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Interaction of the Bacterial Ribonuclease Binase with the Polypeptide Inhibitor Barstar Based on Kinetic Data on Poly(U) Hydrolysis

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Abstract—The reaction of poly(U) hydrolysis catalyzed by binase while the latter is inhibited by barstar has been investigated. The inhibition constant for barstar and the apparent Michaelis constants for the inhibition by barstar in the presence of ethanol and NaCl have been determined. Both ethanol and NaCl enhance the inhibition by barstar. This suggests that the binding of barstar with binase is probably due to the interaction of hydrophobic sites rather than by electrostatic interaction between amino acid residues.

Key words: binase, barstar, poly(U) hydrolysis, enzymatic catalysis, kinetics, hydrophobic interaction

Binase and barnase are homological bacterial RNases, but the mechanisms of suppression of their toxic intracellular action on the cell in which they are synthesized seem to be different. Whereas barnase is synthesized simultaneously with barstar, a polypeptide inhibitor that inactivates it very rapidly inside the *B. amiloliquefaciens* cell [1], an intracellular inhibitor of binase is still not known. Binding of barnase with barstar, its natural polypeptide inhibitor, is mainly due to electrostatic interaction of charged amino acid residues on the contact interface of the two proteins [2]. Barstar forms an inactive complex not only with barnase, but also with binase, though the affinity for the latter is much lower [3]. The significant difference in affinity of barnase and binase to barstar suggests different physicochemical mechanisms of interaction. The goal of present study of kinetic parameters characterizing barstar binding with binase was to clarify the mechanism of formation of this protein–protein complex, in particular, to clarify the role of electrostatic and hydrophobic interactions.

MATERIALS AND METHODS

Barstar, a polypeptide inhibitor of RNase, was obtained by expression of its gene in *Escherichia coli* BL21 (DE3) cells. pGEMEX/Bst, kindly donated by A. A. Shulga (Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow), was used as a plasmid vector. In this genetic construction based on the pGEMEX-I vector, the barstar gene is positioned after the T7 promotor, providing induction of expression of the barstar gene [4]. Barstar was isolated and purified as described earlier [5].

Binase was obtained from the Institute of Organic Synthesis of the Latvian Academy of Sciences and additionally purified on CM-Sepharose CL6B in potassium phosphate buffer, pH 6.5 [6].

The inhibiting action of barstar on binase was assayed via its effect on binase-catalyzed hydrolysis of polynucleotide. High-molecular-weight poly(U) from Sigma (USA) was used as the hydrolyzed substrate. Preliminarily, we determined the limiting concentrations of neutral salt (100 mM) and ethanol (30%) at which the hypochromic effect in the UV spectrum of poly(U) did not exceed 5% of the maximal intensity. The rate of poly(U) hydrolysis by binase was determined as the slope of the concentration versus time kinetic curve using the initial portion of the curve limited by 10 sec. The kinetics were monitored spectrophotometrically at 286 nm using a Specord M-40 spectrophotometer (Carl Zeiss, Germany) in thermostatted cuvettes at 25°C. The differential spectrum of poly(U) has a minimum at 286 nm, $\Delta\varepsilon = 570\ M^{-1}\cdot cm^{-1}$ [7]; so, the reaction rate was determined by the decrease in absorbance at 286 nm, which is proportional to the accumulation of the products of poly(U) hydrolysis.

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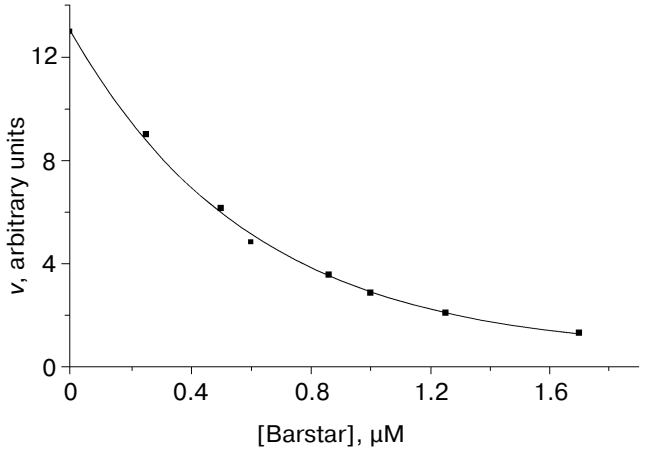

Fig. 1. Rate of poly(U) hydrolysis by binase on inhibition by barstar. Reaction conditions: $2.2 \cdot 10^{-4}$ M poly(U), $4.87 \cdot 10^{-7}$ M binase, 0.05 M Tris-acetate buffer, pH 8.0, 25°C.

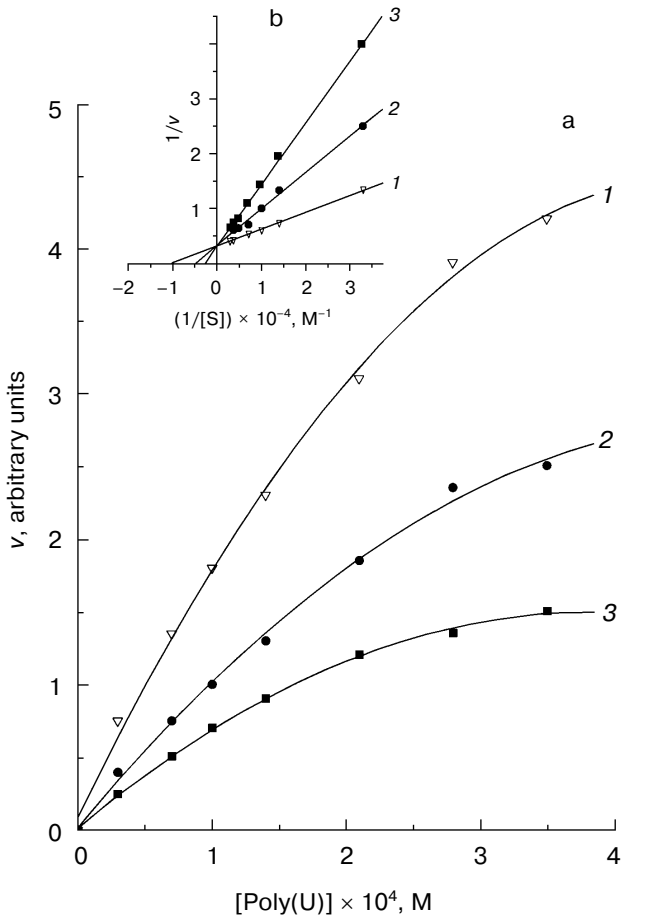

Fig. 2. Initial reaction rate versus poly(U) concentration in {*v*; [S]} (a) and in reciprocal (b) coordinates. Barstar concentration (M): *1*) 0; *2*) $3.12 \cdot 10^{-7}$; *3*) $6.25 \cdot 10^{-7}$. The reaction conditions: $4.87 \cdot 10^{-7}$ M binase, 0.05 M Tris-acetate buffer, pH 8.0, 25°C.

RESULTS AND DISCUSSION

Binase systematically hydrolyzes poly(U), cleaving internucleotide bonds with formation of nucleoside-2′,3′-cyclophosphates and their subsequent cleavage to nucleoside-3′-phosphates. The inhibiting effect of barstar on poly(U) hydrolysis by binase is characterized by dependence of the reaction rate on barstar concentration at constant concentration of the poly(U) substrate. As shown in Fig. 1, barstar inhibits the enzymatic reaction at concentrations comparable with that of binase.

For more precise quantitative evaluation of the inhibiting effect of barstar on the reaction of poly(U) hydrolysis, the inhibition constant was determined from the dependence of the initial reaction rate on poly(U) concentration at various concentrations of barstar. The plots of reaction rate versus substrate concentration are presented in Fig. 2a, and the same data are presented in reciprocal Lineweaver–Burk coordinates in Fig. 2b. The data indicate that inhibition by barstar is of competitive type with inhibition constant $K_i = (3.5 \pm 0.1) \cdot 10^{-7}$ M calculated using the Michaelis equation for competitive inhibition.

For experimental evaluation of the electrostatic contribution to the total energy of the binase–barstar interaction, the changes in kinetic parameters of the reaction at various values of dielectric constant of the reaction medium were studied [8]. Since the interaction of barstar with binase was monitored by changes in kinetic parameters of hydrolysis of poly(U) polyanion, the effects of salts and organic solvent on kinetic parameters of hydrolysis *per se* catalyzed by binase have been preliminarily evaluated. As shown earlier, neutral salts inhibit whereas organic solvents activate hydrolysis of poly(U) by binase [9]. These data indicate that electrostatic interactions are important in poly(U) cleavage by binase; however, it should be added that of neutral salts only anions inhibit the reaction because the identity of the cation does not influence the reaction rate. The absence of any dependence on Na^+ and K^+, which are significantly different in their physicochemical properties, indicates that chloride anions cause the main inhibiting effect on poly(U) hydrolysis. The plots of poly(U) hydrolysis rate versus substrate concentration on inhibition by a neutral salt (NaCl or KCl) are presented in Fig. 3a; the same data in the Lineweaver–Burk reciprocal coordinates are presented in Fig. 3b. As shown, the inhibition by chloride anion is of competitive type, the inhibition constant K_i being 22 ± 0.5 mM. It is probable that competition is caused by specific interaction of Cl^- with the enzyme and that it is for the sites of phosphate binding in the active center of binase having functionally important Arg59 and Arg83 residues [10]; the guanidine group of these residues is able to retain anions.

As shown in Fig. 4, the combined action of barstar and NaCl enhances inhibition of poly(U) hydrolysis. The apparent Michaelis constant of $(1.6 \pm 0.05) \cdot 10^{-4}$ M for

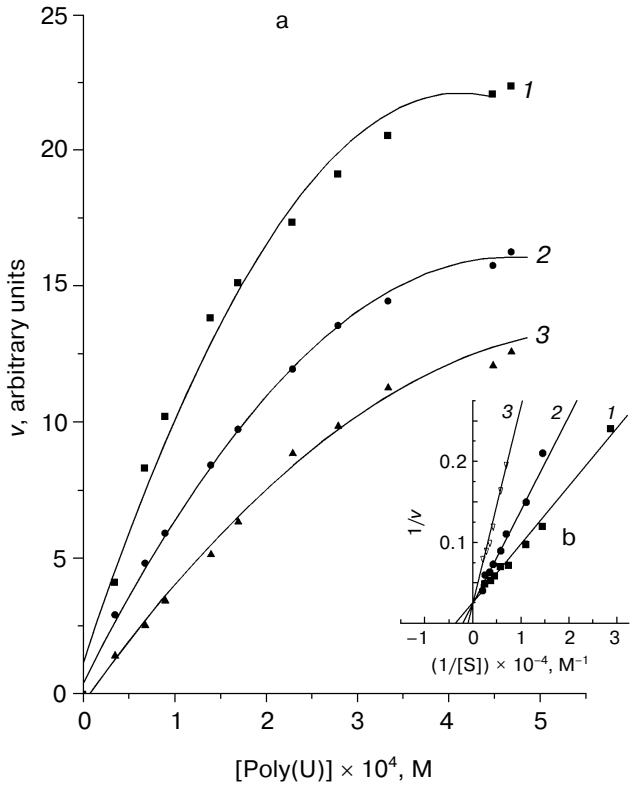

Fig. 3. Initial reaction rate versus poly(U) concentration in {*v*; [S]} (a) and in reciprocal (b) coordinates. Neutral salt (NaCl) concentration (M): *1*) 0; *2*) 0.025; *3*) 0.05. Reaction conditions: $1 \cdot 10^{-6}$ M binase, 0.05 M Tris-acetate buffer, pH 8.0, 25°C.

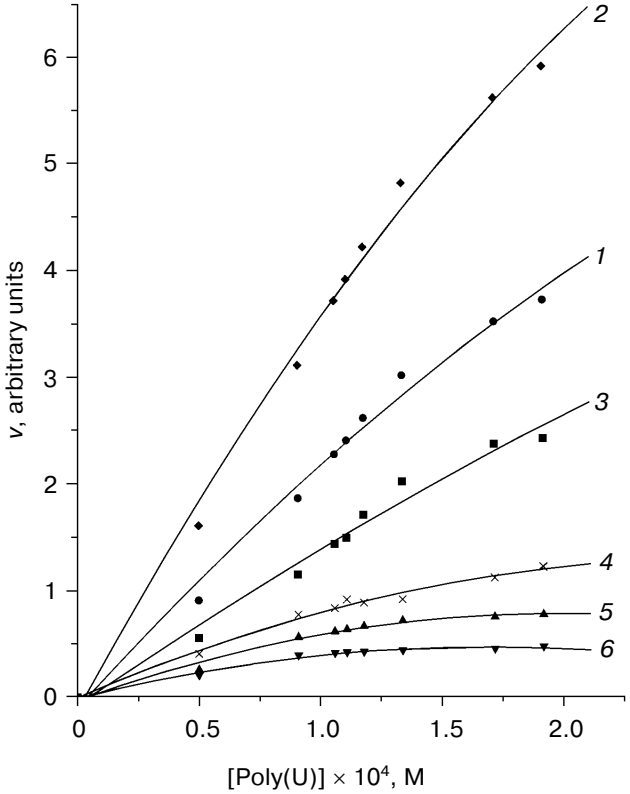

Fig. 4. Initial reaction rate versus poly(U) concentration at various reaction conditions: *1*) without additions; *2*) 30% ethanol; *3*) 100 mM NaCl; *4*) $7 \cdot 10^{-7}$ M barstar; *5*) $7 \cdot 10^{-7}$ M barstar, 100 mM NaCl; *6*) $7 \cdot 10^{-7}$ M barstar, 30% ethanol. Binase concentration, $4.87 \cdot 10^{-7}$ M; 0.05 M Tris-acetate buffer, pH 8.0, 25°C.

inhibition by barstar decreases to $(0.7 \pm 0.03) \cdot 10^{-4}$ M on addition of 100 mM NaCl (Fig. 4, curve *1* and *5*, respectively). However, an unambiguous conclusion about the effect of neutral salt on the interaction of barstar with binase is rather problematic if based only on these data because inhibition can be enhanced by simultaneous but not additive action of salts and barstar on binase-catalyzed hydrolysis. Using only kinetic data without data obtained by other methods, it is rather difficult to determine whether the ions and barstar are bound by the same or by different sites of binase.

Decrease in dielectric constant of the reaction medium on addition of ethanol to 30% concentration significantly activates poly(U) hydrolysis (Fig. 4, curve *2*). It could be expected that activation by ethanol on its addition to the reaction medium during inhibition by barstar will result in decreased inhibition. However, the data indicate that the inhibition by barstar is enhanced in the presence of ethanol. But the apparent Michaelis constant decreases to $(0.4 \pm 0.01) \cdot 10^{-4}$ M (Fig. 4, curve *6*). It seems that the enhancement of inhibition cannot be explained by the action of ethanol on the binase-catalyzed reaction. However, it is obvious that addition of organic solvent results in increased availability of hydrophobic sites of binase and barstar and, possibly, to a tighter barstar binding by binase due to a stronger hydrophobic interaction.

This suggestion is indirectly indicated by the earlier obtained high-resolution NMR data; using this method, the differences in the sites of amino acid sequence of binase and barnase following the conservative loop have been determined; the latter is responsible for guanylic specificity in all microbic RNases [11]. As shown by the authors, in sites contacting barstar the charged amino acid residues Gly64 and Lys65 in barnase are changed for neutral Ser and Ala, respectively, in binase; this results in a lower electrostatic potential in the binase–barstar contact area. The possibility of substrate fixation in the binding site via stacking interaction of its nucleotide part with the aromatic ring of phenylalanine [12] indicates availability of the hydrophobic nucleus of binase. A suggestion of partial availability of the hydrophobic sites of binase corresponds to our kinetic data; the latter indicate that the binase–barstar interaction tends to increase in organic solvents (30% ethanol), which make the hydrophobic sites more available and promote unfolding of the protein globules of binase and barstar.

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