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Dissociation constants and thermal stability of complexes of *Bacillus intermedius* RNase and the protein inhibitor of *Bacillus amyloliquefaciens* RNase

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Abstract **Binase, the extracellular ribonuclease of *Bacillus intermedius*, is inhibited by barstar, the natural protein inhibitor of the homologous RNase, barnase, of *B. intermedius*. The dissociation constants of the binase complexes with barstar and its double $Cys^{40,82}Ala$ mutant are about 10^{-12} M, only 5 to 43 times higher than those of the barnase–barstar complex. As with barnase, the denaturation temperature of binase is raised dramatically in the complex. Calorimetric studies of the formation and stability of the binase–barstar complex show that the binase reaction with barstar is qualitatively similar to that of barnase but some significant quantitative differences are reported.**

Key words: Binase–barstar complex; Dissociation constant; Heat denaturation; Scanning microcalorimetry; Isothermal microcalorimetry

1. Introduction

A low-molecular weight RNase, binase, is produced and secreted by the sporogenic bacteria *Bacillus intermedius*. The enzyme consists of a single polypeptide chain of 109 amino acids with no disulfide bonds [1] and belongs to a large family of homologous microbial ribonucleases [2]. To protect themselves from the lethal effect of binase expression, *Bacillus intermedius* cells synthesize an intracellular inhibitor [3]. The purified inhibitor is extremely unstable [3]. For this reason the details of its complex formation with binase have not been studied. Binase closely resembles the homologous *Bacillus amyloliquefaciens* RNase, barnase, with 84% sequence identity and essentially the same folded structure [4,5]. This probably explains why barnase inhibitor, barstar, produced by *Bacillus amyloliquefaciens* is also active against binase. This barstar property was used to design a plasmid for production of a recombinant binase and its mutant forms [6]. However, the characteristics of the binase–barstar interaction have not been determined previously.

This work deals with the parameters of binase and barstar complex formation and the thermal stability of such complexes.

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2. Materials and methods

Binase was purified by the procedure described in [5]. Barnase was prepared from *E. coli* (strain JM107) containing plasmid pMT416 [7] and purified according to [5]. Barstar and its double $Cys^{40,82}Ala$ mutant, barstar A, were obtained from culture of *E. coli* (strain HB101) carrying appropriate expression plasmids [8]. Protein concentration was determined spectrophotometrically at 280 nm, assuming $E^{0.1\%} = 1.83$ for binase [5], $E^{0.1\%} = 2.21$ for barnase [9] and $E^{0.1\%} = 2.22$ for barstar and barstar A [10].

Dissociation constants for the barstar complexes with binase and barnase were determined by ribonuclease titration with the inhibitor [8] using poly(I) as a substrate. As the protein concentrations in these titrations are of the order of 10^{-11} M, curvature of the end point would be detectable if the dissociation constants were as high as about 10^{-14} M. All enzymic reactions were carried out as follows. The solutions of ribonuclease at a concentration of about 10^{-11} M and at ten different barstar concentrations were incubated for about 24 h in a thermostat at 25°C. Then 2 ml aliquots of incubated solutions were placed in the spectrophotometric cells and the reactions were started by adding 30 μl of 3.8 mM poly(I). The steady-state rates obtained were used to calculate the dissociation constants as described in [8]. All solutions contained 10^{-3} M EDTA and 0.2 mg/ml human serum albumin.

Isothermal flow microcalorimetric measurements were carried out using a ThermoMetric 2277 Thermal Activity Monitor (Sweden) according to [11]. The enthalpy of binase and barnase binding to barstar was determined at 25°C and 37°C in 0.01 M PIPES containing 0.05 M NaCl to prevent protein adsorption on the microcalorimeter feeding tube walls. Scanning microcalorimetric measurements were carried out on a differential scanning microcalorimeter DASM-4 (NPO Biopribor, Pushchino, Russian Federation) in 0.48 ml cells at a heating rate of 1 K/min. Protein concentrations varied from 0.4 to 0.8 mg/ml. Denaturation temperature (T_d), calorimetric denaturation enthalpy (ΔH_{cal}), and van't Hoff's denaturation enthalpy (ΔH_{eff}) were determined as described in [12]. To analyze functions of excess heat capacity we used the software package THERMCALC developed at the Institute of Protein Research (Pushchino, Russian Federation) [12]. The buffers used were 0.01 M Na-acetate, 0.05 M NaCl, pH 6.2 and 0.01 M PIPES, 0.05 M NaCl, pH 8.0.

3. Results and discussion

Dissociation constants of binase complexes with barstar and its double mutant $Cys^{40,82}Ala$ (barstar A) are given in Table 1. For comparison, corresponding values for barnase and barstar complexes were measured. The dissociation constants for barnase complexes with barstar and barstar A practically coincide with those previously obtained at the same conditions (0.2 M NH_4-acetate, pH 8.0) using the barstar titration of the mixture of the native barnase and its inactive mutants [8]. The dissociation constants for binase complexes with barstar and barstar A in 0.2 M NH_4-acetate at pH 8.0 are equal to $4.3 \cdot 10^{-12}$ M and

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Table 1
Dissociation constants for complexes of barstar with barnase and binase

Inhibitor	Conditions	Dissociation constants (M)	
		Binase	Barnase
Barstar	0.2 M NH_4Ac, pH 8.0	$(4.3 \pm 0.9) \cdot 10^{-12}$	$(1.0 \pm 0.2) \cdot 10^{-13}$
Barstar A	0.2 M NH_4Ac, pH 8.0	$(3.2 \pm 0.2) \cdot 10^{-12}$	$(6.6 \pm 0.4) \cdot 10^{-13}$
Barstar	0.01 M PIPES 0.05 M NaCl, pH 8.0	$(6.1 \pm 0.5) \cdot 10^{-12}$	$(3.1 \pm 0.4) \cdot 10^{-13}$
Barstar	0.01 M PIPES, 0.05 M NaCl, pH 6.2	$(1.6 \pm 0.2) \cdot 10^{-11}$	$(9.9 \pm 0.4) \cdot 10^{-13}$

Table 2
Thermodynamic parameters of binase and barnase association with barstar (0.01 M PIPES, 0.05 M NaCl)

Sample	T (°C)	ΔH_a* (kcal/mol)	ΔS_a (cal/K·mol)
Binase + barstar			
pH 8.0	25	−17.3	− 7.0
pH 6.2	25	−19.0	−14.6
pH 6.2	37	−19.8	
Barnase + barstar			
pH 8.0	25	−24.7	−25.9
pH 6.2	25	−26.4	−33.9
pH 6.2	37	−21.7	

*ΔH_a values are accurate within ± 5%.

Table 3
Parameters of thermal denaturation of binase and its complex with barstar*

Sample	T_d (°C)	ΔH_{cal} (kcal/mol)	ΔH_{eff} (kcal/mol)	R**
Binase + barstar				
pH 6.2	81.2	155	72	2.15
pH 8.0	83.4	156	85	1.84
Binase				
pH 6.2	55.3	95	101	0.94
pH 8.0	54.6	59	104	0.57

*ΔH_{cal} and ΔH_{eff} values are accurate within ± 6%, and T_d values are accurate within ± 0.3°C.
**$R = \Delta H_{cal} / \Delta H_{eff}$.

Table 4
Thermodynamic parameters for transitions obtained by deconvolution of excess heat capacity function for binase-barstar complex*

pH	T_d^1 (°C)	ΔH_d^1 (kcal/mol)	T_d^2 (°C)	ΔH_d^2 (kcal/mol)
pH 6.2	73.3	70	80.3	99
pH 8.0	75.7	62	82.8	111

*ΔH_d are accurate within ± 10%, and T_d are accurate within ± 1.2°C.

$3.2 \cdot 10^{-12}$ M, respectively, only 43 and 5 times higher than those for barnase. On decreasing pH from 8.0 to 6.2 the dissociation constants for complexes of binase and barnase with barstar increase by the factor of three.

Most of the 14 barnase residues involved in interaction with barstar are conserved in binase [13]. The exceptions are Ser^{85} and Gln^{104}, involved in van der Waals contacts and replaced by alanines in binase. Such a high degree of conservation suggests that the barnase–barstar and binase–barstar complexes are structurally very similar. The closeness of the dissociation constants for binase and barnase confirms this supposition. However, the enthalpy values for the binase–barstar complex formation are 7.4 kcal/mol lower both at pH 8.0 and 6.2 than for the barnase–barstar complex (Table 2). The absence of at least three hydrogen bonds in the binase-barstar complex may explain this difference. A decrease in the binase-barstar complex formation enthalpy is compensated by a smaller decrease in entropy, probably because of fewer mobile amino acid side chains 'frozen' in the binase–barstar complex as compared with barnase–barstar. Most likely, the number of amino acid residues in the contact zone of binase–barstar complex is lower than in barnase–barstar complex.

Fig. 1 shows the temperature dependence of the binase–barstar complex partial molar heat capacity at pH 6.2 and 8.0 (Panel A) and that of free binase at pH 6.2 (Panel B). Melting of the complex was irreversible. Reversibility of binase denaturation was equal to 33% at pH 6.2 and the process was completely irreversible at pH 8.0. Probably, low binase denaturation reversibility is associated with irreversibility melting of the binase–barstar complex. The calorimetric curves for binase–barstar complex melting are clearly asymmetrical (Fig. 1A). The ratio $R = \Delta H_{cal} / \Delta H_{eff}$ for the binase–barstar complex melting is close to two (Table 3). This fact, along with the peak asymmetry, indicates that there is more than one cooperative transition [12]. To prove this we carried out deconvolution of calorimetric curves for the binase–barnase complex. The curves can be resolved into two overlapping two-state transitions (Fig. 2). Earlier, we obtained the same results for melting of the barnase–barstar complex [14]. The first deconvoluted peak was shown to correspond to barstar denaturation, while the second was assigned to barnase melting. The relation between the

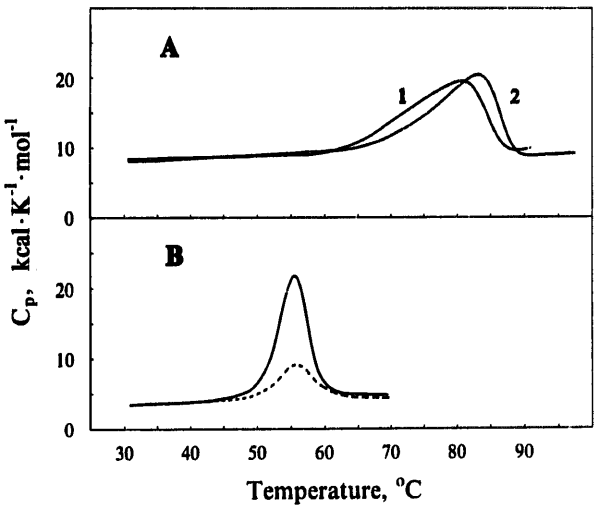

Fig. 1. (A) Temperature dependence of partial molar heat capacity of binase complex with barstar at pH 6.2 (1) and pH 8.0 (2). (B) Temperature dependence of partial molar heat capacity of binase at pH 6.2. Dotted line shows melting reversibility.

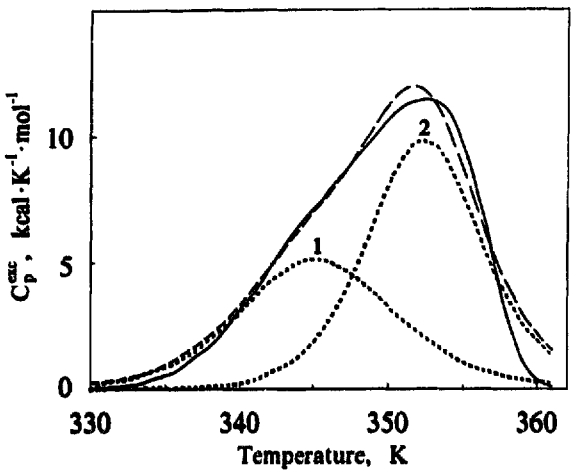

Fig. 2. Computer deconvolution (dotted lines) of the transition excess heat capacity of binase complex with barstar (solid line) at pH 6.2.

values of enthalpies and temperatures for binase and barstar melting in the complex and in the free state allows for the conclusion that the same assignment of deconvolution peaks is valid in this case. As follows from the comparison of the deeconvolution data with those for free enzyme melting (Tables 3,4), at pH 8.0 the enthalpy of binase melting within the complex is double that of free binase melting. Probably, barstar in the complex protects binase from self-association.

When pH is increased from 6.2 to 8.0 T_d values increase for both barstar and binase (Table 4), whereas for the barnase–barstar complex, the pH increase causes T_d to decrease for both proteins [14]. The binase–barstar complex melting temperature is higher by 5.7°C than that for barnase–barstar complex at pH 6.2, although denaturation temperatures for the free RNases are very close [14]. The fact that the enthalpy change on binase binding to barstar is temperature independent and that on barnase complex formation with barstar decreases as temperature increases (Table 2) can explain the higher values of binase–barstar complex melting temperature. The difference in the properties of these complexes is also reflected in the higher denaturation temperature of barstar complexed with binase (Table 4) compared with the T_d of free barstar (73.3°C vs. 71°C at pH 6.2 and 75.7°C vs. 68°C at pH 8.0), whereas the melting temperature for barstar does not change when it is complexed with barnase [14].

That the denaturation temperatures of binase and barnase [14] increase on barstar binding can be explained by their interface region, which include the active sites, being also the regions of initial unfolding during heat denaturation. Stabilization of this region by interaction with the inhibitor would then be expected to raise the T_d as observed. It is noteworthy that crystallographic studies [13] indicated an ordering of the side chains of both proteins in the barnase–barstar interface. It is possible that structural stability in this region of the enzymes might be sacrificed to some extent in favor of both catalytic efficiency and inhibitor binding.

In conclusion, the RNase inhibitor protein of *B. amyloliquefaciens* effectively binds to and inhibits binase, the homologous RNase of *B. intermedius* with a dissociation constant of about 10^{-12} M. The denaturation temperature of binase in complex with barstar is pH-dependent and equals 80–83°C, which is 25–28°C higher than for free binase.

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