PROTEIN ENGINEERING OF RIBONUCLEASE A

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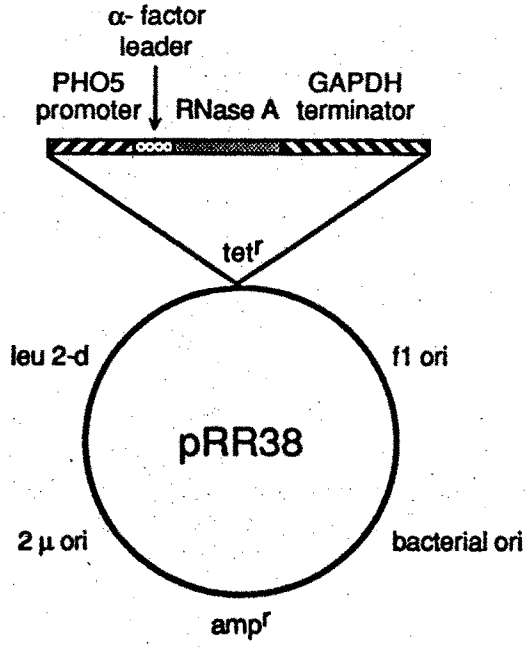
ABSTRACT: The cDNA coding for cow pancreatic ribonuclease A has been cloned and expressed in the yeast *S. cerevisiae*. Site-directed and random mutagenesis are being used to probe catalysis by and folding of the enzyme.

The high ribonuclease activity in the pancreas of ruminants has led to the detailed characterization of ribonuclease A (RNase A; EC 3.1.27.5), the major ribonuclease in cow pancreas. RNase A has been a widely used subject for protein folding studies; and its structure, its protein chemistry, and the mechanism and energetics of its catalysis are all well-defined (RICHARDS and WYCKOFF, 1971; BLACKBURN and MOORE, 1982; BEINTEMA, 1987). Although RNase A may be better characterized than any other enzyme, it has never been the object of study by molecular biology. Two obstacles have been encountered. First, the cDNA of RNase A is difficult to clone because the corresponding RNA must be obtained from the pancreas, an organ rich in ribonuclease. Secondly, RNase A is cytotoxic, making its *in vivo* expression problematic.

Using methods developed in this laboratory (CHIRGWIN et al., 1979; HAN et al., 1987), we have been able to construct a cow pancreas cDNA library, from which we have isolated a clone containing the sequence (CARSANA et al., 1988) coding for RNase A. We have also been able to express RNase A in the yeast *S. cerevisiae*, under the control of the repressible acid phosphatase (PHO5) promoter and the

glyceraldehype-3-phosphate dehydrogenase (GAPDH) transcription terminator (ROSENBERG et al., 1984). In our expression system (shown below), RNase A is synthesized as a hybrid with the yeast α-factor leader peptide (BRAKE et al., 1984). The hybrid protein is processed efficiently by endogenous yeast proteases, and active RNase A identical to that isolated from cow pancreas is secreted into the media in a quantity (~2 mg/L) adequate for stoichiometric as well as catalytic studies. The RNase A produced is readily purified by affinity chromatography on single-stranded DNA~Sepharose. We thus have the ability to make substantial quantities of RNase A, mutated at any amino acid residue.



Probing Ribonuclease A Catalysis using Site-Directed Mutagenesis

To understand the relationship between enzyme structure and function and to evaluate the role of particular amino acid side chains in catalysis, it is now common practice to observe the functional consequences of changing active-site residues using recombinant DNA (KNOWLES, 1987). Still, such observations lead to chemical interpretations only when the three-dimensional structure of the enzyme is known. RNase A was first crystallized fifty years ago (KUNITZ, 1939), and structures of both RNase A and the subtilisin-cleaved form, RNase S, and complexes of these with various inhibitors and substrate analogues have been determined by X-ray crystallography and NMR spectroscopy. Equiped with this structural information, we are probing RNase A catalysis using site-directed mutagenesis.

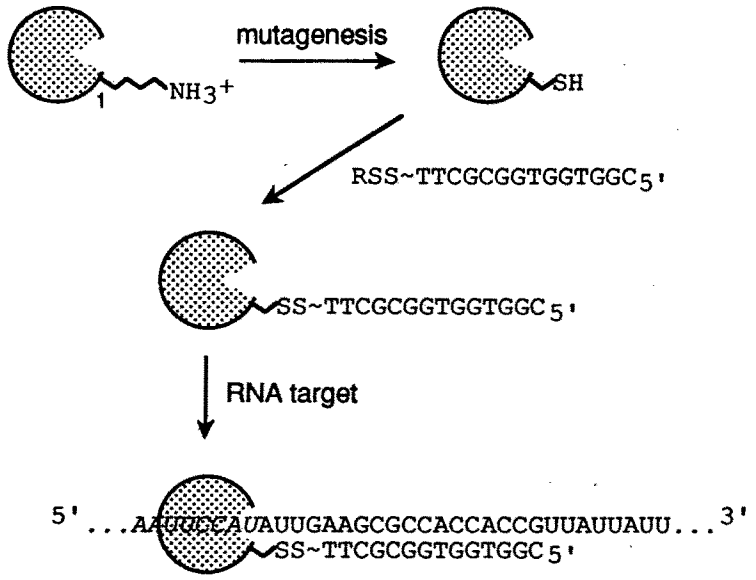
Forty years ago, Linus Pauling said: 'I think that enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyse' (PAULING, 1948). Lys 41 of RNase A may be an exquisite example of a residue that fulfills Pauling's dictum, since the integral role of Lys 41 in RNase A catalysis that is suggested by chemical modification studies (RICHARDS and WYCKOFF, 1971;

BLACKBURN and MOORE, 1982) and by its conservation in all 41 sequenced pancreatic ribonucleases (BEINTEMA, 1987) seems to be evident only in the X-ray structure of the complex with uridine vanadate, a transition state analogue (ALBER et al., 1982). There, the N_ε of Lys 41 is hydrogen-bonded to an oxygen atom of the vanadate.

To explore the role of Lys 41 in RNase A catalysis, we have changed it to Arg or Met. With UpA as substrate (IPATA and FELICIOLI, 1968), the k_{cat} for the Arg 41 mutant is about 100-fold lower than that of the wild-type enzyme and the K_m is some 3-fold lower. The k_{cat} for the Met 41 mutant is $>10^4$-fold lower than that of wild-type RNase A. The K_i of uridine vanadate is some 10-fold higher for the Arg 41 mutant than for the wild type enzyme, a decrease in binding similar to that seen in k_{cat}/K_m (but not in K_m). These data are consistent with the side-chain of residue 41 binding (almost) exclusively to the rate-limiting transition state.

A Hybrid Sequence-Specific Ribonuclease A

We have constructed (as shown below) a semi-synthetic RNase A having a



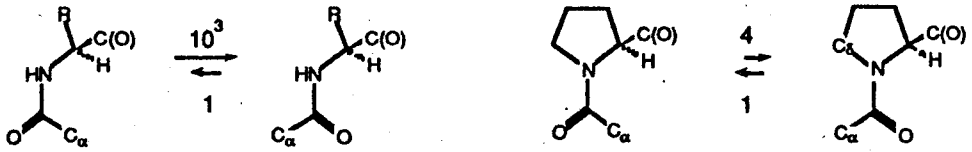
covalently bound DNA oligonucleotide that is complementary to a sequence beginning at the fifth nucleotide downstream from a desired RNA cleavage site. [Results with RNase S (ZUCKERMANN and SCHULTZ, 1988) and staphylococcal nuclease

(ZUCKERMANN et al., 1988) support the efficacy of this approach.] To allow the oligonucleotide to be attached readily and at a spatially appropriate position, we have replaced Lys 1 with Cys, to yield a fully active ribonuclease with a unique sulfhydral group. Preliminary results suggest that our semi-synthetic RNase A has similar sequence specificity but greater thermal stability than that of semi-synthetic RNase S.

Probing Ribonuclease A Folding using Site-Directed Mutagenesis

Making *ab initio* predictions of tertiary structure is likely to require a thorough understanding of folding pathways, including the kinetics and thermodynamics of the folding process and the structure of folding intermediates. The ability to change amino acids selectively provides a means of measuring the contribution of a particular residue to the folding pathway and to the tertiary structure of the protein. We are using site-directed mutagenesis to probe the folding of RNase A.

The *trans* form of a typical peptide bond is greatly favored over the *cis* form (CREIGHTON, 1984). In contrast, a *trans* bond preceding a proline residue is only slightly favored (as shown below), and its conversion to *cis* can be slow on the time



scale of protein folding. In native RNase A, Pro 93 and Pro 114 are *cis*, suggesting that isomerization of one [probably Pro 93 (SCHMID et al., 1986)] or both of these peptide bonds may limit the rate of RNase A folding. To probe the role of proline isomerization in the folding of RNase A, we have changed Pro 93 or Pro 114 to Gly, the most flexible amino acid residue with the largest range of allowable main-chain dihedral angles. We are now studying the folding kinetics of these mutant proteins, which are fully active ribonucleases.

Random Mutagenesis of RNase A

Efficient methods (MYERS et al., 1985; LEHTOVAARA et al., 1988; REIDHAAR-OLSON and SAUER, 1988) have been developed for introducing random base substitutions into specific DNA sequences. Such random mutants can be used to

discern rapidly those amino acid residues that are critical to a particular screen or selection. We have developed a plate screen for ribonuclease activity (HOLLOMAN and DEKKER, 1971). Specifically, yeast cells engineered to secrete RNase A are spread on a plate containing high molecular weight RNA. After growth of colonies, the plate is washed with acid, which precipitates high molecular weight RNA. Since low molecular weight RNA is acid soluble, the presence of active RNase A is indicated by a clear plaque on a white background. The sensitivity of the screen is readily enhanced by allowing longer periods for yeast colony growth and concomitant RNase A production and RNA hydrolysis. We are using this screen to determine the importance of particular amino acid residues to the activity of RNase A.

'A journey of a thousand miles must begin with a single step' (*The Way of Lao-tzu*, 64). Described above are the first steps on what we believe will be a most exciting and informative expedition.

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