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Temperature-dependent change in the rate-determining step in a reaction catalyzed by a hammerhead ribozyme

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Abstract To characterize the reaction catalyzed by a hammerhead ribozyme, the dependence on temperature of the reaction was examined. An Arrhenius plot revealed a transition that indicated a temperature-dependent change in the activation energy at around 25°C. Thermodynamic parameters of the reaction were estimated at 10 and 35°C. The analyses led to the following conclusions. At 25–50°C, the chemical cleavage step (k_{cleav}) was the rate-determining step, and the cleaved fragments dissociated from the ribozyme at a higher rate than the rate of the chemical reaction. When the temperature was below 25°C, the cleaved fragments adhered to the ribozyme more tightly and the product dissociation step became the rate-determining step. Above 50°C, the rate of the reaction decreased because, at such high temperatures, the formation of the Michaelis–Menten complex (duplex formation) was hampered by thermal melting. A conformational change in the ribozyme–substrate complex was not the rate-determining step at any of the temperatures examined.

Key words: Ribozyme; Hammerhead; Arrhenius plot; Kinetics; Thermodynamics

1. Introduction

The hammerhead ribozyme is one of the smallest RNA enzymes [1–3]. Because of its small size and potential utility as an antiviral agent, it has been extensively investigated in terms of the mechanism of its action and possible applications in vivo [1–7]. In naturally occurring hammerhead ribozymes, reactions are catalyzed in *cis* (intramolecularly), with the target and catalytic strands being part of a single RNA molecule. The *trans*-acting hammerhead ribozyme developed by Haseloff and Gerlach [3] consists of an antisense section (stems I and III) and a catalytic domain with a flanking stem II/loop section (Fig. 1). The minimum reaction scheme can be described as shown in Fig. 2. First, the substrate (and Mg^{2+} ions) binds to the ribozyme to form a Michaelis–Menten complex via formation of base pairs with stems I and III (k_{assoc}). Then, a specific phosphodiester bond in the bound substrate is cleaved by the action of Mg^{2+} ions (k_{cleav}; the ribozyme is recognized to function as a metalloenzyme [5,8–14]). This cleavage produces products with 2′,3′-cyclic phosphate and 5′-hydroxyl groups. Finally, the cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events (k_{diss}).

In the reactions catalyzed by many protein enzymes, the kinetics are not as simple as those described above since, in many cases, a conformational change can become the rate-determining step [15]. In this study, we examined the possibility of a rate-determining conformational change in a hammerhead ribozyme at low temperature by measuring the dependence on temperature of k_{cat}. An Arrhenius plot revealed distinct changes in the rate-determining step. A combination of single- and multiple-turnover kinetics in the Arrhenius plot revealed that a conformational change was not the rate-determining step.

2. Materials and methods

2.1. Synthesis of the ribozyme and its substrate

The ribozyme and its corresponding substrate were synthesized on an ABI DNA/RNA synthesizer (model 392; Applied Biosystems, Foster City, CA) and purified by HPLC and electrophoresis in a polyacrylamide gel as described previously [13,16,17]. RNA reagents were purchased from American Bionetics Inc. (ABN; Hayward, CA). Other reagents were purchased either from ABI or ABN. Purification of the synthesized oligonucleotides was performed as described in the ABI user bulletin (no. 53; 1989) with minor modifications.

2.2. Kinetic measurements

The 5′ terminus of the substrate was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Reaction rates were measured in 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0; adjusted at each temperature), either (i) under ribozyme-saturating (single-turnover) conditions at 0°C or (ii) under substrate-saturating (multiple-turnover) conditions over a range of temperatures from 15 to 60°C. In all cases, kinetic measurements were made under conditions where all the available ribozyme or substrate was expected to form a Michaelis–Menten complex. These conditions were achieved by employing high concentrations of either the ribozyme (< 3.8 μM) or the substrate (< 1.1 μM). The K_m value of the ribozyme for its substrate was 0.02 μM at 37°C under the present conditions [13,16,17].

Reactions were stopped by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equal volume of a solution of 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% Bromophenol blue. Substrates and 5′-cleaved products were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and product with a Bio-Image Analyzer (BA100 or BAS2000; Fuji Film, Tokyo).

3. Results and discussion

The activation energy for a reaction can be determined by measuring the reaction rate constant (k) at different temperatures and plotting $\ln k$ vs. $1/T$ (to yield a so-called Arrhenius plot). The Arrhenius plot itself may be non-linear if different steps become the rate-determining step at different tempera-

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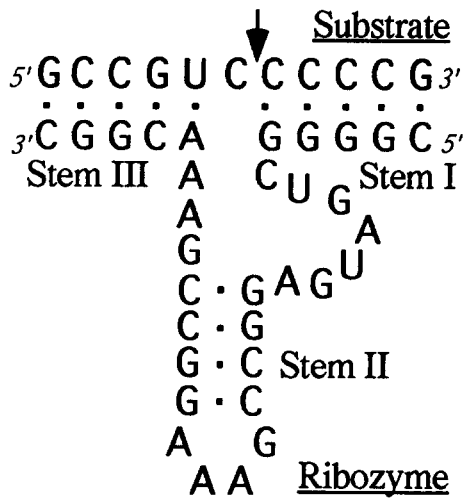

Fig. 1. Secondary structure of the hammerhead-type ribozyme and the substrate used in this experiment. The arrow indicates the cleavage site.

tures. In some cases, the plot may show a sharp change in slope at a temperature ('transition temperature') at which the rate-determining step changes from one to another. Arrhenius plots have been used to detect such changes in standard enzyme-catalyzed reactions [18]. There has been only one reported study to our knowledge in which such a plot has been used to characterize ribozyme-catalyzed reactions [2]. However, no conspicuous change in the slope of the plot was recognized. We wondered whether a sharp change in slope might be detectable with a better-defined ribozyme system [13,16,17] and whether a specific step in the ribozyme-catalyzed reaction could be correlated with such a change.

The results of our analysis are shown in Fig. 3 in which distinct changes in the slope of the plot can be recognized. The plot provides evidence for three different rate-determining steps in the reaction. Arrhenius activation energies were calculated to be 16.0 kcal/mol at mid-range temperatures (25–50°C) and 47.7 kcal/mol at lower temperatures (<25°C). At 25–50°C, the chemical cleavage step (k_{cleav}) was clearly the rate-determining step because no burst kinetics were detected at the measurement temperature of 37°C [17]. Therefore, (i) the cleaved fragments dissociated from the ribozyme at a higher rate than the rate of the chemical reaction, and (ii) any conformational change prior to the cleavage reaction, if such a change occurred, was also rapid ($k_{cleav} < k_{conf}, k_{diss}$). When the temperature of the reaction was below 25°C, a change in the rate-determining step was recognized. The rate-determining step at lower temperatures could reflect either a conformational change or it could correspond to the product dissociation step. In order to distinguish between these two possibilities, k_{cat} was measured at 0°C under conditions of an excess of ribozyme ([ribozyme] >> [substrate]: single-turnover conditions). Under these conditions the product dissociation step becomes irrelevant. If the rate-determining step below 25°C is a conformational change (k_{conf}), required for the formation of the activated complex, the k_{cat} value at 0°C would fall on the extrapolated line at temperatures between 25–0°C. By contrast, if the conformational change occurred more rapidly than the chemical cleavage step ($k_{conf} > k_{cleav}$) and if the rate-determining step below 25°C is the product dissociation step, then the observed k_{cat} at 0°C would fall on the line extrapolated from the region that corresponds to 50–25°C. The measured k_{cat} value at 0°C, indicated by the open square in Fig. 3, supports the latter possibility: the rate-determining step changes upon a decrease in the reaction temperature from the chemical cleavage step to the product dissociation step without the involvement of a rate-determining conformational change. Schematic energy diagrams for these two putative processes are shown in Fig. 4.

Above 50°C, the rate of the reaction decreased, probably because, at such high temperatures, the formation of the Michaelis–Menten complex was hampered by thermal melting (therefore, the rate of the reaction above 50°C does not reflect k_{cat}). The melting temperature (T_m) of stem II of this ribozyme was 58–60°C (data not shown).

Parameters from the Arrhenius plot were converted to thermodynamic activation parameters by application of transition state theory [15]. The free energy of activation, $\Delta G^{\neq}$, is directly related to the reaction rate. $\Delta G^{\neq}$ is given by $-RT\ln(kh/k_BT)$

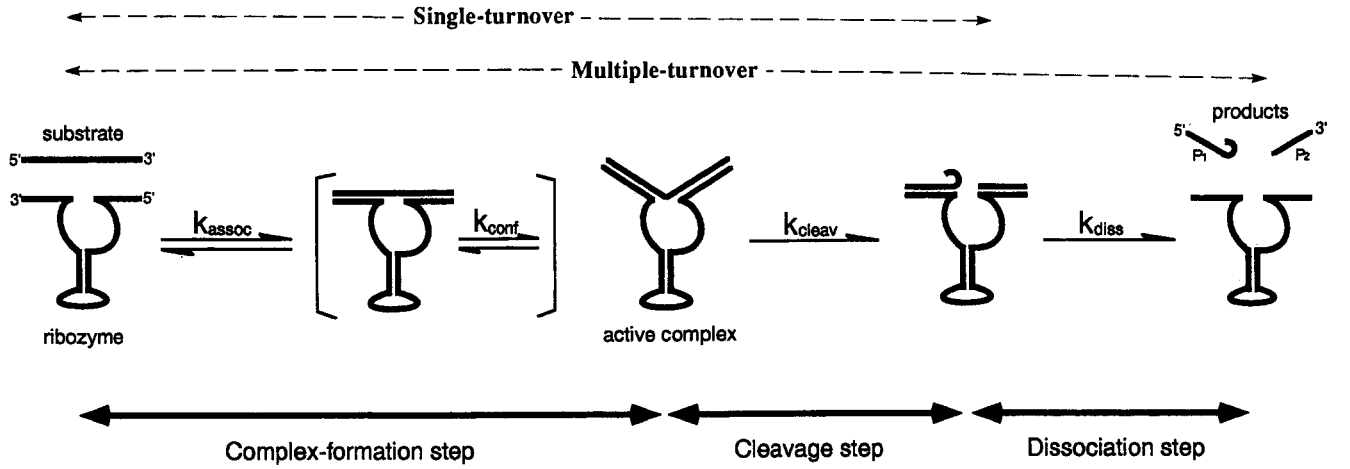

Fig. 2. Schematic representation of the kinetics of the ribozyme-catalyzed reaction. The reaction catalyzed by the hammerhead ribozyme consists of at least three steps. The substrate (and Mg^{2+} ions) first binds to the ribozyme (k_{assoc}). The phosphodiester bond of the bound substrate is cleaved by the action of Mg^{2+} ions (k_{cleav}). The cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events (k_{diss}).

where k is the rate constant at temperature T; h is Planck's constant and k_B is Boltzmann's constant. The enthalpy of activation, $\Delta H^{\neq}$, is a measure of the energy barrier that must be overcome by reacting molecules. $\Delta H^{\neq}$ is given by $E_a - RT$ where R is the gas constant and E_a is the energy of activation. The entropy of activation, $\Delta S^{\neq}$, is a measure of the fraction of reactants with sufficient activation enthalpy that can actually react; it includes, for example, concentration and solvent effects, steric requirements and orientational requirements. $\Delta S^{\neq}$ is equivalent to $(\Delta H^{\neq} - \Delta G^{\neq})/T$.

The energy parameters for the multiple-turnover ribozyme-catalyzed reaction at 10°C, where the dissociation step is the rate-determining step, and at 35°C, where the cleavage step is the rate-determining step, were calculated and are shown in Table 1. Naturally, $\Delta G^{\neq}$ (k_{cat}) incorporates $\Delta H^{\neq}$ and $T\Delta S^{\neq}$. $\Delta H^{\neq}$, at a reaction temperature of 35°C, with the cleavage step being the rate-determining step, was calculated to be 15.4 kcal/mol. $\Delta H^{\neq}$, at a reaction temperature of 10°C, at which the dissociation step was the rate-determining step (47.1 kcal/mol), turned out to be significantly larger than the corresponding

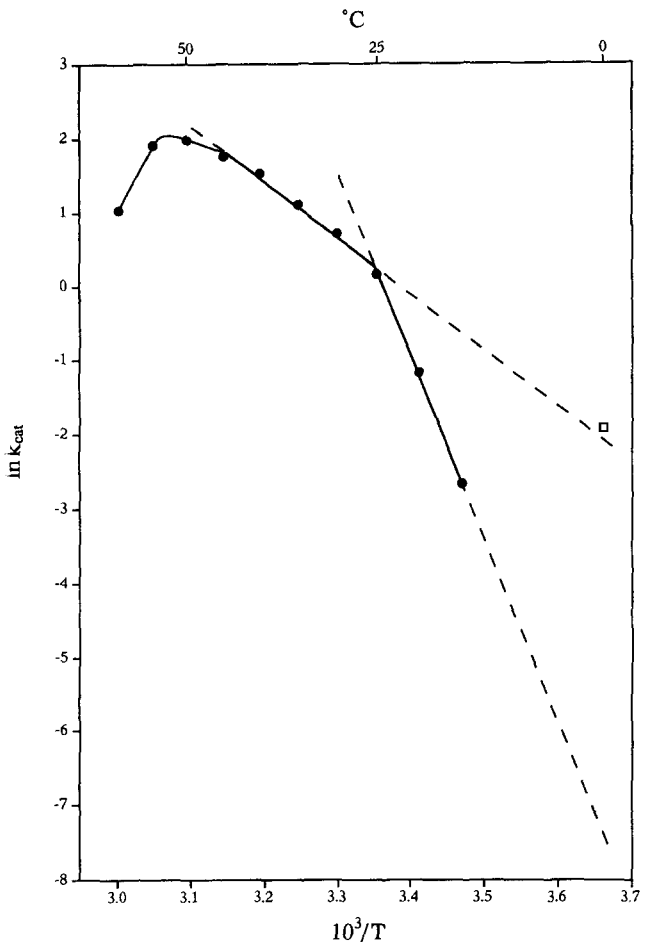

Fig. 3. Arrhenius plot of the ribozyme-catalyzed reaction under multiple-turnover conditions. Arrhenius activation energies were calculated to be 16.0 kcal/mol at mid-range temperatures and 47.7 kcal/mol at lower temperatures. Reactions were carried out with 0.0024 μM ribozyme and 1.1 μM substrate (●) or 3.8 μM ribozyme and 0.1 μM substrate (□). The optimal temperature for the ribozyme-catalyzed reaction was about 50°C.

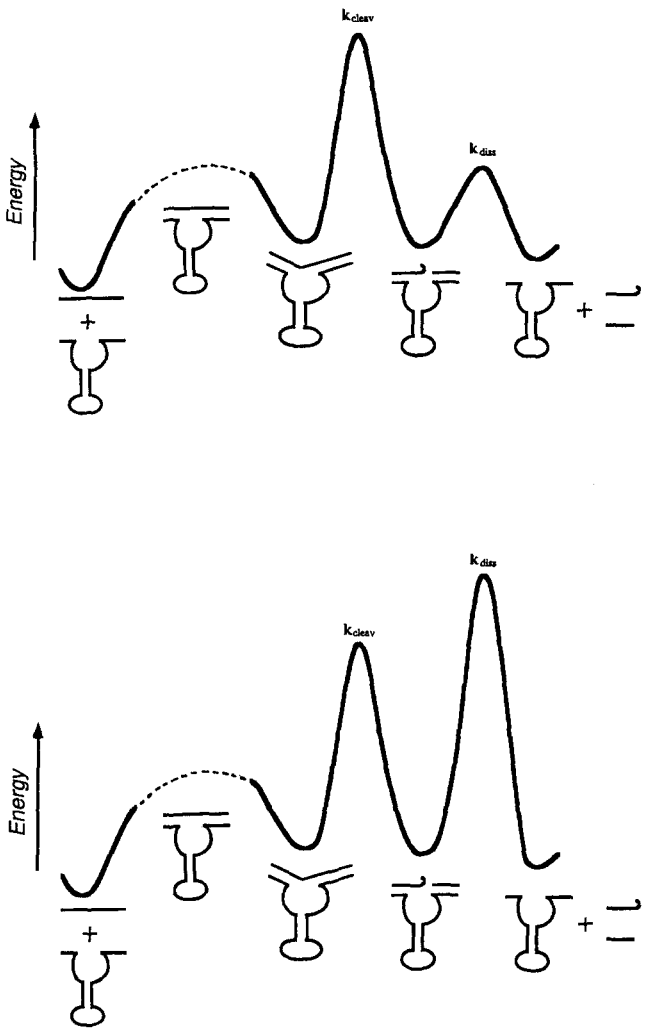

Fig. 4. Qualitative energy diagrams for the ribozyme-catalyzed reaction. The rate-determining step changes upon a decrease in the reaction temperature from the chemical cleavage step (top) to the product-dissociation step (bottom) without the appearance of a rate-determining change in conformation.

value of $\Delta H^{\neq}$ at 35°C (15.4 kcal/mol). In agreement with the above assignment of the rate-determining steps, (i) $\Delta S^{\neq}$ at 35°C is negative (−14.6 eu), a result that suggests the existence, during the transition state of the chemical cleavage process, of some ordered structure that involves, for example, 'Mg^{2+}-mediated torsion' around the cleavage site in ribozyme-substrate complex [14]; whereas (ii) $\Delta S^{\neq}$ at 10°C, at which the product dissociation step is the rate-determining step, is positive (+91.6 eu), reflecting the partial release of the cleavage products from the ribozyme.

The conclusion derived from the thermodynamic parameters (Table 1) is pertinent to that obtained from Fig. 3: the rate-determining step changes, upon a decrease in the reaction temperature, from the chemical cleavage step to the product dissociation step (reflected by positive values of $\Delta S^{\neq}$ below 25°C) without involvement of a rate-determining change in conformation.

In conclusion, in the reaction catalyzed by the hammerhead ribozyme, the rate-determining steps were as follows. (i) At 25–50°C, the chemical cleavage step (k_{cleav}) was the rate-deter-

Table 1
Thermodynamic parameters

Reaction temperature (°C)	$\Delta G^{\neq}$ (kcal/mol)	$\Delta H^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (eu)
10	21.2	47.1	+ 91.6
35	19.9	15.4	− 14.6

The calculations are based on the transition state theory. Entropy values are given in eu (cal/mol·°K).

mining step; the cleaved fragments dissociated from the ribozyme at a higher rate than the rate of the chemical reaction and the transition state had an ordered structure, as reflected by a negative value of $\Delta S^{\neq}$. (ii) When the temperature was below 25°C, the cleaved fragments adhered to the ribozyme more tightly and the product dissociation step became the rate-determining step (reflected by a positive value of $\Delta S^{\neq}$) without the appearance of a rate-determining conformational change, because the apparent k_{cat} measured at 0°C in the single-turnover experiment fell almost on the extrapolated line from the slope between 15°C and 25°C;. (iii) Above 50°C, the rate of the reaction decreased because, at high temperatures, the formation of the Michaelis–Menten complex (duplex formation) was hampered by thermal melting. This kind of analysis should be useful in characterizing the reactions catalyzed by other types of ribozyme, including engineered ribozymes.

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