A Calorimetric Study of the Thermal Stability of Barnase and Its Interaction with 3′GMP†

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ABSTRACT: We have used high-sensitivity differential scanning calorimetry to characterize the thermal stability of barnase from *Bacillus amyloliquefaciens* in the pH range 2.0–5.0. The energetics of the interaction between barnase and its inhibitor 3′GMP have been studied by isothermal titration calorimetry in the temperature range 15–30 °C. Scanning calorimetry experiments were also made with the protein in the presence of various concentrations of 3′GMP at pH 4.5. A novel, simple procedure is proposed to obtain binding parameters from scanning calorimetry data. This method is based on the calculation of the partition functions of the free and the ligand-bound protein. Isothermal calorimetry shows that at 25 °C 3′GMP binds to a single site in barnase with a ΔC_p of −250 ± 50 J/(K·mol). Both free barnase and ligand-bound barnase undergo a highly reversible, two-state thermal unfolding process under our experimental conditions. ΔG and ΔC_p unfolding values are similar to others found for globular proteins, whereas ΔH and ΔS unfolding values are unusually high at the denaturation temperature of barnase. We have also found unexpectedly that the thermodynamic unfolding parameters of barnase fit neither the trend of values described in the literature for the correlation between ΔC_p and ΔH nor the limiting specific enthalpy value in the correlation between ΔH and T_m for globular proteins. These discrepancies might be related to particular features of the folded and/or unfolded states of the protein.

The study of protein folding/unfolding and stability is today of paramount importance both in the academic field and in biotechnology. The equilibrium transition between the folded and unfolded states of small proteins is usually a two-state one (Privalov, 1979); i.e., the protein is either in the native, folded state or in the unfolded, denatured state. The stability of the folded state is only about 20–60 kJ/mol higher than that of the unfolded conformation. This small value is the result of a compromise balance between large enthalpy and entropy values. Protein stability can be affected by many factors, such as the binding to specific effectors, an almost ubiquitous process in *in vivo* protein function. This stability change is obviously related to the energetics of the protein–ligand interaction.

High-sensitivity differential scanning calorimetry (DSC)[1] is a very suitable technique for characterizing the thermal stability of proteins since it provides the calorimetric and van't Hoff enthalpy and other thermodynamic values, and may lead to the analysis of multidomain proteins, as long as the denaturation process occurs under equilibrium conditions (Privalov, 1979, 1982). As for isothermal calorimetry, it allows one the most direct thermodynamic analysis of protein–ligand interactions.

We have applied here both DSC and isothermal titration calorimetry to study barnase and its binding to the inhibitor 3′GMP. Barnase is a single-domain, monomeric, extracellular ribonuclease from *Bacillus amyloliquefaciens*. It consists of a single polypeptide chain with 110 residues, M_r 12 382, without cysteines or methionines and with three *trans*-prolines (Hartley & Barker, 1972). The gene for this enzyme has been cloned and can be expressed in *Escherichia coli* (Paddon & Hartley, 1987). Barnase secondary structure contains two α-helices and a five-stranded antiparallel β-sheet. The X-ray crystal structure has been solved at 2-Å resolution (Mauguen et al., 1982), and the structure in solution has been determined by NMR (Bycroft et al., 1991). The protein undergoes reversible, two-state denaturation induced either by solvent or by heat (Hartley, 1968; Kellis et al., 1989). Barnase is a very appropriate model for studying protein stability and folding in solution, and a large amount of physicochemical data has been published, including detailed information on the thermodynamics and kinetics of the folding of wild-type barnase and many of its mutants (Kellis et al., 1988, 1989; Matouschek et al., 1990, 1992; Serrano et al., 1990, 1992a,b; Fersht et al., 1992).

In this paper, we describe the DSC thermodynamic characterization of the thermal stability of the free protein in the pH range 2.0–5.0, and that of barnase in the presence of increasing concentrations of 3′GMP at pH 4.5. In addition, the interaction of the protein with 3′GMP has been studied by isothermal titration calorimetry at pH 4.5 in the temperature range 15–30 °C. We also propose here a new method of estimating binding parameters by obtaining the partition functions of the free and liganded protein from DSC data. Barnase thermal denaturation follows a reversible, two-state transition; nevertheless, its high unfolding enthalpy does not fit some correlations described in the literature for compact globular proteins.

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[1] Abbreviation: DSC, differential scanning calorimetry.

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EXPERIMENTAL PROCEDURES

Biochemicals and Calorimetric Experiments. Wild-type barnase was expressed and purified as described elsewhere (Serrano et al., 1990). Before calorimetric experiments, the protein was extensively dialyzed against the appropriate buffer. The concentration of barnase was calculated from the UV absorbance of the solution at 280 nm, using an extinction coefficient of 2.21 mg^{-1}·mL·cm^{-1} (2.74 × 10^4 M^{-1}·cm^{-1}; Loewenthal et al., 1991).

In the DSC experiments with 3′GMP (Sigma), the ligand dissolved in buffer at a concentration of 50 mM was added to the dialyzed protein sample to give the desired concentrations of protein and of 3′GMP. If not specified otherwise, the solutions contained either 50 mM sodium or potassium acetate or 50 mM glycine (Sigma).

Scanning calorimetry was performed in a computerized version of the DASM-4 microcalorimeter (cell volume 0.47 mL; Privalov & Potekhin, 1986) at heating rates of 2 and 1 K/min with protein concentrations of about 1.5 mg/mL (a wider range of concentrations from 0.8 up to 4 mg/mL was checked at pH 4.5, used for 3′GMP studies, and also at pH 2.5). In addition, some DSC experiments were made in the DASM-1M microcalorimeter (Privalov et al., 1975) with a different cell configuration and volume (1 mL). An extra pressure of 1.5 atm was maintained during all DSC runs to prevent possible degassing of the solutions on heating. The molar partial heat capacity of the protein, C_p, was evaluated according to standard procedures (Privalov & Potekhin, 1986), taking 0.73 mL/g for the partial specific volume (an average value for globular proteins, confirmed by calculations according to Makhatadze et al., 1990) and 12.4 kDa for the molecular mass of barnase (Hartley & Barker, 1972). The reversibility of the unfolding was checked routinely by sample reheating after cooling inside the calorimetric cell.

Isothermal calorimetric titration experiments were conducted using a mixing calorimeter designed and built at the University of Granada according to McKinnon et al. (1984). The heat of mixing barnase with 3′GMP was measured by adding 10-μL portions of the 0.6 mM ligand stock solution to the calorimetric cell (volume 0.2 mL) containing the protein solution at about 0.2 mM concentration. To compensate for dilution of the enzyme, the 10-μL injection solution had the same protein concentration as that in the cell. Experimental heat binding values were corrected for thermal contributions of the stirring effect and that of the ligand dilution, which were always very small (≅8 μJ). The experiments were performed in 50 mM potassium acetate, pH 4.5, between 15 and 30 °C.

Analysis of Calorimetric Titration Data. Prior to any data analysis, the number of protons exchanged upon binding was obtained by potentiometric titration of the protein with 3′GMP. We found that about 0.12 proton was released by the system within the temperature range 15–30 °C. Given the small heat of ionization of acetate, this thermal correction was almost negligible. The dependence of the heat effect on the total amount of ligand added was recorded and analyzed to evaluate the number of binding sites n_b, binding enthalpy ΔH_b, and binding constant K_b, assuming a noncooperative binding of the ligand. An iterative, nonlinear, least-squares fitting of the molar enthalpy change, ΔH, for a given free-ligand concentration, [L], was used according to the equation:

$$\Delta H = n_b \Delta H_b K_b [L]/(1 + K_b[L]) \quad (1)$$

The free-ligand concentration was obtained from the total ligand, $[L_0]$, and protein, $[P_0]$, concentrations according to

$$[L] = \{\sqrt{(1 - K_b[L_0] + n_b K_b[P_0])^2 + 4K_b[L_0]} + K_b[L_0] - n_b K_b[P_0] - 1\}/2K_b \quad (1a)$$

The results calculated at each temperature (15, 20, 25, and 30 °C) were further analyzed to determine the temperature dependence of thermodynamic functions. For that purpose, the data on binding enthalpy have been fitted using a linear least-squares regression to estimate the ΔC_p of binding, taking into account that only one binding site for 3′GMP was found. After estimating $\Delta C_{p,b}$, the final fitting of the observed temperature dependence of binding parameters was made according to the equations:

$$\Delta H_b = \Delta H_{b,0} + \Delta C_{p,b}(T - T_0) \quad (2)$$

$$\Delta S_b = \Delta S_{b,0} + \Delta C_{p,b} \ln(T/T_0) \quad (3)$$

$$\Delta G_b = -RT \ln K_b \quad (4)$$

The adjustable parameters were $\Delta H_{b,0}$ and $\Delta S_{b,0}$, the enthalpy and entropy of binding at a given temperature, T_0 (normally 288.2 K).

Analysis of Scanning Calorimetry Data. We have also tried to use scanning calorimetry not only to characterize protein unfolding but also to evaluate the parameters of barnase interaction with 3′GMP. The applicability of DSC to protein–ligand binding studies has been analyzed recently by a number of authors (Robert et al., 1988; Shrake & Ross, 1990, 1992; Brandts & Lin, 1990; Straume & Freire, 1992). Thus, it has been shown that scanning calorimetry is an appropriate technique for measuring very strong affinity constants. As concerns weak binding, such as the one taking place on the interaction of 3′GMP with barnase, the traditional isothermal techniques give more precise results below 50 °C. Nevertheless, even in such cases, scanning calorimetry should be more useful to evaluate binding parameters at higher temperatures since with DSC long extrapolations of thermodynamic functions from low to high temperature are unnecessary. Here we show that by using a combination of the two techniques the ligand binding parameters can be determined with sufficient accuracy over a wide temperature range.

We have extensively checked several analytical procedures used for calculating thermodynamic parameters from scanning calorimetry data. First, we applied nonlinear fitting algorithms to calculate the binding and unfolding parameters from the temperature dependence of C_p. Manipulating such parameters as the van't Hoff enthalpy and its ratio to the calorimetry heat effect can be avoided by using modern analytical software. These parameters are only rough estimates but are widely used in the literature due to the simplicity of their determination from calorimetric data. It can be shown, however, that the van't Hoff enthalpy, which is usually estimated from peak sharpness, might be misleading if the melting profile is distorted by factors that do not alter the height-to-area ratio. Therefore, the computer global fitting of calorimetric curves *over the whole temperature range of the transition*, according to a suitable model, seems to be a more precise and informative procedure for data evaluation and analysis. To check the validity of a two-state model for barnase heat denaturation, as well as to calculate the thermodynamic parameters of unfolding, we used the computer programs developed by Filimonov et al. (1982) at the Institute of Protein Research (Pushchino, Russia). An example of the excellent fitting of

Table 1: Thermodynamic Parameters of 3'GMP Binding to Barnase at pH 4.5 Calculated from Isothermal Titration Calorimetry Data

T (K)	$n_b{}^a$ (sites)	$\Delta H_b{}^a$ (kJ/mol)	$\Delta H_b{}^b$ (kJ/mol)	$K_b{}^a \times 10^{-5}$ (M^{-1})	$K_b{}^b \times 10^{-5}$ (M^{-1})	$\Delta G_b{}^a$ (kJ/mol)	$\Delta G_b{}^b$ (kJ/mol)	$\Delta S_b{}^a$ [J/(K·mol)]	$\Delta S_b{}^b$ [J/(K·mol)]
288.2	0.98	−46.9 ± 0.4	−46.7	7.4 ± 0.8	6.98	−32.4 ± 0.3	−32.7	−49.8	−48.6
293.2	1.00	−47.3 ± 0.6	−47.9	4.7 ± 0.6	5.01	−31.8 ± 0.3	−32.4	−52.3	−52.8
298.2	0.92	−49.8 ± 0.6	−49.2	3.4 ± 0.3	3.60	−31.6 ± 0.2	−32.1	−60.7	−57.3
303.2	0.99	−50.2 ± 0.8	−50.4	2.2 ± 0.3	2.68	−31.0 ± 0.3	−31.9	−64.0	−60.9

[a] Parameters calculated by fitting the experimental titration curves to eq 1 and 1a. [b] Values obtained by further fitting of the parameters[a] to eqs 2–4 as described in the text; a linear regression through enthalpy (eq 2) corresponded to $\Delta H_b{}^b(T) = -46.7 - 0.25(T - 288.2)$; therefore, the value −0.25 kJ/(K·mol) was taken as the $\Delta C_{p,b}$ for subsequent calculations.

the calorimetric records at pH 2.0 and pH 4.5 with two-state heat absorbance peaks is shown in the upper half of Figure 1.

To calculate binding parameters from calorimetric data, we adopted two main approaches. First, we have developed and applied a novel and rather simple procedure to evaluate the binding constant from the melting curves of free and ligand-bound protein, as will be described under Results. The advantage of this approach is that no parameter has to be assumed *a priori* except for the scheme of the reaction. In addition, this simple procedure permits the direct estimation of the temperature dependence of the binding constant as soon as the calorimetric melting profiles are accurately recorded.

Second, we performed a more complex global fitting analysis of the DSC data, as suggested by Brandts and Lin (1990). For this purpose, we mainly used the curve-fitting option of the SigmaPlot software (Jandel Co.) and a program for a nonlinear multidimensional fitting developed by Freire at Johns Hopkins University (Straume & Freire, 1992). The fitting has the advantage of not being constrained by the conditions when the free ligand, [L], is in great excess, since free ligand concentration is explicitly expressed with a formula similar to eq 1a. On the other hand, the cases where [L] changes during melting are much more informative and interesting (Brandts & Lin, 1990; Straume & Freire, 1992; Shrake & Ross, 1992). The general treatment of the expressions (eq 6–8) leads to simple quadratic equations describing populations of each state of the protein. After these equations are solved in analytical form, the functions of $\langle \Delta H(T) \rangle$ can be simulated and fitted to the experimental data. A multidimensional fitting was performed simultaneously over the set of $C_p(T)$ curves recorded at different total ligand concentrations to adjust $\Delta H_d(T_m)$ and $K_d(T_m)$ while $\Delta C_{p,d}$ was fixed at 300 J/(K·mol) as explained under Results. The reaction scheme can also be extended to the case of multiple binding, which will, however, simultaneously increase the number of adjustable parameters and make the fitting of the results more ambiguous. Finally, we applied the first approach to estimate roughly the binding parameters and then used them as the initial values to make the global fitting of the DSC traces.

RESULTS

Determination of Binding Parameters by Mixing Calorimetry. The parameters calculated from the isothermal calorimetry data at pH 4.5, as described under Experimental Procedures, appear in Table 1. This set of thermodynamic parameters corresponds closely with those calculated from the scanning calorimetry results (see below). The greatest uncertainty was in the determination of the ΔC_p of binding since the titration data were measured over a rather small temperature range with an accuracy comparable to the heat capacity change itself.

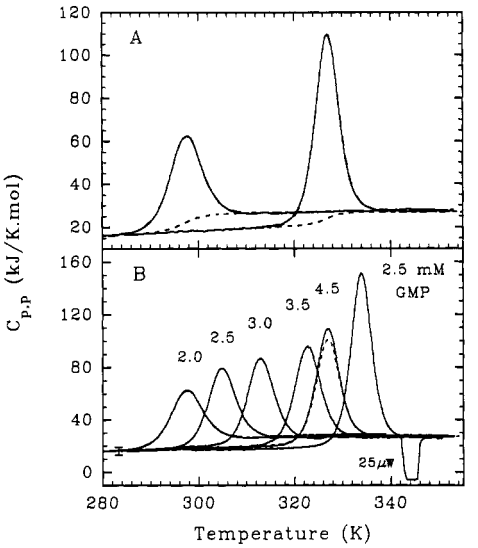

FIGURE 1: (A) DSC records of the molar partial heat capacity of barnase at pH 2.0 and 4.5 (solid lines) and their best fits to a two-state transition (dashed lines). The dashed sigmoid lines correspond to the internal or chemical heat capacity calculated during fitting. (B) Temperature dependencies of the molar partial heat capacity of barnase at various pH values (marked at the tops of the peaks). Curves corresponding to pH 2.5, 3.5, and 4.5 were registered at a heating rate of 1 K/min, while the others were at 2 K/min. Concentrations of protein were between 1 and 2 mg/mL, while for the pH 4.5 curve the concentration was 3.6 mg/mL. The dashed line represents the curve recorded for sample reheating at pH 4.5. The calibration mark of 25 μW [31.9 kJ/(K·mol)] was recorded at a heating rate of 1 K/min. The error bar at 283 K shows the scatter in the position of the curves on the C_p scale. The melting curve of barnase in the presence of 2.5 mM 3'GMP, pH 4.5, is also shown for comparison.

Thermal Unfolding of Barnase. The thermal unfolding of barnase is a highly reversible process below pH 5.0 and above pH 10.0, while at neutral pH the reversibility of melting is much lower, presumably because of aggregation of the unfolded molecules with a net charge close to zero. The alkaline region is much less suitable for temperature unfolding studies because of the high ionization heat effects of the amino groups and the high temperature dependence of the pH of the buffers. For this reason, we have varied protein stability by changing the pH within the acid range. Between pH 2.0 and 5.0 and at 50 mM buffer concentration, the melting curves of barnase do not depend upon the heating rate or protein concentration and follow the two-state model very closely (Figure 1A). Thus, the values of ΔH_m obtained by the fitting procedure (see Figure 2 and Table 2) coincide within the limits of experimental error both with the "calorimetric" and with the "van't Hoff" enthalpies evaluated in a traditional way, *i.e.*, from the area and sharpness of the individual peaks [cf. Privalov and Potekhin (1986)]. The melting curves obtained with fresh barnase preparations (Figure 1) are not distorted in any way, while C_p functions recorded at sample

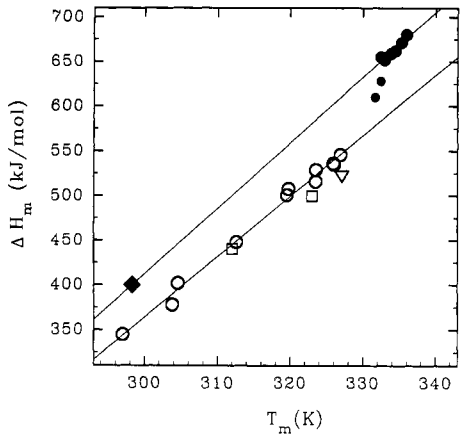

FIGURE 2: Dependence of ΔH_m on T_m for free barnase (open symbols) and ligand-bound protein (filled symbols) according to this study (circles and diamond) and the literature. The data for ligand-bound protein were obtained at a total 3′GMP concentration of between 0.2 and 10 mM, pH 4.5, and at a protein concentration of about 0.15 mM. For comparison, the van't Hoff enthalpies reported by Kellis et al. (1989) (squares) and Hartley (1968) (triangle) are also shown. The straight lines correspond to the least-squares fitting of our own values. The $\Delta H_L(298)$, calculated as described in the text, is shown by the filled diamond.

Table 2: Thermodynamic Parameters for the Thermal Unfolding of Barnase as a Function of pH

pH	T_m (K)	$\Delta H_m(T_m)$ (kJ/mol)	$\Delta S_m(298)^a$ (kJ/(K·mol)]	$\Delta G_m(298)^a$ (kJ/mol)	$\Delta G_m(298)^b$ (kJ/mol)
2.0	297.0	345.0	1.189	−3.4	−1.9
2.5	304.6	394.0	1.151	7.8	7.6
3.0	312.6	449.0	1.115	18.4	21.7
3.5	319.7	497.0	1.082	28.4	31.7
4.0	323.5	523.0	1.063	34.0	35.6
4.5	326.0	540.0	1.051	37.7	37.9
5.0	326.9	546.0	1.046	39.1	38.7

[a] These parameters were calculated from linear regression of experimental data according to eq 5 and the experimental values of T_m as a function of pH. [b] The Gibbs energy changes calculated with temperature-dependent $\Delta C_{p,m}$ specified by eq 17 at $\Delta H_m(298.2)$ = 344 kJ/mol (best fit value for polynomial regression).

reheating or after long storage exhibited a small pretransition shoulder.

We determined ΔH_m to an accuracy of 5%, although the $\Delta C_{p,m}$ values calculated directly from the calorimetric records were more scattered. When melting is completely reversible, such a scatter usually arises from an uncertainty in the extrapolation of the initial C_p over the whole transition range and becomes larger when T_m decreases. Since below pH 3.5 barnase starts to unfold at room temperature, there are difficulties in the estimation of the initial heat capacity slope. For this reason and, probably, due to the temperature dependence of $\Delta C_{p,m}$ (see Discussion), the uncertainty of its direct determination from calorimetric records was about 13%, with the average value of $\Delta C_{p,m}$ being about 6.2 kJ/(K·mol). This is only 10% lower than the slope of the least-squares regression line (Figure 2), which corresponds to the following empirical dependence of ΔH_m on temperature:

$$\Delta H_m(T) = \Delta H_{m,0} + \Delta C_{p,m}(T - T_0) = 351 + 6.8(T - 298.2)\ \text{(kJ/mol)} \quad (5)$$

Using this function, we have calculated the changes of the thermodynamic parameters at different pH values (Table 2).

Thermal Unfolding of Barnase in the Presence of 3′GMP. As was mentioned under Experimental Procedures, we have developed and used a simple approach for estimating binding parameters from DSC data. To describe this approach, we shall use the formulations originally suggested by Freire and Biltonen (1978). We shall consider here a rather simple case, but similar equations can be deduced for more complex multistate schemes.

Let us assume that protein unfolding in the presence of a ligand obeys a simple two-step model and that there is only one binding site for the ligand on the native protein:

$$NL \overset{K_d}{\rightleftarrows} N + L \overset{K_m}{\rightleftarrows} U + L \quad (6)$$

If [NL] stands for the concentration of the protein–ligand complex, [N] and [U] for the concentrations of native and unfolded proteins, respectively, and [L] for the concentration of the free ligand, the dissociation, K_d, and unfolding, K_m, constants, can be expressed as

$$K_d = [N][L]/[NL];\ K_m = [U]/[N] \quad (7)$$

For the total protein $[P_0]$ and ligand $[L_0]$ concentrations, we have

$$[P_0] = [NL] + [N] + [U];\ [L_0] = [NL] + [L] \quad (8)$$

If $[L_0] \gg [P_0]$, then $[L] \approx [L_0]$ = constant, and K_d can be replaced by an effective monomolecular constant, $K_e = K_d/[L_0] = [N]/[NL]$. Hence, the partition function, Q_L, for eq 6 with the liganded native protein taken as the reference state, may be written as

$$Q_L = 1 + K_e + K_eK_m = 1 + K_e(1 + K_m) = 1 + K_eQ \quad (9)$$

Thus

$$K_e = (Q_L - 1)/Q \quad (10)$$

where Q is a partition function for the free protein under the same solvent conditions. Both Q_L and Q are easily calculated from scanning calorimetry data since (Freire & Biltonen, 1978):

$$(\partial \ln Q_L/\partial T)_L = \langle \Delta H_L \rangle / RT^2;\ \partial \ln Q/\partial T = \langle \Delta H_m \rangle / RT^2 \quad (11)$$

and $\langle \Delta H(T) \rangle = H(T) - H_0(T)$ can be found correspondingly from $\langle \Delta C_p(T) \rangle = C_p(T) - C_{p,0}(T)$, since

$$d\langle \Delta H(T) \rangle / dT = \langle \Delta C_p \rangle \quad (12)$$

where subscript "0" refers to the initial state of the protein and ΔH_L is the total heat of unfolding in the presence of the ligand. In practice, to obtain the partition function, a double integration of the $C_p(T)$ function has to be made after subtracting the heat capacity of the initial state. This approach is straightforward, although some complications are again caused by having to approximate the heat capacity of the initial state over the whole transition range. Its extrapolation and subtraction from the total C_p according to standard procedures introduce some errors which are amplified during the evaluation of Q by the double integration procedure. As a result, the factor $Q_L - 1$ is knowable with satisfactory precision almost at the midpoint of protein unfolding. Therefore, $K_d(T)$ calculated in this way is reliably known only at $T > T_m$ of the ligand-bound protein (Figure 3). To overcome this difficulty, we have carried out a global fitting of all individual K_d functions, calculated at each concentration

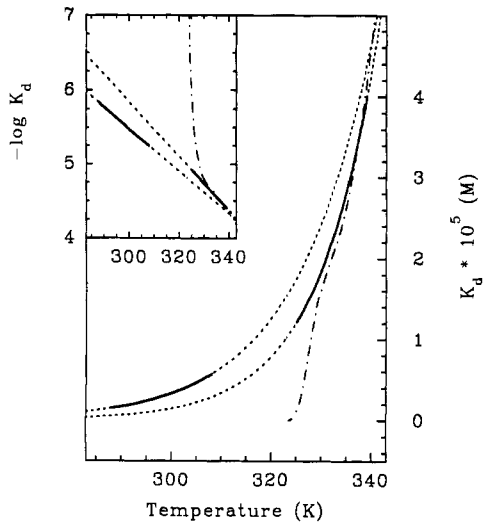

FIGURE 3: Temperature dependencies of the dissociation constant, K_d, of 3'GMP from barnase at pH 4.5, calculated from isothermal titration data (solid line at low temperature extrapolated by dotted line), by applying the partition function method at 5 mM 3'GMP concentration (eq 10, dash-and-dot line) and by global fitting of DSC data in the presence of ligand (solid line at high temperature extrapolated by dotted line). The insert shows the same data in logarithmic scale. Solid lines correspond to the working temperature range for each method.

of the ligand, to the overall analytical expression for the dissociation constant assuming a constant $\Delta C_{p,d}$. This $K_d(T)$ is also shown in Figure 3 together with the temperature dependence of the dissociation constant determined from the mixing calorimetry experiments.

The ΔH_L values measured at pH 4.5 and increasing concentrations of 3'GMP are shown in Figure 2, together with the data for free protein. From a comparison between the two correlations, it is clear that any difference between the two sets of data can be put down exclusively to the effect of ligand binding. With a high excess of ligand, the heat capacity peak is adequately fitted by a two-state unfolding transition, since under these conditions the intermediate state, N in eq 6, is only slightly populated; when the total ligand concentrations were comparable or even smaller than $[P_0]$, the melting curves were somewhat distorted, in full accordance with theory (Brandts & Lin, 1990; Straume & Freire, 1992; Shrake & Ross, 1992). The linear regression line for the ligand-bound protein in Figure 2 is plotted only through the points corresponding to saturating conditions (more than 10-fold molar excess of the ligand over the protein). As can be seen from Figure 2, the points obtained in the presence of ligand are too close to each other to provide a reliable linear regression through them. To decrease the uncertainty in the slope of the regression line, we have plotted on the same graph the value $\Delta H_L(298) = \Delta H_m(298) + \Delta H_d(298)$ (filled diamond in Figure 2). The first term in the sum is the unfolding enthalpy calculated for free protein from eq 5, and the second one is the dissociation heat measured directly with mixing calorimetry (Table 1). The regression line, drawn as shown in Figure 2, has a slope of 7.3 kJ/(K·mol) and corresponds to the equation:

$$\Delta H_L(T) = \Delta H_m + \Delta H_d = 400 + 7.3(T - 298.2) \text{ (kJ/mol)} \quad (13)$$

The difference between eq 13 and 5 should obviously correspond to the temperature dependence of ΔH_d, which is

$$\Delta H_d(T) = 49 + 0.5(T - 298.2) \text{ (kJ/mol)} \quad (14)$$

The value 0.5 in the above equation is twice as high as the $\Delta C_{p,d}$ found from isothermal calorimetry (Table 1). From the global fitting of the $K_d(T)$ functions calculated with the partition function approach (Figure 3), we also got a $\Delta C_{p,d}$ equal to 0.45 kJ/(K·mol). These two $\Delta C_{p,d}$ values estimated from the preliminary analysis of the DSC data compare well. Nevertheless, taking into account the rather large error in their determination [about ±0.35 kJ/(K·mol) in both cases] during the final multidimensional global fitting of the DSC curves, $\Delta C_{p,d}$ was fixed at a weighted-average value of 0.3 kJ/(K·mol), which is clearly closer to the more precise parameter found by isothermal calorimetry. For this multidimensional fitting, the T_m, ΔH_m, and $\Delta C_{p,m}$ values (Table 2) were also fixed, whereas $K_d(T_m)$ and $\Delta H_d(T_m)$ obtained with the partition function approach were used as initial, adjustable parameters. The results of this final fitting are shown in Table 3 and Figure 4.

DISCUSSION

The energetics of the interaction of 3'GMP with barnase have been studied by both isothermal titration and differential scanning microcalorimetry. The direct isothermal experiments have shown that the affinity of the ligand to its single site on the protein is not very high and comparable to that found for other nucleotide binding proteins, in particular that for ribonucleases (Flogel et al., 1975). Taking into account the pH dependence of the binding constant, our value of 3.6×10^5 M^{-1} found for K_b at 298 K and pH 4.5 is in close accord with the value of 4.35×10^5 M^{-1}, determined by Sancho et al. (1991) from kinetic studies at pH 6.3 and the same temperature.

The value of $\Delta C_{p,b}$ found by both techniques has a negative sign, as might be expected from the data reported for ligand-to-protein binding (Sturtevant, 1977; Wiesinger & Hinz, 1986). The magnitude of $\Delta C_{p,b}$ is, however, slightly lower than the values found for the binding of various nucleotides and nucleoside monophosphates to other proteins (Wiesinger & Hinz, 1986; Mateo et al., 1986; Baron et al., 1989).

The effect of 3'GMP binding on the stability of barnase against heat denaturation is similar to the stabilizing effect of other ligands described in the literature (Fukada et al., 1983; Brandts & Lin, 1990; Straume & Freire, 1992). It can easily be explained by the mass action principle without any recourse to "ligand-induced stabilizing conformational changes".

We have found that the most precise and reliable determination of the binding parameters from scanning calorimetry data is provided by the global fitting of the C_p records obtained at various ligand concentrations. Nevertheless, a new approach based on the evaluation of K_d and ΔH_d from the partition functions of the ligand-bound and free proteins provides a very good approximation to the temperature dependence of the binding parameters. This approach, followed by the fitting of the $C_p(T)$ curves (see Results), is recommended for determining the binding parameters when not enough material is available to make a complete calorimetric study with changing ligand concentrations, or when there are problems with ligand solubility.

It must be pointed out, however, that only K_d was determined from our DSC data analysis with any reasonable precision. ΔH_d and, in particular, $\Delta C_{p,d}$ are known much less accurately for several reasons, the most important of which is that ΔH_d, being much smaller than the overall heat of unfolding, is

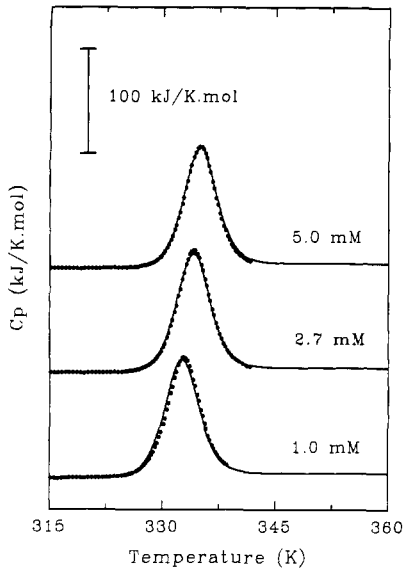

FIGURE 4: Results of nonlinear least-squares global fitting (solid line) of heat capacity excess functions (dots) for barnase in the presence of different concentrations (mM) of 3'GMP.

calculated as the difference between two large values. Therefore, the standard error in determining ΔH_m and ΔH_L, which is about 15 kJ/mol, must serve as the lower limit of the standard error for ΔH_b calculated from DSC data. An even more uncertain situation occurs with $\Delta C_{p,d}$ since (as we know from isothermal calorimetric titrations) it is of the same magnitude and even smaller than the error of direct determination of ΔC_p from DSC records. On the other hand, the overall shape and $T_{m,L}$ of each individual melting curve in the presence of the ligand excess seem to depend mainly on $K_d(T)$ and $\Delta H_d(T_{m,L})$ and much less so on $\Delta C_{p,d}$. For this reason, for example, on making a global fitting of DSC data for liganded protein, we got practically the same results by fixing $\Delta C_{p,d}$ either at 0.3 or at 0.25 kJ/(K·mol). To overcome these problems, one has to determine $\Delta C_{p,d}$ from the temperature dependence of ΔH_d. Nevertheless, because of solubility problems we could not vary the ligand concentration over sufficiently wide limits. So the melting temperature for the liganded protein in our experiments changed within a narrow temperature range, thus introducing a large error into our $\Delta C_{p,d}$ determination from the $\Delta H_d(T)$ slope, unless the independent mixing calorimetry data were used to extend the working temperature range. Finally, with the current levels of DSC experimental error, it is practically impossible to detect any temperature dependence of $\Delta C_{p,d}$, although it can be expected that its relative variation may be as high as the temperature-induced relative changes in $\Delta C_{p,m}$.

The thermodynamic parameters of barnase unfolding, either with or without a ligand, unambiguously show that under our experimental conditions barnase behaves as a single-domain protein, the structure of which melts in an essentially two-state way; *i.e.*, under equilibrium conditions, all intermediate states are much less populated than the native and unfolded ones. This conclusion agrees with previous noncalorimetric results (Hartley, 1968). Recently, however, Makarov et al. (1992) have shown that the C_p endotherm becomes asymmetric when barnase is heated at pH 2.4 in a buffer of low ionic strength (10 mM). We have also found an asymmetry in the calorimetric traces at pH 2.5 and 10 mM buffer. It seems that under these conditions the distortion of the melting peak is caused by a slow, nonspecific oligomerization of the native protein, as was found by analytical methods at the Cambridge laboratory. The oligomerization is reversible and is suppressed by an increase of the ionic strength to above 50 mM and/or of pH to above 3.5. The slow kinetics of nonmonomolecular processes responsible for curve distortion were confirmed by our observation that at low ionic strength the peak shape and position were heating rate dependent and much more evident at sample heating in the cylindrical cells of the DASM-1M calorimeter (this model was the one actually used by Makarov et al.) than in the capillary cells of the DASM-4 (results not shown).

Barnase does not belong to the protein group that have a particularly stable native conformation, as can be seen from the analysis of Gibbs energy changes accompanying unfolding (see Table 2). The ΔG_m values obtained here by DSC agree very well with measurements made using other techniques (Kellis et al., 1988, 1989; Clarke & Fersht, 1993).

Since barnase appears to be a typical representative of the family of small, single-domain globular proteins, it was interesting to compare its specific thermodynamic parameters of unfolding with those of other members of the family. Thus, its specific heat capacity of unfolding, 0.55 J/(K·g) [0.13 cal/(K·g)], compares very well with that of other compact globular proteins (Privalov, 1979). Some time ago Privalov (1979) also found that the extrapolated values of specific heats of unfolding (Δh_m) for globular proteins intersect close to 385 K, where the Δh_m for all of them is about 54 J/g. The exceptions are either very small proteins, extensively cross-linked by S–S bridges, or those with an elongated rather than globular structure.

Initially those extrapolations were made under the assumption of a constant $\Delta C_{p,m}$, but later on Privalov et al. (1989) suggested that $\Delta C_{p,m}$ would be a sort of bell-shaped function of temperature with a maximum close to 310 K and reaching zero at about 413 K. With such a ΔC_p function, the specific heats of unfolding would approach the same value of 54 J/q asymptotically instead of intersecting with it at 373 K. Nevertheless, since the top of the "bell" is rather wide, the absolute variations of $\Delta C_{p,m}$ in the temperature range of protein melting are relatively small, and, consequently, the plots of ΔH_m versus T_m might fit the linear functions very well, with a slope corresponding to an average value of the actual $\Delta C_{p,m}$ (Figure 5). When calculated directly from the calorimetric records, however, the latter must depend on T_m, and these variations might account, at least partly, for the observed scatter of the $\Delta C_{p,m}$ values.

Bearing in mind that the long extrapolations made by Privalov et al. (1989) might be ambiguous, it is also interesting to look for other correlations, which eventually might be more reliable. Murphy et al. (1990) have pointed out that such behavior of the Δh_m dependence on temperature should also provide a strong correlation between $\Delta C_{p,m}$ and Δh_m calculated per mole of amino acid residue at a standard temperature of 298 K, as they have indeed found for more than 10 proteins (Figure 6). Later on, the list of proteins obeying this correlation was extended by Murphy et al. (1992) and Spolar et al. (1992). The latter authors have only found the single exception of ribonuclease T1, the Δh_m vs $\Delta C_{p,m}$ point of which [calculated from the DSC data of Hu et al. (1992)] did not fit into the above-mentioned correlation, primarily due to the high ΔC_p value. Nevertheless, further analysis of the DSC curves for T1 made by Plaza del Pino et al. (1992) gave much a lower $\Delta C_{p,m}$ value [1.16 kcal/(K·mol) or 11.15 cal/(K·mol-residue) instead of 1.59 kcal/(K·mol)], which, in combination with 573 cal/(K·mol-residue) for the $\Delta h_m(298)$, fits perfectly well into Murphy's plot.

Table 3: Thermodynamic Parameters of 3'GMP Binding to Barnase at pH 4.5 and $T = T_m = 326$ K Obtained by Global Fitting of the Temperature Dependencies of the Molar Heat Absorbance with and without Ligand Added to the Protein Solution

T_m (K)	ΔH_m (kJ/mol)	$\Delta C_{p,m}$ [kJ/(K·mol)]	ΔH_d (kJ/mol)	$\Delta C_{p,d}$ [J/(K·mol)]	$K_d \times 10^5$ (M)	$K_b \times 10^{-4}$ (M^{-1})
326.0[a]	540 ± 15[a]	6.8 ± 0.4[a]	59 ± 15[b]	300 ± 100[b]	1.1[b]	9.1[b]
			56 ± 1.5[c]	250 ± 50[c]	1.7[c]	5.9[c]

[a] Parameters defined from linear fitting of DSC data for free protein according to eq 5. [b] Parameters of 3'GMP binding found by fitting the DSC data for ligand-bound protein according to eqs 6–8 as described in the text. The $\Delta C_{p,d}$ value was fixed during fitting. [c] Parameters calculated by extrapolation of mixing calorimetry values to 326 K.

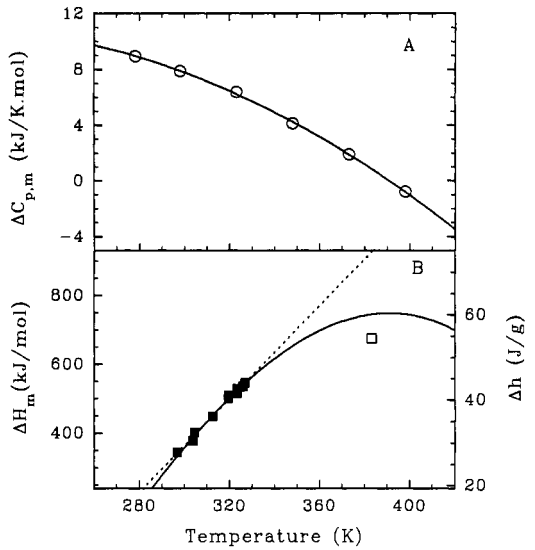

FIGURE 5: (A) Temperature dependence of $\Delta C_{p,m}$, calculated as described in the text. (B) Experimental dependence of ΔH_m on T_m for barnase (solid squares) plotted together with $\Delta H_m(T)$ functions simulated assuming a constant $\Delta C_{p,m}$ of 6.8 kJ/(K·mol) (dotted line) and a variable $\Delta C_{p,m}$ (eq 17, part A of this figure; solid line). The open square corresponds to the Privalov's "limiting value" of 54 J/g at 383 K.

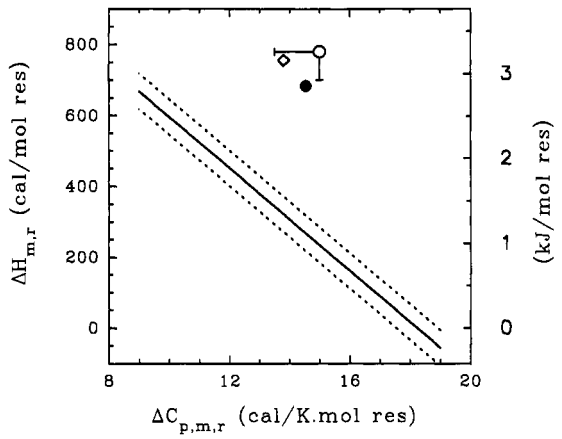

FIGURE 6: Linear correlation between $\Delta H_{m,r}(298)$ and $\Delta C_{p,m,r}$ (both calculated per mole of amino acid residue) as found by Murphy et al. (1990) for more than 10 globular proteins (solid line). The dotted lines show the limits into which all the data for those proteins fit. The open circle with error bars shows our data for barnase. The filled circle corresponds to the value for wild-type barnase, and the opened diamond shows an average of the values for eight single barnase mutants, both calculated from the unpublished data of Matouschek and Fersht.

We have added our data to the plots of both Privalov et al. (1989) and Murphy et al. (1990) and found very unexpected results. First, *at any temperature* barnase has the highest Δh_m of *all globular proteins* studied so far. As a result, the linear extrapolation of Δh_m to 373 K gives a value close to 75 J/g, which is about 40% higher than the universal value of 54 J/g (Figure 5).

We tried to find out then how such an extrapolation would work when a temperature-dependent function of ΔC_p is assumed instead of a constant one. To do this, we estimated the ΔC_p by the procedure suggested by Privalov and Makhatadze (1990). We calculated the C_p of the denatured state from the amino acid content of barnase, which turned out to be a nonlinear function of temperature:

$$C_{p,u}(T) = -14.4 + 0.218T - 0.00028T^2 \text{ [kJ/(K·mol)]} \quad (15)$$

Then, analyzing our calorimetric data we found that an average temperature dependence of C_p for the native state of barnase can be adequately approximated by the *linear* function

$$C_{p,n}(T) = 17.8 + 0.11(T - 298.2) \quad (16)$$

(where each of the numerical parameters is known to 10% accuracy). The difference between the last two functions gives the following empiric formula for $\Delta C_{p,m}$:

$$\Delta C_{p,m}(T) = 0.6 + 0.108T - 0.00028T^2 \quad (17)$$

It can be seen from Figure 5 that the function $\Delta H_m(T)$ simulated with a nonconstant $\Delta C_{p,m}$, using

$$d\Delta H_m/dT = \Delta C_{p,m} \quad (18)$$

fits our experimental data even more precisely than the linear regression. Nevertheless, despite the fact that the extrapolated value of Δh_m at 383 K decreased somewhat it did not reach the universal intersection point. Hence, the temperature dependence of $\Delta C_{p,m}$ cannot account for our observation.

An even higher discrepancy was observed after adding our data point to the plot of Murphy et al. (1990). Again, the barnase data lie far from the correlation found by these authors for other proteins (Figure 6). To obey that correlation, barnase must have either about a 3-fold lower Δh_m per residue or about a 2-fold lower $\Delta C_{p,m}$ value, or lower values for both parameters simultaneously.

Could such a big discrepancy be caused by errors in the determination of the thermodynamic parameters for barnase unfolding? It is very unlikely for the following reasons. First, as was pointed out above, under our experimental conditions the melting of barnase is a highly reversible process and is two-state in character. This observation rules out the possibility of high errors in concentration measurements, which frequently arise from using wrong extinction coefficients [besides, the extinction coefficient used here is in very close agreement with the value of 2.6×10^4 M^{-1}·cm^{-1} reported recently by Hartley (1993)]. Second, under our solvent conditions, the melting curves of barnase are highly symmetrical and did not depend either on heating rate or on protein concentration, which excludes any nonmonomolecular unfolding mechanism. Third, some estimations of the van't Hoff unfolding enthalpy from noncalorimetric measurements are available in the literature, and, as can be seen in Figure 2, they are adequately close to our data. In addition, there are also unpublished DSC data of Matouschek and Fersht which fit the linear dependence of ΔH_m on T_m with a slope

corresponding to a $\Delta C_{p,m}$ value of 6.7 kJ/(K·mol) and a $\Delta H_m(298)$ of 325 kJ/mol (for a comparison, see eq 5 and Figure 6). Finally, the Gibbs energy changes calculated from ΔH_m, T_m, and $\Delta C_{p,m}$ at different pH values (see Table 2) correspond very well with those obtained from urea denaturation studies, usually carried out at much lower protein concentrations because protein unfolding was monitored by fluorescence (Kellis et al., 1988, 1989; Pace et al., 1991; Clarke & Fersht, 1993).

Thus, it is impossible to fit the melting parameters of barnase into the correlations of Privalov et al. (1989) and Murphy et al. (1990). In addition to these simple correlations, different groups working in the field, including those mentioned above, have recently developed methods and algorithms for evaluating the thermodynamic parameters of unfolding from protein structure based on calculations of the buried hydrophobic and hydrophilic areas, the number of internal hydrogen bonds, and so on. So far we have not developed our own methods of structural analysis to form a coherent explanation for our observations. We believe, though, that such an explanation might lie in the properties of the native and unfolded states of barnase. It is highly probable, for example, that the temperature-induced unfolded state of barnase has much less residual structure than that of other proteins *even at low temperature*. This means that its unfolded state should be very specific and very close to random coil. If this were to be the case, a cooperative melting of all structural elements, including the secondary structure, would be accompanied by higher than average enthalpy and entropy changes.

Recently, the X-ray structures of barnase in complex with 3′GMP (Guillet et al., 1993b) and barstar (Guillet et al., 1993a) have been published. The authors of the latter publication have made a very interesting observation about water molecules trapped inside the barnase–barstar complex. It turns out that barstar does not enter the hydrophobic guanine binding pocket of barnase, which appears to be filled with structured water molecules. Since water might be structured in a similar way by unliganded barnase, the melting of this protein–water cluster on protein unfolding may well increase both the enthalpy and entropy of unfolding.

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