Steady-state kinetic studies of the inhibitory action of Zn^{2+} on ribonuclease T_1 catalysis

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The kinetic mechanism of specific inhibition by Zn^{2+} of ribonuclease T_1 catalysis was studied by steady-state kinetic analysis of transphosphorylation of dinucleotides, GpCp(3'), GpUp(2') and GpUp(3'), and dinucleoside monophosphates, GpC and GpU. The inhibition was not simply competitive, non-competitive or uncompetitive, but the kinetic data were compatible with a mechanism of 'fully mixed inhibition' in which a fully non-competitive action was associated with a partially competitive action. Apparent equilibrium quotients involved in this model of inhibition were determined for the dinucleotide substrates, and we found that binding of either of Zn^{2+} and substrate was facilitated when the other was bound. The location of Zn^{2+} was suggested to be near His-40 and/or His-92 of the ribonuclease T_1 molecule.

Ribonuclease T_1 (RNAase T_1) is the ribonuclease 3'-guanylo-oligonucleotido-hydrolase from *Aspergillus oryzae* (EC 3.1.27.3). RNAase T_1 is a small single polypeptide (molecular weight 11085) and the amino acid sequence is established (Takahashi, 1965). Different metal ions are reported to vary in their inhibitory effects on the RNAase T_1 activity when RNA was used as a substrate, and it has been shown that the catalytic activity is drastically lowered by the presence of Zn^{2+} (Uchida & Egami, 1971). In this behaviour Zn^{2+} differs from many other univalent and bivalent ions. Interaction of the more commonly known enzyme, bovine pancreatic RNAase A, with certain metal ions has also been observed by a number of investigators (Breslow & Girotti, 1966; Girotti & Breslow, 1968; Eichhorn *et al.*, 1969; Fan & Bersohn, 1975), and Cu^{2+} and Zn^{2+} are known to be strong non-competitive inhibitors of this enzyme (Takahashi *et al.*, 1967). There have been a couple of Zn^{2+} -binding studies in the past (Takahashi, 1972*a,b*), but, because of the qualitative nature of the measurements, the exact mode of the inhibitory effect of Zn^{2+} on RNAase T_1 activity could not be identified. It should be noted that a specific inhibitory effect of Zn^{2+} on kinetic processes has been reported for several classes of enzymes, among which are, for example, prolyl hydroxylase (Tuderman *et al.*, 1977), glutathione

peroxidase (Splittgerber & Tappel, 1979), lysyl hydroxylase (Puistola *et al.*, 1980) and RNA polymerase III (Vaisius & Horgen, 1980).

The objective of the present investigation was to conduct systematic studies on the inhibitory effect of Zn^{2+} on the rate of transphosphorylation of several dinucleotides and dinucleoside monophosphates [GpCp(3'), GpUp(2'), GpUp(3'), GpC and GpU] and to explore the mode of the inhibition by Zn^{2+} of RNAase T_1 activity. In this present work we have used conventional steady-state kinetic analysis of the initial velocity to elucidate the kinetic mechanism of the inhibition, and the various equilibrium quotients involved were computed from the experimental data by applying curve-fitting programs.

From the results of this investigation we have concluded that (1) in contrast with the case of RNAase A, the inhibition is not simply of non-competitive nature but of an inhibitory mode referred to as 'fully mixed inhibition', for which theoretical formulation was first made by Friedenwald & Maengwyn-Davies (1954); (2) Zn^{2+} binds tightly in the active site and facilitates the binding of substrate to form a catalytically inactive RNAase $\text{T}_1 \cdot \text{Zn}^{2+} \cdot \text{substrate}$ ternary complex; (3) a basic group, in its deprotonated form, on the active site of the enzyme is essential in order that the ternary complex can be formed, and the terminal phosphate group of the dinucleotide substrates studied is involved in stabilization of the complex.

Abbreviation used: RNAase, ribonuclease.

Materials and methods

Materials

The dinucleotides GpCp(3'), GpUp(3') and GpUp(2') and the dinucleoside monophosphates GpC and GpU were prepared as described in previous papers (Aoyagi & Inoue, 1968; Ikenaga & Inoue, 1974). GpC was also prepared by the reverse action of RNAase T₁ from guanosine 2':3'-cyclic phosphate and cytidine (Rowe & Smith, 1970). The purity of these substrates was checked by paper chromatography in the solvent system propan-2-ol/water/aq. conc. NH₃ (sp.gr. 0.880) (7:2:1, by vol.) immediately before use. Solutions containing the transition-metal ion Zn²⁺ were prepared by dissolving analytical-grade ZnSO₄·5H₂O in deionized quartz-distilled water. Highly purified RNAase T₁ was generously given by Dr. H. Okazaki, Sankyo Co., Tokyo, Japan. Enzyme activity was assayed spectrophotometrically on GpC. All other materials were of analytical grade.

Determination of difference absorption coefficients at different [Zn²⁺]/[dinucleotide] ratios

U.v. difference spectra due to the transphosphorylation of the dinucleotide substrates by RNAase T₁ were measured and used for calculating the difference molar absorption coefficients ($\Delta\epsilon$) at the appropriate wavelength where the greatest value of $\Delta\epsilon$ was observed at 25°C and pH 7.50. The dependence of ϵ and $\Delta\epsilon$ on Zn²⁺ concentration for all the dimer substrates was examined, and both ϵ and $\Delta\epsilon$ were found to be independent of [Zn²⁺] in the [Zn²⁺]/[dimer] range 0–3:1. The average values thus determined are: for GpCp(3') and GpC at 280 nm, 1920 and 2475 M⁻¹·cm⁻¹ respectively; for GpUp(3'), GpUp(2') and GpU at 275 nm, 1150, 1130 and 1250 M⁻¹·cm⁻¹ respectively.

Kinetics

The steady-state kinetic techniques were essentially similar to those used before (Yasuda & Inoue, 1981, 1982). The reaction, in 2 ml of substrate solution in 50 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.50, in a 1 cm silica cell placed in the thermostatically controlled cell compartment of a Hitachi 124 spectrophotometer equipped with a Komatsu temperature controller, was initiated by the addition of 20 μ l of RNAase T₁ solution after temperature equilibration (25 $\pm$ 0.1°C). The initial velocity was determined by recording the change in absorbance at the analytical wavelengths shown above. The concentrations used for each of the dinucleotide substrates were in the range 25–110 μ M. The enzyme concentrations were 0.741 nM for GpCp(3') and GpC, and 16.1 nM for GpUp(3'), GpUp(2') and GpU. Inhibition studies were conducted at various Zn²⁺ concentrations. The ranges of [Zn²⁺]/[dimer

substrate] ratios varied depending on the effectiveness of the inhibition: [Zn²⁺]/[GpCp(3')], 0.083–3.23; [Zn²⁺]/[GpC], 0.23–7.13; [Zn²⁺]/[GpUp(3')], 0.28–5.99; [Zn²⁺]/[GpUp(2')], 0.048–2.95; [Zn²⁺]/[GpU], 0.48–14.65. Measurements of initial rate were made in duplicate at four or five different concentrations of each substrate and at least five different Zn²⁺ concentrations. All calculations and fitting of equations to data by the least-squares methods were performed with the aid of a HITAC M-200H computer system of the Computer Centre of the University of Tokyo.

Results and discussion

The first evidence that Zn²⁺ inhibits the catalysis by RNAase T₁ of the degradation of RNA was reported by Egami *et al.* (1964). Later an investigation conducted in our laboratories revealed (a) that the ligands, GMP and Zn²⁺, bind to RNAase T₁ only at the specific binding sites, and (b) that Zn²⁺ strongly inhibits RNAase T₁ activity in both transphosphorylation and hydrolysis (K. Sakurai & Y. Inoue, unpublished work). No other studies have been reported on the inhibitory effect of Zn²⁺ against simple substrates such as guanylyl dinucleotides and guanosine 2':3'-cyclic phosphate. The basic aim of present work was thus to find the kinetic mechanism of inhibition of RNAase T₁-catalysed transphosphorylation by Zn²⁺.

Effect of pH on the inhibition of RNAase T₁-catalysed transphosphorylation by Zn²⁺

The inhibitory effect of Zn²⁺ on RNAase T₁ activity was examined at two selected pH values by steady-state kinetic analysis. Data were interpreted graphically from plots of 1/*v*_i versus Zn²⁺ concentration at pH 7.5 and 5.0 with GpCp(3') and GpC as substrates (Figs. 1a and 1b). The Zn²⁺ inhibitory effect declines rather dramatically as the pH is lowered from 7.5 to 5.0. Considering the similar behaviour observed for both GpCp(3') and GpC, the ionization of the secondary phosphate group (*pK* $\simeq$ 6) of a 3'-terminal phosphate must not be a major factor of the observed effect of pH. Instead the observed decrease in inhibitory power in going from pH 7.5 to 5.0 appears to be the result of the protonation of a particular amino acid residue (presumably His-40 and/or His-92) side chain at the active site of RNAase T₁ (Arata *et al.*, 1976). It is known from n.m.r. studies (Fan & Bersohn, 1975) that addition of Zn²⁺ causes marked changes in the chemical shifts of the imidazole C-2 protons of His-12 and His-119 residues, both of which are located at the active site of RNAase A. For other selected dimer substrates we have also found that the optimal pH for the Zn²⁺ inhibition lies in the neutral

Fig. 1. Inhibition by Zn²⁺ of the RNAse T₁-catalysed transphosphorylation of (a) GpCp(3') and (b) GpC at pH 7.5 (●) and pH 5.0 (○)

Experimental conditions are described in the text.

Fig. 2. Inhibition by Zn²⁺ of the RNAse T₁-catalysed transphosphorylation at 25°C

(a) Double-reciprocal plots of initial velocity versus GpUp(3') concentrations. [Zn²⁺]: ○, 0 mM; ▲, 0.03 mM; △, 0.06 mM; ●, 0.12 mM; ■, 0.16 mM. (b) Double-reciprocal plots with GpU as variable substrate. Zn²⁺ concentration used: ○, 0 mM; ▲, 0.05 mM; △, 0.10 mM; ●, 0.20 mM; ■, 0.40 mM. The continuous lines through the data points show the theoretical fits. Experimental conditions are described in the text.

pH range (results not shown). This is the reason why we have made all our measurements at pH 7.5.

Analysis of steady-state kinetic data

Fig. 2 shows the inhibitory effect of Zn²⁺ by plotting $1/v_i$ against $1/[dimer]$ at several different

Zn²⁺ concentrations. Straight lines in double-reciprocal plots were fitted by linear least-squares analysis of the data points. Both the slope and intercept values vary with [Zn²⁺] and are complicated functions of [Zn²⁺]. The patterns represented in Fig. 2 for GpUp(3') and GpU clearly rule out

classical competitive, non-competitive or uncompetitive mechanism. Therefore we have considered the model shown in Scheme 1 for the analysis of the binding and transphosphorylation of the oligonucleotide substrates to and by RNAase T₁ in the presence of Zn²⁺. The equilibrium constants involved in Scheme 1 are all dissociation constants and may be defined as $K_m^0 = [E][S]/[ES]$, $K_{zn}^0 = [E][Zn^{2+}]/[EZn^{2+}]$, $K'_m = [EZn^{2+}][S]/[ESZn^{2+}]$ and $K'_{zn} = [ES][Zn^{2+}]/[ESZn^{2+}]$. The rate equation of this reaction scheme can be written as:

$$\frac{1}{v_i} = \frac{1}{V_{\max}^0} \cdot \frac{1}{1 + \frac{A}{K'_{zn}} [Zn^{2+}]} \cdot \left(\frac{K_m^0}{[S]} + 1 + \frac{K'_m}{K'_{zn}} \cdot \frac{[Zn^{2+}]}{[S]} + \frac{[Zn^{2+}]}{K'_{zn}} \right) \quad (1)$$

where

$$A = k'_{+2}/k_{+2}$$

and

$$V_{\max}^0 = k_{+2}([E] + [ES] + [ESZn^{2+}] + [EZn^{2+}])$$

The values of K_m^0 , K_{zn}^0 , K'_m , K'_{zn} and A for the reactions defined in Scheme 1 were determined in the following way. First, the value of K_m^0 was assumed to be that experimentally determined from a plot of $1/v_i$ against $1/[S]$ for the RNAase T₁-catalysed transphosphorylation in the absence of Zn²⁺. Next, in the limit as $[S] \rightarrow \infty$, we have:

$$\lim_{[S] \rightarrow \infty} (1/v_i) = \frac{1}{V_{\max}} = \frac{1}{V_{\max}^0} \cdot \frac{1}{1 + \frac{A}{K'_{zn}} [Zn^{2+}]} \cdot \left(1 + \frac{[Zn^{2+}]}{K'_{zn}} \right) \quad (2)$$

Initial rates from experiments at $v_i \rightarrow V_{\max}$ and various $[Zn^{2+}]$ values were plotted in Fig. 3. In every case a plot of $1/V_{\max}$ against $[Zn^{2+}]$ is seen to be linear, strongly indicating $A = 0$ in eqn. (1). The values of $1/V_{\max}$ as a function of $[Zn^{2+}]$ for several values of A are presented in Fig. 3. There is a high degree of coincidence between the straight line obtained by linear least-squares analysis and a hypothetical curve calculated for $A = 0$. The balance of evidence accordingly suggests that $k'_{+2} = 0$, i.e. the ternary complex, $ESZn^{2+}$, would be a non-productive one. Consequently, such a plot as

demonstrated in Fig. 3 may be seen to approximate to a straight line. The slope of each plot, together with the known value of $V_{\max}^0$, was used to obtain the value of K'_{zn} .

Making use of $A = 0$, $1/v_i = 0$ (intercept at $1/v_i$ in a plot of $1/v_i$ against $1/[S]$) and eqn. (1), we can obtain eqn. (3), showing $1/K_m$ as a function of $[Zn^{2+}]$:

$$-\frac{1}{K_m} = \frac{K'_{zn} + [Zn^{2+}]}{K_m^0 \cdot K'_{zn} + K'_m [Zn^{2+}]} \quad (3)$$

With this information and the values of K_m^0 and K'_{zn} , a plot of $-1/K_m$ against $[Zn^{2+}]$ will allow the calculation of K'_m by using eqn. (3). In Fig. 4, plots of $-1/K_m$ versus $[Zn^{2+}]$ for GpUp(3'), GpUp(2') and GpU are shown. A single-parameter non-linear least-squares fit of all the data points to eqn. (3) yielded the best values of K'_m , and the continuous curves in Fig. 4 were calculated by using the values of K'_m thus obtained and listed in Table 1.

In a cyclic equilibrium system as shown in Scheme 1, it follows that $K_m^0 \cdot K'_{zn} = K'_m \cdot K_{zn}^0$. With this relation and the known values for K_m^0 , K'_{zn} and K'_m , we can go on to calculate the value of K_{zn}^0 , the

dissociation constant for the RNAase T₁·Zn²⁺ complex to RNAase T₁ and Zn²⁺. The best values for the equilibrium quotients involved in Scheme 1 were extracted for GpCp(3'), GpC, GpUp(2'), GpUp(3') and GpU and summarized in Table 1. Except for GpU, the binding of either of the two ligands, Zn²⁺ and substrate, is enhanced when the other ligand is bound [$K_{zn}^0/K'_{zn} = K_m^0/K'_m > 1$, i.e. $K_{zn}^0 > K'_{zn}$ and $K_m^0 > K'_m$; cf. Secemski & Lienhard (1974) for lysozyme and Taniuchi & Bohnert (1975) for nuclease T']. It is also true that Zn²⁺ binds to the terminal phosphate group of the dinucleotides used and stabilizes the RNAase T₁·Zn²⁺·dinucleotide ternary complexes.

Figs. 3 and 4, together with the numerical data in Table 1, show that inhibition does depend on the nature of the substrate to be transphosphorylated. Different effects of Zn²⁺ on GpC and GpU transphosphorylation may be related to what is known about their different subsite interactions with RNAase T₁ (Zabinski & Walz, 1976).

In summary, the results of initial-velocity studies reported in the present paper for GpC, GpCp(3'),

Scheme 1. Model for the inhibition by Zn²⁺ of RNAase T₁-catalysed transphosphorylation

K values represent the dissociation constants of binary and ternary complexes involved in the equilibrium scheme for fully mixed inhibition.

Fig. 3. Linear relationship between $1/V_{max}$ and $[Zn^{2+}]$ for RNAase T₁ catalysed transphosphorylation (a) $\blacktriangle$, GpUp(2'); $\bullet$, GpUp(3'); $\circ$, GpU. (b) $\bullet$, GpCp(3'); $\circ$, GpC. The continuous lines represent the best fits of a model [eqn. (2) in which $A = 0$] with the data. Experimental conditions are described in the text. Inset: The family of curves is generated from eqn. (2) for several values of A for (a) GpUp(2') and (b) GpCp(3').

Table 1. Dissociation constants for the equilibria involved in the 'fully mixed inhibition' of RNAase T₁-catalysed transphosphorylation by Zn²⁺ of the dimer substrates at 25°C and pH 7.5

$$\begin{array}{c} E + S + Zn^{2+} \xrightleftharpoons{K_m^0} ES + Zn^{2+} \xrightarrow{k_{+2}} EP + Zn^{2+} \\ \updownarrow K_{zn}^0 \qquad \qquad \qquad \updownarrow K'_{zn} \\ EZn^{2+} + S \xrightleftharpoons{K'_m} ESZn^{2+} \end{array}$$

Substrate	$10^5 \times K_m^0$ (M)	$10^5 \times K'_{zn}$ (M)	$10^5 \times K'_m$ (M)	$10^5 \times K'_{zn}$ (M)
GpCp(3')	17.6 ± 2.9	3.79 ± 0.95	0.520 ± 0.16	0.112 ± 0.001
GpC	22.7 ± 5.9	4.52 ± 0.75	10.0 ± 2.0	1.99 ± 0.09
GpUp(3')	3.39 ± 0.29	4.43 ± 0.10	0.407 ± 0.008	0.532 ± 0.005
GpUp(2')	2.15 ± 0.09	4.11 ± 0.52	0.360 ± 0.070	0.687 ± 0.011
GpU	4.02 ± 0.25	12.2 ± 1.3	22.8 ± 2.1	69.4 ± 1.1

GpUp(2'), GpUp(3') and GpU can be interpreted in terms of a scheme for fully mixed inhibition (Friedenwald & Maengwyn-Davies, 1954; Dixon & Webb, 1979; for a precedent for this type of inhibition see Vaisius & Horgen, 1980) (Scheme 1, in which $k'_{+2} = 0$). Support for such a scheme comes from the following observations: (1) straight lines in double-reciprocal plots of $1/v_1$ versus $1/[S]$ (Fig. 2);

(2) a linear relationship between $1/V_{max}$ and $[Zn^{2+}]$ (Fig. 3); (3) a hyperbolic relationship between $1/K_m$ and $[Zn^{2+}]$ (Fig. 4); (4) a high degree of coincidence of the values of K_{zn}^0 obtained by using different substrates [GpUp(3'), GpUp(2'), GpCp(3') and GpC] (Table 1). It should be noted that Zn²⁺ and Cu²⁺ are known to act as strong non-competitive inhibitors of RNAase A irrespective of reaction

Fig. 4. Dependence on Zn^{2+} concentration of $-1/K_m$ of the RNAase T_1 -catalysed transphosphorylation of the dimer substrates at 25°C of pH 7.5

▲, GpUp(2'); ●, GpUp(3'); ○, GpU. The continuous lines represent the computer-generated curves that provide the best fit of eqn. (3) with the data. Experimental conditions are described in the text.

conditions (Takahashi *et al.*, 1967; Alger, 1970). Our observations for RNAase T_1 are in disagreement with a simple non-competitive nature of the inhibition by Zn^{2+} . At present, however, it is not possible to explain how and why these differences arise. It is also known that addition of Ca^{2+} causes inhibitory effects on RNAase A activity through the formation of RNA- Ca^{2+} complexes (Morrill & Reiss, 1969). This also cannot be the case with the present experimental results, because the dependence of $1/v_i$ on $1/[S]$ cannot be accounted by such a model of inhibition.

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