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

Yasuomi Takagi\textsuperscript{a,}\textsuperscript{**, Kazunari Taira\textsuperscript{a,}\textsuperscript{b,}\textsuperscript{*}

\textsuperscript{a}National Institute of Bioscience and Human Technology, AIST, MITI, Boston, MA, USA
\textsuperscript{b}Institute of Applied Biochemistry, University of Tsukuba, Tsukuba Science City 305, Japan

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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 \((k_{\text{chem}})\) 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 transacting hammerhead ribozyme developed by Haseloff and Gerach [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\textsuperscript{2+} ions) binds to the ribozyme to form a Michaelis–Menten complex via formation of base pairs with stems I and III \((k_{\text{enzyme}})\). Then, a specific phosphodiester bond in the bound substrate is cleaved by the action of Mg\textsuperscript{2+} ions \((k_{\text{chem}})\); 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 \((k_{\text{end}})\).

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 \(k_{\text{cat}}\). 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 Bioinetics 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 \([\gamma-^{32}\text{P}]\text{ATP}\) using T4 polynucleotide kinase. Reaction rates were measured in 25 mM Mg\textsubscript{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-
Fig. 1. Secondary structure of the hammerhead-type ribozyme and the substrate used in this experiment. The arrow indicates the cleavage site.

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 \(k_{\text{assoc}}\). The phosphodiester bond of the bound substrate is cleaved by the action of Mg\(^{2+}\) ions \(k_{\text{cleav}}\). The cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events \(k_{\text{diss}}\).
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, $\Delta H^\ddagger$, is a measure of the energy barrier that must be overcome by reacting molecules. $\Delta H^\ddagger$ is given by $E_a - RT$ where $R$ is the gas constant and $E_a$ is the energy of activation. The entropy of activation, $\Delta S^\ddagger$, 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. $\Delta S^\ddagger$ is equivalent to $(\Delta H^\ddagger - \Delta G^\ddagger)/T$.

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, $\Delta G^\ddagger (k_{cat})$ incorporates $\Delta H^\ddagger$ and $T\Delta S^\ddagger$. $\Delta H^\ddagger$, at a reaction temperature of 35°C, with the cleavage step being the rate-determining step, was calculated to be 15.4 kcal/mol. $\Delta H^\ddagger$, 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 value of $\Delta H^\ddagger$ at 35°C (15.4 kcal/mol). In agreement with the above assignment of the rate-determining steps, (i) $\Delta S^\ddagger$ 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) $\Delta S^\ddagger$ 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 (top) to the product-dissociation step (bottom) without the appearance 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 ($k_{cat}$) was the rate-deter-
Table 1
Thermodynamic parameters

<table>
<thead>
<tr>
<th>Reaction temperature (°C)</th>
<th>ΔG° (kcal/mol)</th>
<th>ΔH° (kcal/mol)</th>
<th>ΔS° (eu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>21.2</td>
<td>47.1</td>
<td>+91.6</td>
</tr>
<tr>
<td>35</td>
<td>19.9</td>
<td>15.4</td>
<td>-14.6</td>
</tr>
</tbody>
</table>

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 ΔS°. (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 ΔS°) without the appearance of a rate-determining conformational change, because the apparent kcat 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.

References