Thermal Perturbation of the Equilibrium System Ribonuclease A-Cytidine 3'-Monophosphate at pH 5.5

Mirna Flögel and Tihana Žanić
Department of Medical Biochemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb
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The equilibrium system ribonuclease A-cytidine 3'-monophosphate at pH 5.5, was submitted to thermal perturbation. A derivative fractional degree of saturation with respect to a temperature raise of twenty degrees was recorded as a function of free ligand concentration. The analysis of the thermal perturbation curve directly yields $\Delta H^\circ$ and $\Delta S^\circ$ of the binding reaction. The evaluated thermodynamic parameters are in good agreement with the values obtained by other methods. The thermal perturbation technique proved to be an elegant, time and material saving experimental method in providing accurate thermodynamic information about ligand-macromolecule interactions.

INTRODUCTION

The thermodynamic description of binding equilibria involving biological macromolecules (e.g. enzyme-substrate reactions, biopolymer-metal ion interactions, allosteric control reactions, drug-receptor reactions and macromolecular self-assembly reactions) is essential to the understanding of the chemical processes of these molecules. Therefore, the experimental measurement and evaluation of binding isotherms is an activity of immense interest. However, most approaches to the problem are concerned with the determination of the apparent binding constants with little effort devoted to the development of a complete thermodynamic characterization of the process. Calorimetric techniques are very useful for obtaining complete thermodynamic information, but they require relatively large amounts of heat in the reaction.

All other methods yield thermodynamic parameters $\Delta H^\circ$ and $\Delta S^\circ$ indirectly from a set of binding isotherms measured at different temperatures. And even then, the deduction of the appropriate thermodynamic mechanism for the binding process most often involves an assumption of a specific model and assessment of its adequacy by a data-fitting procedure. The analysis is usually restricted by the accuracy, precision and quantity of the experimental data. In an effort to introduce an additional experimental method which will supply relevant thermodynamic information and provide sufficiently accurate data fairly rapidly, thermal perturbation has been applied to a thermodynamically well described reaction system and the thermal perturbation curve has been analysed in view of the suggested theoretical framework.
Thermal perturbations are not new. Using t-jumps for kinetic studies by the chemical relaxation method, Eigen and De Maeyer have pointed out that relaxation amplitudes can also yield the equilibrium constant and estimates of $\Delta H$. Thusius has also shown that enthalpy changes and the equilibrium constant can be obtained from relaxation amplitudes. Recently, in a series of papers Billonen et al. presented a theoretical groundwork for the use of derivative binding isotherms in the analysis of ligand binding to macromolecules, based on measurements of the difference in the binding degree of a system at equilibrium at two different temperatures.

**EXPERIMENTAL**

The protein solutions were prepared from a salt free, phosphate free lyophilized Ribonuclease A, obtained from Boehringer Mannheim Corp. Before being used for the spectrophotometric measurements, RNase solutions were adjusted to pH 6.5, thermostated for 10 min at 62 °C to disrupt aggregates, cooled to room temperature, and readjusted to pH 5.5 ± 0.02. The solutions thus obtained were analysed chromatographically on Sephadex G-75 columns and found to be homogeneous. The RNase concentration was determined spectrophotometrically, assuming a molar extinction coefficient of 9800 ml$^{-1}$ dm$^{3}$ cm$^{-1}$ at 277.5 nm at pH 7.6.

Chromatographically pure cytidine 3'-monophosphate (99% pure) was purchased from Boehringer Mannheim Corp. and used without further purification. A molar extinction coefficient of 7600 mol$^{-1}$ dm$^{3}$ cm$^{-1}$ at 260 nm at pH 7.0 was assumed. All solutions contained 0.05 mol dm$^{-3}$ sodium acetate buffer because it has been found that the formation of the enzyme-inhibitor complex involves pH dependent proton absorption and release, the sum of which equals zero at pH 5.5 in 0.05 mol dm$^{-3}$ monovalent salt solution.

A Unicam SP-8-100 Reaction Rate spectrophotometer was used for all optical measurements. Temperature was measured with a Digital thermometer using a thermistor probe placed in the cuvette.

**Thermal Perturbation Experiments**

Solutions of RNase A and 3'CMP were prepared immediately before, the measurement by diluting a 3'CMP stock solution with a constant volume of enzyme solution and with an appropriate volume of 0.05 mol dm$^{-3}$ sodium acetate solution. Only the final concentration of 3'CMP was varied. After mixing, the solution was placed in a 1 cm water jacketed absorption cell in the spectrophotometer. The same solution was used in the reference cell.

The absorption was recorded on the 0.2 O.D. scale. After the equilibration at temperature $T_1$, the change in absorbance at 340 nm, following a temperature change in the reference cell, was recorded.

**RESULTS AND DISCUSSION**

As Sturgill et al. suggested, the simplest approach to understanding the rationale of the thermal perturbation method in order to prove how useful the method is, is to consider a reaction system to which the theory for a simple set of independent sites can be applied. This is why we have used 3'CMP-RNase interaction, known to have 1:1 stoichiometry, as a model system to demonstrate the use of the thermal perturbation technique in evaluation of the thermodynamic quantities associated with the ligand-macromolecule interaction.

The binding of 3'CMP to ribonuclease A was extensively studied by various physical chemical methods, e.g. kinetic, potentiometric, calorimetric and NMR. By calorimetric and potentiometric binding experiments Flögel et al. demonstrated the validity of a self-consisting thermodynamic model for the binding of 3'CMP to RNase which shows that the binding is thermo-
dynamically coupled to the ionisation of histidine residues 12, 48 and 119 and that only the di-anionic form of the inhibitor is bound. In other words, the formation of the complex 3'CMP-RNase causes a change in the \( pK_a \) values of these groups. The correct representation of \( \Delta G_{op}^o \) is

\[
\Delta G_{op} = \Delta G^o - RT \sum_{j=1}^{n} \ln \left( \frac{1 + [H^+]K_j^{-1}}{1 + [H^+]K_j} \right) + RT \ln (1 + [H^+]K_j)
\]

where \( \Delta G^o \) is the standard, pH independent, Gibbs energy of RNase-3'CMP binding, \( K_j, K_j', \) and \( K_i \) are the proton dissociation constants of the groups involved in the proton release and absorption upon binding, index \( j \) represents the group on enzyme and index \( I \) the phosphate group on 3'CMP, and \([H^+]\) is the hydrogen ion concentration of the reaction medium. When enzyme is in the complex form, the proton ionisation constants are marked as \( K' \).

According to the proton uptake upon RNase-3'CMP complex formation, measured as function of pH at pH 5.5, no proton exchange could be detected when ionic strength was 0.05 mol dm\(^{-3}\). Direct binding isotherms were recorded at two temperatures which will later be used as limiting temperatures of the thermal perturbation. The concentration of enzyme was fixed and the 3'-CMP concentration was varied. The apparent association constant \( K \) for the reaction

\[
E + 3'\text{CMP} \rightleftharpoons E - 3'\text{CMP}
\]

can be evaluated from the expression

\[
K = \frac{[E - 3'\text{CMP}]}{[E][3'\text{CMP}]} = \frac{\Delta A}{\Delta A_{\text{max}}} = \frac{1}{\Delta A_{\text{max}}} - \frac{\Delta A}{\Delta A_{\text{max}}}[3'\text{CMP}]_t
\]

where \( \Delta A \) is the observed change in the absorbancy due to the complex formation, \( \Delta A_{\text{max}} \) is the extrapolated value of \( \Delta A \) at infinite 3'CMP concentration and free ligand concentration equals to

\[
[3'\text{CMP}]_t = [3'\text{CMP}]_I - \frac{\Delta A}{\Delta A_{\text{max}}}[E]_t
\]

where index \( t \) means total concentration of the component.

The practical equation for analysing the experimental data is as follows:

\[
\frac{1}{\Delta A} = \frac{1}{\Delta A_{\text{max}}} + \frac{1}{\Delta A_{\text{max}}(K[3'\text{CMP}]_t - [E]_t\Delta A/\Delta A_{\text{max}})}
\]

A typical binding isotherm for 3'CMP binding to RNase is shown in Figure 1. The presented curve is the best fit of the data obtained by iterative least squares analysis. The average value of \( A_{\text{max}} \) from this and all other RNase titrations with 3'CMP was found to be \( 1.43 \times 10^4 \) mol\(^{-1}\) cm\(^2\) dm\(^3\). The binding constants obtained in this way at 291, 299 and 311 K equal \( 7.4 \times 10^4 \), \( 3.6 \times 10^4 \) and \( 1.3 \times 10^4 \) mol\(^{-1}\), respectively. \( A_{\text{max}} \) seems to be temperature independent.

**Thermal Perturbation Experiments**

If one considers the equilibrium system

RNase + 3'CMP ⇌ RNase · 3'CMP

\[
E + L \rightleftharpoons EL
\]
Figure 1. Differential absorbancy of the reaction solution containing $7 \times 10^{-5}$ mol dm$^{-3}$ RNase A and 0.05 mol dm$^{-3}$ NaAc at pH 5.5, measured as function of 3'CMP concentration at 299 K. The curve is calculated as the best fit to the experimental data.

the enzyme fraction in the bound from

$$f = \frac{[EL]}{[E]+[EL]} = \frac{K[L]}{1+K[L]}$$

can be written as function of $y$ where $y = \ln[L]$:

$$f = Ke^y/(1 + Ke^y)$$

When the system is subjected to a small perturbation, the change in $f$ with temperature is given by

$$\left(\frac{df}{dT}\right)_y = \frac{Ke^y}{(1 + Ke^y)^2} \frac{\Delta H^\circ}{RT^2}$$

where $\Delta H^\circ$ is the apparent enthalpy change per mol of E associated with the binding reaction.

The derivative of the fraction $f$ with respect to $y$ is

$$\phi = \left(\frac{df}{dy}\right)_T = Ke^y/(1 + Ke^y)^2$$

Hence, the thermal perturbation curve (TPC), defined as $(df/dT)_y$ as a function of $y$, is the derivative binding isotherm $\phi$ times $\Delta H^\circ/RT^2$. The function achieves the maximum value when $[L] = 1/K$, since the maximum is achieved when
\[ \frac{\partial \phi}{\partial y} = \frac{K_1 + K_2 \left[ L \right]^2}{\left( 1 + K_1 \left[ L \right] \right)^4} = 0 \quad \text{i.e. when } K_2 \left[ L \right]^2 = 1 \]

Thus if $\phi/\partial T$ is determined as a function of $[L]$, the association constant can be simply estimated from the position of the maximum.

From the experimental point of view, if the system RNase and 3'CMP is subjected to a finite perturbation $\Delta T$, the change in fractional degree of saturation per degree is related to the change in absorbance by the equation:

\[ \frac{\Delta A}{\Delta T} = \frac{\Delta f}{f T} \frac{\Delta A_{\text{max}}}{A_{\text{max}}} \]

The change in absorbance at 340 nm could be linearly related to the magnitude of the temperature perturbation for one choice of concentrations, which has been taken as proof that the approximation $df/\partial T \approx \Delta f/\Delta T$ is valid.

The data in Figure 2 were obtained by direct recording of the change in optical density due to the thermal perturbation from 291—311 degrees. The TPC curve on Figure 2 is obtained by fitting the data by an iterative least squares procedure to $\Phi$, where $\Phi$ is the derivative binding isotherm

\[ \frac{\Delta A}{\Delta T} = C - \frac{K \left[ L \right]}{(1 + K \left[ L \right])^2} \]

and $C$ is the least squares fitting parameter.

![Figure 2. Thermal perturbation curve of RNase-3'CMP system by the temperature raise from 291 to 311 K at pH 3.5 (7 X 10^{-5} mol dm^{-3} NaAc) recorded at 346 nm. The points represent the data and the curve is the calculated best fit of the data to the differential binding isotherm $\Phi$.](image-url)
The value of $K$ was varied and for each $K$ the best fit, following correction of total to free concentration, was made. The graph of the variance of the fit vs. $K$ produced a least-squares surface with one minimum from which the $K$ minimizing the variance was selected. The apparent $K_B$ from the position of the maximum for this experiment at pH 5.5 is $2.85 \times 10^{-5}$ and the maximum amplitude of the change in optical density is $-0.043$. The results of all thermal perturbation experiments on the system were summarized and averaged and the thus obtained value for $K_B$ compared to the values obtained by other methods (Table I.) The apparent $\Delta H^\circ$ for the association is given by the expression (2):

$$
\Delta H_{ap}^\circ = \frac{4RT^2(\Delta A/\Delta T)_{max}}{\Delta A_{max}}
$$

$\Delta A_{max}$ was calculated from the knowledge of $\epsilon$ and $[E]$. As seen from the data in Table I, the obtained binding constant for the 3CMP-RNase interaction is within the limits of the values obtained by other methods. $\Delta H^\circ$ value obtained by thermal perturbation calculated assuming $A_{max}$ of $1.43 \times 10^3$ mol$^{-1}$ cm$^{-1}$ dm$^3$ is $-64.8$ kJ mol$^{-1}$, which is also in good agreement with calorimetric measurements.10,14

**TABLE I**

Comparison of the Binding Constant and Enthalpy Change for 3CMP-RNase A Interaction at pH 5.5, Evaluated from TP Measurements with the Corresponding Literature Values

<table>
<thead>
<tr>
<th>$K_b$ 10$^{-4}$ mol$^{-1}$ dm$^3$</th>
<th>$\Delta H_{ap}^\circ$ kJ mol$^{-1}$</th>
<th>$\mu$ mol dm$^3$</th>
<th>$T$, K</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>64.8 s. d. ± 0.9</td>
<td>0.05 (NaAc)</td>
<td>TP (291—311)</td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td></td>
<td>0.05 (NaAc)</td>
<td>311</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td></td>
<td>0.05 (NaAc)</td>
<td>299</td>
<td></td>
</tr>
<tr>
<td>7.4</td>
<td></td>
<td>0.05 (NaAc)</td>
<td>291</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>65.2</td>
<td>0.05 (NaAc)</td>
<td>298</td>
<td>14</td>
</tr>
<tr>
<td>3.8</td>
<td>66.0</td>
<td>0.05 (KCl)</td>
<td>298</td>
<td>10</td>
</tr>
<tr>
<td>1.21</td>
<td>0.1</td>
<td>298</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>2.68</td>
<td>0.1</td>
<td>298</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>3.9</td>
<td>0.15</td>
<td>298</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

It would be interesting to use the TP method at other pH values where the mechanism of 3CMP binding to RNase is more complex because of the shifts in $pK_a$ values of the several groups being involved in the binding process. The application of TPC in characterization of 3CMP to RNase binding has established once again the advantages of the method, primarily in terms
of great economy of material, since the most important information is obtained at ligand concentrations in the vicinity of $K_D$ and in a simple case such as described, the extrapolation of the observable to the saturation is not required. The dissociation constant can be estimated directly from the position of the maximum of TPC, and $L_{HO}$ from the maximum amplitude, as has been pointed out by Thusius. In addition, the details of TPC shape can be used to test the assumed reaction model.

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REFERENCES

Termodinamička i spektroskočka analiza ravnotežnog sustava ribonukleaza-A-citidin-3’-monofosfat kod pH 5,5

Metodom termičke perturbacije određeni su termodinamički parametri $\Delta G^o$ i $\Delta H^o$ za ravnotežni sustav ribonukleaza-A-citidin-3’-monofosfat kod pH 5,5.

Mjerena je promjena apsorbancije u ovisnosti o koncentraciji 3’CMP koja je promjena stupnja saturacije enzima izazvana temperaturnim skokom od 20 K. Dobivena krivulja termičke perturbacije omogućuje procjenu ravnotežne konstante i određivanje entalpije u koncentracijskom području znatno nižem od saturacijskih koncentracija liganda, što svjedoči da je termička perturbacija kao metoda za dobivanje valjanih termodinamičkih informacija o interakcijama makromolekula-ligand izuzetno prikladna, jer štedi materijal i vrijeme.

IZVOD