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Limits to Catalysis by Ribonuclease A

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Bovine pancreatic ribonuclease A (RNase A) catalyzes the cleavage of the P–$O^{5'}$ bond in RNA. Although this enzyme has been the object of much seminal work in biological chemistry, the nature of its rate-limiting transition state and its catalytic rate enhancement had been unknown. Here, the value of k_{cat}/K_m for the cleavage of UpA by wild-type RNase A was found to be inversely related to the concentration of added glycerol. In contrast, the values of k_{cat}/K_m for the cleavage of UpA by a sluggish mutant of RNase A and the cleavage of the poor substrate $UpOC_6H_4$-*p*-NO_2 by wild-type RNase A were found to be independent of glycerol concentration. Yet, the values of k_{cat}/K_m for UpA cleavage by the wild-type and mutant enzymes were found to have an identical dependence on the concentration of added sucrose. Although both glycerol and sucrose are viscogenic, only glycerol interacts strongly with single-stranded nucleic acids. Catalysis of UpA cleavage by RNase A is therefore limited by substrate desolvation. The rate of UpA cleavage by RNase A is maximal at pH 6.0, where $k_{cat} = 1.4 \times 10^3\ s^{-1}$ and $k_{cat}/K_m = 2.3 \times 10^6\ M^{-1}\ s^{-1}$ at 25°C. At pH 6.0 and 25°C, the uncatalyzed rate of [5,6-^{3}H]Up[3,5,8-^{3}H]A cleavage was found to be $k_{uncat} = 5 \times 10^{-9}\ s^{-1}$ ($t_{1/2}$ = 4 years). Thus, RNase A enhances the rate of UpA cleavage by 3×10^{11}-fold by binding to the transition state for P–$O^{5'}$ bond cleavage with a dissociation constant of $<2 \times 10^{-15}$ M.

INTRODUCTION

Catalysis has its limits. On one extreme, the rate of a "catalyzed" reaction must exceed that of the same reaction in the absence of the catalyst. At the other extreme, the rate of a catalyzed reaction cannot exceed the rate at which the catalyst binds to its substrate or releases its product.

The energetics of enzymatic catalysis are defined by these limits. Enzymes catalyze reactions by binding to the transition states for the chemical interconversion of substrate and product (*1–3*). The ability of an enzyme to bind to this transition state is given by the ratio of k_{cat}/K_m for the enzyme-catalyzed reaction and k_{uncat} for the nonenzymatic reaction (*4*). This analysis requires, however, that the catalyzed and uncatalyzed reactions have the same rate-limiting transition state. Thus, calculating the transition state binding affinity from the value of k_{cat}/K_m requires that catalysis be limited by the conversion of enzyme-bound substrates to enzyme-bound products. This constraint does not always (or even often) hold (*5, 6*). If the free energy of the transition state for substrate binding or product release exceeds that for the interconversion of Michaelis complexes, then the decrease in k_{cat}/K_m reflects only a fraction of the ability of an enzyme to bind to the chemical transition state.

Despite its considerable importance to biological chemistry, information on the

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uncatalyzed rates of metabolic reactions is now sparse (*7*). The information coffers are relatively full, however, for the uncatalyzed cleavage of biopolmers. For example, the rate constant for the uncatalyzed cleavage of a peptide bond has been measured at 25°C and near-neutral pH (*8*). Although too slow to measure directly, the rate constant for the uncatalyzed cleavage of DNA can been inferred from data for the hydrolysis of dimethyl phosphate (*7*). Here, we measure the rate constant for the cleavage of a P–$O^{5'}$ bond in RNA at 25°C and near-neutral pH in the absence of a catalyst. We also elucidate the nature of the rate-limiting transition state in catalysis by bovine pancreatic ribonuclease A (RNase A^1; EC 3.1.27.5),

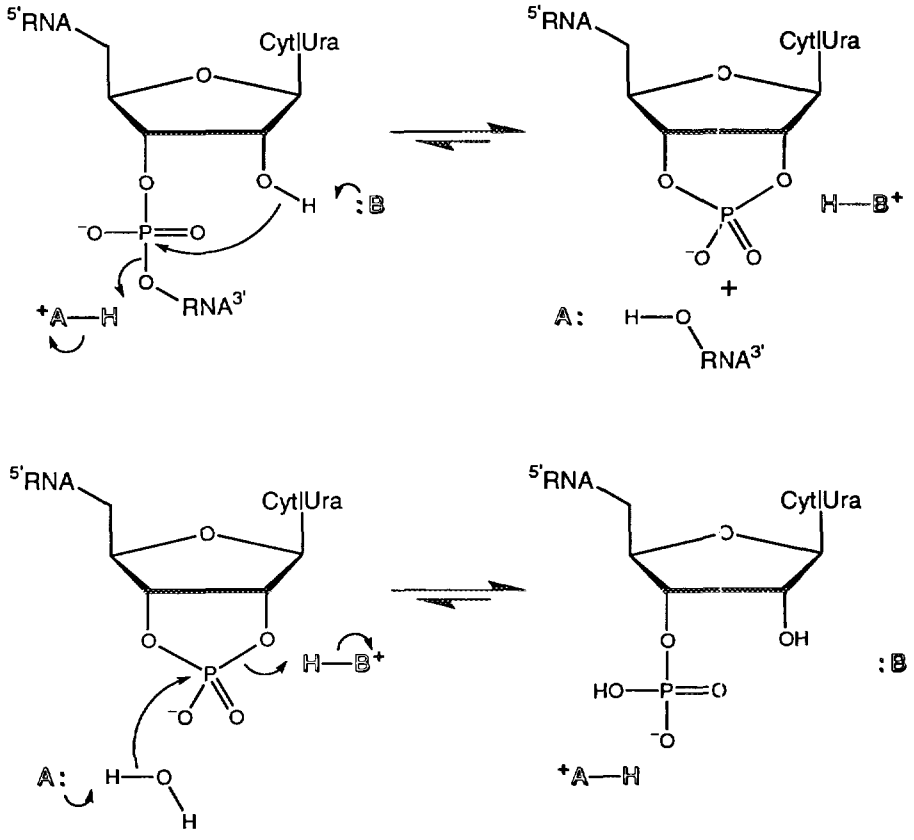

FIG. 1. Simplest mechanism for the transphosphorylation (top) and hydrolysis (bottom) reactions catalyzed by ribonuclease A that is consistent with existing data (*9*).

[1] Abbreviations used: A, adenosine; CpA, cytidylyl (3'→5') adenosine; 5'-AMP, adenosine 5'-monophosphate; DEPC, diethylpyrocarbonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; PEI, poly(ethylenimine); RNase, ribonuclease; SDS, sodium dodecyl sulfate; TBAP, tetrabutylammonium phosphate; TLC, thin-layer chromatography; UpA, uridylyl(3'→5')adenosine; U>p, uridine 2',3'-cyclic phosphate; 2'-UMP, uridine 2'-monophosphate; 3'-UMP, uridine 3'-monophosphate.

which catalyzes the cleavage of this same P–$O^{5'}$ bond in RNA (Fig. 1). Together, these results enable us to reveal the limits to catalysis by RNase A, as well as to compare the energetics of catalysis by RNase A, its mutants, and other ribonucleases.

METHODS

General

UpA was synthesized by the methods of Ogilvie *et al.* (*10*) and Caruthers and Beaucage (*11*). $UpOC_6H_4$-*p*-NO_2 was synthesized by the method of Williams and co-workers (*12*). RNase A (type X-A) was from Sigma Chemical (St. Louis, MO). K41A RNase A was prepared as described for other mutants of RNase A (*13*). [5,6-^{3}H]UTP and [3,5,8-^{3}H]ATP were from Amersham (Arlington Heights, IL).

Ultraviolet absorbance measurements were made on a Cary Model 3 spectrophotometer equipped with a Cary temperature controller (Varian; Sugar Land, TX). ^{3}H content was determined in Biosafe scintillation counting fluid (Research Products International; Mount Prospect, IL) with a TriCarb 1900CA liquid scintillation analyzer (Packard; Meridan, CT).

Cosolvent Dependence of k_{cat}/K_m

The nature of the rate-limiting transition state in catalysis by RNase A was probed by the addition of cosolvents (*6*), here, glycerol and sucrose. The enzyme-catalyzed cleavage of UpA and $UpOC_6H_4$-*p*-NO_2 was monitored directly at 286 and 330 nm, respectively. Substrate concentrations were determined by uv absorption using ε_{260} = 24,600 M^{-1} cm^{-1} at pH 7.0 for UpA (*14*) and ε_{408} = 18,300 M^{-1} cm^{-1} in 0.4 M NaOH for $UpOC_6H_4$-*p*-NO_2. Ribonuclease concentration was determined by using $\varepsilon_{1cm}^{0.1\%}$ = 0.72 at 277.5 nm (*15*). Each reaction contained (in 0.8 ml) substrate [UpA, 0.10–0.50 mM; $UpOC_6H_4$-*p*-NO_2, 25 μM–0.15 mM] in 50 mM Mes–HCl buffer, pH 6.0, containing NaCl (0.10 M) and glycerol (0, 10, 20, or 30% w/v) or sucrose (0 or 30% w/v).

In the absence of cosolvent, $\Delta\varepsilon_{286}$ = −620 M^{-1} cm^{-1} for the cleavage of UpA and $\Delta\varepsilon_{330}$ = 4560 M^{-1} cm^{-1} for the cleavage of $UpOC_6H_4$-*p*-NO_2. New values of $\Delta\varepsilon_{286}$ and $\Delta\varepsilon_{330}$ for the cleavage reactions were determined for each buffer. In each buffer, reaction velocities for at least five concentrations of substrate were determined by monitoring the initial changes in absorbance with time at 25°C. These data were fit to the Michaelis–Menten equation with the program HYPERO (*16*) to obtain values of k_{cat}/K_m.

Synthesis of [5,6-^{3}H]Up[3,5,8-^{3}H]A

[5,6-^{3}H]Up[3,5,8-^{3}H]A was synthesized by an enzymatic and chemical route analogous to that described previously for the synthesis of [5,6-^{3}H]UpA (*17*). Here, both [5,6-^{3}H]UTP (100 μCi) and [3,5,8-^{3}H]ATP (100 μCi) were included in the run-off transcription reaction, along with ATP (12 μM) and UTP (12 μM). Reactions were

monitored by TLC on PEI-cellulose plates (Alltech; Waukegan, IL) as described (*17*).

[5,6-^{3}H]Up[3,5,8-^{3}H]A was purified by HPLC using instrumentation from Waters (Millipore; Bedford, MA). To facilitate its purification, the radiolabeled material was mixed with a small amount of unlabeled UpA. The resulting mixture was injected onto a Novapak C-8 reversed-phase column that had been equilibrated with aqueous ammonium acetate (0.10 M). The loaded column was eluted isocratically with aqueous ammonium acetate (0.10 M) at a flow rate of 0.8 ml/min. Under these conditions, UpA eluted at 2.6 min.

Uncatalyzed Rate of UpA Cleavage

The rate of UpA cleavage in the absence of a catalyst was determined by using HPLC to monitor the time-course of the degradation of [5,6-^{3}H]Up[3,5,8-^{3}H]A. To eliminate contamination from ribonucleases, glassware and plasticware used in this experiment were soaked overnight in aqueous SDS (1% w/v), rinsed with absolute ethanol, and then dried. Likewise, the buffer used in the incubation was treated overnight with DEPC (0.05% v/v), and then autoclaved.

[5,6-^{3}H]Up[3,5,8-^{3}H]A (5 μCi, 15 nmol) was dissolved in DEPC-treated 50 mM Mes–HCl buffer, pH 6.0, containing NaCl (0.10 M), and the resulting solution was incubated at 25°C. At timed intervals, aliquots (50 μl) were removed and combined with an equimolar mixture of seven unlabeled carriers. These carriers were UpA, the four products that could result from the cleavage of the P–O$^{5'}$ and P–O$^{3'}$ bonds of UpA (U>p + A and U + 5′-AMP), and the two isomeric products that could result from the hydrolysis of U>p (3′-UMP and 2′-UMP). The resulting mixture was injected onto a C-18 reverse-phase column that had been equilibrated with 12 mM potassium phosphate buffer, pH 7.0, containing TBAP (4 mM) as an ion-pairing agent. The loaded column was eluted at 0.8 ml/min with 18.0 ml of equilibration buffer, a linear gradient (1.0 ml + 1.0 ml) from equilibration buffer to methanol–water (1/1, v/v), and 8.0 ml of methanol–water (1/1, v/v). Under these conditions, the retention times were: U, 1 min; U>p, 4 min; A, 7 min; 5′-AMP, 13 min; 3′-UMP, 19 min; 2′-UMP, 21 min; and UpA, 25 min. Fractions of each unlabeled carrier were collected. Solvent was removed from each fraction with a vacuum centrifuge. The resulting residues were dissolved in water, and their ^{3}H content was determined.

RESULTS

Cosolvent Dependence of k_{cat}/K_m

The value of $k_{cat}/K_m = 2.3 \times 10^6$ M^{-1} s^{-1} (*18*) for the cleavage of UpA by wild-type RNase A is typical of that for substrate association (*41*). In contrast, the value of $k_{cat}/K_m = 200$ M^{-1} s^{-1} (Messmore and Raines, unpublished results) for the cleavage of UpA by K41A RNase A is indicative of a reaction that is limited by a chemical transition state. As shown in Fig. 2A, the value of k_{cat}/K_m for the cleavage of UpA by wild-type but not K41A RNase A was inversely related to the concentration of added glycerol. The glycerol-dependence of k_{cat}/K_m for the cleavage of

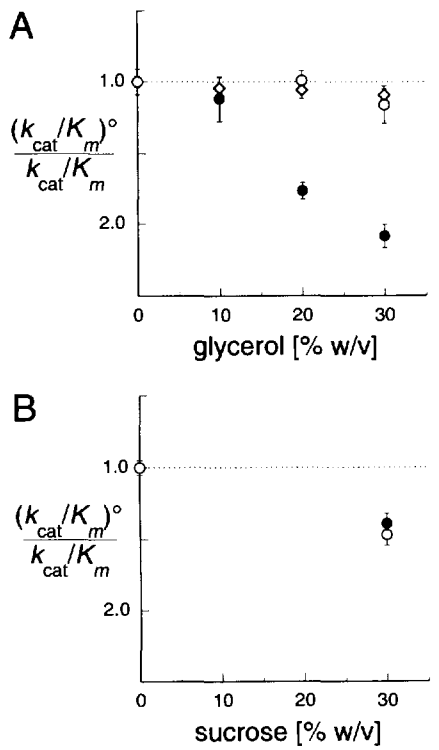

FIG. 2. Plots of the glycerol-dependence (A) and sucrose-dependence (B) of the relative second-order rate constant for the transphosphorylation of UpA by wild-type RNase A (●) and K41A RNase A (○), and for the transphosphorylation of $UpOC_6H_4$-*p*-NO_2 by wild-type RNase A (◇). (k_{cat}/K_m) refers to the absence of cosolvent. The dashed lines have slope 0.

poly(C) by wild-type RNase A is within error of that for the cleavage of UpA (data not shown). If catalysis by the sluggish mutant enzyme is indeed limited by a chemical transition state, then the glycerol-dependent change in the value of k_{cat}/K_m for catalysis by the wild-type enzyme indicates that a transition state other than that for the conversion of bound substrate to bound products limits the rate of catalysis for the cleavage of UpA.

The value of $k_{cat}/K_m = 5.7 \times 10^4$ M^{-1} s^{-1} (*19*) for the RNase A-catalyzed cleavage of $UpOC_6H_4$-*p*-NO_2 is approximately 40-fold lower than that for cleavage of UpA. As shown in Fig. 2A, added glycerol had little effect on the value of k_{cat}/K_m for the cleavage of $UpOC_6H_4$-*p*-NO_2 by wild-type RNase A. Again, the lack of a glycerol-dependence indicates that the rate of this slow reaction is limited by a chemical transition state.

In contrast to the results with glycerol, the sucrose dependence of k_{cat}/K_m is identical for catalysis by wild-type and K41A RNase A, as shown in Fig. 2B. Thus, the rate-limiting transition state for catalysis by wild-type RNase A is altered differently by glycerol and sucrose. This finding indicates that catalysis by the wild-