On the Reaction between the Extracellular Ribonuclease of Bacillus amyloliquefaciens (Barnase) and Its Intracellular Inhibitor (Barstar)

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SUMMARY

It is shown that the interaction between barnase and its inhibitor barstar involves the formation of a one-to-one complex which dissociates only in the presence of strongly dissociating solvents.

Barnase, the ribonuclease that is excreted by various strains of Bacillus amyloliquefaciens, has been under investigation in this laboratory for several years. Our particular interest has been to develop this enzyme as a model system for study of sequence-determined folding in proteins. It is small, consisting of a single chain of 110 amino acids, molecular weight, 12,383. It contains no disulfide bonds, divalent metals, or other non-peptide components (1, 2). Upon heating or with the addition of denaturants, it unfolds completely (or nearly so) in a highly cooperative, reversible transition (3, 4). Its amino acid sequence has been reported (2) and shows no obvious homology to any other known protein. X-ray diffraction work on barnase crystals is under way in another laboratory.

Barstar (5, 6) is a protein which specifically inhibits barnase. It is produced, intracellularly, by the same organism. It is an even smaller protein than barnase with a molecular weight of about 10,000 (approximately 59 amino acids in a single chain). It also has no disulfide bonds. Indeed, there is no sulfur at all in either protein. Nor has it any metal requirements or other non-peptide moieties.

In the original report of Smeaton and Elliott (5), it was shown that the enzyme-inhibitor reaction was of a stoichiometric nature, suggesting the formation of an inactive complex. Our current barstar purification procedure (6) utilizes its specific binding to an agarose column containing covalently bound barnase, followed by elution with 5 M guanidine HCl. Successful purification by this procedure makes it clear that such a complex exists and that covalent bonds are almost certainly not involved.

In this communication, we establish the equimolar stoichiometry of the barnase-barstar complex and show that active barnase, as well as barstar, can be recovered from the complex by separation in a dissociating solvent.

METHODS AND MATERIALS

Barnase and barstar were isolated from B. amyloliquefaciens, strain H2, as described previously (1, 6). Quantitative and qualitative assays for both activities have also been described (1, 6, 7). The assay used in this work involved spotting of appropriate mixtures on RNA-agar for the all-or-none detection of ribonuclease activity (1, 6). RNA, 0.5% (yeast sodium-RNA, used as obtained from Schwarz-Mann), is incorporated into agar plates (2% agar) along with 0.1 M ammonium acetate, pH 8.2. Fifty to 60 samples per 10-cm diameter plate are spotted with the aid of a platinum loop. After incubation for 15 to 60 min at 39°, the plates are developed by adding a few milliliters of 2 N H2SO4. Precipitated RNA turns the agar milky white and clear spots indicate the presence of ribonuclease.

A surplus of barnase over barstar of 0.5 µg per ml yields a good spot and by titrating a known concentration of barnase with barstar or vice versa, a precision of ±3% is readily obtained.

Isolation of Barnase-Barstar Complex—To 1.6 X 106 units of barstar (inhibits 1.6 x 106 units or 0.76 mg of barnase) in 0.8 ml of 1.0 M ammonium acetate, pH 8.2, were added 2.1 x 106 units (1.0 mg) of barnase in 0.2 ml. A positive spot test for ribonuclease activity showed that barnase was in fact in excess. The mixture was applied to a column (0.64 x 183 cm) packed with Sephadex G-75 (superfine) equilibrated with the same buffer. The same column and fluorescence monitor were used for analytical runs of each of the three molecular entities with and without 5 M guanidine HCl.

Polyacrylamide gel electrophoresis of barnase, barstar, and their complex was performed in the presence of 0.1% SDS by the method of Weber and Osborn (8). Equilibrium ultracentrifugation of the three species was performed in a four hole AN-F rotor in a Beckman/Spinco model E ultracentrifuge with 5 M guanidine HCl.

The abbreviation used is: SDS, sodium dodecyl sulfate.
automatic scanner operated at 280 nm. Each sample was diluted with an appropriate quantity of water and 1 m ammonium acetate, pH 8.2, to bring its $A_{260}$ to about 0.5 and its salt concentration to 0.25 M. Equilibrium absorption patterns were obtained at 15,000 rpm. Molecular weights were obtained from plots of log $C$ versus $X^2$

$$M = \frac{2RT}{\omega^2(1 - \rho \bar{p})} \times \frac{d(ln C)}{d(X^2)}$$

where $C$ is $A_{260}$, $X$ is distance from the axis of rotation, $M$ is the molecular weight, $\bar{p}$ the partial specific volume, $\rho$ the solvent density, $R$ the gas constant, $T$ the absolute temperature, and $\omega$ the angular velocity. Values of $\bar{p}$ were estimated from amino acid composition (1, 6) by the method of Cohn and Edsall (9). They were 0.728, 0.749, and 0.738 for barnase, barstar, and the complex, respectively.

**RESULTS**

The equilibrium ultracentrifugal results are illustrated in Fig. 1. The straight lines indicate that each preparation was monodisperse. Molecular weights determined were 11,500, 9,100, and 22,000 for barnase, barstar, and their complex, respectively. As the sum of the first two (20,600) is within experimental error of the last, it is clear that the complex is composed of 1 molecule of barnase and 1 molecule of barstar. The somewhat higher chemical molecular weights (12,383 for barnase and 10,212 for barstar) (1, 6) are to be preferred because of the uncertainty of the partial specific volumes and in view of the fact that the entire barnase sequence is known (2).

When chromatographed independently, in the absence of guanidine HCl, barnase and barstar both emerge from the Sephadex G-75 column (0.64 × 183 cm) at an eluate volume of 39 ml. Together, as their complex, they emerge at 29 ml. When chromatographed on the same column in 5 M guanidine HCl, the results shown in Fig. 2 were obtained. While the two components of the complex (middle pattern) were not resolved completely, the outer halves of the first and second peak revealed (on dilution) appropriate levels of enzyme and inhibitor activity, respectively.

**DISCUSSION**

It is clear from these results that 1 molecule of barstar binds to 1 molecule of barnase, thereby abolishing its ribonuclease activity. No dissociation of the complex could be seen in the ultracentrifuge. That the complex is very stable is further indicated by the fact that only a slight excess of barstar is necessary to eliminate all barnase activity even at high concentrations. Dissociation of the complex in guanidine or SDS suggests that covalent bonds are not involved and that there must be a complementary fit of the 2 molecules analogous to that of an antigen with a monovalent antibody. The exact nature of this reaction can be best approached by determination of the three-dimensional structure of all three entities. The sequencing of barstar and attempts to grow useful crystals of both barstar and the complex are under way.
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