ANALYTICAL BIOCHEMISTRY 179, 131–137 (1989)

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

Thomas Wiseman,* Samuel Williston,* John F. Brandts,*,†,[1] and Lung-Nan Lin†

*MicroCal, Inc., 22 Industrial Drive East, Northampton, Massachusetts 01060, and †Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

Received October 14, 1988

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, $\Delta H°$, $\Delta S°$, and the binding constant K. Sample through-put for the instrument is high, and under favorable conditions binding constants as large as 10^8 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, $\Delta H°$, 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.,

$$\begin{aligned} M + X &= MX \\ MX + X &= MX_2 \\ &\vdots \quad\quad \vdots \\ MX_{n-1} + X &= MX_n, \end{aligned} \qquad [1]$$

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

$$\Delta G° = -RT \ln K = \Delta H° - T\Delta S°. \qquad [2]$$

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, $\Delta H°$, $\Delta S°$, 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-

[1] To whom correspondence should be addressed.

0003-2697/89 $3.00

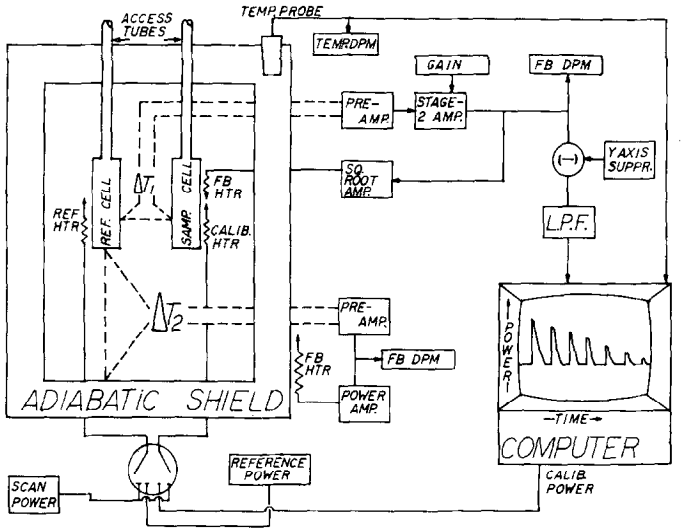

FIG. 1. Block diagram of the titration calorimeter showing the matched reference and sample cells with their access tubes, adiabatic shield, and electronic components which interface to an IBM PC/XT/AT or Series 2 computer. Although a preamplifier in the cell feedback circuit is contained within the instrument, better sensitivity results if a Keithley null detector is inserted to bypass the existing preamplifier.

ical interactions. The instrument design, performance specifications, and software are discussed and extensive data on the binding of 2'CMP[2] to the active site of ribonuclease A are presented.

INSTRUMENT DESCRIPTION

The block diagram for the titration calorimeter is shown in Fig. 1, and a scale drawing of the injector system, adiabatic shield, and matched reference and sample cells (hastelloy C, 1.4 ml) is shown in Fig. 2. The cells are of the total-fill type, lollipop in shape, with long narrow access tubes through which samples are introduced or removed using long-needled syringes. Each cell has two photoetched heaters distributed uniformly over the outer flat surface with a special thermoelectric device, containing 264 oriented crystals of bismuth telluride, sandwiched between the two inner surfaces to measure the temperature difference ΔT_1. A 20 junction wire thermopile goes between the adiabatic shield and the outer circumference of the two cells to produce a signal ΔT_2 which is amplified in a feedback circuit and fed to the feedback heater on the jacket to maintain its temperature very close to that of the cells.

During an experiment, a small, constant power of less than a milliwatt is dissipated in the heater of the reference cell, which activates the cell feedback circuit to drive ΔT_1 back to 0. In the absence of a reaction, the feedback power will be constant at the resting baseline value. Exothermic reactions will temporarily decrease and endothermic reactions temporarily increase feedback power; the reaction heats are readily obtained by computer integration of these deflections from the resting baseline.

Injection syringes (25–250 μl) are precision-bore glass with long stainless needles having a stir paddle attached to the extreme end (Fig. 2). The injection syringe is seated firmly in a low friction bearing assembly which contains an attached timing wheel. This syringe/stirring assembly is easily engaged into the Teflon loading barrel (Fig. 2) and the timing wheel coupled to a precision stirring motor (not shown). This entire assembly is continuously rotated during an experiment at ca. 400 rpm (monitored with a digital tachometer) and produces complete mixing in the cell within a few seconds after an injection, as determined in visual tests. This baseline, with stirring, is used as the resting baseline so that no corrections for heat of stirring are necessary.

The syringe plunger is mechanically coupled to a precise (6400 steps/in.) digital stepping motor. An injection schedule (number of injections, volume per injection, and time between injections) is set up with interactive software, and this schedule is carried out with no opera-

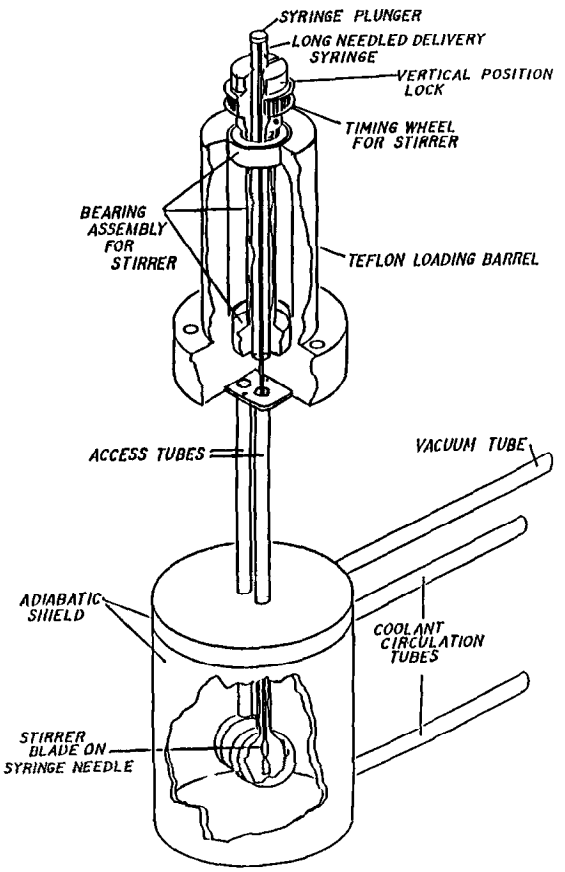

FIG. 2. Drawing of the calorimeter cells, adiabatic shield, and injector/stirrer assembly. For working below room temperature, coolant from an external circulator is passed through a series of machined veins in the wall of the adiabatic shield and an external pump attached to draw a vacuum inside the shield to prevent any condensation.

[2] Abbreviations used: RNase, bovine ribonuclease A; 2'CMP, cytidine 2'-monophosphate; rhs, right hand side.

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 concentrations are determined after corrections for volume displacement in the total-fill cell) to arrive at final data for deconvolution. Deconvolution is based on interative nonlinear least-squares, using either automatic or manual initialization of parameters (n, $\Delta H°$, 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 averaged 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 detector as the preamplifier in the cell feedback circuit. Calibration 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 injection 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 potentiometer which controls the feedback gain. The best performance (fastest response with no overshoot) is obtained with a 6- to 7-s half-time response. This is adequate 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, $\Delta H°$, 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 exceedingly high.

There are two sensitivity parameters which are important when comparing calorimeters. The absolute detection 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-normalized 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 discussed 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 calorimeter 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)

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

where X_{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 $\Delta H°$ is the molar heat of binding. The rhs of Eq. [3] contains two unitless parameters which depend on the total ligand concentration and the total macromolecule concentration (M_{tot}),

$$1/r = c = M_{tot}K \quad [4]$$

$$X_r = X_{tot}/M_{tot}. \quad [5]$$

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

Binding curves simulated from Eq. [3] can be generated from the software for any selected parameter values, and some of these are shown in Fig. 3. For very tight binding ($c = \infty$) all added ligand is bound until saturation occurs so that a rectangular curve of height $\Delta H°$ 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 $\Delta H°$ but this parameter is still easily obtained by deconvolution from the total area under the curve and its shape. Very weak binding (cf. $c = 0.1$) yields a nearly

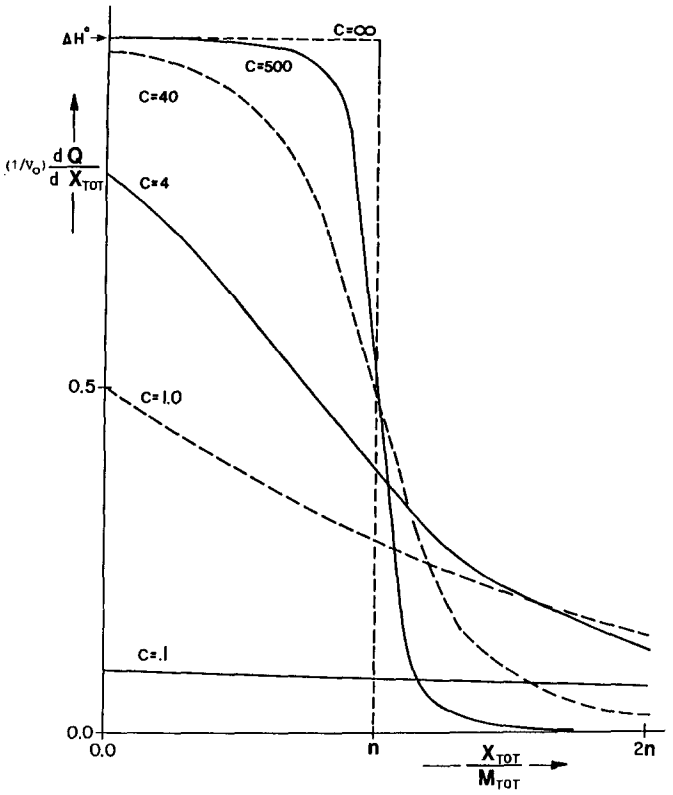

FIG. 3. Simulated binding isotherms for various values of the parameter c (equal to the product of the binding constant times the total macromolecule concentration), presented in derivative format. See text for details.

horizontal trace which again, like very tight binding, yields little information of the precise value of K.

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 ΔH° may be determined but not K. Where this point occurs depends on the S/V sensitivity of the calorimeter and the value of ΔH°. For the present instrument, at least a portion of the K window remains available for K values up to 10^8 if the ΔH° 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 ΔH° and not K is available, binding strength among a related family of molecules frequently parallels changes in ΔH°, while its temperature coefficient ΔC_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 (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-

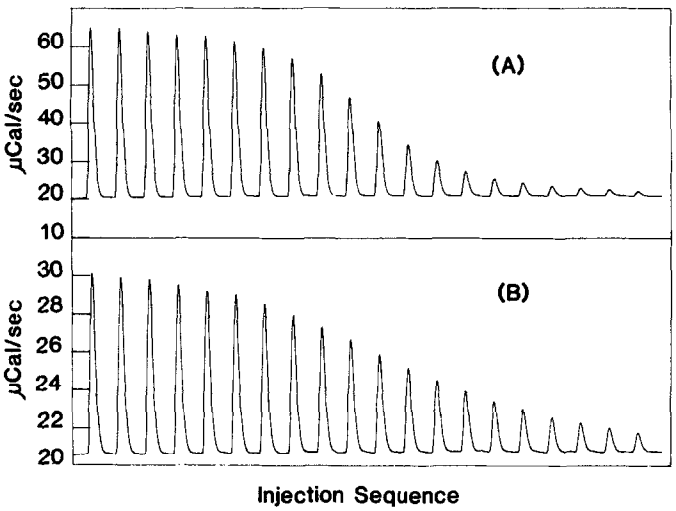

FIG. 4. Raw data obtained for 20 automatic injections, each of 4 μl, of 2′CMP solution into the sample cell containing RNase solution at a concentration of 0.651 mM (A) or 0.177 mM (B). The concentration of 2′CMP solution in the injection syringe was ca. 25 times higher than the RNase concentration in 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.

perimental 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,

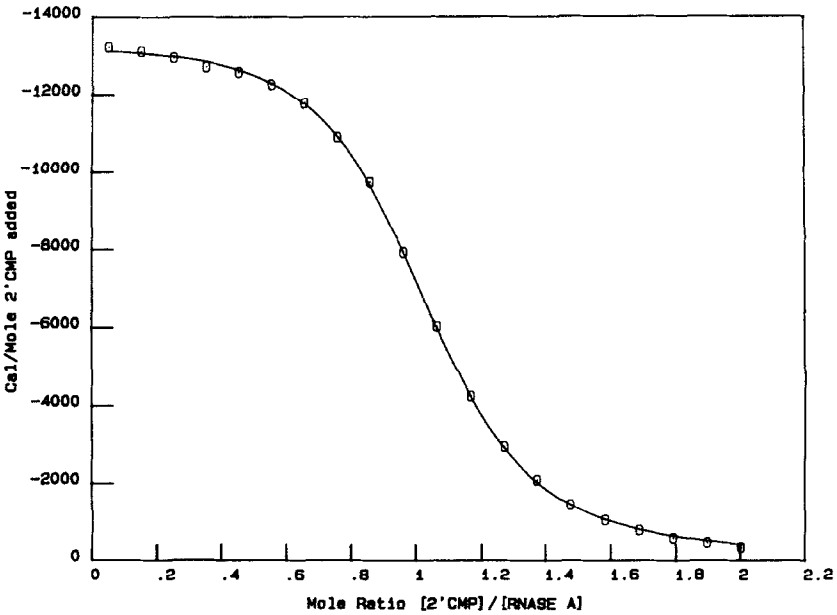

FIG. 5. Plot of processed data (corresponding to raw data of Fig. 4A) in the derivative format previously used for simulated data in Fig. 3. The points are experimental and the solid line corresponds to the best-fit curve obtained by least-squares deconvolution. The best values of the fitting parameters 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.

TABLE 1

The Binding of 2'CMP to Ribonuclease A at 28°C[a]

RNase (mM)	n	K (M^{-1})	$-\Delta H$ (cal/mol)	SD (%)
0.634	0.95	80,500	12,400	0.043
0.633	0.98	79,500	12,300	0.032
0.608	1.00	83,600	12,200	0.034
0.604	1.01	80,000	12,800	0.043
0.745	1.05	83,700	11,600	0.087
0.722	1.00	88,700	12,700	0.048
Av value	1.00 ± 0.02	82,700 ± 2700	12,300 ± 300	0.048
0.175	0.98	100,000	12,000	0.090
0.175	0.99	95,800	12,500	0.070
0.157	0.99	95,700	12,100	0.105
0.172	0.94	100,500	12,600	0.049
Av value	0.98 ± 0.015	98,000 ± 2200	12,300 ± 250	0.079
0.0446	0.99	115,000	11,500	0.14
0.0444	0.96	127,000	12,300	0.11
0.0473	1.00	109,000	12,300	0.18
0.0478	0.94	132,000	12,700	0.18
0.0410	1.00	121,000	11,900	0.07
0.0407	0.99	105,000	12,100	0.14
Av value	0.98 ± 0.02	118,000 ± 8500	12,100 ± 300	0.14
0.0145	1.00	135,000	12,800	0.36
	(0.89)	(114,000)	(14,900)	(0.26)

[a] Experimental values for the binding constant, heat of binding, and stoichiometric ratio are from deconvolution using nonlinear least-squares minimization.

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 K values showed a 2.5-fold change from 400,000 at 0.65 mM to 1,000,000 at 0.045 mM with repeat determinations again showing excellent agreement. Thus, if aggregation of RNase is responsible for the concentration dependence of K values, our results suggest that the aggregation is enhanced considerably in low salt buffers.

To check on the reliability of binding constants obtained by titration calorimetry relative to those from absorbance measurements, we did a single determination under conditions (0.05 M KAc, 0.1 M KCl, 0.18 mM RNase) very similar to those of Anderson *et al.* (12), and obtained n, $\Delta H°$, and K values of 1.03, −13,200, and 233,000 at 28.5°C, with a SD of 0.09%. Corrected to 25°C, this K value becomes 302,000 compared to the reported value of 296,000 from absorbance measurements, which corresponds to only 2% discrepancy.

Less extensive studies were carried out at a higher temperature of 38°C, with results shown in Table 2. The agreement among repeat determinations at the two highest concentrations appears to be equally good to those shown earlier in Table 1, but agreement is not as good at the lowest concentration of 0.047 mM, perhaps because the c value is smaller (ca. 2) due to the generally lower binding constants at this higher temperature. As mentioned in connection with the data at 28°C when the c value was 2, the third experiment at 0.047 mM showed two nearly equivalent minima with different n values of 1.0 and 0.90 and only the first is included in Table 2.

The binding is more exothermic at 38°C with an average heat of binding of −13,600 cal/mol compared to −12,300 at 28°C. 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 $\Delta H°$ values at the two temperatures suggest a $\Delta C_p°$ 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°C and the $\Delta C_p°$ 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 2

The Binding of 2'CMP to Ribonuclease A at 38°C[a]

RNase (mM)	n	K (M^{-1})	$-\Delta H$ (cal/mol)	SD	K (M^{-1})-calcd
0.651	1.05	48,800	13,700	0.047	
0.642	1.03	48,100	13,800	0.035	
Av value	1.04	48,450	13,750	0.041	46,050
0.177	1.04	52,700	13,900	0.085	
0.176	1.02	50,600	14,200	0.095	
Av value	1.03	51,650	14,050	0.090	54,580
0.0476	1.03	79,400	12,200	0.150	
0.0473	1.06	67,800	12,600	0.030	
0.0476	1.00	60,200	12,900	0.160	
Av value	1.03	69,100	12,600	0.110	65,714

[a] Experimental values for the binding constant, heat of binding, and stoichiometric ratio are from deconvolution using nonlinear least-squares minimization. Also included is the calculated value of the binding constant at 38°C obtained from data at 28°C as described in text.

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 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 $M + X = MX$:

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

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

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

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

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

whose only real root is

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

where

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

$$c = M_{tot}X_{tot}. \qquad [VII]$$

Differentiation and rearrangement of Eq. [V] then gives

$$\frac{d(MX)}{dX_{tot}} = \frac{1}{2} + \frac{1 - (1 + r)/2 - X_r/2}{(X_r^2 - 2X_r(1 - r) + (1 + r)^2)^{1/2}}, \qquad [VIII]$$

where r is equal to $1/(KM_{tot})$ and X_r is equal to X_{tot}/M_{tot}. The change in MX concentration can be related to the heat change as

$$dQ = d(MX) \cdot \Delta H^\circ \cdot V_0 \qquad [IX]$$

where ΔH° is the molar enthalpy of binding and V_0 is the cell volume. The substitution of Eq. [IX] into Eq. [VIII] then yields the final equation (i.e., Eq. [3]) used in the text of this article.

ACKNOWLEDGMENTS

The design and development of this instrument was financed from SBIR Grants GM 35577 and RR 03674 to MicroCal, Inc., from NIH. The study of the binding of 2'CMP to RNase was partially financed by NIH Grant GM 11071.

REFERENCES

1. Connors, K. A. (1987). Binding Constants, pp. 171–373, Wiley, New York.
2. Langerman, N., and Biltonen, R. L. (1979) *in* Methods in Enzymology (Hirs, C. H. W., and Timasheff, S. N., Eds.), Vol. 61, p. 261, Academic Press, New York.
3. Biltonen, R. L., and Langerman, N. (1979) *in* Methods in Enzymology (Hirs, C. H. W., and Timasheff, S. N., Eds.), Vol. 61, p. 287, Academic Press, New York.
4. Eatough, D. J., Lewis, E. A., and Hansen, L. D. (1985) *in* Analytical Solution Calorimetry (Grime, J. K., Ed.), p. 137, Wiley, New York.
5. Beaudette, N. V., and Langerman, N. (1978) *Anal. Biochem.* **90,** 693.
6. Bolen, D. W., Flogel, M., and Biltonen, R. L. (1971) *Biochemistry* **10,** 4136.
7. McKinnon, I. R., Fall, L., Parody-Morreale, A., and Gill, S. J. (1984) *Anal. Biochem.* **139,** 134.
8. Brandts, J. F. (1964) *J. Amer. Chem. Soc.* **86,** 4302.
9. Tanford, C. (1980). The Hydrophobic Effect, Wiley, New York.
10. Privalov, P. L. (1979) *Adv. Protein Chem.* **33,** 167.
11. Privalov, P. L., and Gill, S. J. (1988) *Adv. Protein Chem.* **39,** 191–234.
12. Anderson, D. G., Hammes, G. G., and Walz, F. G., Jr. (1968) *Biochemistry* **1,** 1637.
13. Eatough, D. J. (1970) *Anal. Chem.* **42,** 635.
14. Hansen, L. D., Lewis, E. A., and Eatough, D. J. (1985) *in* Analytical Solution Calorimetry (Grime, J. K., Ed.), p. 57, Wiley, New York.
15. Bevington, P. R. (1969). Data Reduction and Error Analysis for the Physical Sciences, p. 235, McGraw-Hill, New York.