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Thermodynamics of denaturation of complexes of barnase and binase with barstar☆

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Abstract

Differential scanning calorimetry was used to study the thermodynamics of denaturation of protein complexes for which the free energy stabilizing the complexes varied between −8 and −16 kcal/mol. The proteins studied were the ribonucleases barnase and binase, their inhibitor barstar and mutants thereof, and complexes between the two. The results are in good agreement with the model developed by Brandts and Lin for studying the thermodynamics of denaturation for tight complexes between two proteins which undergo two-state thermal unfolding transitions.

Keywords: Barnase; Binase; Barstar; Protein–protein complex; Electrostatic interactions; Thermal stability

1. Introduction

Proteins often bind very tightly and specifically to another protein to form a complex, in order to regulate a biological process. In this article, we report studies of the thermodynamics of denaturation of a series of protein complexes in which mutations are used to alter the stability and affinity of one of the participants in the complex. This information will be useful in understanding the stability of these complexes. The results show that a model developed by Brandts and Lin [1] can successfully analyze these types of systems.

Barnase is a ribonuclease synthesized by *Bacillus amyloliquefaciens* and then secreted into the growth medium [2]. The same cells synthesize barstar, a potent inhibitor of barnase ($K_d = 1.3\times10^{-14}$ M, $\Delta G^{\circ} = -19$ kcal/mol at pH 8),

Abbreviations: Barstar A, Cys 40,82 Ala barstar; DSC, differential scanning calorimetry; CD, circular dichroism.

☆ We are delighted to dedicate this paper to Dr Walter Kauzmann for his many important contributions that improved our understanding of protein stability.

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and retain it inside the cell to inhibit any barnase that is not secreted [2]. Mammalian cells synthesize trypsin inhibitor and ribonuclease inhibitor in all of their cells for the same purpose. Barstar A is the Cys 40,82 Ala mutant of barstar. The affinity of barstar A is only slightly less than barstar and the structure of a complex between barnase and barstar A has been determined [2,3]. The total solvent-accessible surface buried upon forming the complex is 1630 $Å^2$, and the surface contributed by barnase is positively charged and that by barstar A is negatively charged [3]. Three barstar A residues that contribute to the stability of the complex are Asp 39 that forms two salt bridges, Glu 76 that forms a salt bridge, and Asp 35 that forms a hydrogen bond [3]. Binase is another microbial ribonuclease synthesized by *Bacillus intermedius* that binds barstar A with a K_d value approximately 10-fold less than barnase [4]. We report here studies of thermal denaturation of barnase and binase in complex with barstar A, and three mutants of barstar A with lower affinity: Asp35Ala, Asp39Ala, and Glu76Ala.

2. Experimental

Recombinant barnase and binase were obtained as described in [5]. The mutant barstar genes were constructed using PCR with the barstar A gene as DNA template. Thus, barstars with substitutions Asp35Ala, Asp39Ala, and Glu76Ala are mutant derivatives of barstar A. Base sequence of genes within the expression vectors was determined by two DNA strands. Bacterial expression and purification of barstars was carried out as described elsewhere [6]. The isolation protocol included cell disruption by ultrasound, fractional salting out of proteins with ammonium sulfate (40–80% saturation), gel filtration on Sephadex G-75, and anion exchange chromatography on MonoQ HR 10/10 (Pharmacia, Sweden). Mass spectrometry has shown that barstar and its mutants still have an N-terminal methionine. All experiments were carried out in 10 mM Na-acetate buffer, 50 mM NaCl, pH 6.2. Protein concentration was determined on a Jasco V-550 spectrophotometer, assuming a molar extinction coefficient at 280 nm (in $M^{-1}cm^{-1}$) of 27410 for barnase and binase, 22960 for barstar, 22715 for barstar A and its mutants Asp35Ala, Asp39Ala and Glu76Ala [6]. After mixing ribonuclease with inhibitor, the solution was incubated for at least 2 h at room temperature for complex formation. The molar concentration of inhibitor always exceeded that of ribonuclease by $\approx 4\%$.

Microcalorimetric measurements were carried out on a DASM-4 instrument (NPO Biopribor, Pushchino, Russia) in 0.48 ml cells at a heating rate of 1 K/min on 0.4–0.9 mg/ml proteins (unless otherwise stated). Curves were corrected for the instrumental baseline obtained by heating the solvent. The reversibility of denaturation was checked routinely by sample reheating after cooling in the calorimetric cell. The partial molar heat capacity of the protein (C_p), denaturation temperature (T_d), calorimetric denaturation enthalpy (ΔH_{cal}), and effective or van't Hoff denaturation enthalpy (ΔH_{eff}) were determined as described elsewhere [7], with a partial specific volume of 0.73 $cm^3\ g^{-1}$ for barnase and binase and 0.71 $cm^3\ g^{-1}$ for the barstars calculated according to [8]. To analyze the DSC data, the SCAL2 software package developed at the Institute of Protein Research (Pushchino, Russia) was used. The accuracy of the calorimetric and effective enthalpies was $\pm 6\%$, and that of T_d was ± 0.2 °C.

CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with thermostated water-jacketed cells and a Neslab RTE-111 programmable bath in a 0.02 cm cell on 0.25 mg/ml (unless otherwise stated) protein solutions. Protein melting was carried out at the same heating rate as in microcalorimetry (1 K/min). Denaturation temperatures were determined from the peaks in the first temperature derivatives of the melting profiles (accuracy ± 0.3 °C). The results were expressed as molar ellipticity, $[\Theta]$ (deg $cm^2\ dmol^{-1}$), based on a mean amino acid residue weight (MRW) of 114.2 for barstar A, 113.6 for Glu76Ala barstar A, and 113.8 for Asp35Ala and Asp39Ala barstar A. The molar ellipticity was determined as $[\Theta] = (\theta \times 100\ \mathrm{MRW})/(cl)$, where c is the protein concentration in mg/ml, l is the light path in centimeters, and θ is the measured ellipticity in degrees.

Dissociation constants (K_d) of ribonuclease complexes with barstar and its mutants were deter-

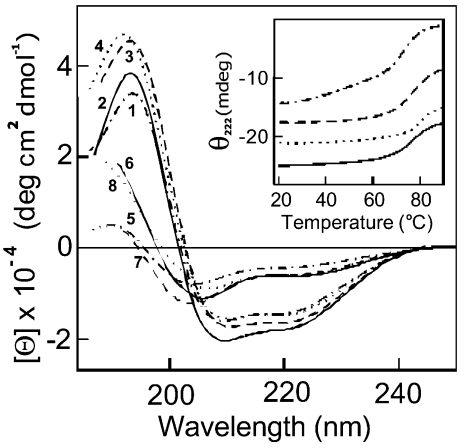

Fig. 1. CD spectra of barstar A (.-.-.-) and its mutants Asp39Ala (—), Asp35Ala (---) and Glu76Ala (…) in the far-UV region at 20 °C (1–4) and 90 °C (5–8) at pH 6.2. Inset: temperature dependence of CD at 222 nm for barstar A and its mutants.

mined by the enzyme titration with inhibitor using poly(I) as substrate [4]. Solutions of ribonuclease at a concentration of approximately 10^{-10} M were incubated for 24 h at 25 °C in the presence of barstar at different concentrations. These solutions were then placed in a spectrophotometer cell, poly(I) was added, and the initial rates of substrate hydrolysis were measured. The rates obtained were used to calculate dissociation constants as described in [4]. Standard free energy changes were then calculated using $\Delta G^{\circ} = -RT \ln K_d$.

3. Results

3.1. Heat denaturation of barstar and its mutants

The characteristic α-helical far-UV CD spectra of barstar A and its mutants Asp35Ala, Asp39Ala, and Glu76Ala are shown in Fig. 1. There are small changes in the magnitude of the spectra, but the shapes are similar suggesting that the secondary structures of the proteins are not changed appreciably. The differences may reflect a rearrangement of the charged groups at the active site of the barstar mutants that distorts the geometry of the α-helical regions and alters the CD amplitude [9]. The CD spectra recorded at 90 °C do not differ significantly. The inset in Fig. 1 shows thermal denaturation curves of the proteins followed by measuring the change in CD at 222 nm. The denaturation temperatures, T_d, are given in Table 1 and show that all three of the mutants are more stable than wild type barstar. A similar increase in stability for these mutants was observed using urea denaturation by Schreiber et al. [10].

Typical DSC scans of barstar A and the mutants are shown in Fig. 2 and the thermodynamic parameters characterizing the curves are given in Table 1. The T_d values from DSC are close to those obtained from CD thermal denaturation curves (Table 1). The T_d and ΔH_{cal} values for barstar and barnase are in good agreement with the values reported previously by Wintrode et al. [11] and Griko et al. [12]. The heat denaturation of the proteins is fully reversible. The $\Delta H_{\mathrm{cal}}/\Delta H_{\mathrm{eff}}$ ratio is equal to 1 for barstar A and Glu76Ala mutant and less than 1 for the Asp35Ala and Asp39Ala mutants. This may indicate that there is a partial association of molecules in the denatured state that lowers the values of ΔH_{cal} observed for D35A and D39A.

3.2. Dissociation constants of barstar mutants complexed with barnase and binase

The dissociation constants of barnase and binase complexed with barstar, barstar A and three mutants are listed in Table 2. It is clear that the mutations in barstar A markedly decrease the affinity of the inhibitors for barnase and binase. Therefore, the increase in thermal stability of the barstar mutants is accompanied by a decrease in their affinity for barnase and binase.

3.3. Heat denaturation of barstar mutants complexed with barnase and binase

Fig. 3 shows the temperature dependences of the partial molar heat capacity of binase complexed with barstar A mutants: Asp39Ala, Asp35Ala, and Glu76Ala. The melting curves of barnase complexes with these mutants are similar. All of the samples were heated to ≈100 °C and a single cooperative peak was observed. The decrease in heat capacity at higher temperatures for Curve 1

Table 1
Thermal denaturation parameters for binase, barnase, barstar, barstar A and its Asp35Ala, Asp39Ala and Glu76Ala mutants, and ribonuclease-inhibitor complexes at pH 6.2

Sample	T_d (°C)	ΔH_{cal} (kcal/mol)	ΔH_{eff} (kcal/mol)	R[a]
Barstar[b]	73.3 (73.0)[c]	53	76	0.7
Barstar A	73.6 (73.7)[c]	73	71	1.0
Asp35Ala barstar A	74.7 (77.0)[c]	46	66	0.7
Asp39Ala barstar A	76.9 (77.1)[c]	42	60	0.7
Glu76Ala barstar A	78.8 (80.0)[c]	65	65	1.0
Barnase	54.5	129	130	1.0
Binase	56.5	124	126	1.0
Barnase-barstar A[b]	74.5	203	167	1.2
Barnase-Asp39Ala barstar A	58.1	110	122	0.9
Barnase-Asp35Ala barstar A	64.6 (63.5/74)[d]	99	146	0.7
Barnase-Glu76Ala barstar A	71.1	115	146	0.8
Binase-barstar A[b]	76.6	191	181	1.1
Binase-Asp39Ala barstar A	60.6	105		
Binase-Asp35Ala barstar A	66.1(66.5/74)[d]	104		
Binase-Glu76Ala barstar A	72.2	112		

[a] $R = \Delta H_{cal}/\Delta H_{eff}$.
[b] [6].
[c] Values of denaturation temperature obtained from the CD change at 222 nm.
[d] Values of denaturation temperatures obtained from DSC for complexes at a concentration 0.14 mg/ml.

in Fig. 3 may indicate aggregation of the unfolded protein. Repeated heating of the samples revealed 15–20% reversibility of denaturation for the barnase complexes or its complete absence for the binase complexes, even if the solution was cooled immediately after the heat absorption peak. The irreversibility is probably due to protein aggregation after denaturation. The melting curves of the complexes are asymmetric. The asymmetry may be due to denaturation being a multistage process and/or kinetic characteristics of the transition [13,14]. Using approaches developed by Conejero-Lara et al. [13] and Potekhin et al. [14] for the treatment of irreversible non-equilibrium denaturation processes, we have shown that the experimental data coincide with theoretical curves for a model describing an irreversible transition between two states. An example is shown in the inset of Fig. 3.

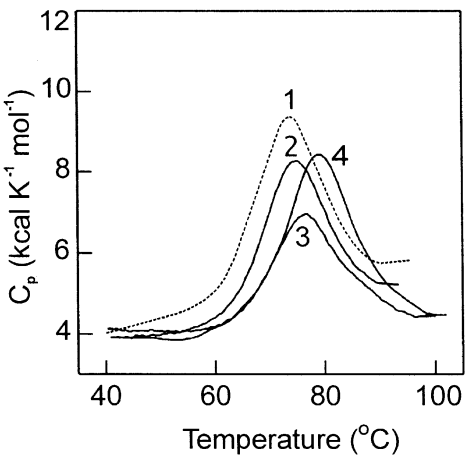

Fig. 2. Temperature dependence of the partial molar heat capacity of barstar A (1) and its mutants Asp35Ala, (2) Asp39Ala and (3) Glu76Ala (4) at pH 6.2.

4. Discussion

4.1. Removing negative charges at the binding site stabilizes barstar and reduces its affinity for barnase and binase

Barstar binds to the active site of barnase and binase and completely inhibits the activity [2]. The barnase active site has several positive charges that aid in the binding of RNA [3]. Consequently,

it is not surprising that the binding site of barstar has several negative charges that interact electrostatically with the positive charges on barnase and contribute to the stability of the complex. When the side chains contributing a negative charge at the binding site of barstar are removed, the stability of the protein increases (Table 1). This results because electrostatic repulsion among the negatively charged groups at the binding site is reduced. As expected, these mutations also reduce the affinity of barstar for barnase and binase (Table 2).

4.2. Binding of barstar to barnase or binase increases the stability of the ribonuclease

The melting temperatures of the barstar complexes are 3.6 to 26.8 °C higher than those of the free ribonucleases (Table 1). The T_d values increase as the affinity of the inhibitor for the enzyme increases. In fact, there is an excellent linear relationship between the T_d values (Table 1) and the ΔG° values (Table 2): $T_d = 38 - 2.5$ (ΔG°) for barnase and $T_d = 38 - 2.9$ (ΔG°) for binase and for both the correlation coefficient is 0.98. These are the expected results when a small or large molecule binds with greater affinity to the folded state of a protein than to the unfolded state [1].

4.3. The Brandts and Lin model correctly predicts the relationship between the denaturation temperature of the ribonuclease-barstar complexes and the free energy of complex formation

Brandts and Lin [1] developed a theory to predict the DSC scans expected for heat denaturation of a 1:1 complex formed between two molecules both of which undergo two-state heat denaturation transitions. The calculations predicted that the observed DSC scan would depend on the tightness of binding between the two molecules. Using their simulation equations and the data in Tables 1 and 2, we calculated the expected DSC scans for binase and barstar A. The results are shown in Fig. 4. Similar data were obtained for barnase. At low binding affinities, two DSC peaks are expected: one for the complex and the second

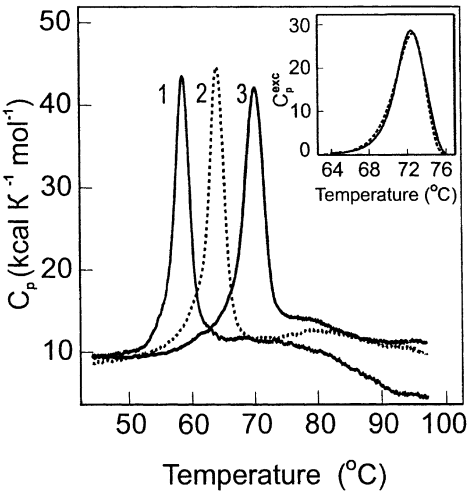

Fig. 3. Temperature dependence of the partial molar heat capacity of binase complexes with barstar A mutants (1) Asp39Ala, (2) Asp35Ala and (3) Glu76Ala at pH 6.2 at a concentration of 0.7 to 0.9 mg/ml. Inset: best fit (---) of the experimental excess heat capacity of binase-Asp35Ala barstar A complex (—) using a model of two-state irreversible transition from Potekhin et al. [14].

Table 2
Dissociation constants and standard free energy changes for complexes of barstar and barstar A and its mutants with barnase and binase at pH 6.2 and 25 °C

	Barnase		Binase	
Inhibitor	K_d (M)	ΔG° (kcal/mol)	K_d (M)	ΔG° (kcal/mol)
Barstar	$1.3(\pm 0.3)\times 10^{-12}$	−16.2	$1.8(\pm 0.3)\times 10^{-11}$	−14.6
Barstar A	$8.1(\pm 0.7)\times 10^{-12}$	−15.1	$7.5(\pm 0.7)\times 10^{-11}$	−13.8
Glu76Ala barstar A	$5.1(\pm 0.5)\times 10^{-10}$	−12.7	$2.9(\pm 0.4)\times 10^{-9}$	−11.6
Asp35Ala barstar A	$3.7(\pm 0.4)\times 10^{-8}$	−10.1	$1.2(\pm 0.3)\times 10^{-7}$	− 9.4
Asp39Ala barstar A	$3.9(\pm 0.3)\times 10^{-7}$	− 8.7	$2.9(\pm 0.2)\times 10^{-6}$	− 7.6

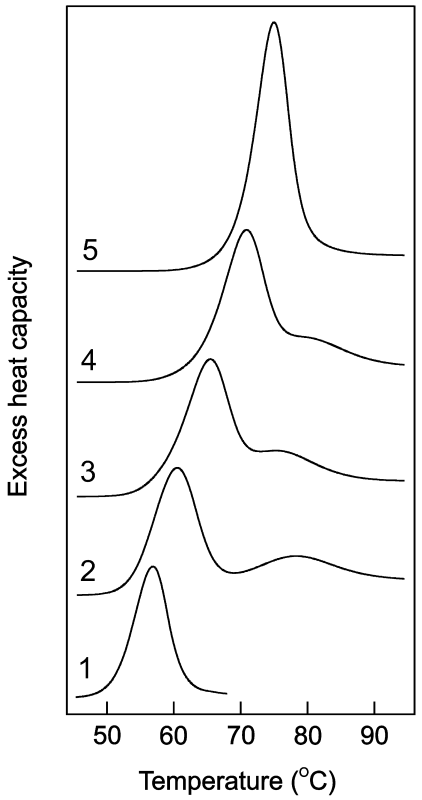

Fig. 4. Simulated DSC melting curves of binase complexes with barstar A and its mutants according to the Brandts and Lin model [1]: (1) free binase; (2) binase-Asp39Ala barstar A; (3) binase-Asp35Ala barstar A; (4) binase-Glu76Ala barstar A; (5) binase-barstar A. The following parameters were used to describe our experimental data using this model. T_d and ΔH_{eff} for binase, barstar A and its mutants were taken from Table 1. The difference between heat capacities of native and denatured protein states, $\Delta_d C_p$, was taken as 1.5 kcal K^{-1} mol^{-1} for binase, and as 1.0 kcal K^{-1} mol^{-1} for barstar A and its mutants. Values of the dissociation constants (K_d) of the complexes were taken from Table 2. Enthalpies of complex formation (ΔH) were calculated from the free energy of complex formation (Table 2), and the entropy of complex formation (ΔS), which was assumed to be fixed and equal to 14.6 cal K^{-1} mol^{-1} for binase–barstar complexes [4]. The heat capacity of complex formation (ΔC_p) was taken as 0.36 kcal K^{-1} mol^{-1} [15]. The binase concentration as well as those of barstar A and its mutants were assumed to be equal to $4 \cdot 10^{-5}$ M each. It should be noted that varying the $\Delta_d C_p$, ΔC_p, and ΔH parameters by as much as 50% only slightly changes the pattern of the excess heat capacity temperature dependence, and has no marked effect on the T_d values.

for the free barstar, which has a T_d value greater than that of the complex. Thus, the first peak reflects the melting of the ribonuclease in the complex and the second peak reflects the melting of the free inhibitor. In this case, the ΔH_{cal} values for the complex are lower because only the enthalpies for ribonuclease unfolding and the binding reaction contribute. At higher binding affinities, only one DSC peak is expected as the T_d value for the complex approaches that of the free barstar. Now the ΔH_{cal} values are considerably higher, because the enthalpies for the unfolding of both proteins as well as the binding reaction contribute. It is not clear that the observed results in Fig. 3 agree with these simulations, because the baselines above the transition for complex melting indicate that some aggregation of the denatured state occurs. To reduce the aggregation, we studied the heat denaturation at lower protein concentrations, and the results are shown in Fig. 5. Now it is clear, in accord with the Brandts and Lin theory [1] that two transitions are visible in both the

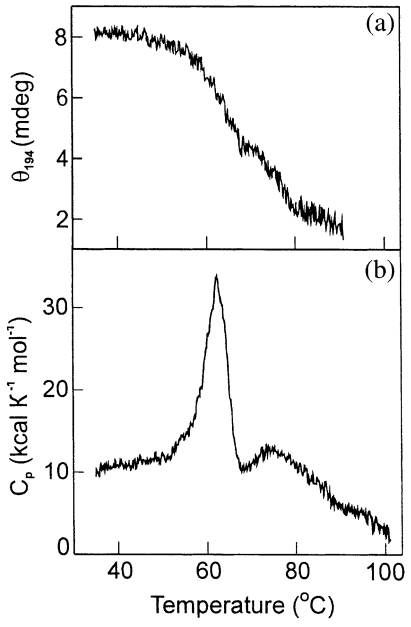

Fig. 5. Thermal denaturation of barnase-Asp35Ala barstar A complex (concentration of complex 0.14 mg/ml): (a) temperature dependence of CD at 194 nm and (b) partial molar heat capacity.

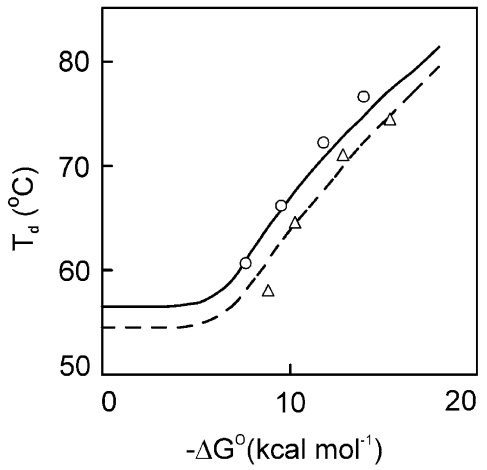

Fig. 6. The dependence of the binase (—) and barnase (---) denaturation temperature in their complexes with barstar A on free energy of complex formation, obtained by simulation of the complex melting curves at different values of association constants in accordance with the Brandts and Lin model [1]. Δ(o) – experimental data for barnase (binase) complexes with barstar A and its mutants Asp39Ala, Asp35Ala and Glu76Ala. The parameters used in the calculations were summarized in the legend for Fig. 4.

thermal denaturation curve followed with CD and in the DSC scan. The first transition is the denaturation of the ribonuclease in the complex, and the second is the denaturation of the free barstar which has a higher melting temperature than the complex under these conditions.

To test the Brandts and Lin model further, we calculated the expected dependence of T_d on the binding affinity, ΔG°, using the data in Tables 1 and 2, and the results are shown in Fig. 6. There is good agreement between the calculated and the experimental results. Thus, our studies of binase and barnase complexes with barstar and its derivatives show that the melting temperature of the ribonucleases within complexes is dependent on the free energy of complex formation and that this dependence can be described by the thermodynamic model of two interacting proteins which was developed by Brandts and Lin [1].

Acknowledgments

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