# Correlative variations of the free energies for enzyme–substrate complex formation and the transition-state stabilization for RNases

Gennady I. Yakovlev, Alexander L. Bocharov and Gennady P. Moiseyev

*Institute of Molecular Biology, USSR Academy of Sciences, 117984, Vavilov street 32, Moscow, USSR*

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It was found for RNases of different specificities that changes in the free energy for substrate–enzyme binding induced by variations in the nucleotide base structure are accompanied by proportional changes in $k_{cat}/K_m$. This was considered to be a consequence of the strain in the enzyme–substrate complex.

*RNase* *Transition state* *Enzyme–substrate complex*

## 1. INTRODUCTION

The catalytic power of enzymes in terms of equilibrium thermodynamics is associated with structural complementarity of their active sites to the transition state of the appropriate uncatalyzed reaction [1,2]. The $k_E/k_{NE}$ ratio of rate constants for enzymatic and non-enzymatic reactions is expressed as

$$\frac{k_E}{k_{NE}} = \frac{K_S}{K_{TS}} \quad (1)$$

where $K_S$ and $K_{TS}$ are dissociation constants for the enzyme–substrate complex at the ground and transition states. In order to comprehend mechanisms of enzyme action, one has to know in detail what makes the ratio $K_{TS} \ll K_S$ be realized.

Nucleotide heterocyclic bases are a necessary structural component of RNase substrates [3,4]. As was demonstrated in this study for the 3 RNases of different specificity, the change of free energy for substrate–enzyme binding $\Delta(\Delta G_S)$ induced by structural rearrangements in nucleotide bases correlates with variations in the stabilization energy of the enzyme–substrate complex transition state $\Delta(\Delta G_{TS} - \Delta G_S)$.

## 2. MATERIALS AND METHODS

Electrophoretically pure samples of pyrimidine-specific RNase A, *B. intermedius* 7P guanyl-specific RNase and *P. brevicompactum* non-specific RNase were prepared as described in [5–7]. Nucleoside cyclophosphates with natural bases were obtained by cyclization of a 2′- and 3′-phosphate mixture [8]. The synthesis and properties of nucleotides with modified bases are described in detail in [9].

The rate of enzymatic hydrolysis of cyclophosphates was measured using pH-stat method (Radiometer pH-stat) by measuring the rate of acid production induced by the formation of nucleoside 3′-phosphates and the secondary ionization of their phosphate groups. Automatic pH-titration was performed by adding a $5.6 \times 10^{-4}$ M KOH solution into a cuvette with an ABU-12 burette. The solution was stirred with a magnet mixer. The cuvette was thermostatted at 25°C, the reaction mixture volume being 2.05–2.10 ml. The volume of the KOH solution added at the pH-stating was not more then 0.2 ml. The concentration of substrates was determined using a Beckman 26 (USA) spectrophotometer. The molar extinction coefficients were taken according to [9,10]. The initial reaction



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rates were found from the kinetic curves registered within 5–10 min. In order to obtain more accurate kinetic parameters, the reaction rate was calculated at zero time as in [11]. The incomplete secondary ionization of phosphate groups at the given pH was calculated, p$K$ being taken as 5.9. The inaccuracy of the assumed p$K$ values 0.2 yields erroneous $k_{cat}$ values less than 7%.

## 3. RESULTS AND DISCUSSION

One can see from fig. 1 and 2 that, for hydrolysis of nucleoside 2′,3′-cyclophosphates by each RNase, ln ($k_{cat}/K_m$) and ln$K_m$ are related linearly.

It may be assumed that there is a small difference in the rate constants $k_{NE}$ for congruent non-enzymatic reactions in the series of cyclophosphates for each RNase. This assumption is supported by the small difference in rate constants of acid- or base-catalysed hydrolysis of Cyt- and Ado-2′,3′-P [12]. Then, taking into account that in the enzymatic hydrolysis of nucleoside-2′,3′-cyclophosphates $K_M = K_S$ [3,6,7] it can be obtained in accordance with eq. (1), that the tangent of the slope angle $\xi$ for the straight lines in fig. 1 and 2 is

$$\xi = -\frac{\Delta(\ln (k_{cat}/K_m))}{\Delta(\ln K_m)} = \frac{\Delta(\ln K_{TS})}{\Delta(\ln K_S)} = \frac{\Delta(\Delta G_{TS})}{\Delta(\Delta G_S)} \quad (2)$$

where $\Delta G_{TS} = RT \ln K_{TS}$ and $\Delta G_S = RT \ln K_S$.

The $\xi$ values for RNase A and *B. intermedius* 7P RNase were found to be 1.23 and 1.25 and for *P.*

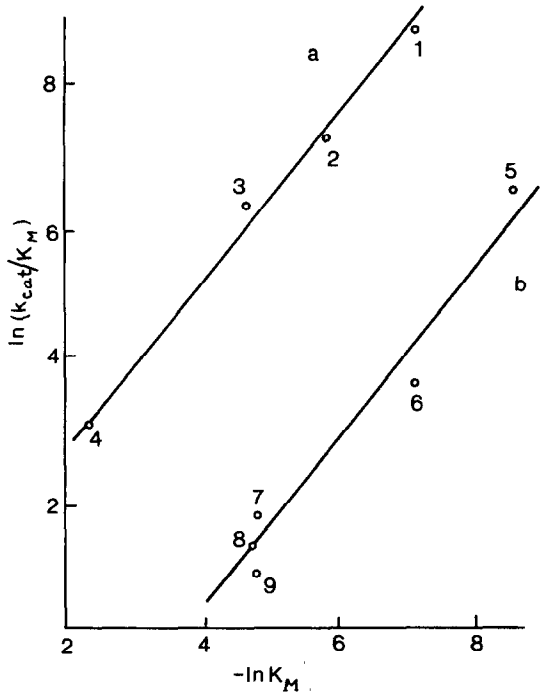


Fig. 1. The dependence of ln ($k_{cat}/K_m$) *vs* ln $K_m$ in the hydrolysis of nucleoside 2′,3′-cyclophosphates with RNase A at pH 6.5 (a) and *B. intermedius* 7P RNase at pH 7.0 (b). I = 0.2 M NaCl. Nucleosides: 1, cytidine; 2, uridine; 3, 2-pyrimidone-β-D-ribofuranoside; 4, 2-pyridone-β-D-ribofuranoside; 5, guanosine; 6, inosine; 7, xantosine; 8, 1-Me-inosine; 9, virasole.

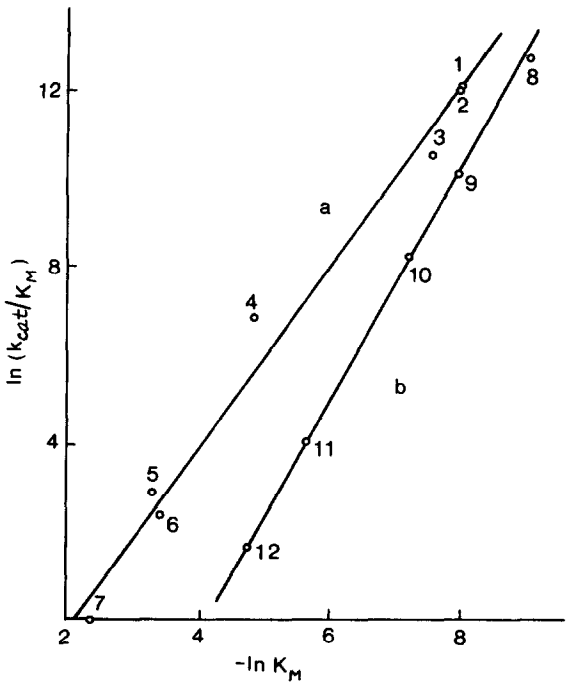


Fig. 2. The dependence of ln ($k_{cat}/K_m$) *vs* ln$K_m$ in the hydrolysis of pyrimidine (a) and purine (b) nucleoside 2′,3′-cyclophosphates with *P. brevicompactum* RNase at pH 5.7 and I = 0.2 M NaCl. Nucleosides: 1, cytidine; 2, uridine; 3, 2-pyrimidone-β-D-ribofuranoside; 4, 2-pyridone-β-D-ribofuranoside; 5, 4-pyrimidone-β-D-ribofuranoside; 6, 4-pyridone-β-D-ribofuranoside; 7, urea-β-D-ribofuranoside; 8, adenosine; 9, guanosine; 10, inosine; 11, xantosine; 12, benzimidasole-β-D-ribofuranoside.

*brevicompactum* RNase 2.14 (pyrimidines) and 2.56 (purines). Thus, the free energy change of transition state stabilisation, $\Delta(\Delta G_{TS} - \Delta G_S)$, induced by the interaction of enzyme and substrate base is $[(\xi-1)/\xi]\Delta(\Delta G_S)$.

Upon hydrolysis of nucleoside 2′,3′-cyclophosphates the structure of the transition state is close to that of corresponding 2′,3′-cyclophosphoranes [13]. Evidently, in the absence of strain in the transition state of enzyme-substrate complex, the change in the energy for the interaction of substrate bases with protein $\Delta(\Delta G_{base})$ is accompanied by an identical change in $\Delta(\Delta G_{TS})$. On the other hand, $\Delta(\Delta G_{base}) = \xi \cdot \Delta(\Delta G_S)$. Then the fact that the value $\xi$ exceeds unity proves the existence of strains in the enzyme-substrate complex in the ground state. This implies that some part of the energy for the interaction between a nucleotide and a protein expressed as $(\xi-1)/\xi$ is conserved in the form of the enzyme-substrate complex strain. The linear dependence of $\ln(k_{cat}/K_m)$ vs $\ln K_m$ can then be assumed to result from the substrate strain in the enzyme-substrate complex.

The change of $\Delta G_S$, when some base groups of natural nucleosides are eliminated, demonstrates that these groups interact with the protein in the ground state of enzyme-substrate complex. In the case of RNase A, the elimination of the exocyclic group at position 4 of the pyrimidine base or the replacement of the nitrogen atom at position 3 for the carbon atom makes the $K_m$ increase. This proves that these groups are involved in the formation of the Michaelis complex, which is doubted by the authors of [14,15].

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