Rapid Measurement of Binding Constants and Heats of Binding Using a New Titration Calorimeter

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A new titration calorimeter is described and results are presented for the binding of cytidine 2'-monophosphate (2'CMP) to the active site of ribonuclease A. The instrument characteristics include very high sensitivity, rapid calorimetric response, and fast thermal equilibration. Convenient software is available for instrument operation, data collection, data reduction, and deconvolution to obtain least-squares estimates of binding parameters \( n \), \( \Delta H^\circ \), \( \Delta S^\circ \), and the binding constant \( K \). Sample throughput for the instrument is high, and under favorable conditions binding constants as large as \( 10^6 \) M\(^{-1} \) can be measured. The bovine ribonuclease A (RNase)/2'CMP system was studied over a 50-fold range of RNase concentration and at two different temperatures. The binding constants were in the \( 10^5 \) to \( 10^6 \) M\(^{-1} \) range, depending on conditions, and heats of binding ca. -15,000 cal/mol. Repeat determinations suggested errors of only a few percent in \( n \), \( \Delta H^\circ \), and \( K \) values over the most favorable concentration range.

When characterizing interactions between a biological macromolecule \( M \) and a small ligand \( X \), or between two macromolecules, i.e.,

\[
M + X = MX \\
MX + X = MX_2 \\
\vdots \\
MX_{n-1} + X = MX_n,
\]

the single-site binding constant \( K \), the heat of binding \( \Delta H^\circ \), and the number of sites \( n \) in the set are the independent variables of thermodynamic interest. The entropy \( \Delta S^\circ \) and free energy \( \Delta G^\circ \) of binding are dependent variables obtained by the calculation

\[
\Delta G^\circ = -RT \ln K = \Delta H^\circ - T \Delta S^\circ. \tag{2}
\]

Most of the commonly used methods (e.g., equilibrium dialysis, ultrafiltration, gel exclusion chromatography) for measuring binding constants (1) involve partitioning between macromolecule and free ligand and, although this enables both free and bound ligands to be measured directly, these methods are inefficient of both time and material as they necessitate sample reloading and lengthy equilibration for each point obtained on the binding isotherm. Measurements become simpler when using ligands or macromolecules which are able to "signal" occurrence of the binding process and thereby avoid the need to partition. Spectroscopic signaling from chromophores or fluorophores whose properties differ in the free and bound states is the most popular of these methods but it is not universally applicable since most systems lack such a signaling group.

Although not frequently used for this purpose in the past (2–6, and references therein) ultrasensitive titration calorimetry offers many potential advantages over other techniques for characterizing biological interactions by signaling. The heat signal is a nearly universal property of binding reactions, so it is applicable to most ligand–macromolecule or macromolecule–macromolecule interactions. Since it measures heat directly, it is the only technique which allows simultaneous determination of all binding parameters (\( K \), \( \Delta H^\circ \), \( \Delta S^\circ \), and \( n \)) in a single experiment. Finally, the signal-to-noise ratio can be very favorable in high precision calorimetry, as will be seen later, which permits study at the high dilutions necessary to measure very strong binding constants.

We describe in this paper a new computerized titration calorimeter that was designed specifically for measuring binding constants and heats of binding for biolog-
Figure 1. Block diagram of the titration calorimeter showing the matched reference and sample cells with their access tubes, adiabatic shield, and electronic components which interface to an IBM PC/XT/AT or Series 2 computer. Although a preamplifier in the cell feedback circuit is contained within the instrument, better sensitivity results if a Keithley null detector is inserted to bypass the existing preamplifier.

Figure 2. Drawing of the calorimeter cells, adiabatic shield, and injector/stirrer assembly. For working below room temperature, coolant from an external circulator is passed through a series of machined veins in the wall of the adiabatic shield and an external pump attached to draw a vacuum inside the shield to prevent any condensation.
tor involvement; all data are stored on disk. Areas for all
injections may be determined either simultaneously or
peak-by-peak by computer integration and the resulting
area table may be quickly edited for heats of dilution,
if necessary, and processed (differential heats, integral
heats, and total ligand and macromolecule concentra-
tions are determined after corrections for volume dis-
placement in the total-fill cell) to arrive at final data for
deconvolution. Deconvolution is based on iterative
nonlinear least-squares, using either automatic or man-
ual initialization of parameters ($n, \Delta H^\circ$, and $K$ for
either one or two sets of sites). The operator may select which
parameters to float during minimization and which
should stay fixed. The software for data collection and
analysis is compatible with IBM PC/XT/AT/Series 2
computers.

**INSTRUMENT PERFORMANCE**

The differential power signal from the cell feedback
circuit is sampled digitally 30 times a second and aver-
egaged over the filter time specified by the operator, with
each averaged point being recorded. For a 2-s filter, the
1-min RMS noise (always available from a function key)
is typically 5–10 ncal/s when using a Keithley null detec-
tor as the preamplifier in the cell feedback circuit. Cali-
bration of the differential power axis may be carried out
either electrically by keyboard entries which activate the
calibration heater of the sample cell or chemically by in-
jection of solutions producing known heats. Agreement
between the two methods is generally within 1%, but
electrical calibration is more convenient.

For small electrical pulses of 5 µcal, the standard error
for 15 repeat determinations was 0.28 µcal. Ten successive
injections of 10 µl each from a 100-µl syringe, using a
titrant producing large heat effects, were consistent with
a standard error of 0.015 µl for each injection.

The response time is adjustable from a digital poten-
tiometer which controls the feedback gain. The best per-
formance (fastest response with no overshoot) is ob-
tained with a 6- to 7-s half-time response. This is ade-
quately so that baseline-to-baseline equilibration for
fast reactions initiated by injection is about 1.5 min. A
complete binding isotherm may then be determined in
ca. 25 min (i.e., 10 injections spaced at 2-min intervals).
After an injection sequence has been completed, on-
screen area analysis, data processing, and deconvolution
require an additional 15–25 min before the $n, \Delta H^\circ$, and
$K$ values are obtained.

Turnaround time between experiments is also short.
When samples are introduced within a couple degrees of
cell temperature, complete equilibration requires only a
few minutes. Introduction of the syringe assembly and
stirring equilibration also requires just a few minutes.
Because of the fast equilibration times, rapid titrations,
and convenient data reduction and analysis, the sample
through-put capabilities of the instrument are exceed-
ingly high.

There are two sensitivity parameters which are impor-
tant when comparing calorimeters. The absolute detec-
tion limit $S$ (µcal) is proportional to the minimum total
mass of macromolecular solute which must be used to
produce a detectable signal, while the volume-normal-
ized sensitivity $S/V$ (µcal/ml) is proportional to the
minimum concentration of solute necessary to produce a
detectable signal. It will be seen in the next section that
the lower the concentration of the macromolecule that
can be used, the larger the binding constant which can be
measured. Of the batch and titration calorimeters dis-
cussed by Hansen et al. (Ref. (14, Table 3.2)), $S$ values
range from 13 to 750 and $S/V$ values from 9 to 390. The
present instrument has an $S$ of 0.3 and an $S/V$ of 0.2
which are both 40–1000 times more favorable than those
of earlier instruments. The excellent titration calorim-
eter built recently in Stan Gill’s lab (7) has an $S$ value
about 3 times larger than ours, but an $S/V$ 20 times
higher due to the small cell volume of 0.2 ml.

**BINDING SIMULATIONS**

Choosing a binding reaction with 1:1 stoichiometry for
simplicity, it can be easily shown that (see Appendix)

\[
1 / V_0 (dQ/dX_{\text{tot}}) = \Delta H^\circ \left( \frac{1}{2} + \frac{1 - (1 + r)/2 - X_r/2}{(X_r^2 - 2X_r(1 - r) + (1 + r)^2)^{1/2}} \right), \tag{3}
\]

where $X_{\text{tot}}$ is the total ligand concentration, free plus
bound, in the reaction cell of volume $V_0$, $Q$ is the heat
absorbed or evolved, and $\Delta H^\circ$ is the molar heat of bind-
ing. The rhs of Eq. [3] contains two unitless parameters
which depend on the total ligand concentration and the
total macromolecule concentration ($M_{\text{tot}}$),

\[
1/r = c = M_{\text{tot}} K \tag{4}
\]

\[
X_r = X_{\text{tot}} / M_{\text{tot}}. \tag{5}
\]

The experimental parameter determined in the titration
calorimeter is the differential heat $dQ/dX_{\text{tot}}$ (actually
$\Delta Q/\Delta X_{\text{tot}}$) and it is seen from Eqs. [3]–[5] that this de-
dpends not on the absolute value of $M_{\text{tot}}$ but only on its
value relative to $K$ and relative to $X_{\text{tot}}$.

Binding curves simulated from Eq. [3] can be gener-
ated from the software for any selected parameter val-
ues, and some of these are shown in Fig. 3. For very tight
binding ($c = \infty$) all added ligand is bound until satura-
tion occurs so that a rectangular curve of height $\Delta H^\circ$ is
seen. For moderately tight binding with $c$ values between
1 and 1000 the shape of the binding isotherms are very
sensitive to small changes in $c$ values. The intercept of
these curves on the ordinate is no longer exactly equal
to $\Delta H^\circ$ but this parameter is still easily obtained by de-
convolution from the total area under the curve and its
shape. Very weak binding (cf. $c = 0.1$) yields a nearly
It is only in the range of $c$ values from ca. 1 to 1000 that isotherms of the type shown in Fig. 3 can be deconvoluted to obtain accurate $K$ values. We will refer to this interval as the experimental $K$ window and it is obvious that reactions with large $K$ must be studied at low macromolecule concentration and those with small $K$ at high concentration in order to fall within this window. For reactions with very large $K$ the point will eventually be reached where the detection limit of the calorimeter precludes studying the reaction at low enough concentration to fall within the $K$ window and in these cases only $n$ and $\Delta H^*$ may be determined but not $K$. Where this point occurs depends on the $S/V$ sensitivity of the calorimeter and the value of $\Delta H^*$. For the present instrument, at least a portion of the $K$ window remains available for $K$ values up to $10^5$ if the $\Delta H^*$ is 10,000 cal/mol or larger. This means that almost all macromolecule/small ligand reactions are potentially accessible for study as well as many protein/protein or protein/polynucleotide reactions. In cases where only $\Delta H^*$ and not $K$ is available, binding strength among a related family of molecules frequently parallels changes in $\Delta H^*$, while its temperature coefficient $\Delta C_p$ may be used to obtain information of the relative importance of hydrophobic bonding (8-11).

**EXPERIMENTAL METHODS**

The RNase (No. R5500) and 2'CMP (No. C7137) were from Sigma Chemical Co. and used without further purification. All other chemicals were of the highest available grade.

The RNase concentration was determined spectrophotometrically at 277.5 nm, using a coefficient of 9800 cm$^{-1}$ M$^{-1}$. The concentration of 2'CMP was measured at 260 nm and pH 7, using a coefficient of 7400 cm$^{-1}$ M$^{-1}$ which was determined by dry weight analysis (105°C).

All calorimetric experiments were carried out using the OMEGA titration calorimeter from MicroCal, Inc. (Northampton, MA), using the 100-μl injection syringe while stirring at 400 rpm. The concentration of 2'CMP in the syringe was generally ca. 25-30 times higher than the RNase concentration in the reaction cell. Since the reference cell of the calorimeter acts only as a thermal reference to the sample cell, it was filled with water containing 0.01% azide.

**EXPERIMENTAL RESULTS**

The ligand 2'CMP is a strong competitive inhibitor of substrates such as 2',3'CMP that bind to the active site of ribonuclease A. Its interaction with the enzyme has previously been characterized in Hammes' lab (12), using changes in absorbance of 2'CMP as the binding signal. In the present study, complete binding isotherms have been obtained at two temperatures (28 and 38°C) at pH 5.5 (0.2 M KAc, 0.2 M KCl) and over a 50-fold range of ribonuclease concentration from 0.015 to 0.70 mM.

Two sets of raw data are shown in Fig. 4 (0.651 mM RNase in (A) and 0.177 mM in (B)) for an automated sequence of 20 injections, each of 4.0 μl, spaced at 2-min intervals. The duration of each injection was 15 s. These data may be directly compared with those of the simulated curves in Fig. 3 since the peak height for each injection is nearly proportional to the area is exactly proportional to the $Y$ axis in Fig. 3 and the time along the injection axis is proportional to that along the $X$ axis in Fig. 3, with the 1:1 equivalence point coming in the 11th injection. The $c$ value is 31 in Fig. 4A and 9 in Fig. 4B so it is easy to see the effect of binding strength on curve shape.

Data such as these were corrected for ligand heats of dilution (which were only significant in experiments at the highest concentration), processed on the computer, and then deconvoluted using an algorithm based on the Marquardt method (15). Results from the deconvolution of one set of data (corresponding to that in Fig. 4A) are shown in Fig. 5, where the points are experimental and the solid line is the calculated best-fit curve using parameter values indicated in the figure legend. As seen, the calculated curve passes very closely through the ex-
**CALORIMETRIC MEASUREMENT OF BINDING CONSTANTS**

FIG. 4. Raw data obtained for 20 automatic injections, each of 4 μl, of 2’CMP solution into the sample cell containing RNase solution at a concentration of 0.651 mM (A) or 0.177 mM (B). The concentration of 2’CMP solution in the injection syringe was ca. 25 times higher than the RNase concentration in each case. Other conditions were the same (38°C, pH 5.5, 0.2 M KAc, 0.2 M KCl) for the two experiments. The total duration of each experiment was 41 min.

Experimental points even though the data are presented in a derivative format which accentuates errors.

For all experiments performed at 28°C, the best values for n, K, and ΔH° are compiled in Table 1, along with the standard deviation of fit expressed as a percentage of the total integral heat. For multiple determinations carried out at approximately the same RNase concentration, the mean value and average deviation from the mean are also given for n, K, and ΔH°. The average deviations are typically 1–3% for all three parameters with the exception of the K value at 0.045 mM, which is 7%. This probably reflects the fact that the c value (ca. 5) is close to the lower edge of the K window (cf. Fig. 3). This becomes more significant for the single determination at the lowest concentration of 0.0145 mM (c = 2) where it was found that there were two nearly equivalent fits with significantly different values of n, K, and ΔH°, as shown in Table 1. This emphasizes the desirability, when forced to work at low c values, of having a priori information on stoichiometry so that deconvolution of resulting isotherms may be carried out with n fixed, floating only K and ΔH° and thereby avoiding problems arising from multiple minima. The problem can also be alleviated by carrying out injections to ligand concentrations higher than those used in the present study (i.e., maximum values of only 2.0 for [2’CMP]/[RNase]) whenever c values are very small.

Although the n and ΔH° parameters show no trend as the RNase concentration is varied over a 50-fold range, the K values change systematically from 83,000 at 0.65 mM to 135,000 at 0.015 mM. Although this is a reasonably small variation, it is much greater than expected in view of the very small deviations in K values seen at any given concentration. Taken at face value, this trend suggests that the binding process could be complicated by dimerization or aggregation of RNase (aggregation of unbound 2’CMP could not cause this effect presumably.

FIG. 5. Plot of processed data (corresponding to raw data of Fig. 4A) in the derivative format previously used for simulated data in Fig. 3. The points are experimental and the solid line corresponds to the best-fit curve obtained by least-squares deconvolution. The best values of the fitting parameters are 1.05 for n, 48,800 M⁻¹ for K, and −13,700 cal/mol for ΔH. The standard deviation of points from the calculated line is 0.047% of the total integral heat for saturating all sites in the RNase sample.
since the concentration of free ligand should depend only on percentage saturation and not on the total RNase concentration) into a form which binds 2'CMP less strongly than monomeric RNase. This trend in $K$ values with concentration was found to be much stronger when the above experiments were repeated in low salt buffers (0.05 M KAc, pH 5.5) where not only the binding constants but also the heats of binding (ca. $17000$ cal/mol) were much larger. The same trend noted above is seen in the variation in $K$ values with concentration, increasing by ca. 40% as RNase concentration is lowered from 0.65 to 0.047 mM. The comparison of average $\Delta H^\circ$ values at the two temperatures suggest a $\Delta C_p^\circ$ of $-140$ cal/deg/mol which is qualitatively in agreement with the idea that there is a decrease in exposure of hydrophobic groups to water when 2'CMP binds to RNase.

As a check on the internal consistency of results at the two temperatures, the binding constant at 38°C may be readily calculated from the binding constant and heat of binding at $28^\circ$C and the $\Delta C_p^\circ$ value. These calculated values are also shown in Table 2 at each of the three concentrations. The average deviation between calculated and measured $K$ values is only about 5%.

**DISCUSSION**

These results on the binding of 2'CMP to the active site of RNase show that computer assisted titration calorimetry, as described here, is a very rapid and accurate method for characterizing biological binding reactions.

| TABLE 1 |
| The Binding of 2'CMP to Ribonuclease A at 38°C |

<table>
<thead>
<tr>
<th>RNase (mM)</th>
<th>$n$</th>
<th>$K$ (M$^{-1}$)</th>
<th>$-\Delta H$ (cal/mol)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.654</td>
<td>0.85</td>
<td>80,500</td>
<td>12,400</td>
<td>0.043</td>
</tr>
<tr>
<td>0.633</td>
<td>0.88</td>
<td>79,500</td>
<td>12,300</td>
<td>0.032</td>
</tr>
<tr>
<td>0.608</td>
<td>1.00</td>
<td>83,600</td>
<td>12,200</td>
<td>0.034</td>
</tr>
<tr>
<td>0.604</td>
<td>1.01</td>
<td>80,800</td>
<td>12,800</td>
<td>0.043</td>
</tr>
<tr>
<td>0.745</td>
<td>1.05</td>
<td>83,700</td>
<td>11,600</td>
<td>0.087</td>
</tr>
<tr>
<td>0.722</td>
<td>1.00</td>
<td>88,700</td>
<td>12,700</td>
<td>0.048</td>
</tr>
</tbody>
</table>

**TABLE 2**

| The Binding of 2'CMP to Ribonuclease A at 38°C |

<table>
<thead>
<tr>
<th>RNase (mM)</th>
<th>$n$</th>
<th>$K$ (M$^{-1}$)</th>
<th>$-\Delta H$ (cal/mol)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0475</td>
<td>1.00</td>
<td>48,800</td>
<td>13,700</td>
<td>0.047</td>
</tr>
<tr>
<td>0.0472</td>
<td>1.03</td>
<td>48,100</td>
<td>13,800</td>
<td>0.035</td>
</tr>
<tr>
<td>0.0478</td>
<td>1.00</td>
<td>109,000</td>
<td>12,300</td>
<td>0.18</td>
</tr>
<tr>
<td>0.0478</td>
<td>0.94</td>
<td>132,000</td>
<td>12,700</td>
<td>0.18</td>
</tr>
<tr>
<td>0.0410</td>
<td>1.00</td>
<td>121,000</td>
<td>11,900</td>
<td>0.07</td>
</tr>
<tr>
<td>0.0407</td>
<td>0.99</td>
<td>105,000</td>
<td>12,100</td>
<td>0.14</td>
</tr>
<tr>
<td>Av value</td>
<td>0.98 ± 0.02</td>
<td>82,700 ± 2700</td>
<td>12,300 ± 300</td>
<td>0.048</td>
</tr>
<tr>
<td>Av value</td>
<td>0.98 ± 0.015</td>
<td>98,000 ± 2200</td>
<td>12,300 ± 250</td>
<td>0.079</td>
</tr>
<tr>
<td>Av value</td>
<td>0.98 ± 0.02</td>
<td>118,000 ± 8500</td>
<td>12,100 ± 300</td>
<td>0.14</td>
</tr>
<tr>
<td>Av value</td>
<td>0.98 ± 0.02</td>
<td>135,000</td>
<td>12,800</td>
<td>0.36</td>
</tr>
<tr>
<td>Av value</td>
<td>0.69 ± 0.02</td>
<td>114,000</td>
<td>14,900</td>
<td>0.26</td>
</tr>
</tbody>
</table>

a Experimental values for the binding constant, heat of binding, and stoichiometric ratio are from deconvolution using nonlinear least-squares minimization.
The precision obtained in duplicate determinations rivals or surpasses other methods commonly used to measure binding constants and does so with considerably less consumption of time and biological materials. Equally important, it provides precise estimates of the heat and entropy of binding in addition to the binding constant, leading to a more complete characterization of the thermodynamics of interaction.

Because of the favorable $S/V$ sensitivity, the instrument extends by a considerable factor, the maximum binding constant which can be measured directly by titration calorimetry. For reactions having a net heat of 10 kcal/mol, $K$ values as large as $10^8 \text{M}^{-1}$ may be estimated using as little as 10 nmol of macromolecule in the cell. As with other calorimeters, this range may be further extended in an indirect way by setting up competitive equilibria involving two ligands and then measuring calorimetrically the equilibrium constant for the competition (13) or by enhancing observed heat effects by using coupled reactions (3).

**APPENDIX**

For a reaction of 1:1 stoichiometry, the following equations describe the binding equilibrium $M + X = MX$:

$$K = \frac{(MX)}{(X)(M)} \quad \text{[I]}$$

$$X_{\text{tot}} = (X) + (MX) \quad \text{[II]}$$

$$M_{\text{tot}} = (MX) + (M) = (MX) + \frac{(MX)}{K(X)} \quad \text{[III]}$$

Equation [II] can be solved for $(X)$ and this then substituted into the rhs of Eq. [III] which can then be rearranged to give the quadratic equation

$$(MX)^2 + (MX)(-M_{\text{tot}} - X_{\text{tot}} - 1/K) + M_{\text{tot}}X_{\text{tot}} \quad \text{[IV]}$$

whose only real root is

$$MX = \frac{-b - (b^2 - 4c)^{1/2}}{2} \quad \text{[V]}$$

where

$$b = -X_{\text{tot}} - M_{\text{tot}} - 1/K \quad \text{[VI]}$$

$$c = M_{\text{tot}}X_{\text{tot}} \quad \text{[VII]}$$

Differentiation and rearrangement of Eq. [V] then gives

$$\frac{d(MX)}{dX_{\text{tot}}} = \frac{1}{2} + \frac{1 - (1 + r)/2 - X_c/2}{(X_c^2 - 2X_c(1 - r) + (1 + r)^2)^{1/2}} \quad \text{[VIII]}$$

where $r$ is equal to $1/(KM_{\text{tot}})$ and $X_c$ is equal to $X_{\text{tot}}/M_{\text{tot}}$.

The change in $MX$ concentration can be related to the heat change as

$$dQ = d(MX) \cdot \Delta H^\circ \cdot V_0 \quad \text{[IX]}$$

where $\Delta H^\circ$ is the molar enthalpy of binding and $V_0$ is the cell volume. The substitution of Eq. [IX] into Eq. [XII] then yields the final equation (i.e., Eq. [3]) used in the text of this article.

**ACKNOWLEDGMENTS**

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