Isolation and Purification of Two Novel Streptomyces RNase Inhibitors, SaI14 and SaI20, and Cloning, Sequencing, and Expression in Escherichia coli of the Gene Coding for SaI14

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Two new RNase inhibitors, SaI14 (M_r ~14,000) and SaI20 (M_r ~20,000), were isolated and purified from a Streptomyces aureofaciens strain. The gene saI14, coding for SaI14 protein, was cloned and expressed in Escherichia coli. The alignment of the deduced amino acid sequence of SaI14 with that of barstar, the RNase inhibitor from Bacillus amyloliquefaciens, showed significant similarity between them, especially in the region which contains most of the residues involved in barnase-barstar complex formation.

A number of investigations have been focused on understanding the nature of the interaction between protein molecules. Natural complexes, such as enzyme-inhibitor and antibody-antigen, have been used in many laboratories to study aspects of protein-protein recognition. One of these is the complex of barnase, an extracellular RNase, and barstar, its intracellular protein inhibitor, both produced by the sporogenic bacterium Bacillus amyloliquefaciens. These proteins form a very tight one-to-one complex with a dissociation constant (K_d) of 10^{-14} M (4, 5, 16).

It was found recently that barstar also inhibits the streptomycete RNases Sa, Sa2, and Sa3 (6). The dissociation constants of the complexes of barstar with RNases Sa and Sa2 have been estimated to be on the order of 10^{-10} M. The barstar-RNase Sa3 complex is even tighter, with a K_d of 10^{-12} M. A practical consequence of inhibition of the streptomycete RNases by barstar is to allow high-level production of the recombinant enzymes when each of their genes is assembled standing the nature of the interaction between protein molecules.

Media and growth conditions. E. coli strains were routinely grown in Luria broth. Selection was made with 100 μg of ampicillin per ml in agar or liquid medium at 37°C. For protein production, superbroth medium was used at 28 to 30°C, and protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactosidase (IPTG).

S. aureofaciens was maintained on Bennett sporulation medium (0.1% yeast extract, 0.1% meat extract, 0.2% tryptone, 1.0% maltose, 1.5% agar) (8). An overnight culture was grown in Niedernberg medium [3.0% saccharose, 2.0% corn steep, 0.2% (NH_4)_2SO_4, 0.7% CaCO_3 (pH 7.0 to 7.2)] (13). Protein production was in 8/8 medium [3.0% saccharose, 2.0% soybean flour, 0.25% NaCl, 0.4% CaCO_3, 0.2% (NH_4)_2SO_4, 0.2% molasses, 0.25% corn steep (pH 5.8)]. Cells for chromosomal DNA isolation were grown in GPY medium (0.3% glucose, 0.3% peptone, 0.4% yeast extract, 1.0% glycine [pH 7.0 to 7.2]). Streptomyces was grown at 30°C.

DNA methods. Chromosomal DNA from S. aureofaciens was isolated according to the method of Hopwood et al. (7). Plasmid DNA was purified with a Wizard purification system (Promega). DNA fragments and PCR products separated on agarose gels were purified from the gel with the Wizard purification system. Restriction digestions, ligations, and transformations were done as described by Sambrook et al. (15).

Protein analysis and assays. Protein concentrations were determined by the method of Bradford (3). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (11) or that of Swank and Munkres (17). Gels were stained for protein with Coomassie brilliant blue R-250 or with silver (2).

The activity of the RNase inhibitor is given as the amount of

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The nucleic acid sequence of the segment of pSaI14 containing the inhibitor gene was determined by primer walking, beginning with the degenerate oligonucleotide primer DK1. As shown in Fig. 1, the open reading frame started with GTG, ended with TGA, and encoded a polypeptide of 126 amino acids with a calculated molecular mass of 14,034 Da. The coding region exhibited an overall G + C content of 71.4%, with an average G + C content at the third codon of 96.8%, which is typical of Streptomyces genes. The deduced amino acid sequence was in agreement with the N-terminal sequence determined experimentally by Edman degradation. Alignment of the deduced amino acid sequences of SaI14 and barstar (Fig. 2) revealed only 29.2% identity, which is about the same as that for barnase and RNase Sa. There are 13 amino acid residues in the region between residues 29 and 46 of barstar which form contacts with barnase in the barnase-barstar complex. In the corresponding region of SaI14 there are 10 residues identical to those of barstar. Among them are Tyr30, Asp36, and Asp40 (equivalent to Tyr29, Asp35, and Asp39 in barstar), whose contributions to the binding energy in the barnase-barstar complex are most important.

**Overexpression of the saI14 RNase inhibitor gene.** In order to facilitate the cloning of the RNase inhibitor gene into the expression vector pTrc 99A, two PCR primers, SaI20.2 (5'-CA CCACACCAAGCITTTCAGCCAAGCGAGGG3') and SaI13 (5'-GCATATCCTCGACCCCATGGCTGTGACTTAT3'), were designed to allow the introduction of an NcoI site on the upstream side of the gene and a HindIII site on the downstream side. The PCR fragment containing the inhibitor gene was then subcloned into the expression vector pTrc 99A, which has both the strong Trc promoter and a strong transcription termination signal. The recombinant plasmid, designated pSaI14.7, was introduced into E. coli DH5α cells. Upon induction by IPTG of expression of the cloned gene, the transformed cells expressed inhibitory activity. We found that decreasing the cultivation temperature from 37°C to 28 to 30°C after induction increased the level of expression fivefold. The inhibitor produced by overexpression of the saI14 gene was purified to homogeneity by a slight modification of the procedure used for the native inhibitor (data not shown). The recombinant SaI14 protein inhibits all three streptomyces RNases, Sa, Sa2, and Sa3, as well as barnase.

The dissociation constant of the RNase Sa-SaI14 inhibitor complex has not yet been determined, but we assume that it will be comparable to the barnase-barstar dissociation constant, because SaI14 was bound to the RNase Sa affinity column very tightly and could only be eluted under strong denaturing conditions, i.e., in the presence of 6 M guanidine-HCl, 6 M urea, or 1% SDS. The comparison of the barstar and streptomyces inhibitors’ dissociation constants could also be interesting from an evolutionary point of view. The interaction of barnase and barstar may be as close as it is because of the high intracellular toxicity of barnase, which has no disulfide bond and can readily fold to its active conformation inside the cells. In contrast, streptomyces RNases have one disulfide bond and may not fold properly in the reducing milieu of the cells. This might explain the lower toxicity of these enzymes. This idea is in agreement with the observation that RNase T1, which has two disulfide bonds, can be produced at a high level by E. coli.
without any inhibitor (6). As a result of evolutionary development, the differences in toxicity of RNases may be reflected in differences in the dissociation constants of the RNase-inhibitor complexes and/or in the levels of inhibitors synthesized. Presumably, the role of inhibitors is to prevent RNase activity prior to secretion, which would be extremely harmful to the cell.

Our next approach will be structural work, which, combined with modern physicochemical techniques and protein engineering, will contribute to an understanding of the interactions between these RNases and their inhibitors. Employment of the three streptomycete RNases, Sa, Sa2, and Sa3, and their two inhibitors, SaI14 and SaI20, as well as others as they become available, together with barnase and barstar, will expand the study of enzyme-inhibitor complexes and should help clarify the details of protein-protein recognition.

Nucleotide sequence accession number. The GenBank accession number for the sequence shown in Fig. 1 is AF 020428.

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REFERENCES


FIG. 1. DNA sequence of the gene encoding the SaI14 RNase inhibitor and its deduced primary structure. The initiation and termination codons are in boldface.

FIG. 2. Alignment of amino acid sequence of barstar and deduced amino acid sequence of SaI14. Alignment was made with PALIGN (12). The identical amino acids are denoted by vertical bars, and similar amino acids are indicated by dots.