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LIGAND-INDUCED BIPHASIC THERMAL DENATURATION OF RNAase A

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Abstract

DSC measurements have been accomplished in aqueous solutions of bovine pancreatic ribonuclease A (RNAase A) in the presence of subsaturating amounts of 3'cytidine monophosphate (3'CMP) and 2' cytidine monophosphate (2'CMP) at *pH* 5.0 and 5.5. In these conditions the experimental profiles do not conform to a one-step unfolding process. It can be emphasized, as a general phenomenon, that a strong linkage between the temperature-induced protein unfolding and the ligand binding, when the ligand is less than the saturation level, causes marked distortions from a two-state transition. A purely equilibrium thermodynamic analysis gives a correct account of this behaviour and allows to simulate calorimetric curves. It is thus possible to obtain, in an indirect manner, information about the thermodynamic parameters concerning the binding process, namely the association constant and the binding enthalpy. The values of K_b and $\Delta_b H$ for 3'CMP and 2'CMP, so determined, are consistent with the literature data.

Keywords: denaturation, micro-DSC, ribonuclease, thermodynamic parameters

Introduction

The thermal denaturation process of RNAase A has proved to be a very good example of a reversible two-state unfolding transition [1–5]. Even performing calorimetric measurements on RNAase A solutions in the presence of saturating amount of 2'CMP or 3'CMP, which are specific ligands for the active site of protein, the denaturation process well conforms to a reversible two-state transition [6, 7]. However, the experimental DSC profiles in the presence of subsaturating levels of these ligands show a broad and complex peak, quite reversible, that is far away from a two-state transition profile. One could say that the transition is nearly biphasic, as an incipient separation between two peaks becomes evident. In principle a biphasic transition can occur for opposite extreme processes: a) the denaturation of more than one domain present in the protein tertiary structure as found for bovine seminal ribonuclease, (RNAase BS) [8], for RNAase A linked to a polymeric matrix [9], or for bovine serum albumin (BSA) [10]. In these cases the total enthalpy of denaturation $\Delta_d H$ results greater

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than the van't Hoff enthalpy change, $\Delta_d H_{v.H.}$, calculated as in the following, this being an indication of the co-presence of more than one cooperative process; b) the denaturation of a mixture of two species, that do not convert each in the other, gives even a biphasic transition: however in this case it results $\Delta_d H/\Delta_d H_{v.H.} < 1$. Such a model was invoked by us to explain the behaviour of the fungine RNAase Tl [11]. Because for subsaturated RNAase A complexes it is found that the previous ratio is greater than unity, a different model must be invoked to rationalize the complex experimental thermal profile. Our experimental results, however, are not unique, but correspond to a general phenomenon occurring when a macromolecule unfolds in the presence of subsaturating amount of a strong ligand [12, 13]. The physical reason of the appearance of a net shoulder (or, in the case of tight binding, of two distinct endotherms) is the substantial increase in free ligand concentration due to the release of the ligand from the denaturing protein molecules, ligand that titrates the binding site on the still native protein molecules. In other words the complex heat capacity profile is due to a strong linkage between the temperature-induced protein denaturation and the ligand binding.

We are able to give a rationalisation of these complex curves on the basis of the equilibrium thermodynamic description of the investigated systems. Using the well established partition function formalism for analysing the calorimetric and binding experiments [14, 15], it is possible to simulate DSC curves for a protein in these conditions. By means of a simulation-fitting procedure of experimental scans, the thermodynamic parameters (association constant, binding enthalpy) characterizing the binding of the ligand to the macromolecule can be determined. For this purpose it has been considered a protein that by itself undergoes a two-state thermal denaturation and shows a ligand binding at a single site on native form only. The goodness of the fit between the experimental and simulated curves is satisfactory and the determined binding parameters are in agreement with those obtained with more direct approaches.

Materials and methods

All the substances used were SIGMA products. The bovine pancreatic ribonuclease (RNAase A) (type XII-A, containing less than 0.14% phosphate in moles) and both mononucleotides 3′ cytidine monophosphate (3′CMP) and 2′ cytidine monophosphate (2′CMP) were used without further purifications. The concentrations were determined spectrophotometrically using an extinction coefficient of 9 800 $M^{-1}\cdot cm^{-1}$ at 278 nm for the protein, 9 200 $M^{-1}\cdot cm^{-1}$ at 270 nm and 8 890 cm^{-1} at 271 nm for 3′CMP and 2′CMP respectively. The 0.1 *M* sodium acetate buffer solution was used for the measurements at both *pH* 5.0 and 5.5. In all cases deionized water, twice distilled and then filtered on Mil-

lipore membranes, was used for the preparation of solutions that were finally equilibrated by dialysis against the buffer solutions which were repeatedly renewed.

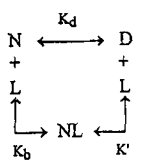
A second-generation Setaram micro-DSC microcalorimeter was used. It is specially designed for studies on dilute aqueous solutions of biological macromolecules. Its temperature program covers the 273–373 K range and its scanning rate can be changed to suit widely differing conditions. The microcalorimeter was connected to a PC for the automatic acquisition of data utilising the MIDAS informatic system, developed in our laboratory. The instrument signal was corrected for the calibration curve and for buffer-buffer reference baseline by the help of the THESEUS program [16]. Finally a consistent baseline was determined assuming the native state as reference so that the apparent molar heat capacity was converted in the excess molar heat capacity $<\Delta C_p>$ with minimum artefacts as possible. The calorimetric enthalpy $\Delta_d H$ was determined by direct integration of the area under the curve and the van't Hoff enthalpy $\Delta_d H_{v.H.}$ was obtained with the standard formula:

$$\Delta_d H_{v.H.} = 4RT^2_{max} < \Delta C_p >_{max} / \Delta_d H \quad (1)$$

where $<\Delta C_p>_{max}$ is the maximum of the excess molar heat capacity function, T_{max} is the temperature at which occurs $<\Delta C_p>_{max}$ and R is the gas constant. The unitary value of the ratio $\Delta_d H/\Delta_d H_{v.H.}$ is a necessary condition that a two-state transition occurs. Other tests have been suggested in a previous paper [8]. A scanning rate of 0.5 deg·min^{-1} was chosen for all the experiments, on the basis of preliminarily trials and our experience.

Thermodynamic description

The analysis follows the ideas and formalism suggested in the stimulating paper of Robert, Gill and Wyman [17]. The thermodynamic model which describes the effect of subsaturating amount of a ligand on the experimental DSC profiles of RNAase A considers a protein that denatures according to a one-step mechanism and is subjected to the binding on a single site of the native conformation, according to the following scheme:



where K_d is the equilibrium constant of denaturation process, K_b is the equilibrium binding constant and K' is the denaturation constant of the stoichiometric complex NL. However it is easy to show that in this triangular equilibrium K' is

not an independent parameter; as it results $K' = K_d/K_b$. Both the equilibrium constants are temperature dependent:

$$K_d = \exp - \{(\Delta_d H/R)[(1/T) - (1/T_d)] + (\Delta_d C_p/R)[1 - (T_d/T) + \ln(T/T_d)]\} \quad (2)$$

where $K_d = 1$ for $T = T_d$, and

$$K_b = K_b^\circ \exp - [(\Delta_b H/R)[(1/T) - (1/T_d)] \quad (3)$$

where $K_b = K_b^\circ$ for $T = T_d$.

In the above Equations $\Delta_d H$ represents the enthalpy change associated to the denaturation process, $\Delta_d C_p$ the net heat capacity change, T_d the midpoint denaturation temperature and $\Delta_b H$ the enthalpy change associated to the binding equilibrium. On the basis of the previous considerations, assuming as a reference the native state the canonical partition for this system is given by:

$$Q(T) = 1 + K_b\,[L] + K_d \quad (4)$$

$Q(T)$ must be normalized with respect to the species selected as reference state for the expression of a specific thermodynamic quantity [18, 19]. In the case of the excess heat capacity function $<\Delta C_p>$, the normalization factor is represented by the binding partition function of native state:

$$Q_o(T) = 1 + K_b[L] \quad (5)$$

The excess enthalpy function $<\Delta H>$ can be readily obtained for the normalized partition function by a well-known statistical mechanical relation:

$$<\Delta H> = RT^2[\partial \ln(Q/Q_o)/\partial T]_{p,[L]} \quad (6)$$

that gives:

$$<\Delta H> = [\Delta_d H + \Delta_d C_p(T - T_d) - \Delta_b H(K_b[L]/Q_o)]\cdot(K_d/Q) \quad (7)$$

When the total ligand concentration is much greater than that of the protein, the free ligand concentration $[L]$ can be assumed as constant at increasing temperature in a DSC measurement. So, in this case, the excess heat capacity is simply given by the derivative of the excess enthalpy function:

$$<\Delta C_p> = \mathrm{d}<\Delta H>/\mathrm{d}T \quad (8)$$

But if the total amount of ligand is lower than the saturation level of protein binding site, it is necessary to consider the mass balance equation for the ligand to determine the free ligand concentration $[L]$:

$$[L]_{tot} = [L] + \overline{[L]}[P]_{tot} \tag{9}$$

where $[L]_{tot}$ and $[P]_{tot}$ are the total ligand and protein concentration respectively and $[\bar{L}]$ represents the binding isotherm that can be calculated from the partition function Q as follows:

$$[\bar{L}] = [\partial \ln(Q/Q_o)/\partial \ln [L]]_{T,p} = K_b[L]/Q \tag{10}$$

In this case the normalization factor is represented by the partition function of unligated protein: $Q_o = 1+K_d$.

The above equations allow to determine the free ligand concentration $[L]$ in the whole investigated temperature range, solving a simple second degree equation:

$$[L] = [- b + \sqrt{(b^2 - 4ac)}]/2a \tag{11}$$

where: $a = K_b$; $b = (1+K_d+K_b^o[P]_{tot}-K_b [L]_{tot})$; and $c = (1+K_d)\ [L]_{tot}$.

In these conditions the free ligand concentration $[L]$ changes in dependence of the linkage with temperature, by the denaturation process, because the ligand can bind only the native protein state.

For this reason, to obtain the $<\Delta C_p>$ function from the excess enthalpy in the case of subsaturating amount of ligand, the free ligand concentration $[L]$ represents a physically significative variable for the thermodynamic description of the system.

Then: $<\Delta H> = f(T,[L])$ and $[L] = f(T)$ and so:

$$<\Delta C_p> = [\partial <\Delta H> /\partial T]_{[L]} + [\partial <\Delta H> /\partial [L]]_T \cdot (d[L]/dT) \tag{12}$$

where $(d[L]/dT)$ is numerically evaluated. Assuming $\Delta_d C_p = \Delta_b H = 0$, this expression results:

$$<\Delta C_p> = (\Delta_d H^2/RT^2)\cdot(K_d Q_o/Q^2) - \Delta_d H\cdot(K_d K_b/Q^2)\cdot(d[L]/dT) \tag{13}$$

The simulations have shown that the maximum distortion of the DSC peak from the two-state transition profile happens when the molar ratio $[L]_{tot}/[P]_{tot}$ is equal to 0.5 (i.e. when the total ligand concentration is half the concentration of binding sites). Moreover, it has been observed that the complex shape of

DSC profile does not depend on the value of the binding constant alone, but markedly depends on the product of the total protein concentration times the association constant at T_d. This product results as an adimensional parameter which will be indicated by C (i.e. $C = K_b^\circ [P]_{tot}$) and appears in the expression of $[L]$, (Eq. 11).

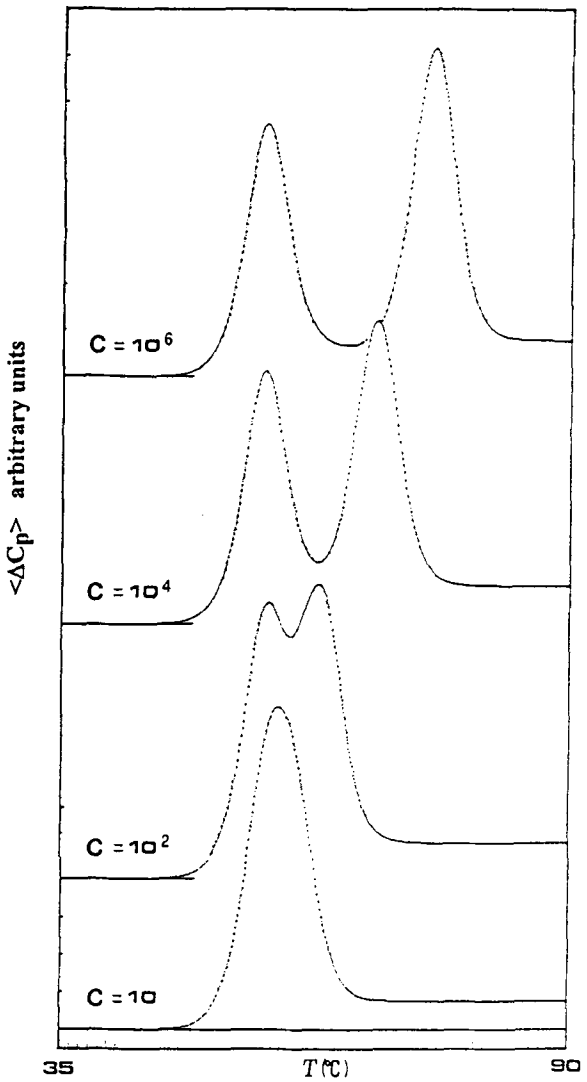


Fig. 1 Simulated DSC profiles according to the described model at different C parameter values. The curves have been shifted along the y-axis for displaying purposes

Simulated DSC profiles for different values of C parameter at $[L]_{tot}/[P]_{tot} = 0.5$ are shown in Fig. 1 for a two-state transition using as parameters $\Delta_d H = 600$ kJ/mol, $T_d = 56.9°C$, $\Delta_d C_p = 8.0$ kJ/mol·K, $\Delta_b H = -40$ kJ/mol, $[P]_{tot} = 1.0$ mM, $[L]_{tot} = 0.5$ mM. It is evident that at increasing the C parameter value, the separation in two peaks becomes enhanced. For example when $C = 10^6$ the maximum of the second peak occurs at a temperature higher than 75°C. It is important to note that even assuming that there is not heat of binding (i.e. $K_b \equiv K_b^\circ$, Eq. (13)) a single two-state transition gives rise to two distinct peaks when the C parameter value is great enough.

However these two peaks do not correspond to the melting of different cooperative subunits nor to two species, unligated native molecules and ligated ones. Indeed as proved by Shrake and Ross [20] the two endotherms are not two state in character and both unligated and ligated native species are responsible for each of the two denaturation peaks. These authors have stated: 'biphasic denaturation derives from titration of the native protein with ligand by decreasing the native protein concentration through thermal denaturation while the total ligand concentration remains fixed'. Up to now this seems the correct physical interpretation of the phenomenon.

Results and discussion

Using the developed thermodynamic model, the analysis of DSC peak distortion allows to obtain all the parameters characterizing the binding process, the association constant K_b and the enthalpy change $\Delta_b H$. This procedure can be applied in a straightforward manner in the case of a very tight binding when two distinct endotherms appear in the DSC profile. However the binding constants of 3'CMP and 2'CMP on RNAase are not large enough to give rise to remarkable distortions of calorimetric curve in the usually employed conditions. Indeed at $[P]_{tot} \approx 0.15$ mM and $K_b^\circ \approx 10^3 \div 10^4$, the value of C parameter is too low for obtaining visible effects. In order to increase the C parameter value, we have used higher protein concentrations (i.e. $[P]_{tot} \approx 2$–3 mM), following the phenomenological indications of the performed simulations. In fact in these conditions, the experimental DSC profiles show marked distortions from the two state transition.

In Fig. 2 are reported the experimental curves obtained at $pH = 5.0$ for RNAase A alone, and for RNAase A in the presence of 3'CMP and 2'CMP at molar ratio equal to 0.50 and 0.66, respectively. In Fig. 3 are reported the experimental curves obtained at $pH = 5.5$ for RNAase A alone, and for RNAase A in the presence of 2'CMP and 2'CMP plus 0.1 M KCl, at molar ratio equal to 0.68 and 0.60, respectively. Clearly in both the figures the curves have been shifted in the y-axis for display purposes.

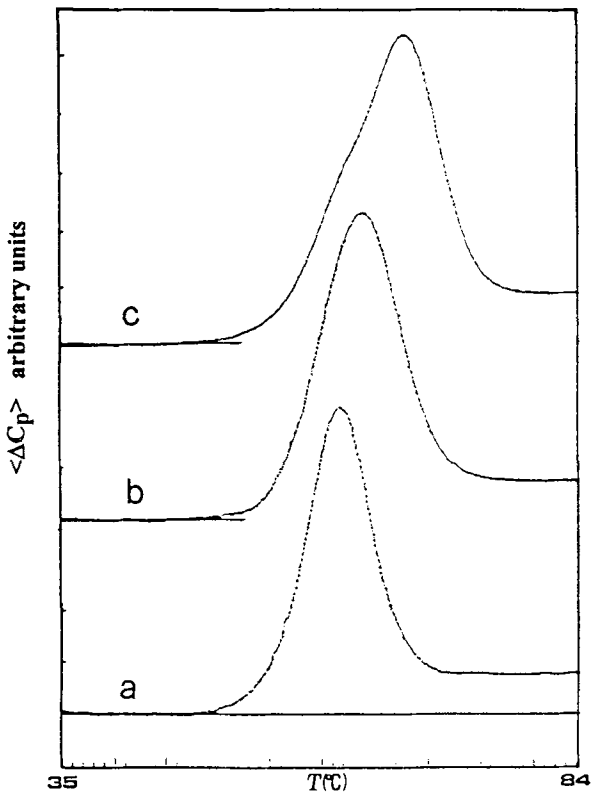


Fig. 2 Experimental DSC curves for a) RNAase A, pH = 5.0, acetate buffer solution; b) RNAase A in the presence of 3'CMP, pH = 5.0, acetate buffer solution; c) RNAase A in the presence of 2'CMP, pH = 5.0, acetate buffer solution

The usual criterion for a complete reversibility is that a reheating of the scanned material after cooling should give a curve that is superposable on the original curve. In our case all the transitions are fully reversible. The thermal profiles obtained for RNAase A in the presence of 2'CMP clearly show a distortion from a two-state transition curve, due to a marked shoulder. Instead in the presence of 3'CMP, the thermal profile is only broader than denaturation peak of RNAase A alone, because the 2'CMP is a stronger ligand than 3'CMP.

In Tables 1 and 2 are reported the experimental values of thermodynamic parameters, T_{max}, $\Delta_d H$, $\Delta_d C_p$, determined from measurements at pH = 5.0 and pH = 5.5, respectively. In the last column it is also reported the value of R, the calorimetric to van't Hoff enthalpy ratio. The unitary value of R for the thermal denaturation of RNAase A solutions confirms that a two-state transition occurs. In the presence of subsaturating levels of 2'CMP or 3'CMP, the R value is

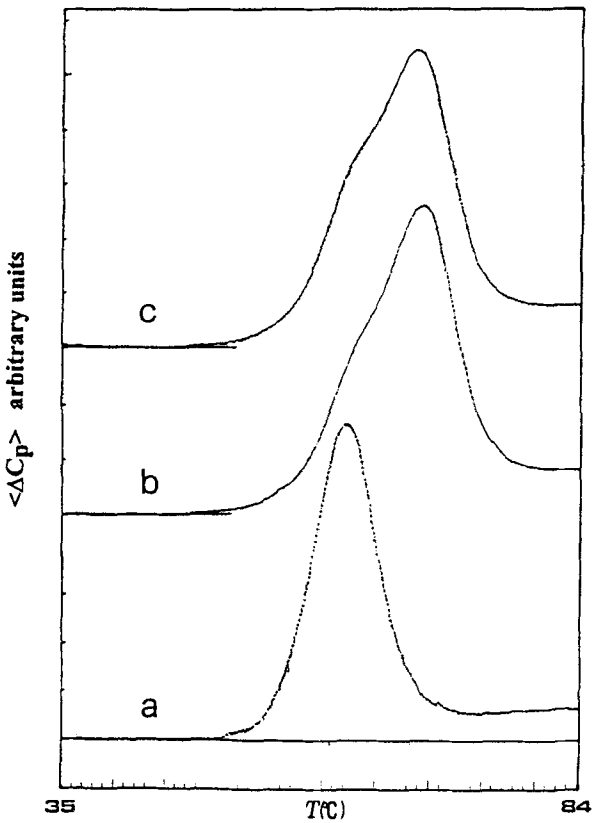


Fig. 3 Experimental DSC curves for a) RNAase A, $pH = 5.5$, acetate buffer solution; b) RNAase A in the presence of 2'CMP, $pH = 5.5$, acetate buffer solution; c) RNAase A in the presence of 2'CMP, $pH = 5.5$ acetate buffer and 0.1 M KCl solution

greater than one (i.e. $R \approx 1.5$), thus indicating that a more complex process occurs. Moreover the values of T_{max} and $\Delta_d H$ increase in a consistent manner, passing, for instance, from 61.3°C and 465 kJ/mol for RNAase A to 68.0°C and 536 kJ/mol for RNAase A with 2'CMP at molar ratio = 0.66 and $pH = 5.0$.

It is possible to characterize the binding equilibrium of 3'CMP and 2'CMP to RNAase A performing a simulation-fitting procedure of the experimental curves with respect to Eq. [12]. A single curve, selected between reproducible measurements, for each set of experimental conditions, has been subjected to this treatment. The results are reported in Tables 3 and 4, and the agreement between simulated and experimental curves is satisfactory as shown in Figs 4a and 4b. The thermodynamic parameters of denaturation process for RNAase A

Table 1 Thermodynamic parameters for thermal denaturation of RNAase A alone and in the presence of subsaturating amount of 3'CMP and 2'CMP at *pH* =5.0, 0.1 *M* acetate buffer solution

	T_{max}/ °C	$\Delta_d H$/ kJ·mol^{-1}	$\Delta_d C_p$/ kJ·mol^{-1}·K^{-1}	*R**	[RNAase A] mM
RNAase A	61.3	465	5.9	1.0	0.39
RNAase A – 3'CMP	63.8	515	7.0	1.3	2.14
RNAase A – 2'CMP	68.0	536	6.7	1.5	2.17

[3'CMP]/[RNAase A] =0.50; [2'CMP]/[RNAase A] =0.66

Table 2 Thermodynamic parameters for thermal denaturation of RNAase A alone and in the presence of subsaturating amount of 2'CMP at *pH* =5.5

	T_{max}/ °C	$\Delta_d H$/ kJ·mol^{-1}	$\Delta_d C_p$/ kJ·mol^{-1}·K^{-1}	*R**	[RNAase A] mM
RNAase A	61.9	475	5.5	1.0	0.54
RNAase A – 2'CMP[a)]	69.0	550	6.8	1.5	3.34
RNAase A – 2'CMP[b)]	68.7	565	6.4	1.6	3.34

[a)] pH 5.5, 0.1 *M* acetate buffer, [2'CMP]/[RNAase A] =0.68

[b)] pH 5.5, 0.1 *M* acetate buffer and 0.1 *M* KCl; [2'CMP]/[RNAase A] =0.60

*$R = (\Delta_d H/\Delta_d H_{vH})$. The unitary value of this ratio confirms that a two-state transition occurs.

Note: reported values are the averages on three or four measurements for each set of experimental conditions. The standard deviations in $\Delta_d H$ and $\Delta_d C_p$ are less than 10 per cent of the reported values. Errors on T_{max} are ±0.2°C

Table 3 Values of the parameters used for the simulation fitting of the DSC profile for the RNAase A solution at pH =5.0

	T_d/ °C	$\Delta_d H$/ kJ·mol^{-1}	$\Delta_d C_p$/ kJ·mol^{-1}·K^{-1}	$\Delta_b H$/ kJ·mol^{-1}	C	σ / J·mol^{-1}·K^{-1}
3'CMP(*)	61.3	460	7.0	–65	10	506
2'CMP(**)	61.3	460	7.0	–65	59	540

(*) 0.1 *M* acetate buffer; protein concentration 2.14 *mM*, ligand concentration 1.07 *mM*
(**) 0.1 *M* acetate buffer; protein concentration 2.18 *mM*, ligand concentration 1.44 *mM*

Table 4 Values of the parameters used for the simulation fitting of the DSC profile for the RNAase A solution in the presence of 2'CMP at pH =5.5

	T_d/ °C	$\Delta_d H$/ kJ·mol^{-1}	$\Delta_d C_p$/ kJ·mol^{-1}·K^{-1}	$\Delta_b H$/ kJ·mol^{-1}	C	σ / J·mol^{-1}·K^{-1}
2'CMP(*)	61.9	470	6.5	–70	59	430
2'CMP(**)	61.9	470	6.5	–85	70	490

(*) 0.1 *M* acetate buffer, protein concentration 3.34 *mM*, ligand concentration 2.27 *mM*
(**) 0.1 *M* acetate buffer in 0.1 *M* KCl, protein concentration 3.34 *mM*, ligand concentration 2.00 *mM*

Note: σ is the standard deviation of the fit calculated by the square root of chi-square to the experimental point number ratio

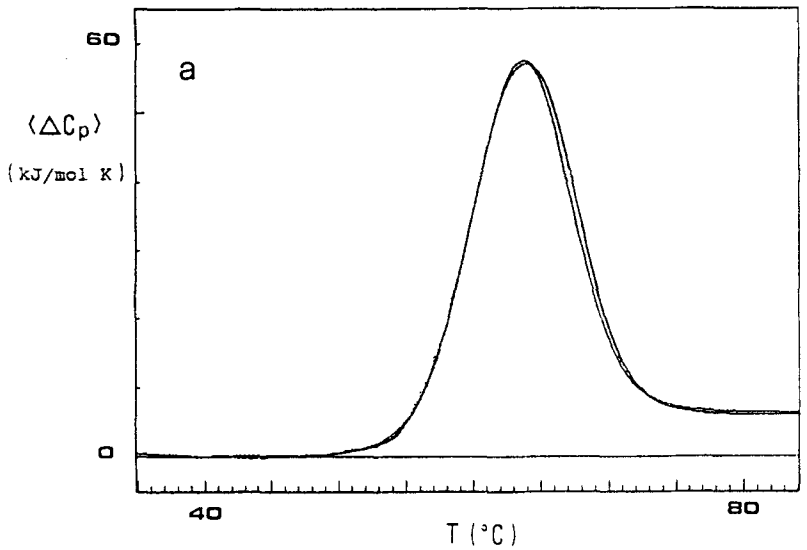


Fig. 4a Experimental and simulated DSC curves for RNAase A in the presence of 3'CMP, pH = 5.0, acetate buffer solution

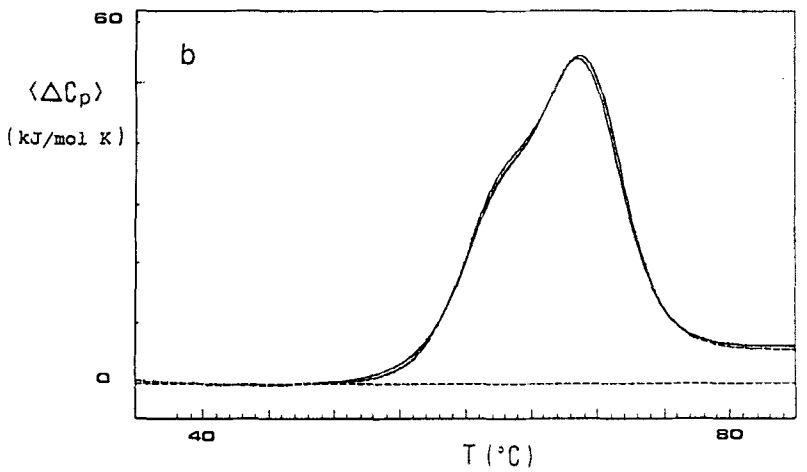


Fig. 4b Experimental and simulated DSC curves for RNAase A in the presence of 2'CMP, pH = 5.5, acetate buffer and 0.1 M KCl solution

alone, determined through direct measurements, have been maintained fixed. The values of $\Delta_b H$, the binding enthalpy assumed independent on temperature, and K_b°, the binding constant at $T = T_d$, have been determined by a non linear regression algorithm [21]. The goodness of the obtained fit convalidates the as-

sumption that there is not heat capacity change associated to the binding process.

For 3'CMP at $pH = 5.0$ it has been obtained: $\Delta_b H = -65$ kJ/mol and $K_b^\circ = 4670\ M^{-1}$; using these data, from the van't Hoff equation it has been calculated $K_b = 80\ 700\ M^{-1}$ at 25°C. For 2'CMP at $pH = 5.0$ it has been determined: $\Delta_b H = -65$ kJ/mol and $K_b^\circ = 27\ 100\ M^{-1}$ and then $K_b = 265\ 000\ M^{-1}$ at 25°C. It is worthnoting that the same values of T_d, $\Delta_d H$ and $\Delta_d C_p$ were used to perform both simulation fitting procedures, obtaining a comparable agreement. The results confirm that 2'CMP is a stronger ligand than 3'CMP for RNAase A. Moreover the calculated association constant and binding enthalpy correspond well to the values determined by isothermal calorimetry [22–25].

For 2'CMP at $pH = 5.5$ it has been determined: $\Delta_b H = -70$ kJ/mol and $K_b^\circ = 17\ 660\ M^{-1}$ and then, from the van't Hoff equation, $K_b = 397\ 000\ M^{-1}$ at 25°C. Instead for 2'CMP at $pH = 5.5$ in 0.1 *M* KCl it has been obtained: $\Delta_b H = -85$ kJ/mol and $K^\circ_b = 20\ 960\ M^{-1}$ and then $K_b = 930\ 000\ M^{-1}$ at 25°C. These values of K_b are very close to those determined by isothermal titration calorimetry [7, 26]. The analysis of DSC measurements shows that, at *pH* 5.5 and in the presence of KCl, the binding of 2'CMP is stronger and more exothermic than in absence of this salt, confirming earlier findings [27]. However the mechanism by which KCl is able to enhance the tightness of the binding is, up to now, unknown. A hypothesis is that the increasing ionic strength raises the activity coefficients of nucleotides.

In conclusion, the determined values of thermodynamic parameters characterizing the binding equilibrium are in good agreement with those resulting from other more direct measurements (spectroscopic and isothermal calorimetric techniques). This confirms the power of DSC, with the aid of the developed equilibrium thermodynamic description, even for the investigation of binding phenomena. Finally, it is worthnoting that a purely thermodynamic analysis of the system accounts correctly for the experimental results, confirming the validity of 'thermodynamic hypothesis' for the description of the physico-chemical behaviour of an aqueous protein solution.

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Zusammenfassung — Bei *pH* 5.0 und 5.5 wurden in Gegenwart von untersättigenden Mengen von 3'Cytidinmonophosphat (3'CMP) und 2'Cytidinmonophosphat (2CMP) DSC-Messungen an einer wäßrigen Lösung von Rinderbauchspeicheldrüsen-Ribonuklease A (RNAase A) durchgeführt. Unter diesen Bedingungen stimmt der experimentelle Verlauf nicht mit einem einstufigen Entfaltungsprozeß überein. Man kann als eine generelle Erscheinung hervorheben, daß – wenn die Liganden unter dem Sättigungspegel liegen – eine enge Verbindung zwischen der temperaturinduzierten Entfaltung des Proteins und der Ligandenbindung bezeichnende Störungen aus einem Zwei-Zustands-Übergang verursachen. Eine reine thermodynamische Gleichgewichtsanalyse ergibt eine korrekte Darstellung dieses Verhaltens und erlaubt die Simulation kalorimetrischer Kurven. Somit ist es möglich, auf indirekte Weise Informationen über die thermodynamischen Parameter (Assoziationskonstante, Bindungsenthalpie) des Bindungsprozesses zu erhalten. Die auf diese Weise ermittelten Werte für K_b und $\Delta_b H$ von 3'CMP und 2'CMP stimmen mit den Literaturangaben überein.