measured the release of protons which accompanies the hydrolysis of cyclic CMP. The reaction mixture was maintained at pH 7.13 by the use of a Radiometer pH-meter 25 in conjunction with a glass electrode (Titron 120 AR) and a Titrator 11 which controlled the addition of CO₂-free NaOH as the reaction proceeded. The reaction was recorded by a Heath recorder, model EUW 20A. The reaction was performed in a titration cell maintained at 23°C under an atmosphere of N₂. N₂ was bubbled through the substrate

solution for 2 min before each assay to remove any traces of CO₂. After the addition of enzyme, N₂ was passed over the surface of the reaction mixture during the experiment. After each reaction the cell and electrodes were washed with HCl (1.0 M) followed by copious quantities of distilled water to prevent the adsorption of RNAase on to the glass.

The reaction mixture consisted of NaCl (0.2 M), RNAase (0.38 μM) and cyclic CMP (5–105 mm).

For each assay system and all substrate concentrations it was noted that plots of product formed versus time remained linear for approx. 20–30% of the reaction. Initial velocities were determined directly from the gradient in these linear regions.

For each assay system the range of enzyme concentration over which velocity was proportional to enzyme concentration was shown to extend beyond the concentrations used in the kinetic studies. For comparative purposes the velocities for each assay are expressed as the millimolar change in product concentration in 1 min catalysed by 1 μM -enzyme.

For each assay, the reaction was normally started by the addition of enzyme.

Chromatographic assay. A chromatographic assay was used to determine the extent of oligonucleotide polymerization catalysed by RNAase. A reaction mixture of cyclic CMP (150 mm) and RNAase (5 nm) was incubated at 4°C. Chromatographic separation of the mixture was carried out at 3 h intervals on cellulose thin-layer plates developed in propan-2-ol-conc. NH₃-water (14:5:1, by vol.) and the spots were detected by means of fluorescence quenching.

Electrophoresis. Electrophoresis of RNAase was carried out on polyacrylamide gel in Tris-EDTA-sodium acetate buffer, pH 7.13 (Fairbanks *et al.*, 1971) and in orthophosphate buffer (0.05 M, pH 6.4) (Fisher, 1969).

Trypsin inactivation. The ability of cytidine 3'-phosphate to protect RNAase was determined. Reaction mixtures containing RNAase (132 μM), cytidine 3'-phosphate (0–10 mm), trypsin (1.2 μM) and Na₂HPO₄-NaH₂PO₄ buffer (pH 7.2; *I* 0.24) were incubated at 60°C and 10 μl samples were removed at 0, 0.5, 1.5 and 3.5 min. The RNAase activity in these samples was determined spectrophotometrically at 284 nm by using 1 ml of substrate solution (2 mm) in Tris-NaCl buffer (pH 7.13; 0.1 M-Tris; *I* 0.2).

The decrease in RNAase activity was linear over 3 min and the initial velocity of tryptic hydrolysis of RNAase was calculated from the gradient of the line. The loss of RNAase activity at each product concentration was expressed as a percentage of the activity of the sample at zero time of tryptic inactivation to take into account the effect of product inhibition on RNAase activity. The resulting initial rate of loss of RNAase activity was a measure of the initial rate of tryptic activity on RNAase and was

expressed as μmol of RNAase inactivated per min.

The effect of cytidine 3'-phosphate on the digestion of casein by trypsin was determined by using a modification of the method of Laskowski (1955). Reaction mixtures containing casein (0.5%), trypsin ($1.2\mu\text{M}$), cytidine 3'-phosphate (0–10mM) and $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$ buffer (pH 7.2) were incubated at 35°C for 2, 4 and 6min. Undigested casein was precipitated by centrifugation and the amount of peptides released by digestion was determined from the increased absorption at 750nm after reaction of the supernatant with Folin–Ciocalteu reagent (Layne, 1957).

Results

Kinetic assays

Initial-velocity measurements at various substrate concentrations obtained by the three assay systems are shown in Fig. 1. In Fig. 1(a) velocity measurements obtained by the spectrophotometric assay over the substrate concentration range 0.175–10.0mM yielded a K_m value of 4.3mM. In Figs. 1(b) and 1(c) velocity measurements obtained with the polarimetric and pH-stat assays over the substrate concentration range 0–25mM yielded K_m values of 13 and 9mM respectively. This increase in the K_m value with substrate concentration is parallel to that shown in Table 1. The V_{max} and K_m values obtained in our experiments varied with the batch of substrate and enzyme used and the difference in the K_m values we quote and those shown in Table 1 may reflect a difference in the small amount (approx. 1%) of hydrolytic by-products which contaminate all preparations of cyclic CMP. Examination of Figs. 1(b) and 1(c), however, shows that as the range of substrate concentration was increased, the enzyme was subjected to an apparent substrate inhibition at 30–40mM-substrate concentration followed by an apparent substrate reactivation. This activation appears to be followed by a further substrate inhibition above 80mM-substrate concentration.

Plots of velocity versus enzyme concentration were shown to be linear over the range of enzyme concentrations used at four substrate concentrations, 5.4, 16.8, 40 and 67mM, indicating that the enzyme was not undergoing association–dissociation reactions.

Although it is possible that trace inhibitors in the substrate may become important at high substrate concentrations it appears unlikely that they could cause inhibition followed by activation. Further, the repetition of velocity versus substrate concentration curves in the presence of a constant amount of product gave the same shaped curve.

Although the data shown in Fig. 1 were obtained using solutions which were of constant ionic strength

before the addition of substrate, repetition of a number of measurements in buffers made up to constant ionic strength, (0.2) after the addition of substrate showed the same trend (Fig. 2). This

Fig. 1. Effect of the concentration of cyclic CMP on the initial rate of its hydrolysis by RNAase

(a) Spectrophotometric data measured at 284nm in cells of 2–5mm pathlength at 23°C . The reaction mixture contained: cyclic CMP (0.175–9.0mM); RNAase (5.0 or $6.7\mu\text{M}$); Tris–NaCl buffer (pH 7.13; 0.1M; I 0.2). (b) Specific rotation data measured at 320nm in cells of 5–50mm pathlength at 17°C . The reaction mixture contained cyclic CMP (4–139mM); RNAase (6.5 to $13\mu\text{M}$); Tris–NaCl buffer (pH 7.13; 0.1M; I 0.2). (c) pH-stat data measured at pH 7.13, and 23°C . The reaction mixture contained: cyclic CMP (5–105mM); RNAase ($0.38\mu\text{M}$); NaCl (0.2M). The curves superimposed on parts (b) and (c) are ----, hyperbolae fitted to the data in the substrate concentration ranges 0–30mM and 40–80mM; —, the curves simulated by the proposed rate equation by using the parameters listed in Table 2.

Table 1. Relationship between the upper limit of the substrate concentration range used and the K_m value obtained for the hydrolysis of cyclic CMP by RNAase

Upper limit of substrate concentration range (mM)	K_m (mM)	Reference
10	0.6	Nelson & Hummel (1961)
	0.75	Litt (1961)
	0.34	Wigler (1968)
	0.99	Hummell <i>et al.</i> (1958)
	0.28	Irie (1968)
60	3.4	Herries <i>et al.</i> (1962)
	2.29	Muller & Talsky (1970)
250	7.2	Ramsden & Laidler (1966)

indicates that the transitions were not artifacts introduced by ionic strength changes caused by the addition of substrate. That the trend was not an artifact caused by a change in pH at high substrate concentrations was shown by the pH-stat assay where pH was maintained constant by the addition of NaOH. The pH of a reaction with a high substrate concentration assayed by the polarimetric method was also measured at the completion of the assay and found to be unchanged. Measurements at a substrate concentration of 40 mM showed that the time-course of the reaction was the same regardless of whether the reaction was initiated by the addition of enzyme or substrate. In an experiment, carried out to test whether the transition in the region 30–40 mM-substrate was reversible, a reaction mixture containing cyclic CMP (42.5 mM), RNAase (1.46 μM) and Tris–NaCl buffer (0.1 M-Tris; pH 7.13; I 0.2) was diluted with Tris–NaCl buffer after 2 min incubation to give a solution of cyclic CMP (32 mM) and RNAase (1.09 μM). This was hydrolysed at the same rate as a solution of cyclic CMP (32 mM) and RNAase (1.09 μM) in which the enzyme had not previously been incubated with 42 mM-substrate, indicating that the transition is completely reversible.

Measurements of initial velocities as a function of substrate concentration have been carried out ten times and on all occasions very similar results have been obtained with the transition occurring over the region 30–40 mM-substrate.

Chromatographic assay

Although it has been reported (Bernfield, 1966) that RNAase can catalyse the synthesis of oligonucleotides from high concentrations of cyclic CMP, chromatographic analysis of the products of RNAase action on this substrate in the present case demonstrated that polymerization was not detectable for the

Fig. 2. Effect of ionic strength on velocity versus substrate concentration curves

○, Initial-velocity measurements obtained by the polarimetric assay with reaction mixtures of variable ionic strengths. The reaction mixtures contained: cyclic CMP (25–75 mM); RNAase (5.86 μM), Tris–NaCl buffer (pH 7.13; 0.1 M-Tris; I 0.2). Δ, Initial-velocity measurements obtained by the polarimetric assay with reaction mixtures of constant ionic strength. The reaction mixtures contained: cyclic CMP (25–75 mM), RNAase (5.86 μM), Tris–NaCl buffers (pH 7.13, 0.1 M-Tris and sufficient NaCl to bring the ionic strength to 0.20).

first 4 h of the reaction. Since Bernfield (1966) has demonstrated that oligonucleotide formation decreases rapidly with increasing temperature and enzyme concentration, it seems unlikely that even chromatographically undetectable concentrations of oligonucleotide could be formed in the 10 min necessary for a kinetic run. Therefore it seems unlikely that these velocities could be attributed to the formation of oligonucleotides with strong optical-rotatory and buffering capacity.

Tryptic inactivation

A plot of μmol of RNAase inactivated per minute during incubation in the presence of trypsin at various concentrations of the ligand, cytidine 3'-phosphate, is shown in Fig. 3. The rate of tryptic inactivation of RNAase decreases sigmoidally with

Fig. 3. Effect of cytidine 3'-phosphate on the digestion of RNAase and casein by trypsin

△, Digestion of RNAase. The reaction mixture contained RNAase (132 μM), trypsin (1.2 μM), cytidine 3'-phosphate (0–10 mM) and Na₂HPO₄–NaH₂PO₄ buffer (pH 7.2). At each cytidine 3'-phosphate concentration the remaining RNAase activity after incubation at 60°C for 0, 0.5, 1.5 and 3.5 min was used to determine the initial rate of trypsin digestion as μmol of RNAase digested/min. ○, Digestion of casein. The reaction mixture contained casein (0.5%), trypsin (1.2 μM), cytidine 3'-phosphate (0–10 mM) and Na₂HPO₄–NaH₂PO₄ buffer (pH 7.2). At each cytidine 3'-phosphate concentration the amount of peptides released during incubation at 35°C for 2, 4 and 6 min was used to determine the initial rate of trypsin digestion as change in absorbation at 750 nm/min.

increasing concentrations of the ligand. This indicates that the ligand protects RNAase from tryptic inactivation and that the protection is highly co-operative in the vicinity of 3 mM concentration of substrate analogue. Similar results were observed at pH 5.6.

Fig. 3 also shows the effect of cytidine 3'-phosphate on the digestion of casein by trypsin. Cytidine 3'-phosphate did not inhibit the action of trypsin on this substrate, indicating that the substrate analogue exerts its influence on RNAase rather than trypsin.

Discussion

The results of the present investigation are clearly not consistent with any conventional kinetic models. The most striking feature is the sharp decrease in initial velocity (v) which occurred as the substrate

concentration ($[S]$) was increased beyond 30 mM. This decrease occurred over a range of $[S]$ too narrow to be accounted for by simple substrate inhibition and so further explanation of this behaviour was sought.

The shape of the plot of v versus $[S]$ (Figs. 1b and 1c) and, in particular, the steepness of the transition at $[S] \div 30$ mM suggests a highly co-operative substrate-induced transition from one enzyme form with a low K_m value to a second form with a higher K_m value. We have constructed a model along these lines which fits the data.

The model

Our model is basically a combination of that proposed by Tanford (1968, 1970) to account for ligand-induced conformational changes and the allosteric model proposed by Monod *et al.* (1965). Tanford's model, in turn, is built on Wyman's concept of linked functions (Edsall & Wyman, 1958; Wyman, 1964).

We propose that RNAase may exist in three forms, E, F and G. E and F are both catalytically active and catalyse the reactions $E + S \rightleftharpoons ES \rightarrow E + P$ and $F + S \rightleftharpoons FS \rightarrow F + P$ respectively. One form, E, with a low K_m value, $K_{s,E}$, predominates at low substrate concentrations but is considered to be at all times in equilibrium with a second form, F, which has a higher K_m value, $K_{s,F}$. It is suggested that the two forms also have different affinities for substrate molecules at sites other than the catalytic site. We call these sites allosteric sites. We use S written to the right of an enzyme form to denote substrate bound to the catalytic site (i.e. ES and FS) and S_i written to the left to denote i molecules of substrate bound to the allosteric sites (i.e. S_iE and S_iF, where $i, j = 0, 1, 2, \dots$).

Since Fig. 1 shows that substrate inhibition occurs at high substrate concentrations, we further propose that form F may be converted into a non-catalytic form, G, by the binding of more substrate molecules at inhibitor sites which prevents either the binding of substrate to the catalytic site or the breakdown of the enzyme-substrate complex. We let S_{j,k}G denote the species with j and k molecules of substrate bound at the allosteric and inhibitor sites respectively.

Thus the completed model may be represented by scheme 1.

According to the theory of linked functions, if a different number of substrate molecules bind to the allosteric sites of E and F, then the apparent equilibrium between the forms E and F will be disturbed as described by the relationship (Wyman, 1964);

$$\frac{d \ln K_{F/E,app.}}{d \ln [S]} = \Delta \bar{\nu}_s \quad (1)$$

Scheme 1. A mechanism for the action of bovine pancreatic RNAase on cyclic CMP

E, F and G denote different conformations of the enzyme; a summation sign denotes all species in a particular conformation with differing numbers of substrate molecules bound at the allosteric and/or inhibitor sites.

where $\Delta\bar{\nu}_S$ is the average excess of substrate binding to form F, and $K_{F/E,app.}$ is the apparent equilibrium constant for the transition $E \rightleftharpoons F$, i.e.

$$K_{F/E,app.} = \frac{\sum_j [S_j F]}{\sum_i [S_i E]} \quad (2)$$

It should be noted that, in general, $\Delta\bar{\nu}_S$ is a function of $[S]$.

If the transition from form E to form F is an all-or-none (two-state) transition, and if all the n allosteric binding sites for substrate molecules are equivalent and independent, eqn. (1) can be integrated to yield:

$$K_{F/E,app.} = K_{F/E}(1 + K_I[S])^n \quad (3)$$

where $K_{F/E}$ is the equilibrium constant in the absence of substrate, and K_I is the intrinsic binding constant for substrate at each of the allosteric sites. Providing $K_I[S] \gg 1$, this equation can be approximated by:

$$K_{F/E,app.} = K_{F/E,int.}[S]^n \quad (3a)$$

where $K_{F/E,int.}$ is a complex 'intrinsic constant' incorporating both $K_{F/E}$ and K_I . It should be noted that eqn. (3a) will only hold under the condition $K_I[S] \gg 1$, i.e. when each of the allosteric sites is almost saturated with substrate. It can be shown that, with the mechanism we are proposing, $K_I[S]$ will in fact be much greater than one throughout most of the transition so that $\Delta\bar{\nu}_S$ in eqn. (1) is approximately equal to n throughout the transition region and the binding appears to be highly co-operative.

Similarly, for the transition $ES \rightleftharpoons FS$:

$$K_{FS/ES,app.} = \frac{\sum_j [S_j FS]}{\sum_i [S_i ES]} \quad (4)$$

$$K_{FS/ES,app.} = K_{FS/ES,int.}[S]^{\Delta\bar{\nu}_S} \quad (5)$$

where $K_{FS/ES,app.}$ and $K_{FS/ES,int.}$ are the apparent and 'intrinsic' equilibrium constants for this transition. We are assuming here that binding at the allosteric sites is independent of binding at the catalytic site.

It should be noted that the apparent and 'intrinsic' constants defined in eqns. (1)–(5) are related to the Michaelis–Menten constants $K_{S,E}$ and $K_{S,F}$ as follows:

$$\frac{K_{F/E,app.}}{K_{FS/ES,app.}} = \frac{K_{F/E,int.}}{K_{FS/ES,int.}} = \frac{K_{S,F}}{K_{S,E}} \quad (6)$$

where:

$$K_{S,E} = \frac{[S] \sum_i [S_i E]}{\sum_i [S_i ES]} \quad (7)$$

$$K_{S,F} = \frac{[S] \sum_j [S_j F]}{\sum_j [S_j FS]} \quad (8)$$

In a similar fashion the equilibrium between forms F and G will be disturbed by the differential binding of substrate molecules to the inhibitor sites as described by the relationship:

$$\frac{d \ln K_{G/F,app.}}{d \ln [S]} = \Delta\bar{\nu}_I \quad (9)$$

where $\Delta\bar{\nu}_I$ is the average number of substrate molecules binding to form G at the inhibitor sites and:

$$K_{G/F,app.} = \frac{\sum_j \sum_k [S_{j,k} G]}{\sum_j [S_j F]} \quad (10)$$

We assume that the binding of substrate molecules to the catalytic and inhibitor sites is mutually exclusive.

When the binding sites are nearly saturated Δv_1 may also be regarded as a constant and eqn. (9) may be integrated to yield:

$$K_{G/F,app.} = K_{G/F,int.}[S]^{\Delta v_1} \quad (11)$$

where $K_{G/F,int.}$ is the 'intrinsic constant' for the $F \rightleftharpoons G$ transition.

To obtain an expression for v in terms of $[S]$, we require two further equations:

$$v = k_E \sum_i [S_i ES] + k_F \sum_j [S_j FS] \quad (12)$$

$$[E_t] = \sum_i [S_i E] + \sum_i [S_i E] + \sum_j [S_j ES] + \sum_j [S_j FS] + \sum_k [S_{j,k} G] \quad (13)$$

where $[E_t]$ is the total concentration of enzyme in the reaction system. Elimination of the summation terms from eqns. (2)–(13) yields the desired equation:

$$v = \frac{(k_E + k_F K_{FS/ES,int.}[S]^{\Delta v_2})[S][E_t]}{K_{S,E} + [S] + \{K_{S,F}(1 + K_{G/F,int.}[S]^{\Delta v_1}) + [S]\} K_{FS/ES,int.}[S]^{\Delta v_2}} \quad (14)$$

Determination of parameters

To obtain estimates of the parameters in eqn. (14) we proceed as follows. As $[S] \rightarrow 0$, $v \rightarrow v_1$ where:

$$v_1 = \frac{k_E[S][E_t]}{[S] + K_{S,E}} \quad (15)$$

With $[S]$ in the range 40–80 mM $v \rightarrow v_2$ where:

$$v_2 = \frac{k_F[S][E_t]}{[S] + K_{S,F}} \quad (16)$$

So, the two regions of substrate concentration range 0–30 mM and 40–80 mM can be fitted by two hyperbolae (see Fig. 1b and 1c) and the parameters k_E , k_F , $K_{S,E}$ and $K_{S,F}$ can be obtained (see Table 2). In the region 0–80 mM-substrate, the inhibition term is negligible and eqn. (14) becomes:

$$v = \frac{(k_E + k_F K_{FS/ES,int.}[S]^{\Delta v_2})[S][E_t]}{K_{S,E} + [S] + (K_{S,F} + [S])K_{FS/ES,int.}[S]^{\Delta v_2}} \quad (17)$$

From eqns. (15), (16) and (17) it can be shown that:

$$\ln \left\{ \frac{(v_1 - v)(K_{S,E} + [S])}{(v - v_2)(K_{S,F} + [S])} \right\} = \Delta \bar{v}_s \ln [S] + \ln K_{FS/ES,int.}$$

The ratio $(v_1 - v)/(v - v_2)$ can be calculated from Figs. 1(b) and 1(c) and a plot of $\ln \{(v_1 - v)(K_{S,E} + [S]) / [(v - v_2)(K_{S,F} + [S])]\}$ against $\ln [S]$ allows $\Delta \bar{v}_s$ to be calculated from the slope (see Fig. 4). The fact that $\Delta \bar{v}_s$ is constant over the whole transition justifies the two-state assumption and hence the integration of eqn. (1) to yield eqn. (3a). In the present case $\Delta \bar{v}_s$ was found to be 18. When $\ln [S] = 0$,

$\ln K_{FS/ES,int.}$ can be calculated to be 1.1×10^{-28} and 0.5×10^{-28} for the data in Figs. 1(b) and 1(c) respectively.

The substrate inhibition at high substrate concentrations is too steep to be fitted by the usual assumption (Laidler & Bunting, 1973) that $FS + S \rightleftharpoons FS_2$ where species FS_2 is inactive. In addition, a plot of $1/v$ versus $[S]$, which would be linear in the inhibition region if only two substrate molecules bound to the enzyme to form a dead-end complex, deviated markedly from linearity. Attempts were also unsuccessful at fitting the data by using a model based on the non-co-operative binding of n substrate molecules to identical and independent inhibitor sites with n ranging from 2 to 20, in the absence of a conformational change. However, with a co-operative conformational change exposing the inhibitor sites, as well as the allosteric sites, the data could be fitted as shown in Figs. 1(b) and 1(c) by using the parameters of Table 2.

Examination of the parameters in Table 2 shows that except for k_E and k_F the values of all the parameters obtained with the two assays systems were similar. Since different batches of enzyme were used in the two assays, the variation in activity of different enzyme preparations and changes in the temperature of the assays probably explains the variation in k_E and k_F .

Further evidence for the model

As an alternative to the present model it might be suggested that the data resulted from the presence of two substrate-inhibited species of enzyme in the preparation. The enzyme preparation, however, migrated as a single species on electrophoresis at both pH 7.13 and 6.4. In addition, such a suggestion would not explain the very strong inhibition at 30–40 mM-substrate concentrations since the logarith-

Table 2. Values for the parameters of the rate equation (eqn. 14) used to simulate the curves shown in Figs. 1(b) and 1(c)

Parameter	Values used in Fig. 1(b)	Values used in Fig. 1(c)
k_E	0.175 min ⁻¹	0.366 min ⁻¹
k_F	0.214 min ⁻¹	0.44 min ⁻¹
$K_{S,E}$	13 mM	9 mM
$K_{S,F}$	64 mM	46 mM
$\Delta \bar{v}_s$	18	18
$\Delta \bar{v}_i$	6	6
$K_{FS/ES,int.}$	1.1×10^{-28}	0.5×10^{-28}
$K_{G/F,int.}$	0.67×10^{-12}	1.04×10^{-12}

Fig. 4. Determination of $\Delta\bar{v}_s$

$\ln\{(v_1-v)(K_{s,E}+[S])/[(v-v_2)(K_{s,F}+[S])]\}$ is plotted against $\ln[S]$ for the data in the transition region (30–40 mM). For details see the text. Δ , Data from Fig. 1(b); $\circ$, data from Fig. 1(c).

mic plots in Fig. 4 show that approx. 18 substrate molecules are involved in the binding. Further, the action of cytidine 3'-phosphate in protecting the enzyme against trypsin inactivation is consistent with the substrate analogue binding to the enzyme in a highly co-operative manner. Since highly co-operative binding of substrate to one enzyme species can explain the data it is unnecessary to postulate the existence of two species.

Markus *et al.* (1968) have studied the ability of analogues of cyclic CMP to protect RNAase against digestion by trypsin at pH 5.6. Cytidine 2'-phosphate and cytidine 3'-phosphate are competitive inhibitors binding at the active site and trypsin can be shown to break a bond of RNAase well removed from the active site. Markus *et al.* (1968) conclude therefore that the concentration-dependent ability of these molecules to protect RNAase against trypsin digestion must be explained in terms of a ligand-induced conformational change which renders RNAase less susceptible to proteolysis.

Examination of the effects of concentrations of cytidine 3'-phosphate higher than those that Markus *et al.* (1968) used at both pH 5.6 and 7.2, would suggest, either that the postulated form F is different from E in conformation or alternatively that the protection by ligand against trypsin inactivation is due to steric hindrance from the 18 ligand molecules bound.

Our assumption of a ligand-induced conformational change occurring in RNAase is supported by the kinetic studies of Rübsamen *et al.* (1974). They have found, on investigating the substrate concentration range 0–12 mM, evidence for a ligand-dependent equilibrium between two conformations. Since we have not examined closely this region of the curve, and since sigmoidicity decreases

with decreasing pH (Rübsamen *et al.* 1974), we have no further evidence to support their claim for a shift in equilibrium in the region of 6 mM-substrate. The idea, however, of changes in the equilibrium between conformations such as the one they postulate occurs at 6 mM-substrate and such as the one we postulate occurs at 30 mM-substrate, fits well with the generally accepted views of globular proteins as a dynamic equilibrium of conformations. Our assumption of co-operative binding of six substrate molecules to the inhibitor sites to cause inhibition is supported by data presented by Lumry *et al.* (1951) and McClure *et al.* (1964) on the kinetics of carboxypeptidase.

These workers, on examining the substrate inhibition obtained for carboxypeptidase, demonstrated that the data show a fourth- to fifth-order dependence on substrate concentration and concluded that four or five molecules of substrate are required to inhibit one enzyme centre with an overall inhibition (dissociation) constant of 7×10^{-6} M.

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, $\Delta\bar{v}_s$ molecules of substrate may associate before addition to the enzyme. The rate equation, however, would be unchanged by this variation in the model.

Although the binding of a large number of substrate molecules to an enzyme may seem foreign to traditional kinetic models it is quite consistent with the strong evidence for conformational changes caused by the binding of large numbers of ligand molecules to proteins. RNAase has been shown by Cann (1971) to bind eleven acetic acid molecules in 'a co-operative phenomenon associated with a change in tertiary structure'. We consider that a similar number of substrate molecules can also bind to RNAase to cause a conformational change.

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