Calorimetric Studies of Protein–Inhibitor Interaction. I. Binding of 3′-Cytidine Monophosphate to Ribonuclease A at pH 5.5*

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ABSTRACT: A calorimetric procedure for determining thermodynamic parameters of ligand binding to proteins is described. Analysis of the calorimetric data permits unambiguous assignment of reaction stoichiometry as well as an estimate of protein purity. For the system 3′-cytidine monophosphate and ribonuclease A the number of binding sites is shown to be one per protein molecule. The described procedure can be generally used to obtain good estimates of the number of sites as well as to provide substantial verification of the independence of sites when applied to systems with multiple binding loci. From the calorimetric data $\Delta G°$, $\Delta H°$, and $\Delta S°$ have been obtained as a function of neutral salt concentration at pH 5.5. Strong arguments are presented to show that chloride and acetate ions do not bind at the inhibitor binding locus of ribonuclease A. This is in contrast to the effect of phosphate ion which presumably interacts with the two histidine residues on the inhibitor site. The effect of added salt on the binding reaction is described.

A number of recent studies have been concerned with the details of the interaction of 3′-cytidine monophosphate (3′CMP)[1] with ribonuclease A (RNase). Nuclear magnetic resonance has been used to monitor the state of the histidine residues (Ruterjans and Witzel, 1969; Meadows *et al.*, 1967, 1969) and temperature-jump experiments have attempted to separate the individual steps in the binding process (Cathou and Hammes, 1964, 1965; Hammes and Schimmel, 1965; French and Hammes, 1965; Anderson *et al.*, 1968; Hammes and Walz, 1969). The obtained data have been interpreted and discussed in terms of rather complicated mechanisms, and speculations with regard to the importance of the three-dimensional structure of RNase and possible conformational change have been made (Hammes and Walz, 1969). Very few reliable thermodynamic data on RNase–inhibitor interaction have been reported and many of the mechanistic conclusions are based on these few estimates. Since the details of 3′CMP–RNase interaction must ultimately agree with the equilibrium thermodynamics of the system, it is essential that a systematic study of equilibrium binding be provided as a function of the variables of interest, *e.g.*, pH, ionic strength, and temperature.

In this communication a calorimetric technique for obtaining complete thermodynamic information ($\Delta G°$, $\Delta H°$, $\Delta S°$) from a single "binding curve" is described. Thermodynamic criteria for the establishment of the stoichiometry of the reaction are developed. The thermodynamic quantities for the binding of 3′CMP to RNase as a function of ionic strength at pH 5.5 are reported and a preliminary discussion of the data with regard to the mechanism is presented.

Experimental Section

Chromatographically pure 3′-cytidine monophosphate (3′CMP) (99% pure) was purchased from Boehringer Mannheim Corp. and used without further purification. A molar extinction coefficient of 7600 at 260 nm, pH 7.0 (Beaven *et al.*, 1955), was assumed. Ribonuclease solutions were prepared from salt-free, phosphate-free, lyophilized RNase obtained from Worthington Biochemicals Co. Before being used for the calorimetric measurements RNase solutions of different salt concentrations were adjusted to pH 6.5, thermostated for 10 min at 62° to disrupt aggregates (Fruchter and Crestfield, 1965), cooled to the room temperature, and readjusted to pH 5.51 ± 0.02. Solutions thus obtained were analyzed chromatographically (Hirs *et al.*, 1953; Kaplan *et al.*, 1956) on a Sephadex G-75 column, and found to be homogeneous. The RNase concentration was determined spectrophotometrically assuming a molar extinction coefficient of 9800 at 277.5 nm at pH 7.6 (Sela and Anfinsen, 1957).

Calorimetric measurements were carried out using an LKB batch microcalorimeter or an LKB flow microcalorimeter. With the former calorimeter the heat evolved on mixing 4 ml of a solution of 3′CMP and 2 ml of a solution of enzyme was measured. The observed heats ranged from 0.1 to 12 mcal. With the flow calorimeter approximately equal volumes of inhibitor and enzyme were mixed. The flow rate of each solution was approximately 0.003 ml/sec and the steady-state heat flux ranged from 1 to 60 μcal per sec. The heats of dilution of the inhibitor and enzyme were measured separately and subtracted from the measured heat of mixing to obtain the heat of reaction. In all experiments the inhibitor and enzyme solutions were identical with respect to salt concentration and pH. The calibration of both calorimeters was performed electrically and checked by measuring the heat of neutralization of HCl and NaOH and the heat of dilution of sucrose.

Since no buffer was used in the experiments a great deal of care was taken to monitor the pH. Prior to placement into the calorimeter the solutions were adjusted to pH 5.5 with dilute HCl or KOH in 0.5 M KCl with a Radiometer TTT 1 pH meter. The pH was taken as soon as possible after mixing and was seldom seen to change more than 0.01 pH unit. Experiments which resulted in pH changes of more than 0.02 pH unit was rejected.

The apparent thermodynamic quantities for the binding reaction, $\Delta H°_B$ and K_B, were obtained by iterative least-

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[1] Abbreviation used is: 3′CMP, 3′-cytidine monophosphate.

squares analysis of the data according to eq 3 or 7. The condition of "best fit" was assumed to be achieved when the change in the estimate of Q_{max} was less then 2%. Each Q was weighted in the appropriate manner by assuming that the absolute random error in each measured Q was the same. To be certain an absolute minimum was achieved by this procedure some of the data was also analyzed by assuming a broad series of values for Q_{max} (in increments of 0.1 kcal/mole) and finding the best-fit value of K_B. Although it was found that the minimum was broad, the estimates of $\Delta H°_B$ and K_B obtained for the minimum sum of residues were in good agreement with those obtained by the former method.

Results and Discussion

Basic Experiment. Upon mixing solutions of the inhibitor and ribonuclease in the calorimeter heat is evolved which is equal to the sum of the heats of dilution of the components and the heat of reaction between inhibitor and enzyme. The enthalpy change calculated per mole of enzyme after subtraction of the heats of dilution is shown for a typical experiment in Figure 1 as a function of total inhibitor concentration. This experimental heat of reaction, Q, is a direct measure of the amount of enzyme–inhibitor complex formed. For a system of n identical and independent binding sites the general expression for extent of association is (Klotz, 1953)

$$\frac{1}{r} = \frac{1}{nK_B[I_F]} + \frac{1}{n} \tag{1}$$

where r = moles of bound I/total moles of E, K_B = intrinsic binding constant/site, and $[I_F]$ = free concentration of inhibitor. The relationship between r and Q is given by

$$r = n\frac{Q}{Q_{max}} \tag{2}$$

where Q_{max} is the heat of association per mole of enzyme for complete saturation of all sites. Thus eq 1 reduces to

$$\frac{1}{Q} = \frac{1}{Q_{max}} + \frac{1}{Q_{max}K_B[I_F]} \tag{3}$$

where

$$[I_F] = [I_t] - n\frac{Q}{Q_{max}}[E_t] \tag{4}$$

$[I_t]$ = total inhibitor concentration and $[E_t]$ = total enzyme concentration. The best values of Q_{max} and K_B were solved by iterative least-squares treatment of the experimental data for assumed values of n. The results of such treatment of the data in Figure 1 are shown in the "double-reciprocal" plot in Figure 2 with $n = 1$. The excellent linear relationship over a 400-fold concentration range shows that the mathematical model is adequate, and hence apparent values of all thermodynamic quantities for the reaction can be calculated from knowledge of $\Delta H°_B$ and K_B using eq 5 and 6 and choosing a standard state of 1 mole/l. Representation of the data for assumed values of $n > 2$ shows curvature outside experimental error. Consequently, the wide concentration range covered is sufficient to exclude models for $n > 2$. Although $n = 1$ represents the data better than $n = 2$, a more stringent test is necessary to determine the stoichiometry. Such a test is provided by obtaining a binding curve at constant inhibitor concentration and varying enzyme concentration. In this case Q' is calculated per mole of inhibitor and the appropriate equation to represent the data is

$$\frac{1}{Q'} = \frac{1}{Q'_{max}} + \frac{1}{Q'_{max}\alpha[E_F]} \tag{7}$$

where Q'_{max} is the heat of association per mole of inhibitor for complete binding of the inhibitor and is equal to Q_{max}/n

$$[E_F] = [E_t] - \frac{Q'[I_t]}{nQ'_{max}} \tag{8}$$

and $\alpha = nK_B$. The best values of Q'_{max} and α can again be solved by an iterative least-squares procedure for assumed values of n. For the case $n = 1$, a linear relationship between $1/Q'$ and $1/[E_F]$ was found as expected. However, the conclusion regarding the exact stoichiometry of the reaction does not rest upon the linearity relationship, but upon comparison

$$\Delta G°_B = -RT \ln nK_B \tag{5}$$

$$\Delta S°_B = \frac{\Delta H°_B - \Delta G°_B}{T} \tag{6}$$

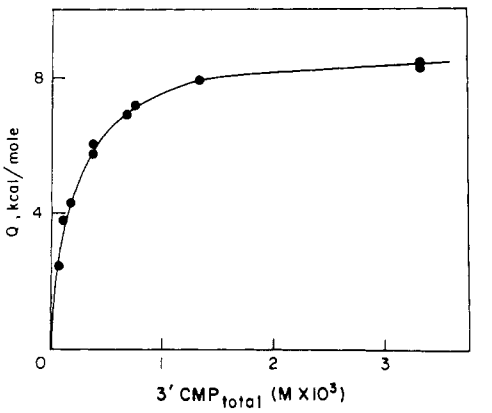

FIGURE 1: Dependence of the heat of reaction between RNase and 3'CMP on the total 3'CMP concentration. [RNase] = 6.63×10^{-5} M; pH 5.52; T 25°.

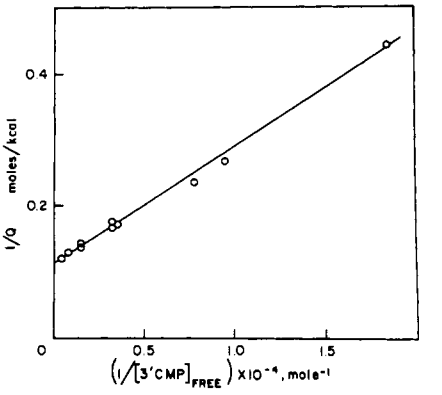

FIGURE 2: Double-reciprocal plot of the data in Figure 1. 1/[3'CMP] is calculated from the free 3'CMP concentration.

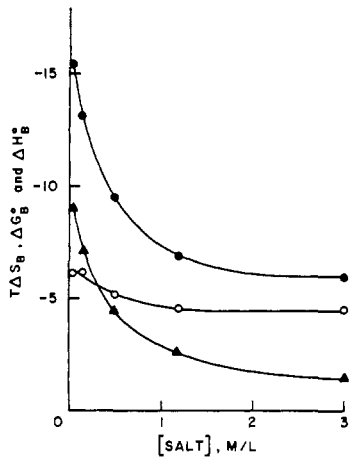

FIGURE 3: Salt dependence of the thermodynamic quantities of the 3′CMP–RNase interaction (●) ΔH°_{B}; (○) ΔG°_{B}; and (▲) $T\Delta S^{\circ}_{B}$.

TABLE I: Values for the Binding Constant, K_B, ΔH°_{B}, and the Number of Binding Sites, n, for the Interaction between RNase and 3′CMP at 25°, pH 5.51 ± 0.02 at ionic strength, $\mu = 0.5$.

	$10^{-3} K_B$ (mole^{-1})	n^b	ΔH°_{B} (kcal/mole)	n^c
[Inhibitor] varied[d]	6.1 ± 1.8		9.7 ± 0.5	
		1.0 ± 0.3		0.99 ± 0.05
[Enzyme] varied[e]	6.1 ± 0.9[a]		9.8 ± 0.2	

[a] Calculated assuming $n = 1$. [b] Calculated from the ratio α/K_B; see text for details. [c] Calculated from the ratio of ΔH°_{B} of the two different types of experiments; see text for details. [d] Average values of the data obtained from four experiments. [e] Average values of the data obtained from two experiments.

of the calculated values of K_B and α in the two different experiments since $\alpha = nK_B$, and upon comparison of the calculated values of Q_{max} and Q'_{max} since $Q_{max} = nQ'_{max}$. The calorimetric results for two sets of such experiments at 0.5 M KCl are summarized in Table I. These results show that n is equal to 1 for the binding of 3′CMP to RNase, a result consistent with previous studies. The error in the estimate of n, calculated from values of the binding constant, is about 25% because of the large errors in the estimate of this "fitting" parameter. However, the error in Q_{max} and Q'_{max} are on the order 5% in the present experiments. For this reason n calculated from the ratio of Q_{max} to Q'_{max} is the best estimate of n.

Any impurity or loss in activity of enzyme would produce a value of $n < 1$, when calculated from the ratio Q_{max}/Q'_{max}. McKie (1969) has recently found that even after heating a solution of lyophilized RNase, as described by Fruchter and Crestfield (1965), approximately 9% of the protein is nonmonomeric. From the data presented in Table I one must conclude that either there is no such nonmonomeric material or, if it is present, that it participates in the binding reaction in a manner equivalent to monomeric RNase. According to our chromatographic analysis the former conclusion is more likely than the latter. Thus the enthalpy ratio test is useful not only for determining reaction stoichiometry but it can also serve as a check on the purity of reactants. In the present study we have been concerned with a relatively simple interacting system and the analysis of the situation is straightforward. This is not the case when n is much larger than 1. To unambiguously determine n in such cases, it is necessary to have a protein preparation of high purity and it is required that all sites be equivalent and independent. However, we are certain the calorimetric technique will prove to be very useful in developing a complete thermodynamic description of a variety of binding processes, particularly when used in conjunction with techniques that directly measure the amount of binding.

An example of how the present experimental approach would be useful in solving apparent discrepancies in experimental results and interpretation is provided by recent experiments of Bjurulf *et al.* (1970) and Vichutinsky *et al.* (1969) on the binding of inhibitors to lysozyme. There is apparent disagreement concerning whether the stoichiometry of the reaction is such that $n = 1$ or $n = 2$. The assessment of the situation is based on the deviation from linearity of "double-reciprocal" plots. Since the two sets of experiments were done under different experimental conditions it is impossible to determine whether the differences are real or experimental "artifacts." However, calorimetry experiments, as described above, could in principle resolve the situation.

Influence of Neutral Salt. The strong influence of neutral salts on the properties of RNase have been widely reported. In most cases this influence has been interpreted either in terms of binding of anions to the positively charged centers of the protein, or in terms of induced changes in conformation of the molecule. Since neutral salts have a significant influence on the binding of 3′CMP to RNase, and since the molecular basis of this influence was not clear, a systematic study of the influence of neutral salt on the binding reaction at pH 5.5 was undertaken. The results are tabulated in Table II. According to these results, there exists a direct relationship between the thermodynamic quantities and the ionic strength as shown in Figure 3. There are two qualitative conclusions which can be made. First, the thermodynamic quantities are only a function of ionic strength since no difference is observed between potassium chloride and sodium acetate. Second, while all thermodynamic quantities change with increasing ionic strength and reach limiting values at high salt concentration, the changes in enthalpy and entropy tend to compensate for one another making the change in free energy relatively small. Similar behavior has previously been observed for the binding of inhibitors to RNase as a function of pH (Hammes, 1964) and for several other types of processes involving protein systems in aqueous solvent (Lumry and Rajender, 1970).

There are several possibilities for the molecular basis of the influence of neutral salts on the binding of 3′CMP to RNase. These include: (A) specific counterion binding to positively charged histidine residues which are known to directly interact with the phosphate group of the inhibitor (Meadows *et al.*, 1969); (B) simple counterion shielding of the electrostatic interaction between the protein histidine residues and the phosphate group of the inhibitor; and (C) ionic strength effects on the apparent pK_a values for the ionizable groups on the protein and inhibitor which are

TABLE II: The Influence of Salt Concentration on the Thermodynamic Parameters of the 3′CMP–RNase Binding Reaction in Water at 25° (pH 5.50 ± 0.02, [RNase] = 3.5×10^{-4} M and [3′CMP] varied from 2×10^{-4} to 2×10^{-2} M).

[Salt], Moles/l.:	0.05		0.15	0.50		1.20		3.00
Salt:	NaAc	KCl	NaAc[a]	NaAc	KCl[b]	NaAc	KCl	NaAc[a]
$10^{-3} K_B$, mole^{-1}	36	38	39	5.3	6.3	1.5 2.5[a]	3.2	2.1
$-\Delta G°_B$, kcal/mole	6.19	6.21	6.24	5.11	5.20	4.62[a] 4.32	4.64	4.53
$-\Delta H°_B$, kcal/mole	15.3	15.8	13.2	9.2	9.8	7.5 6.1[a]	7.0	6.0
$-\Delta S°_B$, cal/(mole deg)	30.6	29.5	23.5	14.0	15.4	10.7 5.0[a]	11.3	4.9

[a] Values calculated from the data obtained at 30° assuming no heat capacity change. [b] Average values of the data obtained from three experiments.

important in the binding reaction. Such an effect could be coupled with a conformational change of the protein.

The data in Table II allow us to present strong arguments that competitive binding of anion to the histidine residues is not responsible for the changes in the thermodynamic parameters as a function of ionic strength. This follows first from the fact that there is no difference in the influence of chloride or acetate anion. One might expect that if direct counterion binding is important a difference in the effect of the two anions would be observed. An even stronger basis for discarding specific counterion binding is the fact that at high ionic strength both $\Delta G°_B$ and $\Delta H°_B$ reach limiting values. If one assumes a direct competition between inhibitor [I] and anion [A^-] the appropriate reaction scheme is the following

$$E + A^- \rightleftarrows EA^-$$

$$E + I \rightleftarrows EI$$

where $K_A = [EA^-]/[E]\,[A^-]$. The apparent dissociation constant of the inhibitor is given by

$$K_{app} = \frac{([E] + [EA^-])[I]}{[EI]} = \frac{[I][E]}{[EI]}(1 + K_A[A^-])$$

or

$$K_{app} = K_0(1 + K_A[A^-]) \quad (9)$$

where K_0 = dissociation constant for the enzyme–inhibitor complex in the absence of competitive anion. In such a reaction scheme one would observe that $\Delta H°_B$ would reach a limiting value at high ionic strength, but $\Delta G°_B$, which is a linear function of ln $(1 + K_A[A^-])$, would continue to increase with increasing salt concentration. Since this is not the case, one must conclude that there is no competitive binding of acetate or chloride ion to the two histidine residues involved in the 3′CMP binding. It should be noted that the present data does not allow the elimination of the possibility that the anions can bind specifically to both the free enzyme and the complex. If this is the case, then

$$K_{app} = K_0\frac{(1 + K_A[A^-])}{(1 + K'_A[A^-])}$$

where K'_A is the dissociation constant for the dissociation of the anion from the ternary complex and both $\Delta G°_{app}$ and $\Delta H°_{app}$ would attain limiting values at high salt concentration. This would seem to be an unlikely possibility, however, since the ternary complex of protein, inhibitor, and anion would have to be thermodynamically identical for both acetate and chloride anion. In addition, extensive studies of the pH dependence of the thermodynamics of the inhibitor–protein interaction clearly show that a major effect of ionic strength is manifested as a shift of pK values of histidine residues involved in the interaction (M. Flögel, D. W. Bolen, and R. Biltonen, in preparation).

It is also to be noted that the comments regarding anion effects on the thermodynamics of inhibitor binding to RNase made here do not pertain to the influence of phosphate ion on the reaction. Studies in our laboratory clearly show that phosphate competes with the inhibitor at the protein binding locus (M. Flögel and R. Biltonen, unpublished observations). A difference between phosphate, on one hand, and acetate or chloride anion, on the other, has previously been shown by electrophoresis studies of Crestfield and Allen (1954), who found that at constant ionic strength the isoelectric point of RNase was shifted to lower pH by about 3 units in the presence of phosphate as compared to other anions. This result is consistent with specific phosphate binding to the protein and hence reduction of the isoelectric point.

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Structure of Immunoglobulin A. I. Interchain Disulfide Bridges of a γA1 Myeloma Protein*

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ABSTRACT: A γA1 κ-light-chain-type myeloma protein was partially reduced and alkylated with [^{14}C]iodoacetic acid in order to study the interchain disulfide bridges. After digestion, six carboxymethylcysteine peptides were obtained (α1 to α6). One of them (α1) was shown to bridge heavy to light chains and its sequence and function was similar to a peptide obtained from a protein belonging to the Am2 (−) genetic variant of γA2-globulins. A second peptide (α2) rich in proline, cysteine, and carbohydrate probably represented the "hinge" region. A third one (α6) was at the C-terminal end of the heavy chain. The location and function of the other three carboxymethylcysteine peptides are not known.

In recent years, immunoglobulin A (IgA) has assumed great significance because although it is a minor constituent of serum it is the predominant immunoglobulin present in external secretions derived from mucosal surfaces, and thus appears to play an important role in local defense mechanisms of the body (Tomasi and Bienenstock, 1968). Like other immunoglobulins, serum IgA appears to consist of four polypeptide chains, two light chains which are common to all immunoglobulins, and two heavy chains (α) which determine the properties characteristic of this class. Two antigenically distinguishable subclasses known as γA1 and γA2 have been recognized (Feinstein and Franklin, 1966; Kunkel and Prendergast, 1966; Vaerman and Heremans, 1966). The antigenic differences are located on the α chains and are independent of light-chain class and monomer–polymer-related structures. In 1968, Grey *et al.* reported that the γA2-immunoglobulins lacked the disulfide bond linking the heavy and light chains in all other classes of immunoglobulins. Instead, the γA2 fraction consists of a pair of disulfide-bonded light chains which are bound to the α chains by noncovalent bonds. More recently, Kunkel *et al.* (1969) and Vyas and Fudenberg (1969) described a genetic marker associated with the γA2 fraction which was called Am2 to conform with the terminology used in the Gm system for γG-globulins. Family studies showed evidence for close linkage of the Am2 marker to the Gm system (Kunkel *et al.*, 1969). Jerry *et al.* (1970) reported that the γA2-immunoglobulins belonging to the genetic variant Am2 (+) were responsible for the previously noted behavior of the γA2 fraction since they were the predominant component and dissociated in the presence of acid or urea into heavy- and light-chain dimers without reduction of the disulfide bonds. In contrast, the rarer Am2 (−) fraction resembled the other immunoglobulins in having the light and heavy chains linked by a single disulfide bond.

IgA present in secretions has a higher molecular weight than serum IgA since it exists as a dimer or trimer linked to an additional polypeptide chain called secretory piece or transport piece, having a molecular weight of approximately 60,000 (Tomasi and Bienenstock, 1968). A new component called J chain present in rabbit and human secretory IgA, as well as in dimeric forms of human IgA myelomas, has been described recently (Halpern and Koshland, 1970; Mestecky *et al.*, 1971). It has been suggested that the J chain might be involved in maintaining the tertiary structure of the polymeric immunoglobulin molecule.

Prior studies of the four subclasses of γG-globulins have clearly demonstrated the value of characterizing the intrachain

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