FEBS Letters 354 (1994) 251–254

FEBS 14740

Thermostability of the barnase–barstar complex

A.A. Makarov[a,*], I.I. Protasevich[a], V.M. Lobachov[a], M.P. Kirpichnikov[a], G.I. Yakovlev[a], R.M. Gilli[b], C.M. Briand[b], R.W. Hartley[c]

[a]*Engelhardt Institute of Molecular Biology, Acad. Sci. Russia, Moscow 117984, Russian Federation*
[b]*Groupe de Recherche sur les Interactions des Protéines en Pharmacologie, Faculté de Pharmacie, Marseille 13385, France*
[c]*National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA*

Received 23 September 1994

Abstract Scanning microcalorimetry was used to study heat denaturation of barnase in complex with its intracellular inhibitor barstar. The heat denaturation of the barnase–barstar complex is well approximated by two two-state transitions with the lower temperature transition corresponding to barstar denaturation and the higher temperature one to barnase denaturation. The temperature of barnase melting in its complex with barstar is 20°C higher than that of the free enzyme. The barstar melting temperature is almost the same in the complex or alone (71°C at pH 6.2 and 68°C at pH 8.0). It seems possible that when barstar unfolds it can remain bound to barnase, while the latter unfolds only on dissociation of the denatured barstar.

Key words: Barnase–barstar complex; Heat denaturation; Scanning microcalorimetry; Isothermal microcalorimetry

1. Introduction

Barnase is the major extracellular ribonuclease produced by *Bacillus amyloliquefaciens* [1–3]. Barstar is a specific intracellular inhibitor of barnase, produced by the same organism. Inhibition involves the formation of a stable, noncovalent, one-to-one complex of the two proteins. The dissociation coefficient of the complex has been estimated to be of the order of 10^{-14} M at pH 8.0 [2,3]. Protein engineering has been used to map residues of barnase and barstar involved in the recognition site [2,3] and the structure of the complex has recently been solved by X-ray crystallography [4]. It was shown that the complex is predominantly stabilized by charge interactions involving positive charges in the active site of the enzyme. The heat denaturation of barnase was studied by scanning microcalorimetry and it was shown that the protein undergoes a two-state thermal transition [5–7]. In this work we report on the thermal stability of the barnase–barstar complex as observed by scanning microcalorimetry and on the thermodynamic parameters of complex formation based on isothermal microcalorimetry measurements.

2. Materials and methods

Barnase was prepared from a culture of *E. coli* (strain JM107) containing the plasmid pMT416 [2]. It was purified by the procedure described in [5]. Barstar was purified from *E. coli* (strain HB101) containing the plasmid pMT316 [2]. Protein concentration was determined spectrophotometrically at 280 nm, assuming $E^{0.1\%} = 2.21$ for barnase [8] and $E^{0.1\%} = 2.02$ for barstar [9].

Scanning microcalorimetric measurements were carried out on a differential scanning microcalorimeter DASM-4 (NPO Biopribor, Russia) in 0.48 ml cells at a heating rate of 1 K/min. Protein concentrations varied from 0.2 to 0.8 mg/ml. Denaturation temperature (T_d), calorimetric denaturation enthalpy (ΔH_{cal}) and effective or van 't Hoff denaturation enthalpy (ΔH_{eff}) were determined as described in [10,11]. To analyze functions of excess heat capacity we used the software package THERMCALC developed at the Institute of Protein Research (Puschino, Russia) [10,11], a modification of a method described in [12].

Isothermal flow microcalorimetric measurements were carried out with a ThermoMetric 2277 Thermal Activity Monitor (Sweden) according to [13]. The enthalpy of barnase binding to barstar was determined at 25°C and 37°C in 0.01 M buffer, containing 0.05 M NaCl to prevent protein sorption on the feeding tubes walls of the microcalorimeter.

Circular dichroism (CD) spectra of barstar in the far UV region were measured on a Jasco J-500A dichrograph (Japan) at the protein concentration of 0.2 mg/ml. The results were expressed in the units of ellipticity for the mean amino acid residue assuming its average mass for barstar to be equal to 114.6.

3. Results and discussion

Fig. 1A shows the temperature dependence of partial molar heat capacity of a barnase–barstar mixture at pH 6.2. The calorimetric curve consists of two peaks with maxima at 54.5°C and 75.5°C, the latter being clearly asymmetric. The denaturation temperature of the low temperature transition and its reversibility coincide with those for barnase melting, obtained in a parallel experiment (Fig. 1B). Thus, the low temperature peak represents melting of free barnase, whereas the high temperature peak reflects melting of the complex. The melting temperature of the complex is higher by 20°C than that for barnase. At barstar concentration 0.2 mg/ml the complex melting reversibility was equal to 55%. However, a three-fold increase of barnase and barstar concentrations had no effect on barnase melting reversibility (54%), whereas melting of the complex became completely irreversible. In this case according to the peak of free barnase melting its amount almost doubled on a second heating. No reversibility of complex melting was observed in the absence of NaCl in solution. Since these factors had no effect on the reversibility of barnase denaturation, the disappearance of reversibility for the complex is due to their influence on the state of barstar molecule at high temperature.

The results of quantitative analysis of calorimetric curves are shown in Table 1. Depending on experimental conditions the ratio $R = \Delta H_{cal}/\Delta H_{eff}$ for melting of the barnase–barstar complex varied from 1.25 to 2.31. This fact and also the peak asymmetry indicate case of more than one cooperative transition [10]. Therefore, we carried out deconvolution of the calorimetric curves for the barnase–barstar complex. The curves can

*Corresponding author. Fax: (7) (095) 1351405.

SSDI 0014-5793(94)01127-3

be presented as two overlapping two-state transitions (Fig. 2, Table 2). The enthalpy of the first peak (ΔH_d^1) increased with NaCl addition. The denaturation enthalpy of the second peak ΔH_d^2) was independent of the presence of NaCl. Analysis of barnase melting has shown no changes in its denaturation enthalpy with NaCl addition (Table 1). Increasing pH from 6.2 to 8.0 decreases the denaturation enthalpy of the second deconvoluted peak of the melting of the complex (Table 2), as was observed earlier for the melting of barnase alone [5]. These data allowed us to conclude that the second peak corresponds to the melting of barnase. Since the dependence of calorimetric enthalpy on denaturation temperature is proportional to the difference between the heat capacities of the native and denatured states ($\Delta_d C_p$) [10], we extrapolated the melting enthalpy of free barnase to the temperature of the second peak maximum, using the $\Delta_d C_p$ value. $\Delta_d C_p$ for free barnase, determined from the calorimetric curve (Fig. 1B), is equal to (1.36 ± 0.14) kcal/K·mol. Then the value of ΔH_{cal}, calculated for T_d^2 = 74.7°C, is 155 kcal/mol which is very close to the ΔH_d^2 value (Table 2). This result confirms that the second peak of complex melting represents barnase denaturation.

At pH 6.2 and barstar concentrations 0.2–0.6 mg/ml, as used when studying complex melting, we did not observe a reliably registered heat effect of barstar denaturation within the temperature interval 5–120°C. Thereafter, we investigated the effect of temperature on the barstar CD spectrum in the far UV region. Temperature dependence of the CD at 208 nm showed a highly cooperative transition with T_d = (71 ± 0.5)°C at pH 6.2 and (68 ± 0.5)°C at pH 8.0 (Fig. 3). Similar melting curves were obtained at 220 nm. Values of barstar denaturation temperatures, obtained by the CD technique, coincide well with the corresponding values of the first deconvoluted peak on the calorimetric melting curve of the complex (T_d^1, Table 2). The absence of a noticeable heat effect during microcalorimetric registration of barstar melting at pH 6.2 might be due to the self-association of denatured protein molecules which is accompanied by a heat effect of the opposite sign [10,14]. At pH 8.0 a small heat effect of barstar melting was registered (Fig. 4A). Increase of NaCl concentration (0–0.2 M) resulted in increases of barstar denaturation enthalpy and R ratio. In all cases the R values are substantially below one, which indicates self-association of protein molecules on heating [14]. The increasing R ratio at higher NaCl concentration points to a lower degree of self-association of unfolded barstar. This explains the effect of

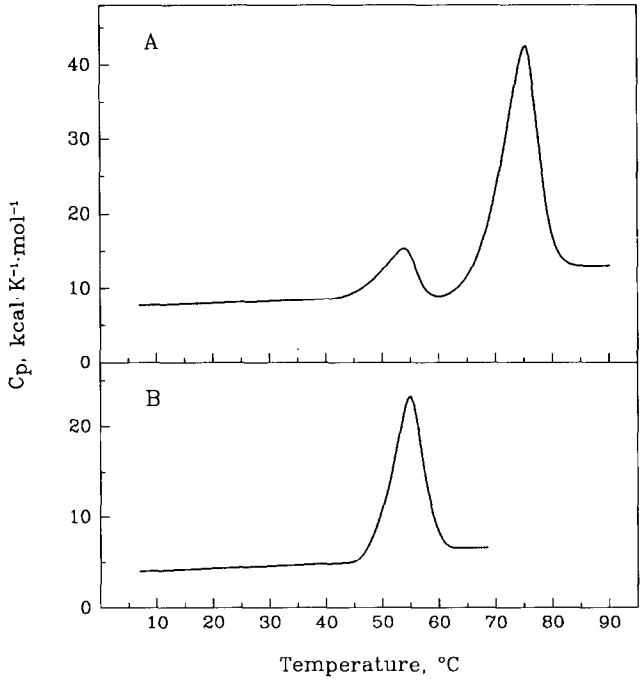

Fig. 1. Temperature dependence of partial molar heat capacity of barnase complex with barstar (A) and barnase (B) at pH 6.2 (0.01 M PIPES, 0.05 M NaCl).

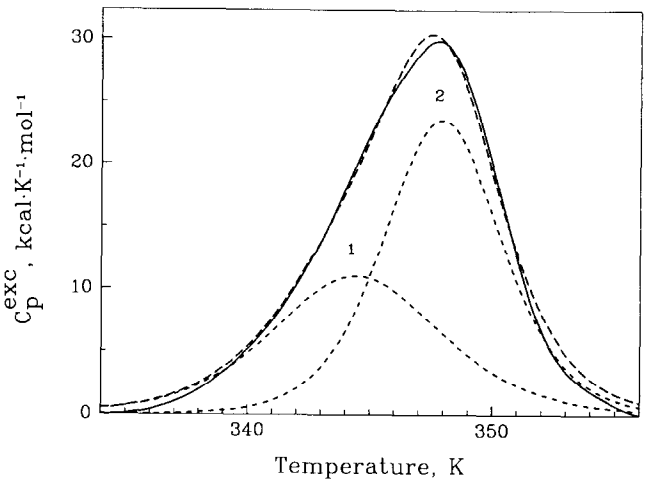

Fig. 2. Computer deconvolution (dotted lines) of the transition excess heat capacity of barnase complex with barstar (solid line) at pH 6.2 (0.01 M PIPES, 0.05 M NaCl).

Table 1
Parameters of thermal denaturation of barnase and its complex with barstar (0.01 M PIPES)

Sample	T_d (°C)	ΔH_{cal} (kcal/mol)	ΔH_{eff} (kcal/mol)	R*
Barnase				
pH 6.2	53.6	135	126	1.07
pH 6.2, 0.05 M NaCl	54.8	128	129	0.99
Barnase + barstar				
pH 6.2	78.0	169	135	1.25
pH 6.2, 0.05 M NaCl	75.5	242	114	2.12
pH 8.0, 0.05 M NaCl	73.5	192	83	2.31

The relative error of the given ΔH_{cal} and ΔH_{eff} values did not exceed ±6%; the absolute error of the given T_d values did not exceed ±0.3°C. *$R = \Delta H_{cal}/\Delta H_{eff}$.

Table 2
Thermodynamic parameters of transitions obtained by deconvolution of excess heat capacity function for barnase–barstar complex (0.01 M PIPES)

Condition	T_d^1 (°C)	ΔH_d^1 (kcal/mol)	T_d^2 (°C)	ΔH_d^2 (kcal/mol)
pH 6.2	72.1	44	77.9	147
pH 6.2, 0.05 M NaCl	71.2	102	74.7	151
pH 8.0, 0.05 M NaCl	67.5	88	72.8	116

The relative error of the given ΔH_d values did not exceed ±10%; the absolute error of the given T_d values did not exceed ±1.2°C.

NaCl on the value of melting enthalpy of the first peak on the complex melting curve (Table 2).

Thus, the first deconvoluted peak indicates denaturation of the barstar globule. This means that barstar undergoes a two-state transition in the complex.

Table 3 shows thermodynamic parameters of barnase association with barstar. The enthalpy of barnase binding to barstar does not depend on pH and decreases slightly upon temperature increase from 25°C to 37°C. Using these results, we have estimated the value of barnase–barstar complex dissociation enthalpy at the melting temperature of the complex as about 10 kcal/mol. Therefore, dissociation enthalpy could not contribute significantly to the observed values of denaturation enthalpy of the complex. Dissociation of the complex is indicated by the increase of the free barnase denaturation peak on repeated heating at pH 6.2, as described above. This is also confirmed by the results of melting the complex at pH 8.0 (Fig. 4B). No low temperature peak of free barnase melting was observed (curve 1) but it appears on the second heating (curve 2), whereas the heat absorption peak of the complex vanishes completely.

A pH decrease from 8.0 to 6.2 causes no change of barnase–barstar binding enthalpy, whereas ΔS_a increases by 1.5 times (Table 3). This shows that a decrease of barnase–barstar association constant on reducing the pH from 8.0 to 6.2 [3] is due mainly to a change in the entropy contribution to the free energy of complex formation. It has been shown [3] that the pH dependence of the barnase–barstar association constant is determined predominantly by change of ionization of the imidazole ring of barnase residue His^{102}. Deprotonation of His^{102} upon pH increase raises the free energy of interaction of the two proteins [3]. Evidently, dehydration of the protonated His^{102} residue in barnase during its complex formation with barstar causes a decrease of the binding constant at the lower pH values.

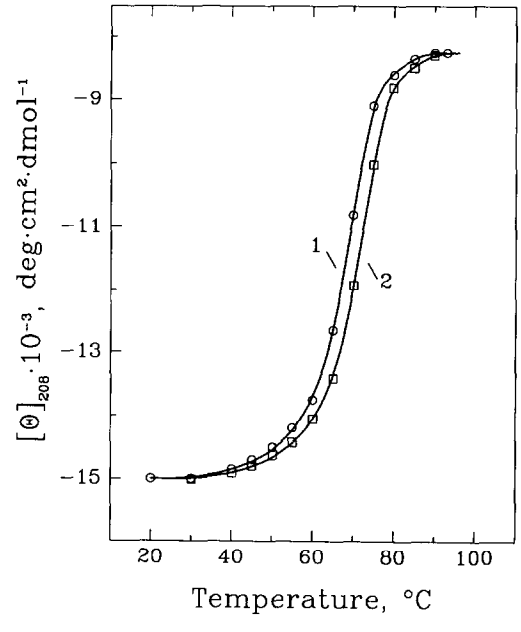

Fig. 3. Temperature dependence of barstar CD value at 208 nm for pH 8.0 (1) and pH 6.2 (2) (0.01 M PIPES).

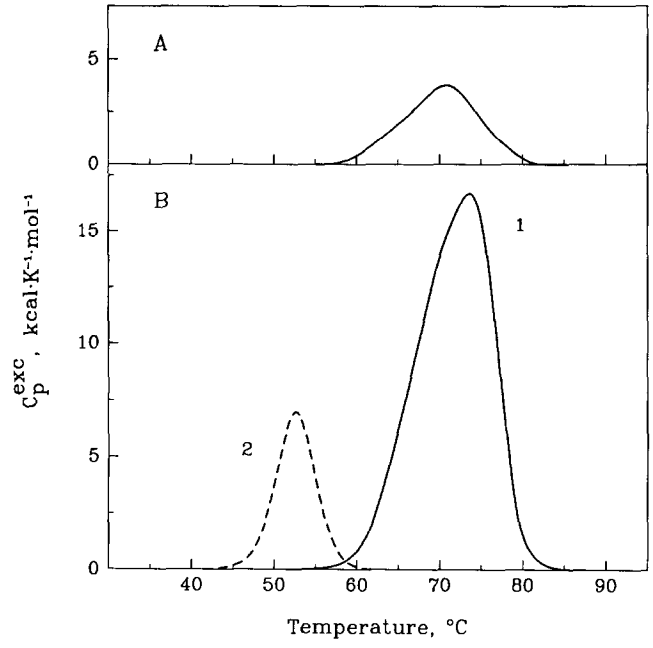

Fig. 4. (Panel A) Transition excess heat capacity vs. temperature for barstar at pH 8.0 (0.01 M PIPES, 0.1 M NaCl). (Panel B) Transition excess heat capacity vs. temperature for barnase complex with barstar at pH 8.0 (0.01 M PIPES, 0.05 M NaCl): (1) first heating, (2) second heating. Curves in both panels were obtained after baseline subtraction, as described in [10,11].

The temperature of barnase melting in its complex with barstar is 20°C higher than that of the free enzyme and 4–5°C higher than the melting temperature of barstar. Barstar melting temperatures are almost the same in the complex and alone. It seems possible that when barstar unfolds, it can remain bound to barnase. It would be this complex which dissociates and unfolds in the second deconvoluted peak. This model explains the fact that barstar denaturation in the complex is apparently associated with a large enthalpy change, while there is little or no detectable enthalpy change with free barstar. Barnase, remaining bound to the unfolded barstar, prevents its self-association. This also makes structural sense since barnase binds to barstar residues located on a continuous polypeptide fragment (residues 29–46) [4] so that the binding region could remain intact independently of the rest of the protein. It explains why, in the complex, barnase might unfold at a higher temperature than barstar.

Table 3
Thermodynamic parameters of barnase association with barstar (0.01 M PIPES, 0.05 M NaCl)

pH	T (°C)	ΔH_a (kcal/mol)	ΔS_a (cal/K·mol)
8.0	25	−24.7	−22.1*
6.2	25	−26.4	−32.9*
6.2	37	−21.7	

The relative error of the given ΔH_a values did not exceed ±5%. *For ΔS_a calculation values of ΔG_a were taken from [3].

Acknowledgements: This work was supported by International Science Foundation grant MF6000, Russian Fund of Fundamental Research grant 93-04-21295, Russian Ministry for Science and Technology and DAGIC grant of the French Ministry of National Education and Research.

References

[1] Hartley, R.W. (1989) Trends Biochem. Sci. 14, 450–454.
[2] Hartley, R.W. (1993) Biochemistry 32, 5978–5984.
[3] Schreiber, G. and Fersht, A.R. (1993) Biochemistry 32, 5145–5150.
[4] Guillet, V., Lapthorn, A., Hartley, R.W. and Mauguen, Y. (1993) Structure 1, 165–176.
[5] Makarov, A.A., Protasevich, I.I., Kuznetsova, N.V., Fedorov, B.B., Leshchinskaya, I.B., Hartley, R.W., Kirpichnikov, M.P., Yakovlev, G.I. and Esipova, N.G. (1993) J. Biomol. Struct. Dyn. 10, 1047–1065.
[6] Griko, Y.V., Makhatadze, G.I., Privalov, P.L. and Hartley, R.W. (1994) Protein Sci. 3, 669–676.
[7] Martinez, J.C., Harrous, M.E., Filimonov, V.V., Mateo, P.L. and Fersht, A.R. (1994) Biochemistry 33, 3919–3926.
[8] Loewenthal, R., Sancho, J. and Fersht, A.R. (1991) Biochemistry 30, 6775–6779.
[9] Fitzgerald, P.C. and Hartley, R.W. (1993) Anal. Biochem. 214, 544–547.
[10] Privalov, P.L. and Potekhin, S.A. (1986) Methods Enzymol. 131, 4–51.
[11] Filimonov, V.V., Prieto, J., Martinez, J.C., Bruix, M., Mateo, P.L. and Serrano, L. (1993) Biochemistry 32, 12906–12921.
[12] Freire, E. and Biltonen, R.L. (1978) Biopolymers 17, 463–479.
[13] Gilli, R.M., Sari, J.C., Sica, L.M. and Briand, C.M. (1988) Biochim. Biophys. Acta 964, 53–60.
[14] Sturtevant, J.M. (1987) Annu. Rev. Phys. Chem. 38, 463–488.