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Toward RNase inhibitors: thermodynamics of 2′-CMP/RNase-A binding in multi-ion buffer

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Abstract

Certain ribonucleases (RNases), such as eosinophil-derived neurotoxin, are associated with pathological conditions (e.g. asthma and inflammatory bowel disease) and can even be overtly cyto(neuro)toxic. It has been proposed that small-molecule inhibitors should have therapeutic utility. We used isothermal titration microcalorimetry to characterize reversible inhibitor cytidine 2′-monophosphate (2′-CMP) binding to RNase-A in a multi-ion buffer at 37° as a representative system. The estimated parameters were: $K_d = 13.9$ μM; $\Delta G^\circ = -6.90$ kcal/mol; $\Delta H^\circ = -15.7$ kcal/mol; and $\Delta S^\circ = -0.028$ kcal/mol-K ('enthalpy-driven' interaction). These data should assist drug design of small-molecule inhibitors of homologous RNase catalytic domains.

Keywords: RNase; Microcalorimetry; Thermodynamics

1. Introduction

RNases are endonucleases that have diverse useful actions. However, in certain situations RNases can be deleterious. For example, angiogenin, a plasma-borne RNase, induces the formation of new blood vessels in tumors; RNase-2 (RNase U_s) is neurotoxic and has been implicated in asthma and in inflammatory bowel disease; bovine seminal RNase is immunosuppressive, embryotoxic, and aspermatogenic; and secretory RNases are cytotoxic if not blocked by endogenous 50-kDa protein RNase inhibitor. Molecular modeling and crystal structures of RNase/inhibitor complexes have led to efforts to design RNase inhibitors using bovine pancreatic RNase-A (EC 3.1.27.5) as a model system [1].

ITC determines the interaction binding constants (affinities) and the thermodynamic parameters of changes in enthalpy (ΔH°), entropy (ΔS°), and Gibbs free energy (ΔG°) by direct measurement of the heats of reaction [2–4]. It is becoming increasingly recognized that thermodynamic parameters complement other interaction data for characterizing and modeling ligand–receptor interactions and for the design of novel therapeutic agents [5]. We recently used ITC to measure the effect of individual ions on the interaction between RNase-A and the competitive inhibitor 2′-CMP [6]. This is the first investigation, to our knowledge, of the 2′-CMP/RNase-A interaction in a multi-ion buffer that more closely approximates some intracellular conditions [7]: Na^+ (10 mM), K^+ (59 mM), Ca^{2+} (0.1 μM), Mg^{2+} (0.4 mM), Cl^- (0.2 mM), phosphate (3 mM), sulfate (0.4 mM), and acetate (60 mM) at 37°.

2. Materials and methods

Bovine pancreatic RNase-A and 2′-CMP free acid (98% purity) were purchased from the Sigma-Aldrich Co. Na^+, K^+, Ca^{2+}, and Mg^{2+} acetate, and glacial acetic acid (ACS or molecular biology grade) were purchased from Fisher Scientific. The RNase was dissolved in deionized (Nanopure®; Barnstead Inc.) water and dialyzed through SpectraPor 8000 Da MWCO membrane (Spectrum Laboratories) 2×4 hr (20 mL solution) in a stirred 1000-mL beaker maintained at 1.5° by immersion in an

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Abbreviations: Ac, acetate; 2′-CMP, cytidine 2′-monophosphate; ITC, isothermal (micro)calorimetry; K_d, dissociation constant; and RNase, ribonuclease.

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ice-bath. The RNase and salt stock solutions (in deionized water) were mixed such that the final salt concentrations were: 3 mM KCl, 0.1 mM $CaCl_2$, 10 mM NaAc, 3 mM K_2PO_4, 0.4 mM $MgSO_4$, and 50 mM KAc adjusted to pH 5.5 by dropwise addition of 50 mM HAc. The RNase concentration (0.04 to 0.05 mM), selected so that the *c* value (equal to the product of the binding constant and total molar concentration of RNase) would be between 1 and 500 [6], was determined by quantitative UV spectrophotometry (Hewlett-Packard 8453 diode-array spectrophotometer; 277.5 nm; extinction coefficient $\varepsilon = 9800$ M^{-1} cm^{-1}). The concentration of 2′-CMP (1.2 mM), selected to yield full dose-related interaction with 2′-CMP, was prepared in the same buffers as RNase-A and verified spectrophotometrically (260 nm, $\varepsilon = 7400$ M^{-1} cm^{-1}). Solutions were degassed at 36.5° under filtered vacuum (about 686 mm Hg). The reference cell of the calorimeter (model VP-ITC; MicroCal, Inc.) contained degassed, deionized water. The reaction cell contents were stirred at 400 rpm at 37° throughout the experiment (the frictional heat of stirring is incorporated into the baseline). 2′-CMP was introduced into the reaction cell in a series of thirty-five 4-μL injections, each delivered over 16 sec at 3-min intervals. The equipment automatically adjusts for the change in volume. The data (sampling rate 2 sec^{-1}) were evaluated using ORIGIN® (v. 5.0) software (MicroCal Inc.).

3. Results and discussion

As shown in the isotherm in Fig. 1 (top panel), consistent isotherms with stable baselines were obtained. Maximal output was about −1.5 to −2.5 μcal/sec, the negative deflection indicative of an exothermic reaction. The transposed data were plotted as the integrated heats (kcal/mol of 2′-CMP) for each injection against the 2′-CMP/RNase-A molar ratio, as shown in Fig. 1 (bottom panel). Fitting parameters for the single-site nonlinear regression computer-fit of the raw data points yielded S (stoichiometry of the interaction), K_{eq} (equilibrium constant), and ΔH° (change in standard enthalpy) for each run. The calculated stoichiometry was close to 1:1 [0.99 ± 0.10 (SD)], consistent with a 1 to 1 interaction between 2′-CMP and RNase-A (e.g. Ref. [8]). The other estimated parameters, means (± SD) of triplicate runs, were: $K_d = 13.9$ (±3.9) μM; $\Delta G^\circ = -6.90$ (±0.16) kcal/mol; H° (kcal/mol) = −15.7 (±2.0) kcal/mol; and $\Delta S^\circ = -0.028$ (±0.006) kcal/mol-K. The negative entropy change is consistent with the location of the ribonucleolytic reaction active site within a cleft that binds and cleaves RNA [9]. The interaction proceeds because of the larger contribution of the decrease in enthalpy. The present results, obtained in multi-ion buffer, are notably different from those obtained in single-ion buffer (Table 1). Hence, these results in a multi-ion buffer that more closely approximates some intracellular conditions contribute to the overall goal of understanding the mechanism of inhibitor binding to RNase catalytic domains and the design of selective inhibitors with potential therapeutic utility for asthma, inflammatory bowel disease, and other RNase-associated disorders.

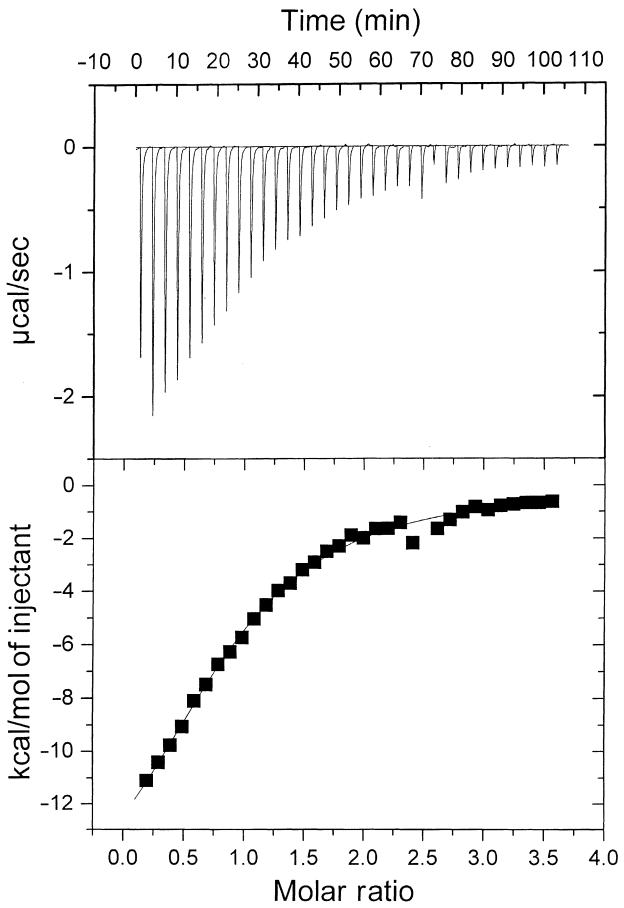

Fig. 1. Isothermal titration microcalorimetry results for the 2′-CMP interaction with bovine pancreatic RNase-A. The top panel displays the raw data output of power (heat output) as a function of time for each 2′-CMP injection (the progressively smaller heat outputs correspond to the progressively greater saturation of RNase-A binding sites by 2′-CMP). The bottom panel displays the heat exchange at each injection, normalized to kcal/mol of 2′-CMP injected, obtained by integration of the area of each 'spike' in the top panel. The curve is the computer-generated best-fit to a single-site binding model.

Table 1
Comparison of the dissociation constant and thermodynamic parameters obtained for the 2′-CMP/RNase-A interaction in multi-ion buffer (present experiment) and in a 50 mM potassium acetate (single-ion) buffer [6]

	Multi-ion[a]	Single-ion[b]
ΔG° (kcal/mol)	−6.90 ± 0.16*	−7.46 ± 0.10
ΔH° (kcal/mol)	−15.7 ± 2.0*	−21.9 ± 0.9
ΔS° (kcal/mol-K)	−0.028 ± 0.006*	−0.047 ± 0.003
K_d(μM)	13.9 ± 3.9*	5.6 ± 1.0

[a] Values are means ± SD, N = 3.
[b] Values from Ref. [6].
* Significant difference ($P < 0.05$).

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