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Calorimetric evidence for conformational transitions of RNase A in the presence of cytidine 2′,3′-cyclic phosphate

M. Gharanfoli, A.A. Moosavi-Movahedi*, S. Safarian, J. Chamani, A.A. Saboury

Institute of Biochemistry and Biophysics, University of Tehran, P.O. Box 13145-1384, Tehran, Iran

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Abstract

Thermal denaturation of ribonuclease A (RNase A) complex with cytidine 3′-monophosphate (3′CMP) was studied by differential scanning calorimetry (DSC). The kinetic and binding studies of RNase A with cytidine 2′,3′-cyclic phosphate (cCMP) as a substrate, and 3′CMP as a ligand were also investigated by difference spectrophotometry. The obtained kinetic saturation curve reveals the occurrence of an anomalous non-hyperbolic shape at high substrate concentrations, and a biphasic binding isotherm. These phenomena indicate that a conformational change is occurring with RNase A during the hydrolysis of cCMP. A combination of kinetic and thermodynamic studies tends to elucidate the reasons for the formation of a non-hyperbolic behavior in a kinetic saturation curve. The thermal profile of the enzyme-3′CMP complexes shows a splitting of two distinct peaks with different structural stabilities of melting points (T_m) of 325 and 337 K. The bifurcate appearance of DSC profile of RNase A-3′CMP complexes manifests a physical view of a light kinetic structural transition. It is worthy to note, the direct binding (not via enzymatic reaction) of enzyme with 3′CMP indicates single DSC profile and monophasic binding isotherm.

Keywords: RNase A; cCMP; 3′CMP; DSC; Kinetic; Binding sites; Ionic strength; Conformational transitions

1. Introduction

Bovine pancreatic ribonuclease A (RNase A) is an endonuclease that cleaves and hydrolysis the single stranded RNA in two distinct steps. The first step involves the cleavage of RNA chain where a transphosphorylation reaction begins from 5′ position of one nucleotide to the 2′ position of the adjacent nucleotide resulting in the formation of a 2′,3′-cyclic phosphodiester. The second step involves the hydrolysis of 2′,3′-cyclic phosphodiester to 3′-nucleotide [1–5]. However, a number of kinetic studies have been carried out following the hydrolysis of cytidine 2′,3′-cyclic phosphate (cCMP), as the substrate. For instance, the rate of hydrolysis at low substrate concentration, in an aqueous environment, seems to follow a hyperbolic behavior; whereas the rate of hydrolysis at a higher substrate concentration does not follow the same pattern, non-hyperbolic behavior [2,6]. This conduct by an enzyme was interpreted in the terms of an allosteric model meaning that a substrate-dependent change in the equilibrium occurs among three pre-existing enzyme conformations where one of which remains inactive [6,7]. Interestingly, the deviation from hyperbolic kinetics for RNase A with cCMP, at a low substrate concentration, was reported by Piccoli and D'Alessio. They have attributed this behavior to the presence of a quaternary structural change [8]. Recently, an alternative explanation based on what is now believed to be a well-documented model of the subsites structure of RNase A has been reported for a non-hyperbolic kinetics of enzymes [2].

A number of investigators have proposed numerous concepts to explain the general catalytic efficiency of enzyme. For example, Eftink and Biltonen [9] and Mitsui et al. [10] believed that one possible source could be linked to the existence of electrostatic subsites in substrate phosphate groups of RNase A, on another hand, Pares et al. [11,12] justified the efficiency of the enzyme to a partially hydrophobic character of purine binding site. However, the interaction between RNase A and a variety of phosphate containing ligands, including cytidine 2′-monophosphate

Abbreviations: cCMP, cytidine 2′,3′-cyclic phosphate; 3′CMP, cytidine 3′-monophosphate; 2′CMP, cytidine 2′-monophosphate; 3′UMP, uridine 3′-monophosphate; BS-RNase, bovine seminal ribonuclease; DSC, differential scanning calorimetry

* Corresponding author. Tel.: +98-21-6403957; fax: +98-21-6404680.
E-mail address: moosavi@ibb.ut.ac.ir (A.A. Moosavi-Movahedi).

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(2′CMP), cytidine 3′-monophosphate (3′CMP) and uridine 3′-monophosphate (3′UMP), were studied by UV differential spectrophotometry and the equilibrium dialysis [13–15]. Nevertheless, Walker et al. [6] demonstrated that a multiple binding of 3′-nucleotide to a molecule of RNase A occurs, and further showed that a number of molecules of 3′CMP bound to RNase A.

In this study, we are proposing an alternative explanation based on two sets of binding sites that is previously reported [16,17]. Our interpretation of the abnormal binding and the kinetics of the RNase A that catalyzes the cCMP is through the existence of two kinds of binding subsites. This would reveal role of cooperative manner of the subsites, which is in the active site of the enzyme.

The thermal denaturation of RNase A and the complex of RNase A with 3′CMP (direct binding) were previously studied by differential scanning calorimetry (DSC) [18]. DSC profile belonging to alone RNase A showed a single transition at $T_m = 337$ K; whereas at the presence of 3′CMP (direct binding) it showed a transition at $T_m = 340$ K. In the present work, we attempt to elucidate the conformational transitions of RNase A in the presence of both substrate (cCMP) and ligand, 3′CMP (as an enzymatic product) by kinetic, thermodynamic, and binding studies.

2. Materials and methods

2.1. Materials

Ribonuclease A (EC 3.1.27.5) and cytidine 2′,3′-cyclic monophosphate (sodium salt) (cCMP) and 3′CMP were obtained from Sigma. All other reagents were of analytical grade. 25 mM ($I = 0.025$ M), 50 mM ($I = 0.05$ M) and 100 mM ($I = 0.1$ M) Tris, 2 mM EDTA, pH 7.5 were used as Tris–EDTA buffers.

2.2. Methods

2.2.1. Kinetic assay

The RNase A samples were prepared from stock solution by dissolving 1 mg of the enzyme into 1 ml of Tris–EDTA buffer. The enzyme was assumed to have a molar extinction coefficient of $\varepsilon_{280} = 9630\,M^{-1}\,cm^{-1}$ [19]. The substrate and product concentrations were determined spectrophotometrically based on the molar absorptivity, substrate: $\varepsilon_{268} = 7950\,M^{-1}\,cm^{-1}$; product: $\varepsilon_{270} = 8400\,M^{-1}\,cm^{-1}$ [6]. The kinetic assay was utilized by differential spectrophotometry based on [20] method. The assay was carried out with a Shimadzu UV-3100 spectrophotometer using quarts cell of 1 cm path length in a thermostatically controlled cell compartment and maintained at 25 °C using a Haake D8 water bath. The rate of enzymatic reaction was calculated automatically during 2 min utilizing the kinetic mode of the instrument. All assays were done in triplet. Activity measurements were expressed as $\Delta A/\Delta\varepsilon$ where ΔA is the maximum difference of absorbance between substrate and product at 284 nm. $\Delta\varepsilon$ value was measured and it was equal $1180\,M^{-1}\,cm^{-1}$ for the difference between the molar absorption coefficients of the product (3′CMP) and the substrate (cCMP) at 284 nm. This absorbance coefficient was determined from the difference between the slopes of two linear lines of the absorbance plots, which were drawn for the product (3′CMP), and the substrate (cCMP). The reaction mixture consisted of Tris–EDTA buffers (25, 50 and 100 mM Tris, 2 mM EDTA, pH 7.5, $I = 0.025$, 0.05 and 0.1 M, respectively), RNase A (10 μM) and cCMP (0–60 mM) concentrations.

2.2.2. Binding measurement

The binding measurement was carried out by a titration method in Tris–EDTA buffer (100 mM Tris, 2 mM EDTA, pH 7.5, $I = 0.1$ M) at 25 °C by two different manners as follows: (1) direct binding (direct mixing of the enzyme and ligand, 3′CMP) and (2) binding via the enzymatic reaction (mixing the enzyme with substrate, cCMP, and allow the reaction to go to completion). (1) In the direct method, the binding of 3′CMP upon interaction with the RNase A was done by difference spectrophotometry. At first, both the cells contained the same amount of enzyme (0.05 mM) and buffer. Then, the 3′CMP solution with concentration range (0–30 mM) was added into the sample cell. In each titration the difference absorbency at 270 nm is related to the RNase A-3′CMP complex and free 3′CMP. The absorption of 3′CMP free was measured separately and the difference absorbance of two experiments were related to the absorbency of 3′CMP-RNase A complex. (2) The binding via enzymatic reaction was done upon the addition of cCMP as substrate (0–30 mM) into the sample cell (containing a constant concentration of RNase A, 0.05 mM) the substrate was hydrolyzed to the final product (3′CMP) within a period of 2 h of incubation (the cCMP is not present at all based on the control test). This experiment was done based on [20] method, which they outlined the RNase A is gone to completion up to 2 h and no substrate indicated. Therewith, the produced 3′CMP (P) bind to the existent enzyme molecules (E) to generate the enzyme-3′CMP complex (EP). The net absorbance for the enzyme-3′CMP complex, A^{EP}, could be obtained by subtracting the absorbance of complete hydrolyzed substrate solution, A^{EP+P}, from the absorbance of the alone 3′CMP solution, A^{P} [1]. The molar concentration of the EP complex was obtained by consuming the molar extinction coefficient of $7800\,M^{-1}\,cm^{-1}$. This value obtained based on the Anderson method at 270 nm [14]. Finally, calculating the important average parameter of $\bar{\nu}$ (net molar concentration of the bound 3′CMP [EP] divided by the total enzyme molar concentration).

Fitting the empirical kinetic data with the theoretical function, and the calculation of the kinetic and binding parameters were all achieved by using a Sigma Plot Scientific Graph System (Jandel Corporation). The good agreement of empirical and computer-calculated data were determined by the typical χ^2 test. All experiments were repeated three times.

2.2.3. DSC measurement

DSC were carried out on a Scal-1 microcalorimeter (Russia), the heating rate was fixed at 1 K min^{-1}. An additional pressure of 1.5 atm was applied during all DSC runs, in order to prevent any possible degassing of the solutions during heating. All experiments were repeated for three times, and the enzyme solution was prepared by dissolving 0.5 mg of the RNase A into 1 ml of Tris–EDTA buffer (100 mM Tris, 2 mM EDTA, $I = 0.1$ M) at pH 7.5. The DSC profiles were obtained for enzyme–3′CMP complex through direct binding and binding via the enzymatic reaction, containing 3′CMP (40 mM) and enzyme concentration of 30 μM.

3. Results and discussion

3.1. Saturation kinetic curve

Fig. 1 shows a typical kinetic saturation curve for RNase A versus cCMP as substrate in the range 0–60 mM at 25 °C and pH 7.5. The characteristic "bump" occurs at the substrate concentration of 14 mM. This illustrates the interchange between two enzymatic states within a narrow range of substrate concentration. The hyperbolic saturation curve occurs at the first enzymatic state, at low substrate concentration, which is in agreement with the literature [2,6,21]. According to the result of the first enzymatic state (cCMP concentration is below 14 mM), K_m was obtained based on Lineweaver–Burk plot and k_{cat} determined by the ratio of $[E]_{total}/V_{max}$. The values of K_m and k_{cat} are equal to 0.4 mM and 2.5 s^{-1}, respectively, and that is near consistent with literature [2,5]. Our investigation of the second enzymatic state at higher substrate concentrations (above 16 mM) shows the catalytic behavior of the enzyme converting to a non-hyperbolic kinetics.

Fig. 2 shows the enzymatic rate versus cCMP concentrations for the hydrolysis of cCMP by RNase A at different

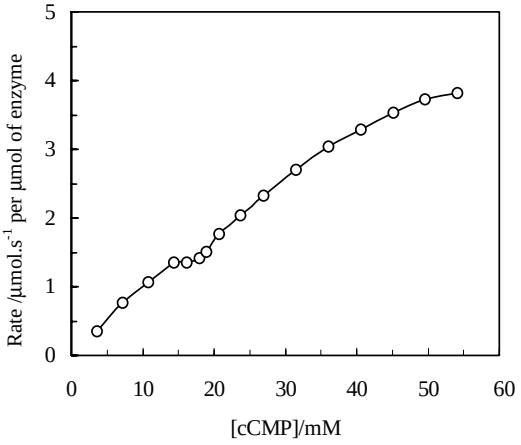

Fig. 1. The saturation kinetic curve of RNase A and cCMP as the substrate. Initial velocities were determined by spectrophotometric assays in Tris–EDTA buffer (100 mM Tris, 2 mM EDTA, $I = 0.1$ M) at 25 °C and pH 7.5.

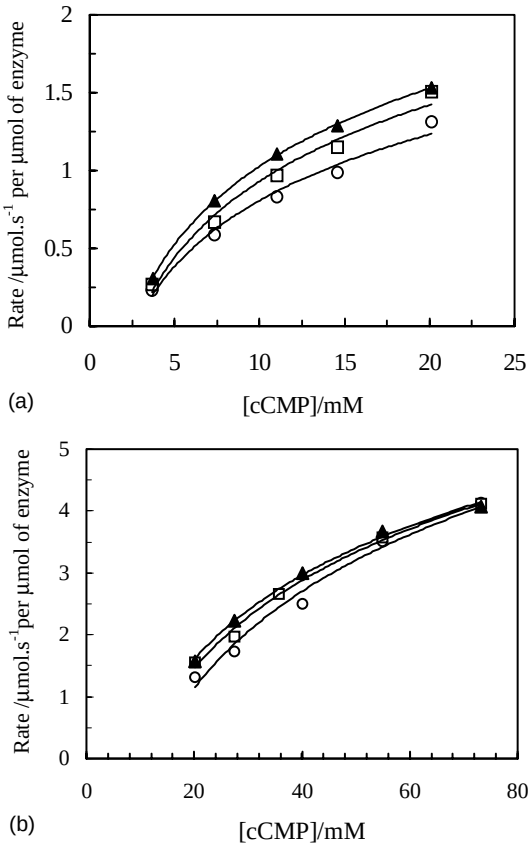

Fig. 2. Ionic strength dependence of the RNase A catalyzed hydrolysis of cCMP at ionic strengths of 0.025 M (25 mM Tris, 2 mM EDTA), 0.05 M (50 mM Tris, 2 mM EDTA) and 0.1 M (100 mM Tris, 2 mM EDTA). (a) At low substrate concentrations (0–14 mM) and (b) at high substrate concentrations (16–70 mM). (▲) $I = 0.025$ M, (□) $I = 0.05$ M, and (○) $I = 0.1$ M.

ionic strengths at 25 °C and pH 7.5. The activities of RNase A in the presence of cCMP were obtained at ionic strengths of 0.1, 0.05 and 0.025 M at two enzymatic transition states (low and high substrate concentrations). Fig. 2a shows that at low substrate concentration, the activity of RNase A increases as the value of ionic strength decreases. Fig. 2b shows that at a high substrate concentration, the increasing of ionic strength has negligible dependence on the activity of the RNase A. The main purpose of this study was aimed to determine the cause of the unusual kinetics behavior of RNase A and the biphasic behavior of binding curve during the interaction of the enzyme with 3′CMP. The following explanations are to clarify the non-hyperbolic behavior of the cited reaction. Moussaoui et al. [2] have stated that the unusual kinetics of the enzymatic reaction of cCMP by RNase A results from the cooperative interaction between different subsites within the same polypeptide chain. On the other hand, DI Donato et al. [22] have outlined the non-hyperbolic saturation curve with an intermediate plateau for the reaction kinetic and the binding of dimeric bovine seminal ribonuclease (BS-RNase) with cCMP. They have explained the abnormal behavior of non-hyperbolic curve in the terms of a

mixed cooperativity through site–site interaction. Crystallographic analysis of complexes between the enzyme and the various ligands also reveals the existence of multiple subsites in RNase [23,24]. A number of the subsites of RNase A and the substrate interactions show electrostatic contribution [9]; while there are others that their interactions (stacking) do not exhibit any electrostatic contribution [11,25]. The electrostatic interactions are revealed by their ionic strength dependence because the activity of electrostatic interactions is the function of their ionic strength [26]. The dependence of the activity of RNase A on ionic strength was noted previously by earlier workers [9,27]. Our work supports the cited literatures regarding the effect of ionic strength on the activity of reaction of RNase A with cCMP. Nevertheless, the results indicate a discrepancy in the rate of RNase A at low substrate concentrations at different ionic strengths; whereas such a distinction is negligible at higher substrate concentrations. According to the activity dependence of the ionic strength, one expects to observe the important function of the electrostatic contribution interaction at low substrate concentrations. It means that at low substrate concentration, the subsite of RNase A acts electrostatically; whereas at high substrate concentration it has non-electrostatic behavior (see Fig. 2).

3.2. Binding isotherm

Fig. 3 illustrates the binding curve of RNase A and 3′CMP at 25 °C in Tris–EDTA buffer (100 mM Tris, 2 mM EDTA, $I = 0.1$ M, pH 7.5) by two manners: (1) direct binding and (2) binding via the enzymatic reaction. (1) Fig. 3 (inset) shows the binding isotherm of RNase A–3′CMP complex (direct binding) that is produced by direct mixing of the enzyme and ligand, 3′CMP. According to the obtained curve, the average number of moles of 3′CMP bound to the mole of enzyme, $\bar{\nu}$, are about 4. It is a monophasic curve and consistent with literature [7]. (2) The binding assay via the enzymatic reaction was carried out in the presence of cCMP, which is producing 3′CMP (the final enzymatic product used as the ligand molecules in binding study). The binding isotherm was plotted as $\bar{\nu}$ (the average mole number of bound ligand to the mole number of enzyme) versus free concentrations of 3′CMP, $[3'\text{CMP}]_{\text{free}}$. The binding isotherm shows the value of $\bar{\nu}$ is about 10 (see Fig. 3). The two phasic shape of this curve indicates two steps of binding sets. The binding data were analyzed through two binding sets, in order to obtain both of binding sets and binding constants that were related to each set of binding. In this study, we followed the Hill equation with more than one term as follows [16]:

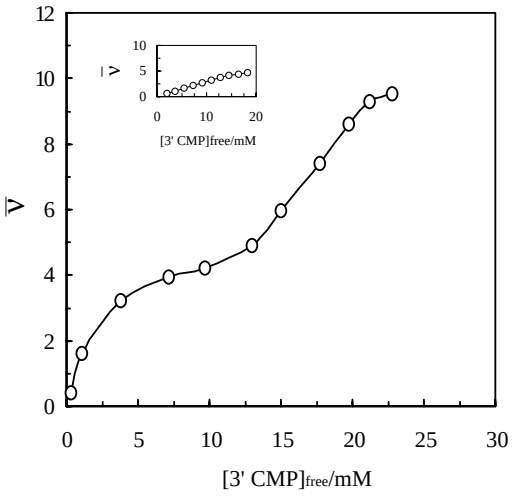

Fig. 3. The binding isotherm curve ($\bar{\nu}$ against free concentrations of 3′CMP) of RNase A and 3′CMP (via enzymatic reaction) in Tris–EDTA buffer (100 mM Tris, 2 mM EDTA, $I = 0.1$ M) at 25 °C and pH 7.5. The assay was carried out in the presence of cCMP, which produces 3′CMP (the final enzymatic product used as the ligand molecules in binding study). Inset shows the binding isotherm curve of RNase A–3′CMP complex (direct binding) that produced by direct mixing of the enzyme and 3′CMP.

$$\bar{\nu} = \frac{g_1(K_1[L_f])^{n_{H_1}}}{1 + (K_1[L_f])^{n_{H_1}}} + \frac{g_2(K_2[L_f])^{n_{H_2}}}{1 + (K_2[L_f])^{n_{H_2}}} \quad (1)$$

where g_1, K_1 and n_{H_1} are the number of binding sites, binding constant, and Hill coefficient for first binding set and g_2, K_2 and n_{H_2} are the corresponding parameters for the second binding set and $[L_f]$ is the free concentrations of ligand. It has been previously shown that the existence of an unusual Scatchard plots rises from two kinds of binding sites having different cooperativity [17,28]. Here the binding isotherm of 3′CMP with RNase A complexes indicates a biphasic saturation curve including an intermediate plateau accompanying with an unusual shape of Scatchard plot (data not shown). The cited binding data have been fitted by Sigma Plot program through Eq. (1). Experimental and calculated parameters derived from Hill equation (Eq. (1)) are as follows: $n_{H_1} = 1$, $g_1 = 4$, $K_1 = 10^3\,\text{M}^{-1}$ and $n_{H_2} = 4.1$, $g_2 = 8$, $K_2 = 60\,\text{M}^{-1}$. Obtaining the Hill coefficients (n_{H_1} and n_{H_2}), where n_{H_1} is equal to 1 at the region of the curve below the plateau, while n_{H_2} is equal to 4.1 at higher concentration of 3′CMP. Hill coefficients are the measure of the cooperativity of the interactions. Accordingly, when $n_H = 1$, it indicates non-cooperativity, if $n_H < 1$, it designates negative cooperativity, and whenever $n_H > 1$, it means positive cooperativity interactions [29]. One may infer that low substrate concentration is linked to the non-cooperativity of the enzyme complex ($n_{H_1} = 1$) and high substrate concentration is relative to a positive cooperativity of 3′CMP ($n_{H_2} = 4.1$) and also literature [8]. It is important to note that at low ligand concentration, RNase A binds to a fewer mononucleotide with strong affinity ($K_1 = 10^3\,\text{M}^{-1}$); while at a higher ligand concentration it binds to the remaining ligand molecules by a cooperative manner with low affinity ($K_2 = 60\,\text{M}^{-1}$).

3.3. Differential scanning calorimetry

Fig. 4a illustrates a typical excess heat capacity scan of the RNase A in the presence of 3′CMP (as a final product

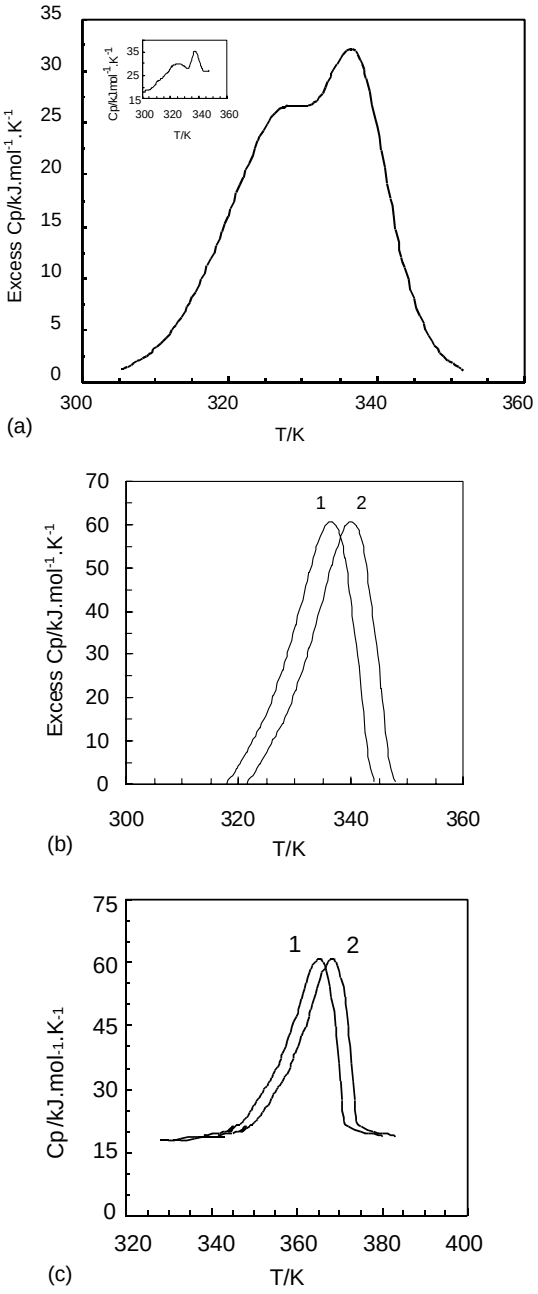

Fig. 4. (a) DSC thermal profile (inset) and excess heat capacity of RNase A upon addition of 40 mM 3′CMP (via enzymatic reaction) in Tris–EDTA buffer (100 mM Tris, 2 mM EDTA, $I = 0.1$ M) at pH 7.5. The experiment was carried out in the presence of cCMP, which produces 3′CMP (the final enzymatic product used as the ligand molecules in DSC study). The concentration of RNase A is 30 μM. (b) Curves 1 and 2 show excess heat capacity profiles of RNase A alone and RNase A–3′CMP complexes (direct binding). (c) Curves 1 and 2 show DSC thermal profiles of RNase A alone and RNase A–3′CMP complexes (direct binding) as the control curves.

of reaction of RNase A with cCMP which is used as a ligand). The sample cell containing a constant concentration of RNase A and cCMP was incubated for a period of 2 h. The substrate was completely hydrolyzed into the final product of 3′CMP. Fig. 4b shows the DSC profiles belonging to RNase A alone and RNase A–3′CMP complexes (direct binding). These experiments were done as the control and consistent with the literature [18].

DSC technique is utilized to obtain additional knowledge for structural transitions through the thermodynamic characterization [30–32]. The thermal denaturation of RNase A–3′CMP complexes (via enzymatic reaction) was studied by DSC and the results were compared with direct binding of 3′CMP with enzyme in the absence of the substrate. The experimental conditions for direct binding and via enzymatic reaction were similar except the presence of substrate for hydrolytic reaction. From hydrolytic pathway, the DSC profile of RNase A–3′CMP complexes (via enzymatic reaction) shows two distinct splitting profiles that confirms the two conformational transitions during the enzyme reaction. It is worthy to note that splitting profile includes two distinct peaks with different melting temperatures (T_m), $T_{m_1} = 325$ K and $T_{m_2} = 337$ K (see Fig. 4a). The DSC profile of RNase A and RNase A–3′CMP complex (direct binding) indicate just one single peak without any splitting (see Fig. 4b). The melting points profile of RNase A and RNase A–3′CMP complexes (direct binding) are 337 and 340 K, respectively. This means that RNase A–3′CMP complex is more stable than RNase A alone. This is worthy to emphasize that the melting point belonging to the right hand of splitting profile (see Fig. 4a) is equal to 337 K (T_{m_2}) and it is identical with T_m of the main conformational structure of RNase A alone. Therefore, the left-hand splitting peak is linked to the conformational structure as a new transition during the enzymatic reaction.

4. Conclusion

A combination of the kinetic and thermodynamic studies were carried out with RNase A with the purpose of elucidating the cause of the non-hyperbolic kinetics of the hydrolysis of cCMP by RNase A. Here, we have observed two intermediates (kinetically) for RNase A via the enzymatic reaction with substrate cCMP. In this paper, we have imposed such intermediates thermodynamically by binding studies and DSC. The binding analysis of RNase A with 3′CMP (via enzymatic reaction) shows two sets of binding of $\bar{\nu}$ near 10 that is including of electrostatic and non-electrostatic interactions. The first set is linked to the first transition state, and the second set is relative to second transition state having a higher cooperativity extent. It is important to note that the binding isotherm and DSC profile of RNase A–3′CMP (direct binding) show only one binding set transition of $\bar{\nu}$ equal to 4 and a single thermal profile for the enzyme structure, respectively. DSC profile for RNase A–3′CMP (via enzymatic reaction) shows a splitting peak with two melting points. This finding is an indication of two physical transitions for the cited complex with different structural stabilities. The existence of two peaks in the DSC profile at $T_m = 325$ and 337 K justifies the presence of two

conformations with different stability. The results show the new conformational structure during the enzymatic reaction that is less stable than the main conformation of RNase A.

Acknowledgements

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