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ABSTRACT: The cDNA that codes for cow pancreatic ribonuclease A has been cloned and expressed in the yeast *S. cerevisiae*. Site-directed mutagenesis of conserved residues Lys_{41} and Asp_{121} has been used to probe catalysis by the enzyme. In addition, mutation of Lys_1 to cysteine has allowed for the covalent attachment of a DNA oligonucleotide that confers sequence specificity on ribonuclease A. Finally, a nutritional selection and an activity screen have been developed to distinguish yeast colonies that secrete active ribonuclease A.

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 predominant 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 only recently been subjected to study by molecular biology (STACKHOUSE et al., 1990). Two obstacles have been encountered. First, the cDNA of
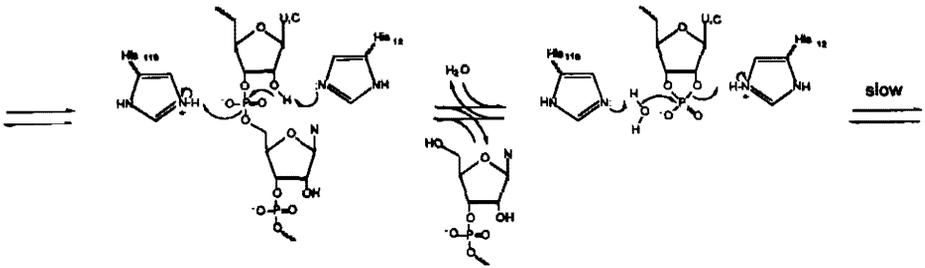
Figure 1. Mechanism of the reaction catalyzed by ribonuclease A. In the transphosphorylation step, His_{12} is a general base and His_{119} is a general acid. In the hydrolysis step, His_{119} is a general base and His_{12} is a general acid.

RNase A is difficult to clone because the corresponding RNA must be isolated from the pancreas, an organ rich in ribonuclease. Secondly, RNase A is cytotoxic, making the *in vivo* expression of its cDNA problematic.

We have been able to construct a cow pancreas cDNA library from which we have isolated a clone containing the sequence that codes for RNase A. (This sequence is identical to that reported for the RNase A gene by CARSANA et al., 1988). We have been able to express this cDNA in the yeast *S. cerevisiae*, under the control of the repressible acid phosphatase (PHO5) promoter and the glyceraldehype-3-phosphate dehydrogenase
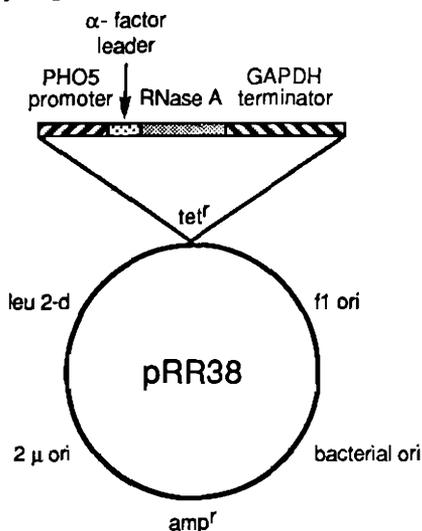

Figure 2. Plasmid for the expression of ribonuclease A in yeast.

(GAPDH) transcription terminator (ROSENBERG et al., 1984). In our expression system (Figure 2), 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 (FULLLER et al., 1989), and active RNase A identical to that isolated from cow pancreas is secreted into the media in a quantity (approximately 5 mg/L) adequate for stoichiometric as well as catalytic studies. The RNase A produced is readily purified by cation-exchange chromatography and affinity chromatography on a single-stranded DNA matrix.

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.

Transition State Stabilization

In 1948 Linus Pauling, in a seminal statement, said: "I think that enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyze" (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 (Figure 3)(ALBER et al., 1982). There, the N_ε of Lys_{41} is hydrogen-bonded to an oxygen atom of the vanadate.
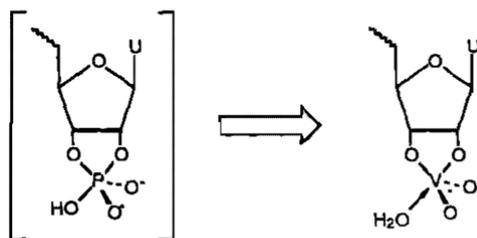
Figure 3. Putative structure of the rate-limiting transition state for the reaction catalyzed by ribonuclease A, and the uridine vanadate analogue of that structure.

To explore the role of Lys_{41} in RNase A catalysis, we have changed it to arginine, histidine, or methionine and assayed catalysis by these mutant enzymes by following the release of A from UpA (IPATA and FELICIOLI, 1968) and the competitive inhibition of catalysis by uridinyl vanadate. Our results indicate that the affinity of a mutant RNase A for the transition state analogue is reflected in its affinity for the rate-limiting transition state (that is, $^{mutant}(k_{cat}/K_m)/^{wild-type}(k_{cat}/K_m) \approx {}^{wild-type}K_i/^{mutant}K_i$) but not in its affinity for ground states (that is, $^{wild-type}K_i/^{mutant}K_i \neq {}^{wild-type}K_m/^{mutant}K_m$). These data are consistent with the side-chain of residue 41 binding exclusively to the rate-limiting transition state. We are proceeding by determining the ability of the mutant enzymes to catalyze each of the half reactions shown in Figure 1.

Catalytic Triad

RNase A may have converged upon a 'catalytic triad' similar to that of the serine proteases. Specifically, Asp_{121} seems to stabilize the tautomer of His_{119} that is appropriate for both the delivery of a proton to the leaving nucleoside and the abstraction of a proton from the water molecule that attacks the cyclic phosphodiester intermediate. [His_{119} and

Asp121 are conserved in all 41 sequenced pancreatic ribonucleases (BEINTEMA, 1987)]. We are beginning to dissect the putative catalytic triad of RNase A by studying enzymes in which Asp121 has been changed to either asparagine (thereby removing the negative charge) or alanine (thereby removing both the negative charge and the ability to hydrogen bond). Our preliminary studies indicate that both of these mutant RNase A's catalyze the release of A from UpA at the same rate, which is only about 10-fold less than that of the wild-type enzyme!

A Hybrid Sequence-Specific Ribonuclease A

We have constructed (as shown in Figure 4) a hybrid RNase A having a covalently-
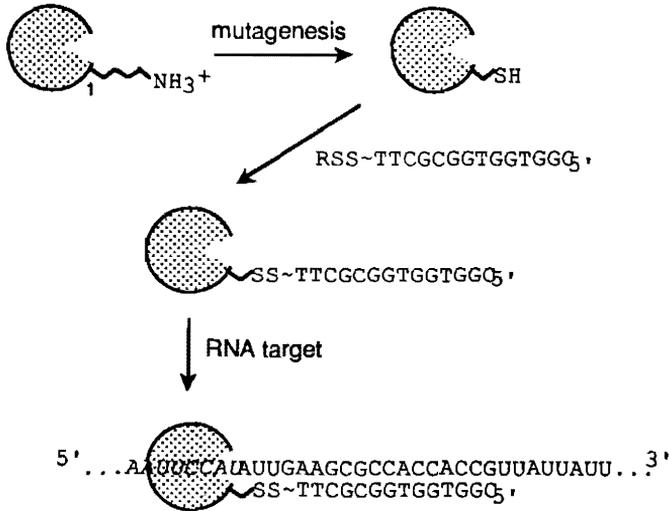
Figure 4. Scheme for the synthesis and reaction of a hybrid ribonuclease A.

bound DNA oligonucleotide that is complementary to a sequence beginning at the fifth nucleotide downstream from a desired RNA cleavage site. To allow the oligonucleotide to be attached readily and at a spatially appropriate position, we have replaced Lys 1 with cysteine, to yield a fully active ribonuclease with a unique sulfhydral group. The sequence-specificity of RNA cleavage of our first-generation hybrid RNase A is improved significantly over that of wild-type RNase A.

Nutritional Selection and Activity Screen for Active Ribonuclease A

Efficient methods have been developed for introducing definably random base substitutions into specific DNA sequences (HERMES et al., 1989). Such random mutants

can be used to discern rapidly those amino acid residues that are critical to a particular screen or selection. We are applying such systematic genetics to RNase A. We have developed a nutritional selection (based on polymeric RNA as the source of uracil for an auxotrophic yeast strain) and an activity screen [based on the acid solubility of monomeric but not polymeric RNA (HOLLOMAN and DEKKER, 1971)] to distinguish yeast clones that secrete active RNase A. We are now searching for mutant enzymes that have a sequence specificity different than that of wild-type RNase A, which cleaves after only after pyrimidine bases.

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