Origin of the 'inactivation' of ribonuclease A at low salt concentration

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Abstract The effect of salt concentration on catalysis by ribonuclease A (RNase A) has been reexamined. At low salt concentration, the enzyme is inhibited by low-level contaminants in common buffers. When an uncontaminated buffer system is used or H12A RNase A, an inactive variant, is added to absorb inhibitory contaminants, enzymatic activity is manifested fully at low salt concentration. Catalysis by RNase A does not have an optimal salt concentration. Instead, k_{cat}/K_M was $>10^9$ $M^{-1}s^{-1}$ for RNA cleavage at low salt concentration. These findings highlight the care that must accompany the determination of meaningful salt-rate profiles for enzymatic catalysis.

Key words: Ribonuclease A; Salt effect; Enzyme inactivation; Enzyme mechanism

1. Introduction

Salt concentration, like pH and temperature, is a solution condition that can have a dramatic affect on enzymatic catalysis. Despite the importance of salt effects on catalysis, their chemical origins are unknown for most enzymes. For decades, bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5 [1,2]) has been the object of much study in this area [3–11]. Numerous workers have reported that the enzyme has a bell-shaped salt-rate profile with a salt concentration optimum. Surprisingly, there has been no consensus as to either the optimal concentration of salt or the origin of the decrease in catalytic activity at low salt concentration.

Various explanations have been proposed to explain the decrease in the catalytic activity of RNase A at low salt concentration. These explanations include intramolecular interactions between the binding sites of the enzyme [5], a conformational change of the enzyme or substrates [8], a decrease in 'specific' interactions with substrates due to increased rigidity of the enzyme [10], and so forth. Here, we use a series of kinetic experiments with a sensitive fluorogenic substrate and enzyme variants to demonstrate that the inactivation of RNase A at low salt concentration is caused by inhibitory contaminants of buffer solutions commonly used for enzymatic assays. Rather than having an optimum, ribonucleolytic activity continues to increase as salt concentration decreases. Moreover, in solutions of low salt concentration, RNase A has $k_{cat}/K_m > 10^9$ $M^{-1}s^{-1}$ for the cleavage of an RNA substrate.

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2. Materials and methods

2.1. Materials

Wild-type RNase A and its H12A and K7A/R10A/K66A variants were produced, folded, and purified as described elsewhere [12,13]. The substrate 6-carboxyfluorescein ~ dAdArUdAdAdA ~ 6-carboxytetramethylrhodamine [6-FAM ~ $(dA)_2rU(dA)_3$ ~ 6-TAMRA] was from Integrated DNA Technologies (Coralville, IA). A large increase in fluorescence occurs upon cleavage of the P–$O^{5'}$ bond on the 3′ side of the single ribonucleotide residue embedded within this substrate [14]. 2-(*N*-Morpholino)ethanesulfonic acid (MES) and citric acid (trisodium salt) were from Sigma Chemical (St. Louis, MO). Bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane (Bistris) was from ICN Biomedicals (Aurora, OH). The concentration of solutions of the wild-type, H12A and K7A/R10A/K66A enzymes was determined spectrophotometrically by using $\varepsilon = 0.72$ ml mg^{-1} cm^{-1} at 277.5 nm [15]. Concentrations of the substrate were also determined spectrophotometrically by using $\varepsilon = 126\,400$ M^{-1} cm^{-1} at 260 nm [14].

2.2. Assays of catalytic activity

Ribonucleolytic activity was assessed at 23°C in 2.00 ml of 10 mM or 50 mM MES–NaOH buffer (pH 6.0), or 25 mM or 50 mM Bistris–HCl buffer (pH 6.0). The buffered solutions contained NaCl (0–1.0 M), 6-FAM ~ $(dA)_2rU(dA)_3$ ~ 6-TAMRA (19–38 nM), and enzyme (0.50–500 pM). Fluorescence was measured with a QuantaMaster 1 photon-counting fluorescence spectrometer from Photon Technology International (South Brunswick, NJ) using quartz or glass cuvettes (1.0 cm pathlength; 3.5 ml volume) from Starna Cells (Atascadero, CA) and 493 nm and 515 nm as the excitation and emission wavelengths, respectively.

Kinetic parameters were determined by regression analysis of the fluorescence intensity change as described previously [14]. Briefly, when the rate was fast enough to reach completion, pseudo-first order rate constants were determined by fitting the observed fluorescence intensity to an exponential equation:

$$F = F_0 + (F_{max} - F_0)(1 - e^{-kt}) \quad (1)$$

where F is the observed fluorescence intensity, F_0 is the initial intensity before the reaction is initiated, F_{max} is the final fluorescence intensity after the reaction has reached completion. The values of F_{max}, F_0, and k were determined by non-linear regression analysis. When substrate concentration is sufficiently smaller than K_m, the pseudo-first order rate constant k from this regression can be regarded as V/K. When a reaction was too slow to achieve completion, V/K was determined with Eq. 2 instead of Eq. 1.

$$V/K = \frac{(\Delta F/\Delta t)}{F_{max} - F_0} \quad (2)$$

where $\Delta F/\Delta t$ is the slope from linear regression. The slope was determined from linear regression analysis of fluorescence intensity change for a linear region. The value of F_{max} was determined by completing the reaction by adding an excess of enzyme. The value of the apparent k_{cat}/K_M was calculated by dividing V/K by the enzyme concentration.

2.3. Rescue of catalytic activity by increasing protein concentration

The effect of protein concentration on the catalytic activity of wild-type RNase A was assessed by measuring the activity at varying concentrations of H12A RNase A. After a reaction was initiated at 23°C with wild-type RNase A (0.5 nM) in 50 mM MES–NaOH buffer (pH 6.0) as described above, the total concentration of protein was increased by adding aliquots of a concentrated solution of H12A RNase

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A dissolved in the assay buffer. In a control experiment with H12A RNase A alone, there was no detectable ribonucleolytic activity under identical reaction conditions.

3. Results and discussion

3.1. Salt-rate profiles of catalysis

The effect of salt concentration on the catalytic activity of wild-type RNase A and the K7A/R10A/K66A variant were determined in MES–NaOH buffer (pH 6.0) containing different concentrations of NaCl. The K7A/R10A/K66A variant has three fewer cationic residues than does the wild-type enzyme [13,16], and is effectively missing two of the four known subsites for phosphoryl group binding [17,18]. The two enzymes appear to have salt-rate profiles of similar shape (Fig. 1). Both enzymes lose their catalytic activity at low salt with an identical steep slope. The apparent salt optima for catalysis by the two enzymes are, however, distinct. The apparent salt optimum for catalysis by wild-type RNase A is 120 mM Na^+, whereas that for K7A/R10A/K66A RNase A is 14 mM Na^+. As a consequence, wild-type RNase A appears to have only 1% of the catalytic activity of the K7A/R10A/K66A variant at low salt concentration. This result is consistent with the previously reported observation that the salt optimum for catalysis by a ribonuclease depends on the basicity of the enzyme [10].

At high salt concentration, the salt-rate profile of the wild-type enzyme has a steeper slope than does that of K7A/R10A/K66A RNase A. The decline in activity at high salt concentration is likely due to the decrease in the strength of the Coulombic interaction between the enzymes and the substrate. This explanation is consistent with our previous observation that the binding of a single-stranded nucleic acid to wild-type RNase A is more sensitive to salt concentration than is the binding to the K7A/R10A/K66A variant [13]. What then is the origin of the observed decline in activity at low salt concentration?

3.2. Dependence of salt-rate profiles on buffer type

Salt effects on catalysis by RNase A were found to be dependent on the buffer system used for an assay. The salt-rate profile in Bistris–HCl buffer (pH 6.0) is markedly different from that in MES–NaOH buffer (pH 6.0) (Fig. 2A). Although the salt-rate profile determined with MES–NaOH buffer has a bell-shaped curve with a salt optimum, the profile with Bistris–HCl buffer does not show a salt optimum. Rather, the rate continues to increase as the salt concentration decreases. In 25 mM Bistris–HCl buffer (pH 6.0) without added NaCl, the apparent value of k_{cat}/K_M was 2.8×10^9 M^{-1} s^{-1}, which is 50-fold greater than the apparent value of k_{cat}/K_M at the salt optimum in MES–NaOH buffer (pH 6.0). The activities measured with the two different buffers coincide only when the salt concentration is >0.10 M. This result suggests that the 'inactivation' of RNase A at low salt concentration is due to buffer contaminants rather than to an intrinsic property of the enzyme. Moreover, the true k_{cat}/K_M for catalysis of RNA cleavage by RNase A does not have a salt optimum.

To demonstrate directly the inhibitory effect of MES–NaOH buffer at low salt, small amounts of MES–NaOH buffer (pH 6.0) were added to an enzymatic reaction occurring in Bistris–HCl buffer (pH 6.0) (Fig. 2B). The reaction was quenched completely by the addition of the MES–NaOH buffer to a final concentration of only 0.5 mM. Citrate–HCl buffer (pH 6.0) also effected a similar inhibition. The reaction in

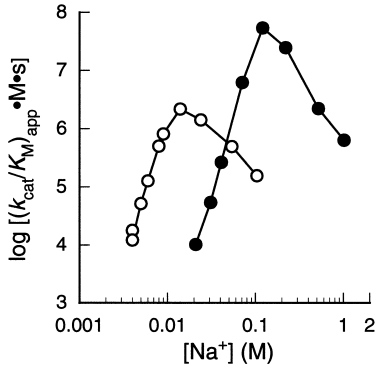

Fig. 1. Salt-rate profiles for catalysis of the cleavage of 6-FAM~$(dA)_2rU(dA)_3$~6-TAMRA by wild-type ribonuclease A (●) in 50 mM MES–NaOH buffer (pH 6.0) and the K7A/R10A/K66A variant (○) in 10 mM MES–NaOH buffer (pH 6.0). Apparent values of k_{cat}/K_M were determined at 23°C in buffer containing substrate (19 nM) and enzyme (0.50 nM). [Na^+] was calculated by summing the contribution from buffer and added salt.

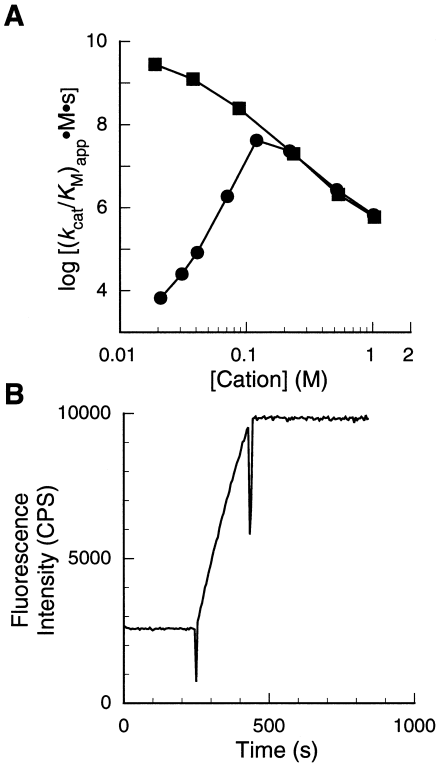

Fig. 2. Effect of buffer on the salt dependence of catalysis of the cleavage of 6-FAM~$(dA)_2rU(dA)_3$~6-TAMRA by wild-type ribonuclease A. A: Salt-rate profiles at 23°C in 25 mM or 50 mM Bistris–HCl buffer (■; pH 6.0) and 50 mM MES–NaOH buffer (●; pH 6.0). [Cation] is the sum of the concentration of Bistris cation and Na^+. B: Inhibition of catalytic activity by MES–NaOH buffer. The reaction was initiated at 23°C by the addition at $t\approx250$ s of wild-type RNase A (1 μl of a 1 nM solution) to a final concentration of 0.50 pM in 2.00 ml of 50 mM Bistris–HCl buffer (pH 6.0) containing substrate (19 nM). At $t\approx450$ s, an aliquot (20 μl) of 50 mM MES–NaOH buffer (pH 6.0) was added to the reaction mixture to give a final MES–NaOH concentration of 0.50 mM.

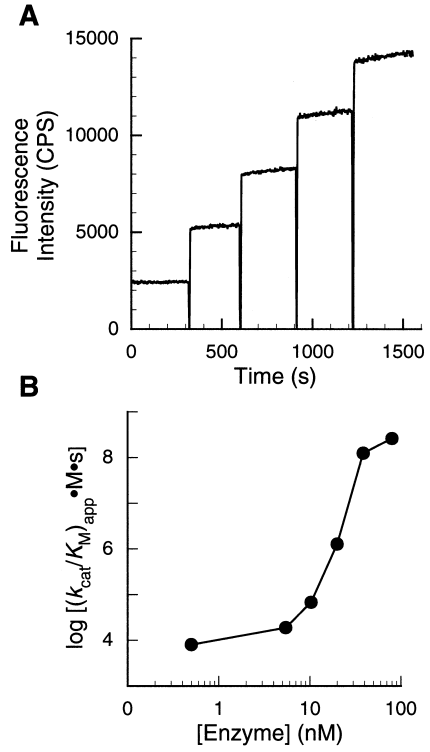

Fig. 3. Effect of enzyme concentration on the 'inactivation' of ribonuclease A at low salt concentration. A: The reaction was performed at 23°C in 50 mM MES–NaOH buffer (pH 6.0) containing 6-FAM ~ $(dA)_2rU(dA)_3$ ~ 6-TAMRA (19 nM). Bursts of catalytic activity were observed upon addition of aliquots of wild-type RNase A (5 μl of a 0.31 nM solution) to the reaction mixture (2.00 ml). B: The reaction was performed at 23°C in 50 mM MES–NaOH buffer (pH 6.0) containing 6-FAM ~ $(dA)_2rU(dA)_3$ ~ 6-TAMRA (19 nM), wild-type RNase A (0.50 nM), and H12A RNase A (0–80 nM). [Enzyme] is the sum of the concentrations of wild-type RNase A and H12A RNase A.

Bistris–HCl buffer (pH 6.0) was quenched when citrate–HCl buffer (pH 6.0) was added to a final concentration of 5 mM (data not shown).

3.3. Dependence of salt-rate profile on enzyme concentration

A distinctive characteristic of the catalytic activity of RNase A in a MES–NaOH buffer (pH 6.0) with a low salt concentration is the presence of a burst. Specifically, the addition of enzyme to a reaction mixture produces a sharp increase in fluorescence intensity followed by a slow phase (Fig. 3A). This burst is consistent with the enzyme being active in the stock solution but becoming inactive quickly upon addition to the reaction mixture. Moreover, the degree of inhibition is related to the concentration of enzyme in the reaction mixture. These results were unexpected because both the stock solution and the reaction mixture were made of the same buffer – 50 mM MES–NaOH buffer (pH 6.0).

H12A RNase A was used to elucidate the origin of the apparent burst kinetics. His12 is an active-site residue of RNase A, and the H12A variant has 10^5-fold less ribonucleolytic activity than does the wild-type enzyme [19]. Hence, the addition of small quantities of H12A RNase A to a reaction mixture increases the total protein concentration without introducing significant catalytic activity. Surprisingly, the H12A variant was able to revive the catalytic activity of wild-type RNase A when added to reactions occurring in 50 mM MES–NaOH buffer (pH 6.0). Addition of H12A RNase A (to 80 nM) increased by $>10^4$-fold the apparent k_{cat}/K_m value for catalysis by the wild-type enzyme (at 0.5 nM) (Fig. 3B). This result indicates that the inhibitor was not the MES–NaOH buffer itself, which was present at 50 mM, but low-level contaminants. In effect, the H12A variant is acting like a sponge that absorbs those contaminants. Because the addition of H12A RNase A to 80 nM enabled the wild-type enzyme to regain almost all of its intrinsic catalytic activity (Figs. 2A and 3B), the concentration of the inhibitory contaminants in 50 mM MES–NaOH buffer must be <80 nM, that is, <2 ppm. The enzyme is active in the stock solution because the enzyme concentration there (unlike in the reaction mixture) far exceeds the concentration of the inhibitory contaminants. Finally, neither RNase A with scrambled disulfide bonds nor the S-protein fragment (residues 21–124) can revive the catalytic activity of RNase A in 50 mM MES–NaOH buffer (pH 6.0) (data not shown). Apparently, binding to the inhibitory contaminants requires the native structure of RNase A.

3.4. Nature of the inhibitory contaminants

The inhibitory contaminants are likely to be highly charged because inhibition is sensitive to salt concentration. They are also likely to be anionic, because RNase A is cationic ($pI = 9.3$ [20]). This proposal is consistent with the observation that a less cationic variant, K7A/R10A/K66A RNase A, has less affinity for the contaminants (Fig. 1). The contaminants do not seem to be polymeric, as the salt dependence of inhibition is similar for wild-type RNase A and the K7A/R10A/K66A variant, which is missing two enzymic subsites for phosphoryl group binding.

4. Conclusions

The often reported 'inactivation' of RNase A at low salt concentration is due to low levels of small anions in common buffer solutions. The degree of inhibition can vary according to the buffer system and enzyme concentration used in assays of catalytic activity. These variations could be responsible for the reported discrepancy in the optimal salt concentration for catalysis [3–8,10,11]. pH-rate profiles have been used often and with great success to reveal the role of acidic and basic functional groups in enzymatic catalysis [21–23]. Likewise, salt-rate profiles can provide valuable insight into the role of Coulombic interactions, but only when proper care is used to avoid artifacts in their determination.

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