Kinetics of inactivation of bovine pancreatic ribonuclease A by bromopyruvic acid

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The kinetic theory of substrate reaction during the modification of enzyme activity [Duggleby (1986) *J. Theor. Biol.* **123**, 67–80; Wang and Tsou (1990) *J. Theor. Biol.* **142**, 531–549] has been applied to a study of the inactivation kinetics of ribonuclease A by bromopyruvic acid. The results show that irreversible inhibition belongs to a non-competitive complexing type inhibition. On the basis of the kinetic equation of substrate reaction in the presence of the inhibitor, all microscopic kinetic constants

for the free enzyme, the enzyme–substrate complex and the enzyme–product complex have been determined. The non-competitive inhibition type indicates that neither the substrate nor the product affects the binding of bromopyruvic acid to the enzyme and that the ionization state of His-119 may be the same in both the enzyme–substrate and the enzyme–product complexes.

INTRODUCTION

The irreversible modification of enzymes has attracted particular attention because of its usefulness in elucidating the nature of the functional groups located at the active site, essential for enzyme activity, and likely to be involved in the catalytic process. Some years ago a systematic study on the kinetics of irreversible modification of enzyme activity was presented [1]. Not only is this method most desirable because during the action of the chemotherapeutic agents *in vivo* the presence of substrate is inevitable, but it can also give information on the interaction between enzyme, substrate and modifier. Moreover, it is easy to operate and capable of fast modification, in contrast with the conventional method.

The kinetics of enzyme modification and denaturation have been treated in two different ways, depending on whether a significant proportion of substrate is consumed in the enzyme-catalysed reaction [2–5] or whether the substrate concentration can be considered to be essentially constant during the period of observation and hence set equal to its initial value in the derivation of integrated rate expressions describing the time dependence of product formation [1,6]. The latter approach has been extended beyond the treatment of relatively simple cases of irreversible modification of an enzyme catalysing a single-intermediate one-substrate reaction to include more complicated models [7–23].

However, in some cases where the inactivation process is slower or there is stronger product inhibition, the assumptions that the substrate concentration remains constant and the effect of the product can be neglected during the modification process are no longer satisfied. Laidler and Bunting [2] considered briefly the decrease in substrate during enzyme inactivation where substrate provides complete protection (competitive) or has no effect (non-competitive) on the inhibition of enzyme activity. Recently such cases were considered thoroughly in theory and several graphical methods were proposed to determine the kinetic constants [3,5]. This method has been applied to study the

stability of alkaline phosphatase during inactivation by EGTA [4].

In the present paper the method was extended to the case of non-competitive complexing inhibition. On the basis of the kinetic equation of substrate reaction in the presence of the inhibitor, a plotting method has been developed for determining the inhibition kinetic constants. As an example, all modification kinetic constants for bovine pancreatic RNase A by bromopyruvic acid have been determined. The results of the present study indicate that both substrate and product binding have little effect on either the binding of the modifier or the irreversible modification of His-119 at the enzyme's active site. Therefore His-119 of RNase A might be involved in the catalytic rather than the substrate-binding step.

MATERIALS AND METHODS

Bovine pancreatic RNase A, cCMP (sodium salt) and bromopyruvic acid were purchased from Sigma. Mes was a Fluka product. All other reagents were local analytical-grade reagents without further purification.

The concentration of RNase A was determined by measuring the absorbance at 280 nm and taking the molar absorption coefficient to be $9800 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The concentration of cCMP was determined with the use of a molar absorption coefficient of $8500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 268 nm.

Enzyme activity was determined at 25 °C by the method of Crook et al. [24] by measuring the change of absorbance at 290 nm with cCMP as the substrate in 0.2 M Mes/NaOH buffer (pH 6.0). The difference in molar absorption coefficient between the substrate and product, $\Delta\epsilon_{290}$, $990 \text{ M}^{-1} \cdot \text{cm}^{-1}$, was determined by monitoring substrate hydrolysis to completion in the presence of excess enzyme. For the kinetics assays, RNase A was added to the preincubated reaction buffer and mixed rapidly (less than 10 s), then the absorbance changes were recorded on a DU7500 spectrophotometer thermostatically maintained at 25 °C. Ex-

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perimental data were stored in a computer and analysed further by a non-linear least-square regression method.

Kinetic analysis

Let us consider the reaction mechanism in Scheme 1, where E, S, P and Y represent the enzyme, substrate, product and inhibitor respectively.

Scheme 1 The reaction mechanism

As will be seen later, the experimental data obtained from the kinetic study of the inactivation of RNase A by bromopyruvic acid are in accordance with this theoretical model. On the basis of Scheme 1, we have:

$$\left. \begin{aligned} [E_T] &= [E] + [ES] + [EP] + [EY] + [ESY] + [EPY] \\ [E_T^*] &= [EY^*] + [ESY^*] + [EPY^*] \\ [E]_0 &= [E_T] + [E_T^*] \end{aligned} \right\} \quad (1)$$

Assuming that the steady state of the substrate reaction is rapidly established, and the formation of EY, ESY and EPY complexes are fast reactions relative to the inactivation step, the following relations hold at any time:

$$\left. \begin{aligned} [E] &= \frac{[E_T]}{\Sigma} & [ESY] &= \frac{[S][Y][E_T]}{K_s K'_i \Sigma} \\ [ES] &= \frac{[S][E_T]}{K_s \Sigma} & [EPY] &= \frac{[Y][P][E_T]}{K'_i K_p \Sigma} \\ [EY] &= \frac{[Y][E_T]}{K_i \Sigma} \end{aligned} \right\} \quad (2)$$

where

$$\Sigma = 1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[Y]}{K_i} + \frac{[Y][S]}{K'_i K_s} + \frac{[Y][P]}{K'_i K_p}$$

K_s and K_p are the dissociation constants of the enzyme for the substrate and product respectively, and K_i , K'_i and K''_i are the dissociation constants for the modifier with different forms of the enzyme, respectively.

The rates of the modification of the enzyme and the depletion of the substrate are given by:

$$\begin{aligned} -\frac{d[E_T]}{dt} &= k_{+0}[EY] + k'_{+0}[ESY] + k''_{+0}[EPY] \\ &= \left(\frac{k_{+0}}{K_i} + \frac{k'_{+0}[S]}{K'_i K_s} + \frac{k''_{+0}[P]}{K''_i K_p} \right) [Y][E_T] / \Sigma \end{aligned} \quad (3)$$

$$-\frac{d[S]}{dt} = k_2[ES] = \frac{k_2[S][E_T]}{K_s \Sigma} \quad (4)$$

Combining eqns. (3) and (4):

$$-\frac{d[E_T]}{d[S]} = \left(\frac{k_{+0}}{K_i} + \frac{k'_{+0}[S]}{K'_i K_s} + \frac{k''_{+0}[P]}{K''_i K_p} \right) [Y] / \left(\frac{k_2[S]}{K_s} \right) \quad (5)$$

Integrating eqn. (5) with the boundary conditions $[S] = [S]_0$, $[E_T] = [E]_0$ gives:

$$\begin{aligned} [E_T^*] &= [E]_0 - [E_T] = \frac{k_{+0} K_s [Y]}{K_i k_2} \ln \frac{[S]_0}{[S]} + \frac{k'_{+0} [Y]}{K'_i k_2} ([S]_0 - [S]) \\ &\quad + \frac{k''_{+0} [Y] K_s [S]_0}{K''_i K_p k_2} \ln \frac{[S]_0}{[S]} - \frac{k''_{+0} K_s [Y]}{K''_i K_p k_2} ([S]_0 - [S]) \end{aligned} \quad (6)$$

If the enzyme is completely inactive when t approaches infinity, we then have $[E_T] = 0$, $[S] = [S]_\infty$, $[P]_\infty = [S]_0 - [S]_\infty$, and eqn. (6) can be written:

$$\begin{aligned} [E]_0 &= \left(\frac{k_{+0} K_p}{K_i} + \frac{k'_{+0} [S]_0}{K'_i} \right) \frac{[Y] K_s}{K_p k_2} \ln \frac{[S]_0}{[S]_0 - [P]_\infty} \\ &\quad + \left(\frac{k'_{+0} K_p}{K'_i} - \frac{k''_{+0} K_s}{K''_i} \right) \frac{[Y]}{K_p k_2} [P]_\infty \end{aligned} \quad (7)$$

which is identical in form with eqn. (26) of Wang and Tsou [5]. To determine the inactivation rate constants, eqn. (7) can be linearized as before:

$$\begin{aligned} \frac{[E]_0}{[P]_\infty} &= \left(\frac{k_{+0} K_p}{K_i} + \frac{k'_{+0} [S]_0}{K'_i} \right) \frac{[Y] K_s}{K_p k_2} \frac{1}{[P]_\infty} \ln \frac{[S]_0}{[S]_0 - [P]_\infty} \\ &\quad + \left(\frac{k'_{+0} K_p}{K'_i} - \frac{k''_{+0} K_s}{K''_i} \right) \frac{[Y]}{K_p k_2} \end{aligned} \quad (8)$$

According to eqn. (8), plots of $[E]_0/[P]_\infty$ against $(1/[P]_\infty) \ln \{[S]_0/([S]_0 - [P]_\infty)\}$ at a series of fixed $[S]_0$ values will give a group of straight lines with slopes of:

$$s = \frac{k_{+0}[Y] K_s}{K_i k_2} + \frac{k'_{+0}[Y] K_s}{K'_i K_p k_2} [S]_0 \quad (9)$$

and ordinate intercepts of:

$$i = \left(\frac{k'_{+0} K_p}{K'_i} - \frac{k''_{+0} K_s}{K''_i} \right) \frac{[Y]}{K_p k_2} \quad (10)$$

A secondary plot of s against $[S]_0$ will also give a straight line with a slope of $k'_{+0}[Y] K_s/(K_p k_2 K'_i)$ and an ordinate intercept of $k_{+0} K_s [Y]/(K_i k_2)$. As K_s , K_p and k_2 can be obtained from experiments without the modifier, k_{+0}/K_i , k'_{+0}/K'_i and k''_{+0}/K''_i can then be determined from eqns. (9) and (10).

Because the reaction of RNase A with bromopyruvic acid occurs through a two-step mechanism, it should be possible to determine the dissociation constants of EY, ESY and EPY

separately. When $t \rightarrow 0$, $[S] \rightarrow [S]_0$, $[P] = 0$ and $[E_T] = [E]_0$, eqn. (4) becomes:

$$v_0 = k_2[S]_0[E]_0 / K_s \left(1 + \frac{[S]_0}{K_s} + \frac{[Y]}{K_I} + \frac{[Y][S]_0}{K'_I K_s} \right) \quad (11)$$

Thus the dissociation constants for the modifiers with the free enzyme and the enzyme-substrate complex, K_I and K'_I , can be determined from the initial velocity (V_0) equation, eqn. (11).

To determine the dissociation constants for the modifier with the enzyme-product complex, K''_I , we should use the velocity equation at a series of fixed production concentrations during the modification. In fact, as the reaction proceeds the product is accumulated in the system. Therefore we can generate a set of velocities to be analysed as though they were initial rates established at various different values of $[S]$ and $[P]$. The velocity will be affected by the product concentration. To obtain the relation, rewrite eqn. (4) as:

$$v_t = \frac{k_2[S][E_T]}{K_s \Sigma} = \frac{k_2([S]_0 - [P])([E]_0 - [E^*])}{K_s \Sigma} \\ = \frac{k_2([S]_0 - [P])[E]_0}{K_s \Sigma} - \frac{k_2([S]_0 - [P])[E^*]}{K_s \Sigma} \quad (12)$$

According to eqn. (12), v_t is proportional to $[E]_0$ at fixed values of $[S]_0$ and $[P]$ because both $[E^*]$ and Σ are constant under these conditions [see eqn. (6)]. The reciprocal of the slope, $1/\gamma$, is a linear function of $1/([S]_0 - [P])$ as:

$$\frac{1}{\gamma} = \frac{K_s \Sigma}{k_2([S]_0 - [P])} \\ = \frac{K_s}{k_2} \left(\frac{1}{[S]} + \frac{1}{K_s} + \frac{[P]}{K_p[S]} + \frac{[Y]}{K_I[S]} + \frac{[Y]}{K_s K'_I} + \frac{[Y][P]}{K'_I K_p[S]} \right) \\ = \frac{K_s}{k_2} \left(\frac{1}{K_s} + \frac{[Y]}{K_s K'_I} + \frac{1}{[S]} + \frac{[Y]}{K_I[S]} + \frac{[S]_0 - [S]}{K_p[S]} + \frac{[Y]([S]_0 - [S])}{K'_I K_p[S]} \right) \\ = \left(1 + \frac{[S]_0}{K_p} + \frac{[Y]}{K_I} + \frac{[Y][S]_0}{K'_I K_p} \right) \frac{K_s}{k_2} \frac{1}{[S]_0 - [P]} \\ + \left\{ \frac{1}{k_2} \left(1 + \frac{[Y]}{K'_I} \right) - \frac{K_s}{K_p k_2} \left(1 + \frac{[Y]}{K'_I} \right) \right\} \quad (13)$$

Once k_2 , K_p and K_I , K'_I have been determined from eqns. (11) and (14), K''_I can be calculated from the slope of the plot of $1/\gamma$ against $1/([S]_0 - [P])$.

RESULTS

Kinetics of the RNase A-catalysed reaction

The steady-state kinetics of RNase A has been extensively studied [25,26]. Under the conditions employed in the present study of the inactivation kinetics [0.2 M Mes/NaOH buffer (pH 6.0) at 25 °C], the kinetic constants for the RNase A-catalysed reaction were determined by analysing the progress curves of the enzyme reaction. The time courses of the enzyme-catalysed reaction at different substrate concentrations are shown in Figure 1. By fitting the progress curves to the integrated Michaelis-Menten equation [27]:

$$t = \left(1 + \frac{[S]_0}{K_p} \right) \frac{K_s}{V} \ln \frac{[S]_0}{[S]_0 - [P]} + \frac{1}{V} \left(1 - \frac{K_s}{K_p} \right) [P] \\ = \alpha \ln \frac{[S]_0}{[S]_0 - [P]} + \beta [P] \quad (14)$$

Figure 1 Hydrolysis of cCMP at different concentrations by RNase A

The enzyme concentration was 1 μ M. Curves 1–3 are progress curves with 0.24, 0.39 and 0.60 mM cCMP respectively. The reaction was performed in 0.2 M Mes/NaOH (pH 6.0) at 25 °C. The dots are experimental points, and the curves are results of curve-fitting by a non-linear regression program in accordance with the integrated Michaelis-Menten equation. The inset shows the determination of K_p , K_s and V . Line 1 is the plot of α against $[S]_0$; the y intercept gives K_s/V , and the slope gives $K_s/(VK_p)$. Line 2 is the relation between β and $[S]_0$.

the values of $\alpha = \{1 + ([S]_0/K_p)\} K_s/V$ and $\beta = \{1 - (K_s/K_p)\}/V$ can be obtained, where $V = k_2[E]_0$. A secondary plot of α against $[S]_0$ gives a straight line with a slope $K_s/(VK_p) = 3153.2 \text{ s} \cdot \text{mM}^{-1}$, and an intercept on the ordinate of $K_s/V = 133.9 \text{ s}$. From these values all the kinetic constants K_s , k_2 and K_p can be determined as 0.57 mM, 4.3 s^{-1} and 0.042 mM respectively. These values are in agreement with those reported previously [25].

Figure 2 Plot of $\ln a$ against time

This is the kinetic study of inactivation of RNase A by bromopyruvic acid; a is the fraction of activity remaining. A mixture of the enzyme (8 μ M) and bromopyruvic acid in 0.2 M Mes/NaOH buffer (pH 6.0) was incubated at 25 °C. At different time intervals as indicated, 20 μ l portions were taken for activity determination in 1.0 ml of reaction mixture containing 0.4 mM cCMP in the same buffer. Curves 1–5 are time courses with 3, 4, 6, 10 and 20 mM bromopyruvic acid respectively. The inset shows a double-reciprocal plot of k_{obs} against concentration of bromopyruvic acid (Y).

Figure 3 Double-reciprocal plots for the determination of the dissociation constants for bromopyruvic acid with RNase A and the RNase A-cCMP complex

The bromopyruvic acid concentrations for curves 1–5 were 0, 4, 8, 12 and 16 mM respectively. The inset shows a secondary plot. Line 1 is the plot of the slopes against [Y], and line 2 is the plot of the ordinate intercepts of the double-reciprocal plots against [Y].

Kinetic study of the inactivation of RNase A by conventional methods

The inactivation of RNase A by bromopyruvic acid was first studied by conventional methods. Aliquots were taken from the incubated mixture at different time intervals for the measurement of the remaining activity. The fraction activity, a , decreases exponentially with time and thus the plot of $\ln a$ against time gives a series of lines at different modifier concentrations (Figure 2). The apparent inactivation rate constants were determined from the slopes of the lines. The relation between the apparent inactivation rate constant and the concentration of bromopyruvic acid indicates that the inactivation reaction is in agreement with a two-step mechanism:

For such a two-step mechanism, the apparent inactivation rate constant, k_{obs} , is given by:

$$k_{\text{obs}} = \frac{k_{+0}[Y]}{K_1 + [Y]} \quad (15)$$

The plot of $1/k_{\text{obs}}$ against $1/[Y]$ gives a straight line (the inset to Figure 2). The values of K_1 and k_{+0} were obtained from the slope and the ordinate intercept as 14.8 ± 2.5 mM and $(8.25 \pm 0.65) \times 10^{-4} \text{ s}^{-1}$ respectively.

Kinetics of substrate reaction in the presence of bromopyruvic acid

It is difficult to determine the initial rates accurately from the linear parts of the experimental curves because of inhibition by the product. Therefore the initial rates were estimated by fitting the initial parts (from 10 to 180 s) of the experimental curves to

Table 1 Binding and rate constants for inhibition of RNase A by bromopyruvic acid

	Conventional method		Present method	
	K_1 (mM)	$10^4 k$ (s^{-1})	K_1 (mM)	$10^4 k$ (s^{-1})
E	14.8 ± 2.5	8.25 ± 0.65	7.8 ± 1.4	11.9 ± 0.42
ES	—	—	15.4 ± 2.7	19.5 ± 0.46
EP	—	—	11.4 ± 1.8	7.3 ± 0.47

Figure 4 Time course of hydrolysis of cCMP at different enzyme concentrations in the presence of 1 mM bromopyruvic acid

Experimental conditions were as for Figure 1 except that 1 mM bromopyruvic acid was included in the reaction system. The cCMP concentration was 0.27 mM; the concentration of RNase A in curves 1–4 was 50, 100, 150 and 250 nM respectively.

second-order polynomial equations in product concentration [28]:

$$t = a + b[P] + c[P]^2 + \dots \quad (16)$$

The initial rate, $(d[P]/dt)_{t=0}$, was taken to be a measure of $1/b$ [29] (where b is the reciprocal of V_0).

As with reversible inhibition, eqn. (11) can be rewritten:

$$\frac{1}{v_0} = \frac{K_s}{k_2[E]_0} \left(1 + \frac{[Y]}{K_1} \right) \frac{1}{[S]_0} + \frac{1}{k_2[E]_0} \left(1 + \frac{[Y]}{K'_1} \right) \quad (17)$$

Figure 3 shows the double-reciprocal plots of $1/v_0$ against $1/[S]_0$ at different concentrations of bromopyruvic acid. Both the slopes and the ordinate intercepts increase with increasing [Y]. Secondary plots of the slopes and the ordinate intercepts against [Y] both give straight lines (the inset to Figure 3). From the slopes and intercepts of the secondary plots, the values of K_1 and K'_1 were calculated and are listed in Table 1.

Figure 4 shows the time courses of substrate reaction during modification at different enzyme concentrations. To determine the value of K'_1 , the reaction rates at a fixed product concentration, v_i , were calculated from the tangents of the experimental curves. It can be seen from eqn. (12) that the value of v_i is proportional to $[E]_0$ (Figure 5) and the slopes decrease as the product concentrations increase. The inset to Figure 5 shows a secondary plot of $1/\gamma$ against $1/([S]_0 - [P])$. Because K_s , K_P , K_1 and K'_1 have been determined previously, K'_1 can be calculated as 11.4 ± 1.8 mM from the slope, by using eqn. (13).

Figure 5 Plots of velocity against enzyme concentration at a series of fixed product concentrations

The product concentrations in curves 1–4 were 0.12, 0.13, 0.14 and 0.17 mM respectively. The inset is a double-reciprocal plot of the slopes against $([S]_0 - [P])$.

Figure 6 Plot of $[P]_{\infty}$ against $[E]_0$

The cCMP concentrations in curves 1–5 are 0.2, 0.3, 0.4, 0.5 and 0.6 mM respectively. The dots are experimental points; the curves are fitted results.

Determination of the inactivation rate constants

It can be seen from Figure 4 that with increasing reaction time the concentrations of product formed approach finite values, $[P]_{\infty}$. Figure 6 shows plots of $[P]_{\infty}$ against $[E]_0$ in the presence of 1 mM bromopyruvic acid at different cCMP concentrations. The plots of $[E]_0/[P]_{\infty}$ against $(1/[P]_{\infty}) \ln \{[S]_0/([S]_0 - [P]_{\infty})\}$ give a series of straight lines (Figure 7) as predicted by eqn. (8). According to eqn. (9), a secondary plot of s against $[S]_0$ also gives a straight line (the inset to Figure 7). The value of k_{+0}/K_I was calculated as $0.153 \text{ M}^{-1} \cdot \text{s}^{-1}$ from the ordinate intercept; k''_{+0}/K_I'' was calculated as $0.064 \text{ M}^{-1} \cdot \text{s}^{-1}$ from the slope. From the value of i , k'_{+0}/K_I' was calculated as $0.127 \text{ M}^{-1} \cdot \text{s}^{-1}$ by using eqn. (10). Therefore all the values of the inactivation rate constants can be determined separately; they are listed in Table 1.

Figure 7 Plots of $[E]_0/[P]_{\infty}$ against $(1/[P]_{\infty}) \ln \{[S]_0/([S]_0 - [P]_{\infty})\}$ to determine the kinetic constants in accordance with eqn. (8)

Curves 1–3 correspond to curves 1, 3 and 5 respectively in Figure 6. The inset shows a secondary plot of the slopes against $[S]_0$.

An alternative way is to determine the inactivation constants by fitting the relation between $[E]_0$ and $[P]_{\infty}$ to eqn. (7). The solid lines in Figure 6 are fitted results. The values of s and i are in agreement with those determined by the plotting method.

DISCUSSION

The conventional method for determining the rate constant for the irreversible modification of enzyme activity is to take aliquots from an enzyme–modifier incubation mixture at fixed time intervals and assay for the enzyme activity. This method is laborious and not easily applied to fast reactions with a half-life of less than 1 min. During the past decade the kinetics of the irreversible modification of enzyme activity has been studied systematically. Experimental studies have produced useful results in this and other laboratories [6,16,17,20,21,23,30–37]. In comparison with the conventional method, the advantage of the progress-curve method is not only its usefulness in the study of fast modification reactions but its convenience in the study of substrate effects on the interaction between the modifier and the enzyme.

Most of the previous studies on the kinetics of the irreversible modification of enzyme activity were performed under the conditions in which the change in substrate concentration can be neglected and no product inhibition exists. The cases where the conditions are not satisfied were considered theoretically some years ago [3,5]. In the present paper, the inactivation kinetics of RNase A by bromopyruvic acid, where strong product inhibition exists, has been studied by the latter method. Our result indicates that the inactivation follows a non-competitive complexing mechanism (Scheme 1). This type of inhibition has been reported recently in the study of modification of the aminoacylase thiol group by 2-chloromercuri-4-nitrophenol [23]. As pointed out above, for the non-competitive complexing mechanism it is very difficult to obtain all information on the reaction of the modifier with the enzyme by the conventional method.

The mechanism of RNase A that catalyses the hydrolysis of

Figure 8 Schematic representation of the proposed structure of the enzyme (a), the enzyme-cCMP complex (b), the enzyme-transition state (c) and the enzyme-3'-CMP complex (d)

single-stranded ribonucleotide phosphodiester is believed to consist of a transphosphorylation by which the 3',5'-phosphodiester is transesterified to a cyclic 2',3'-phosphate and a hydrolysis by which the cyclic 2',3'-phosphate is converted to a 3'-phosphate [26,38,39]. In the hydrolysis step, His-119 acts as a base to accept a proton from water and then attacks the 2',3'-cyclic phosphate; His-12 then acts as an acid to donate a proton to the O2' atom, thus forming the 3'-nucleotide. Note that according to this mechanism, the imidazole group of His-119 is protonated in the enzyme-substrate complex, but deprotonated in both the enzyme-transition state and the enzyme-product complex (see Fig. 12 of [26]).

The modification of His-119 of RNase A by haloacids has been widely studied [38,39]. The mechanism is thought to be a nucleophilic reaction in which the deprotonated imidazole of His-119 is attacked by the modifier [40,41]. The nucleophilic deprotonated form of His-119 can facilitate the reaction, whereas the protonation of His-119 would reduce the rate of modification. Thus it would be expected that His-119 in the free enzyme and in the enzyme-substrate and enzyme-product complexes should react with bromopyruvic acid at different rates. In the present study, however, we found that the modification reaction is affected by the binding of neither the substrate nor the product.

This result suggests that (1) His-119 might be involved in catalysis and not in substrate binding, and (2) the ionization state of the imidazole of His-119 might be the same in both the enzyme-substrate and enzyme-product complexes. Therefore the catalytic mechanism of RNase A presented by Eftink and Biltonen [26] can be revised as shown in Figure 8. According to this mechanism, the pK_a of the protonated imidazole hydrogen ion of His-119 is not affected by binding of either the substrate or the product, and thus at a given pH the concentration of the unprotonated His-119 is unaffected by the substrate or the product. The ionization state will change transiently during catalysis if His-119 acts as a general base and will then re-equilibrate to the dissociation equilibrium position dictated by the pH and pK_a .

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