# Engineering ribonuclease A: production, purification and characterization of wild-type enzyme and mutants at Gln11

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Bovine pancreatic ribonuclease A (RNase A) has been the object of much landmark work in biological chemistry. Yet the application of the techniques of protein engineering to RNase A has been limited by problems inherent in the isolation and heterologous expression of its gene. A cDNA library was prepared from cow pancreas, and from this library the cDNA that codes for RNase A was isolated. This cDNA was inserted into expression plasmids that then directed the production of RNase A in Saccharomyces cerevisiae (fused to a modified  $\alpha$ -factor leader sequence) or Escherichia coli (fused to the pelB signal sequence). RNase A secreted into the medium by S.cerevisiae was an active but highly glycosylated enzyme that was recoverable at 1 mg/l of culture. RNase A produced by E.coli was in an insoluble fraction of the cell lysate. Oxidation of the reduced and denatured protein produced active enzyme which was isolated at 50 mg/l of culture. The bacterial expression system is ideal for the large-scale production of mutants of RNase A. This system was used to substitute alanine, asparagine or histidine for Gln11, a conserved residue that donates a hydrogen bond to the reactive phosphoryl group of bound substrate. Analysis of the binding and turnover of natural and synthetic substrates by the wild-type and mutant enzymes shows that the primary role of Gln11 is to prevent the non-productive binding of substrate.

Key words: E.coli/glycosylation/inclusion body/non-productive binding/Saccharomyces cerevisiae

#### Introduction

Together, the  $10^6$  extant unrelated enzymes represent an insignificant fraction of the number of possible amino acid sequences. Changing the residues in a protein and analyzing the consequences of these changes can therefore be a powerful method of probing the role of particular functional groups in protein folding, stability and function (Knowles, 1987). However, the scientific harvest from this approach is more bountiful if the wild-type and mutant proteins are studied in detail.

Ribonucleases, the enzymes that catalyze the degradation of RNA, are an essential component of all living cells. The ribonuclease activity in the pancreas of ruminants is particularly high, presumably to enable digestion of the large amount of RNA produced by stomach microflora (Barnard, 1969). This high level of activity has led to the detailed characterization of bovine pancreatic ribonuclease A (RNase A: EC 3.1.27.5). This enzyme has been the object of landmark work on the folding, stability and chemistry of proteins, on enzymology and on molecular evolution (Richards and Wyckoff, 1971; Karpeisky and Yakovlev, 1981; Blackburn and Moore, 1982; Wlodawer, 1985; Eftink and Biltonen, 1987; Beintema et al., 1988), RNase A was first crystallized >50 years ago (Kunitz, 1939, 1940), and the crystals were shown to diffract to a resolution of 2 Å (Fankuchen, 1941). Structures of both RNase A and the subtilisin cleavage product RNase S, and complexes of these with various inhibitors and substrate analogs, have been determined by X-ray diffraction analysis (Wlodawer, 1985). Finally, the use of nuclear magnetic resonance (NMR) spectroscopy in determining both protein structure (Saunders et al., 1957) and protein folding pathways (Udgaonkar and Baldwin, 1988) has been developed with RNase A.

Despite extensive study, the precise role of each active-site residue in catalysis by RNase A is uncertain. RNase A is a small protein (124 amino acid residues, 13.7 kDa) that catalyzes the cleavage of the P-O5' bond of RNA specifically after pyrimidine residues. Figure 1 depicts a mechanism of catalysis that is consistent with all known data (Findlay et al., 1961). For other proposed mechanisms see Witzel (1963), Hammes (1968), Wang (1968) and Anslyn and Breslow (1989).] In the mechanism shown in Figure 1, the side chain of His12 acts as a general base that abstracts a proton from the 2'-oxygen of a substrate molecule, thereby facilitating its attack on the phosphorus atom (Thompson and Raines, 1994). This attack proceeds in-line to displace a nucleoside (Usher et al., 1970, 1972). The side chain of His119 acts as a general acid that protonates the 5"-oxygen to facilitate its displacement (Thompson and Raines, 1994). Both products are released to solvent. The slow hydrolysis of the 2',3'-cyclic phosphate occurs separately and resembles the reverse of transphosphorylation (Thompson et al., 1994). Both reactions shown in Figure 1 probably occur via transition states with a pentavalent phosphorus atom. The side chain of Lys41 enhances catalysis by stabilizing this transition state (Trautwein et al., 1991; J.M.Messmore, D.N.Fuchs and R.T.Raines, manuscript submitted).

Uridine 2',3'-cyclic vanadate (U>v) is a stable isosteric analog of the transition state for the hydrolysis reaction in Figure 1 (Lienhard *et al.*, 1971; Lindquist *et al.*, 1973). The high-resolution structure of the crystalline complex of RNase A and U>v obtained by joint X-ray/neutron diffraction analysis has provided invaluable insight into the catalytic mechanism of RNase A (Wlodawer *et al.*, 1983). A part of this structure is shown in Figure 2. In the crystalline complex the side chains of His12, Lys41 and His119 are proximal to the vanadyl group of bound U>v, as expected. This structure shows, however, that two other amino acid residues also interact intimately with the vanadyl group. The side-chain nitrogen of Gln11



Fig. 1. Mechanism of the transphosphorylation (top) and hydrolysis (bottom) reactions catalyzed by RNase A. 'B' is His 12; 'A' is His 119.

forms a hydrogen bond with the non-bridging oxygen,  $O_{IV}$  ( $N_{\delta 2}-O_{IV}$  distance = 2.6 Å,  $N_{\delta 2}-H-O_{IV}$  angle = 140°), and the main-chain nitrogen of Phe120 forms a hydrogen bond with a non-bridging oxygen,  $O_{3V}$  ( $N-O_{3V}$  distance = 2.9 Å,  $N-H-O_{3V}$  angle = 162°). This arrangement has led to the recent suggestion that these residues stabilize a phosphorane intermediate (Gerlt and Gassman, 1993b). A study of semisynthetic mutants of RNase S' having various residues at Gln11 has also ascribed a significant role for Gln11 in catalysis (Marchiori *et al.*, 1974). Do Gln11 and Phe120 polarize the phosphoryl group and thereby make the phosphorus atom more electrophilic? Do they stabilize negative charge that may accumulate on the non-bridging oxygens in the pentavalent transition state?

We are interested in using protein engineering to understand in detail catalysis by RNase A (Raines and Rutter, 1989). Accordingly, we have prepared a cDNA library from cow pancreas, cloned the cDNA that codes for RNase A, and expressed this cDNA in a novel *Saccharomyces cerevisiae* expression system and in the popular *Escherichia coli* T7 RNA polymerase expression system. The yeast system secretes active ribonuclease into the medium with an isolated yield of 1 mg/l of culture. Like the isolate from bovine pancreas, the isolate from yeast contains a mixture of glycosylated forms, along with the unmodified enzyme. The bacterial system produces RNase A in an insoluble form that can be renatured with an isolated yield of 50 mg/l of culture. The kinetic parameters obtained for the bacterial isolate are similar to those of RNase A isolated from bovine pancreas. The parameters for



**Fig. 2.** Structure of the active site of RNase A bound to uridine 2',3'-cyclic vanadate, a transition state analog (Wlodawer *et al.*, 1983). The structure was refined at 2.0 Å from X-ray (*R* factor = 0.191) and neutron (*R* factor = 0.207) diffraction data collected from crystals grown at pH 5.3. The side chain of Phe120 and the base of U>v are not shown.

the yeast isolate, which suffers extensive modification, vary somewhat from those of RNase A.

Using the bacterial expression system, we have created and characterized mutants of RNase A in which Gln11 has been changed to a histidine, asparagine or alanine residue. Although  $N_{\delta 2}$  of the side chain of Gln11 donates a hydrogen bond to the vanadyl group of bound U>v, replacing Gln11 does not affect the ability of the enzyme to bind to the rate-limiting transition state. We discuss the implications of these results for the catalytic mechanism of RNase A.

#### Materials and methods

#### Materials

S.cerevisiae strain BJ2168 (MAT a prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52) was obtained from the Yeast Genetic Stock Center (Berkeley, CA). E.coli strain BL21(DE3) (F<sup>-</sup> ompT r<sub>B</sub>m<sub>B</sub>-; Studier and Moffatt, 1986) was acquired from Novagen (Madison, WI). Escherichia coli strain CJ236 and helper phage M13K07 were purchased from Bio-Rad (Richmond, CA) and E.coli strain Y1088 (Hanahan, 1983) was the generous gift of D.Hanahan. Plasmid  $\lambda$ gt11 was obtained from New England Biolabs (Beverly, MA). Plasmids pC1/1 (Rosenberg *et al.*, 1984), pPHO5, pCB124 and pMP36 (Phillips *et al.*, 1990), and pRS304 (Sikorski and Heiter, 1989) were generous gifts from S.Rosenberg, A.J.Brake, M.A.Phillips and R.S.Sikorski, respectively. Plasmid pET22B(+) was purchased from Novagen.

All enzymes for molecular biology were obtained from Promega (Madison, WI), except for restriction endonucleases PvuII, StuI, ScaI, BspHI and concentrated T4 DNA ligase, which were acquired from New England Biolabs. Reagents for DNA synthesis were purchased from Applied Biosystems (Foster City, CA), except for acetonitrile which was ordered from Baxter Healthcare (McGaw Park, IL). Agarose was obtained from Life Technologies (Gaithersburg, MD). Acrylamide and N,N'-methylene-bis-acrylamide were purchased from Bio-Rad. Spectra/Por dialysis membranes (3500  $M_r$  cutoff) were obtained from Spectrum Medical Industries (Los Angeles, CA). DE52 diethylaminoethyl cellulose anion exchange resin was acquired from Whatman (Maidstone, UK) and S-Sepharose cation exchange resin was from Pharmacia LKB (Piscataway, NJ).

Yeast minimal medium (SD) contained (in 1 l) Bacto yeast nitrogen base without amino acids (YNB, 6.7 g), dextrose (2% w/v) and a supplemental nutrient mix (Ausubel et al., 1989). Variations of SD medium were also used. For example,  $2 \times S$ 4% D-trp medium contained twice the concentrations of YNB and dextrose but lacked tryptophan in the supplemental nutrient mix. Yeast rich medium (YEPD) contained (in 1 l) Bacto yeast extract (10 g), Bacto peptone (20 g) and dextrose (2% w/v). Yeast-rich medium depleted of inorganic phosphate (YEPD -P<sub>i</sub>) was prepared as described (Rubin, 1974). Bacterial Luria broth (LB) contained (in 1 l) Bacto tryptone (10 g), Bacto yeast extract (5 g) and NaCl (10 g). Bacterial terrific broth (TB; Maniatis et al., 1989) contained (in 1 l) Bacto tryptone (12 g), Bacto yeast extract (24 g), glycerol (4 ml), KH<sub>2</sub>PO<sub>4</sub> (2.31 g) and K<sub>2</sub>HPO<sub>4</sub> (12.54 g). All media were prepared in distilled deionized water and autoclaved.

Bovine pancreatic ribonuclease (type III-A or type X-A) was obtained from Sigma Chemical Co. (St Louis, MO) and was used without further purification. Adenosine deaminase,

endoglycosidase-F and *O*-glycosidase were purchased from Boehringer Mannheim (Indianapolis, IN). Poly(C) was acquired from Midland Reagent (Midland, TX).

Uridylyl(3' $\rightarrow$ 5')adenosine (UpA) was synthesized by J.E.Thompson using the methods of Ogilvie (1978) and Beaucage and Caruthers (1981), or was obtained from Sigma Chemical Co. UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> was synthesized by J.E.Thompson and T.G.Kutateladze using the method of Davis *et al.* (1988). Toluidine blue, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and yeast RNA were purchased from Sigma Chemical Co. Bacto yeast extract, Bacto tryptone, Bacto peptone, Bacto agar and Bacto yeast nitrogen base without amino acids were obtained from Difco (Detroit, MI). All other chemicals were of reagent grade or better and were used without further purification.

#### General methods

Reactions involving restriction enzymes, T4 DNA ligase or T4 polynucleotide kinase were performed in the buffers provided by their suppliers. DNA restriction fragments were purified from bands in agarose (0.7% w/v) gels with a GeneClean II kit from Bio-101 (La Jolla, CA). Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer by using the  $\beta$ -cyanoethyl phosphoramidite method (Sinha *et al.*, 1984), and purified with both Oligo Purification Cartridges (Applied Biosystems) and PAGE. DNA sequences were determined with the Sequenase Version 2.0 kit from US Biochemicals (Cleveland, OH). Site-directed mutagenesis was performed on single-stranded DNA isolated from *E.coli* strain CJ236 using the method of Kunkel *et al.* (1987). Other manipulations of DNA were performed as described (Ausubel *et al.*, 1989).

Proteins were separated by PAGE performed in the presence of SDS (0.1% w/v), as described (Ausubel *et al.*, 1989). Gels were fixed and stained by washing with aqueous methanol (40% v/v) containing acetic acid (10% v/v) and Coomassie brilliant blue (0.1% w/v). M<sub>r</sub> standards were obtained from Bio-Rad: phosphorylase B (97 400 unstained, 106 000 prestained); serum albumin (66 200 unstained, 80 000 prestained); ovalbumin (45 000 unstained, 49 500 prestained); carbonic anhydrase (31 000 unstained, 32 500 prestained); trypsin inhibitor (21 500 unstained, 27 500 prestained) and lysozyme (14 400 unstained, 18 500 prestained).

Ultraviolet and visible absorbance measurements were made with a Cary Model 3 spectrophotometer (Varian, Sugar Land, TX) equipped with a Cary temperature controller. Protein concentrations were determined for crude samples with a Protein Assay kit from Bio-Rad, or for pure samples by using an absorption coefficient of  $\varepsilon_{1 \text{ cm}}^{0.1\%} = 0.72$  at 277.5 nm (Sela *et al.*, 1957). The pH was measured with a Beckman pH meter fitted with a Corning electrode, calibrated at room temperature with standard buffers from Fisher (Chicago, IL). Protein sequences were determined at the University of Wisconsin Biotechnology Center, as described (Rybak *et al.*, 1991).

## Construction of cDNA library

Total RNA was extracted from a freshly removed cow pancreas with a solution of guanidinium isothiocyanate (4 M) and 2mercaptoethanol (0.1 M), which denatures endogenous ribonucleases (Chirgwin *et al.*, 1979). Total poly(A)<sup>+</sup> RNA was purified by oligo(dT)-cellulose column chromatography (Maniatis *et al.*, 1989). The poly(A)<sup>+</sup> RNA was used as a template for single-stranded cDNA synthesis by reverse

Name	Sequence $(5' \rightarrow 3')$	Function
JHH16	TTGCCATTGCAGCTGGCAATCGTGG	destroy PstI site
RRI	AACTACTGCAACCAGATGATGAAGAGCAGGAACCTGACCAAGGAC	probe cDNA library
RR9	CTTTGGATAAAAGAAAGGAAACTGCA	Kpn1-PstI adaptor
RR13	GTTTCCTTTCTTTTATCCAAAGGTAC	KpnI-PstI adaptor
RR16	AACACCATGGGATTTCCTT	create NcoI site
SDI	GCGGATCCGCACGT	BamHI linker/adaptor
SD2	CGTGCGGATCTCTGGTATGGT	destroy BamHI site
SD3	CCAAGGAAACTGCAGCAGCC	5' PCR primer for RNase A
SD4	GGCCTTAGGTCGACTACTACACTGAAGC	3' PCR primer for RNase A
SD5	CACTACCGGTTCCCCATTTAG	destroy KpnI site
SD6	GCTGGAGTCCATATGATGCCGCTCAAACTT	Q11H, create Ndel site
SD7	GCTGGAGTCCATATGGTTCCGCTCAAACTT	Q11N, create Ndel site
SD8	GCTGGAGTCCATATGCGCCCGCTCAAACTT	Q11A, create NdeI site

Table I. DNA oligonucleotides for cloning, expression and mutation of the cDNA that codes for RNase A

transcriptase, and this single-stranded cDNA was used as a template for double-stranded cDNA synthesis by *E.coli* DNA polymerase. The resulting cDNA was methylated with *Eco*RI methylase, ligated to *Eco*RI linkers and digested with *Eco*RI (Gubler and Hoffman, 1983). The restriction fragments containing >600 bp were ligated into plasmid  $\lambda$ gt11, and the resulting plasmids were introduced into *E.coli* strain Y1088 (Hanahan, 1983).

#### Isolation of RNase A cDNA

Transformants (10<sup>5</sup> plaques) were screened by in situ hybridization with either a 406 bp fragment of nick-translated rat ribonuclease cDNA (MacDonald et al., 1982) or oligonucleotide RR1 (Table I), which codes for a region of RNase A in which the codons are relatively non-degenerate. Hybridization was performed in 50 mM Tris-HCl buffer, pH 8.0, containing NaCl (1 M), ethylenediaminetetraacetate (EDTA; 10 mM), bovine serum albumin (0.2% w/v), Ficoll 400 (0.2% w/v), SDS (0.2% w/v) and heat-denatured salmon sperm DNA (100 µg/ml; Yoshimura et al., 1983). The hybridized DNA was washed at 42°C with 30 mM sodium citrate buffer, pH 7.0, containing NaCl (300 mM) and SDS (0.1% w/v). The cDNA insert from a  $\lambda$ gt11 clone that remained hybridized to both the rat ribonuclease cDNA probe and the oligonucleotide probe was isolated by digestion with EcoRI, subcloned into M13mp8, and sequenced.

#### Cassettes for expression of RNase A cDNA in S.cerevisiae

Two expression cassettes were designed that would direct the expression of the RNase A cDNA when carried by a plasmid that propagated in the yeast S.cerevisiae. These cassettes differed only in the promoter used and the length of the 3'untranslated region of the RNase A cDNA. The cassettes were constructed by assembling the BamHI-NcoI fragment from either pMP36 (which contains the ADH2-GAPDH promoter) or pPHO5 (which contains the PHO5 promoter), the NcoI-KpnI fragment from pCB124 (which codes for a modified  $\alpha$ factor leader sequence), a KpnI-PstI adaptor made from oligonucleotides RR9 and RR13; the PstI-Sall fragment from the above M13mp8 clone (which codes for RNase A), and the SalI-BamHI fragment from pPHO5 (which contains the GAPDH terminator). The PHO5 cassette contains 282 bp of the 3'-untranslated region of the RNase cDNA; the ADH2-GAPDH cassette contains only the 3 bp amber codon of this region.

The  $\alpha$ -factor leader sequence encoded by the expression cassette contains two mutations: R2G (due to the creation of a *NcoI* site) and S81P (due to the creation of a *KpnI* site). Also,

the C-terminal (Glu–Ala)<sub>3</sub> sequence, which is not necessary for efficient protein secretion or processing (Brake *et al.*, 1984; Brake, 1989), has been deleted. The expression cassette therefore codes for a leader sequence of 85 amino acids residues, including the C-terminal Lys–Arg.

#### Design of S.cerevisiae expression plasmid pWL

Plasmid pWL (where W and L refer to tryptophan and leucine, respectively) is a shuttle plasmid designed to facilitate the genetic manipulations required in protein engineering. The plasmid carries regions sufficient for replication (ori) and packaging as a single-stranded phagemid (fl) in E.coli, and for replication in [cir<sup>+</sup>] S.cerevisiae (2µ). E.coli transformed with pWL can be selected by ampicillin resistance (Amp<sup>1</sup>). S.cerevisiae transformed with pWL can be selected by the recovery of tryptophan (TRP1) or leucine (LEU2-d) prototrophy. The presence of these two S.cerevisiae genes allows for both ease of transformation (because a single copy of the TRP1 gene is sufficient to confer tryptophan prototrophy) and high-copy number (because many copies of the 5'-truncated LEU2-d gene are necessary to confer leucine prototrophy; Erhart and Hollenberg, 1983). A unique BamHI site located between the Amp<sup>r</sup> and TRP1 regions is a convenient site for the insertion of cassettes directing the expression of a gene of interest.

The plasmids YEpWL.RNase A and YEpWL.Pi.RNase A (where YE refers to yeast expression and Pi refers to inorganic phosphate) are simply pWL carrying either the ADH2-GAPDH or the PHO5 BamHI expression cassettes, described above. S.cerevisiae harboring either YEpWL.RNase A or YEpWL. Pi.RNase A produces RNase A fused to a modified  $\alpha$ -factor leader sequence. The production of  $\alpha$ -factor-RNase A from YEpWL.RNase A is under the transcriptional control of the hybrid ADH2-GAPDH promoter, which is derepressed by the depletion of fermentable carbon sources (Cousens et al., 1987). The production of α-factor~RNase A from YEpWL.Pi.RNase A is under the transcriptional control of the PHO5 promoter, which is derepressed under conditions of low inorganic phosphate (Hinnen et al., 1989; Schneider and Guarente, 1991). Filamentous phage carrying single-stranded copies of either of the two expression plasmids serve as a source of single-stranded DNA useful for oligonucleotide-mediated sitedirected mutagenesis and DNA sequencing.

# Construction of plasmids pWL, YEpWL.RNase A and YEpWL.Pi.RNase A

The plasmid pWL was constructed as follows. The 3952 bp StuI-ScaI restriction fragment from pC1/l carrying the 2µ and

LEU2-d regions was isolated and joined by blunt-end ligation with the band-purified 3825 bp PvuII fragment from pRS304 carrying the fl, ori, TRP1 and Amp<sup>r</sup> regions. An undesired *AatII* site within the pC1/1 fragment was eliminated from the resulting plasmid by digestion at the surrounding *BspHI* sites and ligating the complementary ends of the cleaved plasmid. Finally, a *BamHI* site was positioned between the Amp<sup>r</sup> and TRP1 regions by digestion of the plasmid with *AatII* and insertion of a cassette made from self-complementary oligonucleotide SD1 (Table I), which contains a *BamHI* site. This ligation destroyed the *AatII* site.

YEpWL.RNase A and YEpWL.Pi.RNase A were constructed by the insertion of either the ADH2–GAPDH or the PHO5 *Bam*HI expression cassette into the unique *Bam*HI site of pWL. The orientation of these cassettes was determined by restriction mapping and sequence analysis.

# Construction of plasmid pBXR

Plasmid pBXR (where BXR refers to bacterial expression of RNase A) was constructed as follows. A fragment carrying the cDNA for RNase A that could be inserted between the MscI and SalI sites of pET22B(+) was generated from YEpWL.RNase A using the PCR and priming oligonucleotides SD3 and SD4. The amplified fragment was band-purified, treated with T4 DNA polymerase to remove any overhanging bases left by taq polymerase and digested with Sall. The resulting fragment has the RNase A cDNA flanked on its 5' end by two CG base pairs (which form a blunt end) and on its 3' end by a Sall sticky-end. This fragment was then ligated to the band-purified MscI-Sall fragment of the E.coli expression plasmid pET22B(+) by T4 DNA ligase. A PstI site in the Amp<sup>r</sup> gene was then removed by site-directed mutagenesis using oligonucleotide JHH16, making the PstI site in the RNase A cDNA the only such site in the plasmid. The integrity of this construction was assessed by restriction and sequence analysis.

Plasmids for the production of Q11A, Q11H and Q11N RNase A The CAG codon for the Gln11 of RNase A was changed to GCG (alanine), AAC (asparagine) or CAT (histidine) by oligonucleotide-mediated site-directed mutagenesis of plasmid pYEpWL.RNase A using oligonucleotides SD8, SD7 or SD6 (Table I). Sequenase version 2.0 was used for primer extension in the mutagenesis reactions. The complete cDNA of each mutant was analyzed by sequencing. The genes encoding the mutant enzymes were then ligated into the *PstI-SaII* sites of pBXR. The resulting plasmids are termed pBXR.Q11A, pBXR.Q11N and pBXR.Q11H.

## Plate assay for ribonuclease activity

Plate assays have been described that measure the ability of a microbe to secrete an active ribonuclease (Holloman and Dekker, 1971; Quaas *et al.*, 1988). A plate assay was used to test the ability of pBXR, YEpWL.RNase A and YEpWL.Pi. RNase A to direct the secretion of active RNase A. For bacteria, an aliquot (1  $\mu$ l) of a culture of BL21(DE3) carrying either pBXR or pET22B(+) and grown overnight in LB medium containing ampicillin (400  $\mu$ g/ml) was placed on a plate of the same medium containing Bacto agar (1.5% w/v), yeast RNA (2 mg/ml) and IPTG (1 mM). The plate was incubated for 8 h at 37°C. For *S.cerevisiae*, an aliquot (1  $\mu$ l) of a culture of BJ2168 carrying YEpWL.RNaseA and grown overnight in S 4% D-leu was placed on a plate of YEP 1% D, containing Bacto agar (2% w/v) and yeast RNA (2 mg/ml).

Alternatively, a culture of BJ2168 carrying YEpWL.Pi.RNaseA and grown overnight in SD-leu was placed on a plate of YEPD -Pi, containing Bacto agar (2% w/v) and yeast RNA (2 mg/ml). The plates were incubated at 30°C for 3 days. The plates were then developed by washing with aqueous perchloric acid (10% v/v), which precipitates high  $M_r$  RNA. *S.cerevisiae* colonies that secrete active ribonuclease produced a clearing on an otherwise foggy background.

#### Zymogram electrophoresis

Zymogram electrophoresis is an extremely sensitive assay for ribonuclease activity (Blank et al., 1982; Ribó et al., 1991; Kim and Raines, 1993; delCardayré et al., 1994). This technique can detect the activity of 1 pg (10<sup>-16</sup> mol) of RNase A. Protein samples were separated as usual by SDS-PAGE (Laemmli, 1970), except that the sample buffer contained no reducing agent and the running gel was copolymerized with poly(C)(0.5 mg/ml). After separation, proteins in the gel were renatured by washing the gel twice (for 10 min each) with 10 mM Tris-HCl buffer, pH 7.5, containing isopropanol (20% v/v) to extract the SDS, then twice (for 10 min each) with 10 mM Tris-HCl buffer, pH 7.5, and finally once (for 15 min) with 0.1 M Tris-HCl buffer, pH 7.5. The gel was then stained (for 5 min) with 10 mM Tris-HCl buffer, pH 7.5, containing toluidine blue (0.2% w/v), which binds to high M<sub>r</sub> nucleic acid, and then destained with water. Regions in the gel containing ribonuclease activity appeared as clear bands on a blue background.

#### Zymogram spot assay

A spot assay was developed to assess rapidly the ribonuclease content of fractions produced during the purification of RNase A. A protein sample  $(1 \ \mu$ l) was placed on an agarose gel  $(1\% \ w/v)$  containing poly(C) (0.3 mg/ml) and 10 mM Tris-HCl buffer, pH 7.5. The gel was then incubated at 37°C for 30 min before being stained with 10 mM Tris-HCl buffer, pH 7.5, containing toluidine blue (2 mg/ml), and destained with water. Samples containing ribonuclease activity appeared as a clear spot on a blue background.

## Production and purification of RNase A from S.cerevisiae

RNase A was purified from *S.cerevisiae* strain BJ2168 harboring the plasmid YEpWL.RNase A. Transformed cells were stored as  $-70^{\circ}$ C freezer stocks in glycerol (30% w/v). Frozen cells were rejuvenated by plating onto 2×S 4% D-trp containing Bacto agar (2% w/v) and incubated at 30°C for 2 days. A liquid culture of 2×S 4% D-trp (25 ml) was inoculated with cells from the plate and shaken at 30°C until turbid. A liquid culture of 2×S 4% D-leu (25 ml) was then inoculated with the turbid culture (1 ml) and grown for 24 h, or until turbid. This inoculum was added to YEPD medium (1 l), which was then shaken at 30°C for 96 h.

Cells were removed by centrifugation at 3000 g for 10 min and the supernatant was concentrated to 70 ml with a Minitan<sup>TM</sup> ultrafiltration system (Millipore, MA) using a 5000 M<sub>r</sub> cut-off polysulfone membrane. Acetone was added to the concentrate at a concentration of 60% (v/v) and the resulting precipitate was collected by centrifugation at 27 000 g for 30 min. The precipitate was resuspended in a minimal volume of water and lyophilized. The lyophilsate was resuspended in and dialyzed exhaustively against 25 mM sodium acetate buffer, pH 5.5.

The dialysate was loaded onto a column  $(10.0 \text{ cm} \times 1.8 \text{ cm}^2)$  of S-Sepharose cation exchange resin equilibrated with the same buffer. RNase was then eluted with a linear gradient of

NaCl (0.00–0.35 M) in 25 mM sodium acetate buffer, pH 5.5. Fractions were collected and assayed for ribonuclease activity with the zymogram spot assay. The purity of the active fractions was assessed by SDS–PAGE and zymogram electrophoresis. The active fractions were divided into two pools, a high  $M_r$  pool which contained RNase species with low electrophoretic mobility, and a low  $M_r$  pool which contained RNase species having electrophoretic mobilities closer to that of native RNase A. The two pools were characterized independently.

#### Detection of protein glycosylation

RNase A contains a consensus N-glycosylation sequence (Asn34-Leu35-Thr36) and the enzyme is isolated from bovine pancreas as an assortment of glycosylated forms. To determine whether the recombinant enzyme produced from S.cerevisiae was glycosylated similarly, the high and low M<sub>r</sub> samples purified from S.cerevisiae were analyzed by glycogram electrophoresis with a Glycotrak<sup>TM</sup> kit from Oxford Glycosystems (Rosedale, NY). Briefly, a protein sample was subjected to SDS-PAGE and then transferred to a PVDF membrane. Protein-linked carbohydrates were then oxidized with periodate and the resulting aldehydes were condensed with hydrazine biotin. The membrane was then washed with a solution of Streptavidin-linked alkaline phosphatase, followed by a solution of p-nitroblue tetrazolium (NBT) salt. Regions in the membrane containing glycosylated protein activity appeared as brown bands.

N- and O-linked glycosylation were differentiated in two ways. Cell supernatants from *S.cerevisiae* producing RNase A in the presence of tunicamycin (an inhibitor of *N*-glycosylation) were analyzed by zymogram electrophoresis. Also, the high  $M_r$  RNase A purified from *S.cerevisiae* was treated with endoglycosidase F (which removes *N*-linked glycosylation), O-glycosidase (which removes *O*-linked glycosylation), or both, and then analyzed by zymogram electrophoresis.

#### Production and purification of RNase A from E.coli

The production of RNase A was maximal when cultures were started from either freshly transformed cells or transformed cells grown to mid-log phase ( $A_{600} = 0.5$  OD) in LB medium containing ampicillin (400 µg/l), diluted 1:1 with glycerol, and stored at  $-70^{\circ}$ C. A culture of *E.coli* strain BL21(DE3) (20 ml,  $A_{600} = 0.5$  OD) harboring plasmid pBXR (or a related mutant) in TB medium containing ampicillin (400 µg/ml) was used to inoculate a larger culture (1 l) of the same medium lacking ampicillin. The inoculated culture was shaken (250 r.p.m.) at 37°C until it reached the late log phase ( $A_{600} = 1.9$  OD), and was then induced to express the cDNA that codes for RNase A by the addition of IPTG (to 2 mM). Shaking at 37°C was continued for 4 h, before the cells were harvested by centrifugation for 10 min at 5000 g.

The cell pellet was resuspended in 250 ml of cell lysis buffer, which was 20 mM Tris-HCl buffer, pH 7.8, containing urea (6 M) and EDTA (1 mM), and the suspension was shaken for 20 min at 37°C. This suspension was then centrifuged for 15 min at 30 000 g, the resulting pellet was resuspended in 250 ml of solubilization buffer, which was 20 mM Tris-HCl buffer, pH 7.8, containing urea (6 M), NaCl (0.4 M), dithiothreitol (DTT; 20 mM) and EDTA (1 mM), and the suspension was shaken for 20 min at 37°C. This suspension was then centrifuged for 15 min at 30 000 g. The supernatant was collected and to it was added reduced DTT (0.22 g). The resulting solution was stirred at room temperature for 10 min. Glacial acetic acid was added to lower the pH to 5.0, and the resulting solution was dialyzed exhaustively against 20 mM Tris-acetic acid (AcOH) buffer, pH 5.0, containing NaCI (0.1 M). The insoluble material that accumulated during dialysis was removed by centrifugation. The soluble fraction was then reoxidized by exhaustive dialysis (>24 h) against refolding buffer, which was 50 mM Tris-AcOH buffer, pH 7.8, containing NaCI (0.1 M), reduced glutathione (1 mM) and oxidized glutathione (0.2 mM). The refolded sample was then dialyzed exhaustively against 20 mM Tris-AcOH buffer, pH 8.0.

The dialyzed sample was passed through a column  $(15.0 \text{ cm} \times 4.9 \text{ cm}^2)$  of DE52 anion exchange resin equilibrated with the same buffer. The flow-through was loaded onto a column  $(15.0 \text{ cm} \times 1.8 \text{ cm}^2)$  of S-Sepharose cation exchange resin equilibrated with Tris-AcOH buffer (20 mM), pH 8.0, and the loaded column was washed with the same buffer (100 ml). RNase A was eluted with a linear gradient of NaCl (0.00–0.35 M), in Tris-AcOH buffer, pH 8.0. Fractions were collected and assayed for ribonuclease activity with the zymogram spot assay. The purity of the active fractions was assessed by SDS–PAGE and zymogram electrophoresis. Fractions containing RNase A of >95% purity were pooled and characterized.

#### Steady-state kinetic parameters

Spectrophotometric assays were used to determine the steadystate kinetic parameters for the cleavage of UpA, poly(C) and UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>, and for the hydrolysis of uridine 2',3'-cyclic phosphate (U>p) by wild-type, mutant and glycosylated RNase A. Each assay reaction contained (in 0.8 ml) substrate [UpA, 10  $\mu$ M-0.75 mM; poly(C), 10  $\mu$ M-0.40 mM; UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>, 1  $\mu$ M-1.7 mM; U>p, 50  $\mu$ M-3.0 mM] and enzyme (1 nM-1  $\mu$ M) in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes)-HCl buffer, pH 6.0, containing NaCl (0.1 M). UpA assays also contained adenosine deaminase (25  $\mu$ l of a 22  $\mu$ g/ ml solution). All assays were performed at 25°C.

The cleavage of UpA was coupled to the conversion of the adenosine (A) product to inosine (I) with adenosine deaminase (Ipata and Felicioli, 1968). The  $\Delta\epsilon$  for the conversion of UpA to U>p and I was determined to be  $-6000 \text{ M}^{-1}\text{cm}^{-1}$  at 265 nm in 0.1 M Mes–HCl buffer, pH 6.0, containing NaCl (0.1 M). The  $\Delta\epsilon$  for the cleavage of poly(C), calculated from the difference in molar absorptivity of the polymeric substrate and the mononucleotide cyclic phosphate product, was 2380 M<sup>-1</sup> cm<sup>-1</sup> at 250 nm. The  $\Delta\epsilon$  for the cleavage of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> was 4560 M<sup>-1</sup>cm<sup>-1</sup> at 330 nm (Thompson and Raines, 1994). The  $\Delta\epsilon$  for the hydrolysis of U>p was 600 M<sup>-1</sup>cm<sup>-1</sup> at 286 nm. Samples with excessive absorbance were assayed in cuvettes with 0.2 rather than 1.0 cm path lengths. The values for  $k_{cat}$  and  $K_m$  were determined from initial velocity data with the program HYPERO (Cleland, 1979).

## Results

#### RNase A cDNA

RNA was isolated from a fresh cow pancreas by using methods that limited its degradation by the high concentration of endogenous ribonucleases. A cDNA library containing 10<sup>5</sup> independent transformants was constructed from the isolated RNA. The cDNA that codes for RNase A was cloned by probing this library with a non-degenerate oligonucleotide (which remained hybridized to 2.2% of all plaques) or with a



 $\label{eq:lysAsnValAlaCysLysAsnGlyGlnThrAsnCysTyrGlnSerTyrSerThrMetSer_{80} \\ AAAAATGTTGCCTGCAAGAATGGGCAGACCAATTGCTACCAGAGCTACTCCACCATGAGC \\ \end{tabular}$ 

 $\label{eq:light} IleThrAspCysArgGluThrGlySerSerLysTyrProAsnCysAlaTyrLysThrThr_{100} ATCACCGACTGCCGTGAGACCGGCAGCTCCAAGTACCCCAACTGTGCCTACAAGAACCACC$ 

 $\label{eq:GinAlaAshLysHisIleIleValAlaCysGluGlyAshProTyrValProValHisPhe_{120} CAGGCGAATAAACACATCATTGTGGCTTGTGAGGGAAACCCGTACGTGCCAGTCTTTT$ 

#### AspAlaSerVal AM

Fig. 3. Nucleotide sequence of the cDNA that codes for RNase A, and its translation. Endogenous recognition sites for *Pstl*, *Clal* and *Hincll*; *Eco*RI linkers used during construction of the cDNA library; and Asn34 of the consensus glycosylation site are underlined.





Fig. 4. Map of plasmids YEpWL.RNase A and YEpWL.Pi.RNase A. Plasmid pWL (8605 bp) is a *S.cerevisiae* shuttle plasmid designed to facilitate the genetic manipulations required in protein engineering. YEpWL.RNase A is plasmid pWL containing a cassette (2732 bp) that directs the expression of RNase A under the control of the ADH2–GAPDH promoter. YEpWL.Pi.RNase A is pWL containing a cassette (2393 bp) that directs the expression of RNase A under the control of the PHO5 promoter. *S.cerevisiae* strain BJ2168 harboring either of these plasmids and grown under conditions that derepress expression produces RNase A fused to a modified  $\alpha$ -factor leader sequence (dashed arrow).

fragment of the cDNA that codes for rat pancreatic ribonuclease (which remained hybridized to 2.9% of all plaques). The sequence of this cDNA and its corresponding amino acid sequence are shown in Figure 3. The cDNA sequence is identical to the sequence for the RNase A gene reported previously (Carsana *et al.*, 1988), except for the downstream untranslated region which includes a poly(A) tract interrupted by a single guanylic acid residue.

Plasmids pBXR, YEpWL.RNase A and YEpWL.Pi.RNase A A map of plasmids YEpWL.RNase A and YEpWL.Pi.RNase A are shown in Figure 4. A map of plasmid pBXR is shown



Fig. 5. Map of plasmid pBXR. *E.coli* strain BL21(DE3) harboring pBXR produces RNase A fused to the pelB signal sequence (dashed arrow).

<b>Table II.</b> Steady-state kinetic parameters for the cleavage of UpA by RNase A isolated from <i>E.coli</i> , <i>S.cerevisiae</i> and bovine pancreas <sup>a</sup>						
Source	$k_{\text{cat}}$ (s <sup>-1</sup> )	<i>K</i> <sub>m</sub> (mM)	$\frac{k_{\rm cat}}{(10^6 {\rm M}^{-1} {\rm s}^{-1})}$			
E.coli <sup>b</sup> S cerevisiae	$1440 \pm 150$	$0.62 \pm 0.09$	$2.3 \pm 0.4$			
Low glycosylation	$280 \pm 140$	$2 \pm 1$	$0.2 \pm 0.1$			
High glycosylation	$400 \pm 70$	$0.3 \pm 0.1$	$1.4 \pm 0.6$			
Rovina pancreas	$(920 \pm 130)$	$0.33 \pm 0.05$	$58 \pm 10$			

<sup>a</sup>Data were obtained at  $25^{\circ}$ C in 0.1 M Mes–HCl buffer, pH 6.0, containing NaCl (0.1 M).

<sup>b</sup>Data from delCardayré and Raines (1994).

in Figure 5. The genes in these plasmids were demonstrated to be functional as follows. All three plasmids were propagated in *E.coli* and conferred ampicillin resistance to transformed cells. Single-stranded DNA was produced by *E.coli* strain CJ236 carrying any of the three plasmids and infected with M13K07 helper phage. Plasmids pWL, pWL.RNase A and YEpWL.Pi.RNase A were also propagated in *S.cerevisiae* and allowed transformed cells to recover tryptophan and leucine prototrophy. *S.cerevisiae* cells carrying YEpWL.RNase A but not those carrying pWL tested positive in the ribonuclease plate assay under low glucose conditions. Similarly, cells harboring YEpWL.Pi.RNase A but not pWL tested positive in the ribonuclease plate assay under low phosphate conditions.

#### Purification of RNase A from S.cerevisiae

RNase A was isolated from BJ2168 *S.cerevisiae* cells harboring plasmid YEpWL.RNase A. The RNase produced by these cells was fused to the  $\alpha$ -factor leader sequence, a peptide which directed the secretion of mature enzyme to the medium. SDS–PAGE and zymogram electrophoresis demonstrated that the RNase secreted to the medium was in a variety of forms having diverse electrophoretic mobilities. *S.cerevisiae* cells carrying pWL and exposed to the same conditions produced no detectable RNase activity. Thus, the variety of RNase species observed was likely to be due to disparate post-



Fig. 6. (A) Zymogram electrophoresis gel showing the purification of RNase A from *S.cerevisiae*. Lane 1, RNase A from bovine pancreas; lane 2, acetone precipitate from *S.cerevisiae* strain BJ2168 harboring plasmid YEpWL.RNase A and grown under conditions to derepress heterologous expression; lane 3, RNase A(H), pooled fractions eluting early during cation exchange chromatography; lane 4, RNase A(L), pooled fractions eluting late during cation exchange chromatography. (B) Zymogram electrophoretic analysis of glycosylated RNase produced in *S.cerevisiae*. Lane 1, RNase A from *E.coli*; lane 2, M<sub>r</sub> standards; lane 3, RNase A(H); lane 4, RNase A(H) after treatment with endoglycosidase F; lane 5, RNase A(H) after treatment with 0-glycosidase; lane 6, RNase A(H) after treatment with both endoglycosidase F and 0-glycosidase.

translational modification of  $\alpha$ -factor~RNase A during the secretory process. Cation exchange chromatography separated the RNase species into two fractions corresponding to species of distinct electrophoretic mobility. The early-eluting fractions were termed RNase A(H) and the late-eluting fractions were termed RNase A(L) (where 'H' refers to a high M<sub>p</sub> and 'L' refers to a low M<sub>r</sub>), and are shown in Figure 6A. The kinetic parameters and glycosylation of the two fractions were assessed separately (Table II).

#### Glycosylation of RNase isolated from S.cerevisiae

RNase from the culture medium of *S.cerevisiae* strain BJ2168 harboring plasmid YEpWL.RNase A was isolated as two fractions, RNase A(L) and RNase A(H), which differed in their electrophoretic mobility. Each fraction contained RNase species that ran at an  $M_r$  higher than that expected for RNase A. To discern whether the apparent increase in  $M_r$  was due to post-translational glycosylation, these samples were analyzed for the presence of carbohydrate with glycogram electrophoresis. Glycogram electrophoresis showed that the very high but not the high  $M_r$  species in RNase A(H) contained carbohydrate, and that only the highest  $M_r$  species in RNase A(L) were glycosylated (data not shown). This result is consistent with an observed decrease in the amount of very



**Fig. 7.** SDS–PAGE gel showing the purification of RNase A from *E.coli* strain BL21(DE3) harboring plasmid pBXR. Lane 1,  $M_r$  standards; lane 2, whole-cell lysate of cells before induction; lane 3, whole- cell lysate of cells after induction by IPTG; lane 4, protein extracted with solubilization buffer; lane 5, protein soluble after dialysis against 20 mM Tris–AcOH buffer, pH 5.0, containing NaCl (0.1 M); lane 6, protein soluble after dialysis against refolding buffer; lane 7, pooled fractions from cation exchange chromatography; lane 8, RNase A from bovine pancreas.

high  $M_r$  species but not in that of high and low  $M_r$  species, when tunicamycin was added to the growth medium (data not shown). The treatment of RNase A(H) with endoglycosidase F, *O*-glycosidase, or both, resulted in a collapse of most of the very high  $M_r$  species to low  $M_r$  species (Figure 6B). These data suggest that RNase produced in *S.cerevisiae* is processed inefficiently at its leader sequence, is glycosylated extensively with *N*-linked carbohydrates, and is glycosylated mildly with *O*-linked carbohydrates.

#### Purification of RNase A from E.coli

Plasmid pBXR was designed to direct the secretion of RNase A to the periplasm of *E. coli* strains containing the DE3 lysogen (Studier and Moffatt, 1986). The majority of the RNase A produced by strain BL21(DE3) carrying pBXR was not released from the periplasm upon cold osmotic shock, nor was it found in the soluble fraction of the cell lysate. Rather, RNase A was produced in an insoluble but processed form. The insolubility of the enzyme, though unexpected, proved to facilitate greatly its purification.

Native RNase A is an extremely soluble protein. Methods for the efficient folding/oxidation of denatured/reduced RNase A are well established. Our isolation strategy was therefore to solubilize RNase A selectively under denaturing conditions, and then to fold and oxidize soluble protein under conditions that had been optimized for RNase A (McGeehan and Benner, 1989). The majority of the soluble protein was removed from the induced cells by the cell lysis buffer which contained a high concentration of urea. RNase A remained in the insoluble fraction along with other insoluble cellular constituents. RNase A was then solubilized from this fraction by the addition of solubilization buffer which contained high concentrations of urea, NaCl and DTT. The removal of urea from the solubilized fraction by dialysis against an acidic buffer left RNase A soluble but not reoxidized. In contrast, most of the other proteins were precipitated during dialysis. Oxidation of the soluble dialysate with a glutathione redox buffer resulted in a >1000-fold increase in the ribonuclease activity of this fraction. All of this enzymatic activity flowed through an anion exchange

RNase A	Substrate	$k_{\rm cat}$ (s <sup>-1</sup> )	$K_{\rm m}~({\rm mM})$	$k_{\text{cat}}/K_{\text{m}} (10^6 \text{ M}^{-1} \text{s}^{-1})$
Wild-type	poly(C) <sup>b</sup>	$510 \pm 10$	$0.034 \pm 0.002$	$15 \pm 1$
	UpA <sup>b</sup>	$1400 \pm 150$	$0.62 \pm 0.09$	$2.3 \pm 0.4$
	UpOC <sub>6</sub> H <sub>4</sub> -p-NO <sub>2</sub>	$11 \pm 1$	$0.4 \pm 0.1$	$0.032 \pm 0.008$
	Ū>p <sup>™</sup>	$2.9 \pm 0.4$	$3.2 \pm 0.5$	$0.0009 \pm 0.0002$
Q11A	poly(C)	$155 \pm 2$	$0.017 \pm 0.001$	$9.0 \pm 0.5$
	UpA	$750 \pm 40$	$0.15 \pm 0.02$	$5.0 \pm 0.7$
	UpOC <sub>6</sub> H <sub>4</sub> -p-NO <sub>2</sub>	$0.106 \pm 0.007$	$0.005 \pm 0.002$	$0.021 \pm 0.006$
	U>p	$1.0 \pm 0.4$	$0.74 \pm 0.09$	$0.0013 \pm 0.0005$
Q11N	poly(C)	$95 \pm 2$	$0.006 \pm 0.001$	$17 \pm 2$
-	UpA	$560 \pm 40$	$0.54 \pm 0.07$	$1.0 \pm 0.2$
	UpOC <sub>6</sub> H <sub>4</sub> -p-NO <sub>2</sub>	nd <sup>c</sup>	nd	nd
	U>p	nđ	nd	nd
Q11H	poly(C)	$390 \pm 20$	$0.07 \pm 0.01$	$5.6 \pm 0.8$
	UpA	$290 \pm 50$	$0.8 \pm 0.2$	$0.36 \pm 0.04$
	UpOC <sub>6</sub> H <sub>4</sub> -p-NO <sub>2</sub>	$0.024 \pm 0.005$	$0.08 \pm 0.05$	$0.003 \pm 0.001$
	U>p	$0.21 \pm 0.02$	$0.15 \pm 0.09$	$0.0014 \pm 0.0008$

Table III. Steady-state kinetic parameters for the cleavage of poly(C) and UpA, and for the hydrolysis of U>p by wild-type, Q11A, Q11N and Q11H RNase A<sup>a</sup>

<sup>a</sup>Data were obtained at 25°C in 0.1 M Mes-HCl buffer, pH 6.0, containing NaCl (0.1 M).

<sup>b</sup>Data from delCardayré and Raines (1994).

<sup>c</sup>Not determined.

column. The activity did bind, however, to a cation exchange column, and RNase A of purity >95% eluted from the cation exchange column when the NaCl concentration was 0.15-0.20M. Fractions from the purification of RNase A from *E.coli* are shown in Figure 7. The amino acid sequence of the 14 Nterminal residues of the fraction from the cation exchange column (Figure 7, lane 7) was identical to that of the enzyme isolated from bovine pancreas (Figure 7, lane 8).

#### Steady-state kinetic parameters

The ability of each of the four isolates of RNase A [E.coli, S.cerevisiae (two) and bovine pancreas] to catalyze the cleavage of UpA was determined by using an adenosine deaminasecoupled assay. In this assay, the conversion of UpA to U>p and I is monitored in the presence of excess adenosine deaminase activity. Reported values for the  $K_m$  of this reaction, measured from pH 5 to 7, vary from 0.3 to 1.8 mM (Witzel and Barnard, 1962; Shapiro et al., 1986; Studier and Moffatt, 1986; Trautwein et al., 1991). The assays described here were performed at pH 6.0, where  $k_{cat}/K_m$  appears to be maximal (Richards and Wyckoff, 1971; Shapiro et al., 1986). Although the adenosine deaminase-coupled assay has been used since 1968, we could locate no explicit reference to the value of  $\Delta \epsilon_{265}$  for the conversion of UpA to U>p and I. We determined this value to be  $-6.0 \times 10^3$  OD M<sup>-1</sup> cm<sup>-1</sup> in 0.1 M Mes-HCl buffer, pH 6.0, containing NaCl (0.1 M). The steady-state kinetic parameters for the four isolates of RNase A are shown in Table II.

The kinetic parameters for the cleavage of UpA, poly(C) and UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>, and the hydrolysis of U>p by wildtype, Q11A, Q11N and Q11H RNase A from *E.coli*, are shown in Table III. Replacing the side chain of Gln11 had only a small effect on the value of  $k_{cat}$  and  $K_m$  for the natural substrates UpA, poly(C) and U>p. Mutating Gln11 did, however, have a dramatic effect on these parameters for the cleavage of the non-natural substrate UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>. For all substrates, replacing the side chain of Gln11 resulted in an approximately equal decrease in the values of both  $k_{cat}$  and  $K_m$ , with no significant change in the value of  $k_{cat}/K_m$ . Thus, the mutations enhance substrate binding (lower  $K_m$ ) at the expense of substrate turnover (lower  $k_{cat}$ ). For the Q11A mutant, the decrease for natural substrates was 2- to 5-fold for the natural substrates but 100-fold for  $UpOC_6H_4$ -p-NO<sub>2</sub>.

#### Discussion

Studies on RNase A have been seminal to the current understanding of many aspects of biological chemistry. For example, RNase A was the first enzyme and second protein (after insulin) for which a complete amino acid sequence was determined, and the third enzyme and fourth protein (after myoglobin, lysozyme and carboxypeptidase) whose structure was solved by X-ray diffraction analysis. The ensuing wealth of information has inspired generations of biochemists to make RNase A their model system. This information also makes RNase A an ideal object for exploring the relationship between amino acid sequence and protein function with the techniques of recombinant DNA (Raines and Rutter, 1989).

#### Production and purification of RNase A

RNase A has been a challenging enzyme to produce heterologously. This difficulty has arisen from the evasiveness of its cDNA to cloning, its susceptibility to proteolysis when unfolded and its cytotoxicity when folded. Advances in the technologies of cDNA cloning, heterologous gene expression and protein renaturation have assisted in overcoming these obstacles and have led to the development of several expression systems for RNase A. The first such system was reported by Benner and co-workers, who synthesized a gene for RNase A and expressed this gene in *E.coli* to produce a fusion protein with B-galactosidase (Nambiar et al., 1987). Purifying RNase A from this original system was laborious and inefficient, and these workers later reported an improvement, in which the fusion to  $\beta$ -galactosidase was eliminated (McGeehan and Benner, 1989). The new system produced RNase A with an N-formyl methionine residue. Both of these systems required the RNase A to be denatured and then renatured in the presence of an oxidizing agent. Most recently, Schein et al. (1992) expressed the same synthetic gene fused to a murine signal sequence. The RNase A produced by this system was secreted to the periplasm and was recovered as mature RNase A. This system had the highest yield described to date, allowing ~5

mg of soluble RNase A and 5 mg of insoluble RNase A to be recovered from each liter of fermenter culture.

Other, less effective systems for the heterologous production of RNase A have also been reported. A group at Genex expressed a synthetic gene for RNase A in Bacillus subtilus, and isolated small amounts of an N-formyl methionyl RNase A from the culture medium (Vasantha and Fipula, 1989). Recently, Schultz and Baldwin (1992) expressed the same synthetic gene in E.coli and recovered small amounts of Nmethionyl RNase A from the soluble fraction of the cell lysate. The pINIII system (Masui et al., 1984) that had worked well for the production of RNase T1 (Quaas et al., 1988), failed in the expression of the cDNA for rat pancreatic RNase in E.coli, and Miranda (1990) had to resort to an inefficient human cell culture expression system. Rabin and co-workers have expressed RNase A in E.coli, isolating <0.5 mg of enzyme from the periplasmic fraction of a 1 l culture (Tarragona-Fiol et al., 1992). Most recently, Scheraga and co-workers expressed the synthetic gene from Genex as a fusion protein with the T7 gene10-II protein. After in vitro processing with factor X<sub>a</sub>, these workers isolated 4 mg of RNase A per liter of culture (Laity et al., 1993).

We report here and in Raines and Rutter (1989) the first cloning of the cDNA that codes for RNase A (Figure 3). This cloning was accomplished with methods that optimize the isolation of RNA from tissue rich in ribonuclease activity (Chirgwin *et al.*, 1979). The RNase A cDNA was inserted into expression plasmids that direct its production in *S.cerevisiae* or *E.coli*.

Plasmid pWL is the basis of a novel *S.cerevisiae* expression plasmid designed to facilitate protein engineering. Plasmids YEpWL.RNase A and YEpWL.Pi.RNase A direct the expression of RNase A fused to the  $\alpha$ -factor leader peptide (Figure 4). After being translated,  $\alpha$ -factor leader-RNase A undergoes numerous modifications (Brake, 1989; Innis, 1989). Upon translocation into the endoplasmic reticulum (ER), the signal sequence of the  $\alpha$ -factor leader is removed by signal peptidase. The polypeptide folds and its cysteine residues are oxidized to half-cystines. Finally, the glycosylation machinery of the ER and Golgi (Kukuruzinska *et al.*, 1987) adorns  $\alpha$ -factor-RNase A with a mélange of carbohydrates before the protein is secreted into the medium.

RNase isolated from the culture medium of S.cerevisiae cells harboring YEpWL.RNase A is a mixture of species that differ in the extent of their post-translational modification (Figure 6). Like RNase secreted from bovine pancreas, RNase secreted from S.cerevisiae consists of different glycosylated forms. These forms likely derive from N-linked glycosylation of Asn34 and O-linked glycosylation of serine residues. [Fu et al. (1994) have reported the complete structural characterization of the oligosaccharides from RNase B, which is a mixture of glycosylated forms of bovine pancreatic ribonuclease.] Together, the RNase forms are recoverable at up to 1 mg/l of culture medium and can be separated by chromatography into two fractions that differ primarily in the extent of glycosylation. Conventional chromatographic procedures, however, failed to separate native RNase A from the glycosylated or unprocessed forms. Thus, the YEpWL expression system is not optimal for producing the homogeneous protein necessary for rigorous chemical or kinetic analysis.

The *S.cerevisiae* expression system is useful for studying cellular biochemistry. For example, coupling expression by YEpWL.RNase A with zymogram electrophoresis allowed us

to ascertain readily the effect of tunicamycin on protein glycosylation (data not shown). Also, plasmid pWL and the ribonuclease plate assay have been used to verify that the RNS2 gene product of Arabidopsis thaliana is indeed a ribonuclease (Taylor *et al.*, 1993). In addition, studies that use altered glycosylation patterns as an indicator of the subcellular localization or secretory history of a protein may benefit from this system.

Plasmid pBXR (Figure 5), derived from plasmid pET (Studier et al., 1990), directs the production of RNase A in E.coli (Figure 7). BL21(DE3) cells harboring pBXR produce RNase A in an insoluble form that is recoverable at 50 mg/l of culture. This isolate was >95% pure. Although this expression system is designed to secrete RNase A into the periplasm as a soluble protein, the RNase A produced is insoluble and not recoverable from the periplasm by cold osmotic shock. Interestingly, the insoluble RNase A was mature - it did not contain the pelB signal sequence. This result is unusual because maturation occurs in the periplasm but inclusion bodies usually form in the cytoplasm. This result can be explained if partially folded RNase A aggregated in the periplasm (Mitraki and King, 1989), or if the translocation of RNase A was arrested after signal peptide cleavage. Such inefficient translocation has been observed for lysozyme, which like RNase A has several basic amino acid residues near its N-terminus (Yamane and Mizushima, 1988).

The insolubility of RNase A produced in *E.coli* was useful not only in overcoming the problem of its toxicity, but also in facilitating its purification. Whereas others have succeeded in isolating 5 mg of soluble RNase A per liter of *E.coli* fermenter culture (Schein *et al.*, 1992), we have isolated RNase A at a 10-fold greater level by using the T7 RNA polymerase promoter and isolating enzyme from the insoluble fraction. This increase in recovery makes studies that require large quantities of protein, such as NMR spectroscopy (Stockman and Markley, 1990) and X-ray diffraction analysis, accessible without the need for fermenter growth. The combination of the T7 RNA polymerase promoter and the murine signal sequence (Schein *et al.*, 1992) could increase the production of RNase A in *E.coli* even further.

# Kinetic parameters for wild-type RNase A

Reported values for the kinetic parameters for cleavage of UpA by RNase A vary greatly, as expected in the absence of a standard value for  $\Delta \varepsilon_{265}$ . By comparison, the difference in the kinetic parameters for the enzyme from *E.coli* and from bovine pancreas observed here (Table II) and elsewhere (Trautwein *et al.*, 1991) is small. This small difference is likely to result from deamidation incurred during storage (D.J.Quirk, unpublished results). The  $k_{cat}/K_m$  values for the cleavage of UpA are close to those expected for a reaction that is limited by diffusion (Hammes and Schimmel, 1970).

The kinetic parameters for the two RNase A fractions isolated from *S.cerevisiae* vary from those of the *E.coli* or bovine pancreas isolates (Table II). Both RNase A(L) and RNase A(H) had an ~5-fold decrease in  $k_{cat}$  for UpA cleavage. RNase A(L) also had a 3-fold increase in  $K_m$ . These changes may arise from an increase in steric crowding and a decrease in dynamic flexibility, as has been observed recently for RNase B (Opdenakker *et al.*, 1994; Rudd *et al.*, 1994). RNase A(H), despite its extensive glycosylation, had a  $K_m$  indistinguishable from that of RNase A. RNase A is a basic protein (pI = 9.3) and RNA is an acidic substrate. This reversal of the elevated



**Fig. 8.** Free energy profiles for catalysis of the transphosphorylation of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> (1 M) by wild-type (—) and Q11A (- - ) RNase A. Profiles were constructed by using the data in Table III and the equations:  $\Delta G^{\circ} = -RT \ln K_s$  and  $\Delta G^{\ddagger} = -RT \ln (k_{cat}h/k_BT)$ , and by assuming that  $K_s = K_m$  for this substrate.

 $K_{\rm m}$  of RNase A(L) by increased glycosylation may therefore be the result of a decrease in the dielectric constant in the vicinity of the protein imposed by the large layer of carbohydrate (Beintema, 1987).

#### Role of Gln11 in catalysis by RNase A

One of the most intriguing residues in RNase A is Gln11, which is conserved in all 41 pancreatic ribonucleases of known sequence (Beintema et al., 1988). X-ray diffraction analyses show that the side chain of Gln11 forms a hydrogen bond to substrates, substrate analogs (Mosimann et al., 1994), phosphate ions and sulfate ions bound to the active site of RNase A (Wlodawer, 1985). <sup>1</sup>H NMR spectroscopy provides further evidence for this interaction, as large changes in the resonances NH<sub>e1</sub> and NH<sub>e2</sub> of Gln11 are observed upon binding of pyrimidine nucleotides (Bruix et al., 1991). In the highresolution structure of RNase A complexed with U>v (Figure 2), the N<sub> $\epsilon 2$ </sub> of Gln11 is only 3.9 Å from the vanadium of U>v (Wlodawer, 1985). The distance between the  $N_{\epsilon 2}$  of Gln11 and the closest oxygen of U>v is 2.6 Å, which is a distance expected to produce a low-barrier hydrogen bond (Cleland, 1992; Gerlt and Gassman, 1993a,b; Cleland and Kreevoy, 1994), Together, these data strongly suggest that Gln11 plays an important role in the catalytic mechanism of RNase A.

To investigate the role of Gln11 in catalysis by RNase A, we created Q11A, Q11N and Q11H RNase A and measured the ability of these enzymes to catalyze the cleavage of various substrates. As shown in Table III, replacing Gln11 with an alanine, asparagine or histidine residue resulted in only small changes in the kinetic parameters for the cleavage of poly(C) or UpA, or for the hydrolysis of U>p. Compared with the wild-type RNase A, the mutant enzymes demonstrated a decrease in the values of both  $k_{cat}$  and  $K_m$ , with no significant change in the value of  $k_{cat}/K_m$ . The small effect of altering the side chain of Gln11 suggests that this residue is not essential for catalysis by RNase A, at least if poly(C), UpA or U>p is the substrate. These results allot a somewhat lesser role to Gln11 than do results obtained with analogous semi-synthetic mutants of RNase S' (Marchiori *et al.*, 1974).

RNase A has evolved to bind its substrates by various interactions (Parés et al., 1991; delCardayré and Raines, 1994). For example, the phosphoryl group of UpA appears to be oriented by a hydrogen bond from the main-chain nitrogen of Phe120 and by additional constraints imposed by the binding of its uracil and adenosine bases to the B1 and B2 subsites, respectively. The phosphoryl group of poly(C) is oriented by the same interactions that orient UpA and by additional interactions with the B3 subsite and various basic residues. The phosphoryl group of U>p is constrained by a fivemembered ring, as well as through interactions with Phe120 and the B1 subsite. Although structural data suggest that Gln11 forms a hydrogen bond to a non-bridging phosphoryl oxygen, this hydrogen bond may be superfluous for orienting poly(C), UpA and U>p. If so, only small perturbations in  $k_{cat}$  and  $K_m$ for the reaction of these substrates would be expected because the bound phosphoryl group of poly(C), UpA and U>p would be oriented properly whether or not the side chain of Gln11 was present.

To unmask the role for Gln11 in catalysis, we determined the contribution of this residue to the cleavage of UpOC<sub>6</sub>H<sub>4</sub>p-NO2. This substrate does not contain a 5" nucleoside moiety that can bind to the B2 subsite, nor is its phosphoryl group constrained by a ring. Bound UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> is therefore limited to interactions with Phe120 and the B1 subsite. Previously, we used this substrate to demonstrate that the side chain of His119 serves RNase A as a general acid catalyst (Thompson and Raines, 1994). Here, we reasoned that the proper orientation of the phosphoryl group of UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> may rely strongly on a hydrogen bond donated by the side chain of Gln11. If so, then altering the side chain of Gln11 should have a greater effect on the cleavage of UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> than on that of poly(C), UpA or U>p. As shown in Table III, cleavage of UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> by Q11A RNase A exhibited a 100-fold decrease in the values of both  $k_{cat}$  and  $K_m$ , but no significant change in that of  $k_{cat}/K_m$ . Thus, Gln11 destabilizes bound UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> in the same manner as it does the natural substrates but to a much greater degree.

The effect of altering the side chain of Gln11 on catalysis by RNase A is illustrated by the free energy profile shown in Figure 8. Apparently, Gln11 does not stabilize the rate-limiting transition state during catalysis by RNase A. Rather, Gln11 serves to increase the free energy of the Michaelis complex. The destabilization of ground state complexes is a common event in the evolution of enzymatic efficiency, and can be the result of several molecular scenarios (Fersht, 1985). Here, the increase in the free energy of the ground state appears to be due to a binding interaction that reduces non-productive binding. In the absence of the amide side chain of Gln11, a substrate may bind to the active site but with its phosphoryl group in an improper conformation for in-line attack by the 2'-hydroxyl group (or a water molecule). The increase in the number of substrate binding modes causes a decrease in the value of  $k_{cat}$  and an identical decrease in the value of  $K_m$ , such that the value of  $k_{cat}/K_m$  is unchanged (Fersht, 1985). Thus, a hydrogen bond between the side chain of Gln11 and a phosphoryl oxygen enhances catalysis by orienting the substrate so as to prevent it from binding in a non-productive mode.

Altering the side chain of Gln11 results in only a small ( $\leq 0.6$  kcal/mol) decrease in the value of  $K_m$  for substrates

constrained about their phosphoryl group. In contrast, altering the side chain of Gln11 results in a large (2.6 kcal/mol) decrease in the value of  $K_m$  for UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> (Figure 8), the phosphoryl group of which is not constrained by interaction with the B2 subsite. Page and Jencks (1971) estimated that freezing one internal bond rotation in a five-membered ringforming reaction has an entropic cost of 4.5 cal/mol K. Thus, the ~2 kcal/mol difference between constrained [poly(C), UpA and U>p] and unconstrained (UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub>) substrates corresponds at 25°C to freezing the rotation about one or two bonds. In UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> bound to Q11A RNase A, unfrozen rotations are likely to arise from the bonds between the phosphorus and the two bridging oxygens. A molecular explanation for the small decrease in the value of  $K_m$  for the constrained substrates and mutant enzymes is more elusive. The side chain of Gln11 could eliminate residual rotational degrees of freedom in the constrained substrates, or it could activate the ground state by straining its conformation towards that of the transition state.

The interpretation of the kinetic parameters for Q11N and Q11H RNase A is more complex than that for the Q11A enzyme. Catalysis by both Q11N and Q11H RNase A echoes the trends observed with the Q11A enzyme (Table III), but also displays an ~2- to 10-fold decrease in  $k_{cat}/K_m$ . In native RNase A, position 11 is in the heart of the active site (Figure 2). Although asparagine and histidine are relatively conservative replacements for a glutamine residue, both substitutions relocate heteroatoms. This relocation apparently has effects other than that observed for the simple deletion of a side chain by its replacement with an alanine residue.

What remains intriguing about Gln11 is its absolute conservation, despite the presence of the B2 subsite and the negligible contribution of this residue to stabilizing the ratelimiting transition state. (Of course, the role of Gln11 could extend beyond catalysis. For example, the residue could expedite protein translocation or increase the stability of native protein.) The B2 subsite consists of residues Gln69, Asn71 and Glu111. Like Gln11, Asn71 is conserved in all 41 pancreatic ribonucleases of known sequence. In contrast, Gln69 is conserved in 35 of 41 (three Arg, two Lys and one Asn) and Glu111 is conserved in 38 of 41 (one Asp, one Gly and one Val). Mutation of the residues of the B2 subsite shows that Asn71 (but not Gln69 or Glu111) is indeed important for the cleavage of dinucleotide substrates (Tarragona-Fiol et al., 1993). The residues of RNase A appear to have evolved to catalyze the rapid cleavage of RNA (Thompson et al., 1994). Perhaps the evolution of both Gln11 and the B2 subsite has allowed RNase A to cleave a broader spectrum of RNA substrates. UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> is not a natural substrate. However, natural substrates that retain considerable rotational freedom when bound to Q11A RNase A do exist. For example, pyrimidine dinucleotides such as UpU bind strongly to the B1 subsite but only weakly to the B2 subsite (Wlodawer et al., 1993), which has a strong preference for adenine bases (Parés et al., 1991). Similarly, a pyrimidine residue upstream from secondary structure could bind to the B1 subsite but could not make contact with the B2 subsite. The side chain of Gln11 provides a third point of contact with acyclic phosphodiester substrates that would otherwise interact only with Phe120 and the B1 subsite.

#### Implications for catalysis by RNase A

An important, unresolved aspect of the reactions in Figure 1 is the nature of the pentavalent phosphorus species through

which an in-line mechanism must pass. Theoretical arguments have been used to suggest that this species is a pentavalent transition state (Deakyne and Allen, 1979) or a phosphorane intermediate (Gerlt and Gassman, 1993b). Our results suggest that the side chain of Gln11 does not contribute significantly to the stability of a pentavalent phosphorus species, regardless of its nature. This finding is consistent with the ability of a simple primary amide ( $pK_a = 15.1$ ; Bordwell, 1988) to donate a hydrogen bond, being similar to that of water ( $pK_a = 15.7$ ). Still, several viable mechanisms exist for the reactions in Figure 1. The pentavalent phosphorus species could be an associative transition state or a phosphorane intermediate. Then, negative charge would accumulate on the non-bridging oxygens but would be stabilized largely by residues other than Gln11. Alternatively, the reaction could proceed by a dissociative or an  $S_N$ 2-like transition state in which negative charge would not accumulate on the non-bridging oxygens. This latter mechanism would obviate the role usually assigned to Lys41. An alternative role for Lys41 could then be to lower the  $pK_a$  of the 2'-hydroxyl group.

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