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# Site-directed mutagenesis of bovine pancreatic ribonuclease: lysine-41 and aspartate-121

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Chemical modification studies suggest that two residues of bovine pancreatic ribonuclease A (RNase A), Lys-41 and Asp-121, are important for catalysis. Three mutants of RNase A have been prepared, two point mutants with Lys-41 altered to Arg-41 and Asp-121 altered to Glu-121, and a double mutant where both residues are altered. The Lys-41 Arg mutant has ca. 2% the catalytic activity ($k_{cat}/K_m$) of the native protein, while the Asp-121Glu mutant has ca. 17% the catalytic activity of the native protein. The double mutant has catalytic activity comparable to the Lys-41Arg mutant.

Bovine pancreatic ribonuclease; Active site site-directed mutagenesis; Synthetic gene; Catalytic antisense oligonucleotide

## 1. INTRODUCTION

RNase is a valuable system for studying the general aspects of structure and catalysis in proteins [1,2]. Furthermore, some extracellular homologs of RNase A have interesting biological properties [3a,b,c and 4] and, together with extracellular RNA as their substrate [5a,b] may play a role in the regulation of cell growth [6a,b]. Also, an understanding of the mechanism by which RNase catalyzes the hydrolysis of phosphodiester bonds can guide efforts to develop 'catalytic antisense' oligonucleotide analogs, analogs that are stable to chemical and biological degradation, penetrate biological barriers, bind to complementary mRNA and, via attached functional groups, catalyze the hydrolysis of the bound mRNA [7a,b,c]. Such oligonucleotide analogs may be able to disrupt the expression of specific mRNA molecules without the assistance of RNase H [7a,b,c].

In the reaction catalyzed by RNase A, roles for His-12 and His-119 as general acid and general base catalysts are well established [1]. However, model studies have shown that simple molecules bearing two imidazole rings are far poorer catalysts than RNase A itself for the hydrolysis of RNA [8]. Thus, other residues in the active site of RNase A must be important for catalysis, both in the enzyme, and in many molecules designed to mimic the catalytic properties of the enzyme.

Two candidates for such residues are Lys-41 and Asp-121 [9]. Chemical modification of Lys-41 (see [1] for a review) invariably yields an enzyme with greatly reduced catalytic power, although the modifications often appear to differently affect the rates of various steps in the catalytic cycle (in particular, the formation and the hydrolysis of the 2′,3′-cyclic phosphate intermediate) [10] and the rates at which different substrates are processed [11a,b]. Crystallographic studies have shown that when RNase is bound to the vanadate complex of uridine, Lys-41 appears to interact with the negatively charged vanadate oxygen [12]. These studies imply that the positive charge of the ε-amino group of Lys-41 stabilizes the negatively charged oxygen on the pentacoordinate phosphate in the transition state.

Catalytic roles for Asp-121 are less clear, although the residue may form hydrogen bonds with His-119, perhaps acting in a 'charge relay' fashion [13]. When the C-terminal residues of RNase (residues 121–124) are removed by proteolysis, only 0.5% to 4% of the catalytic activity towards various substrates is retained [14,15]; further digestion removes all traces of catalytic activity. However, catalytic activity can be restored by adding synthetic peptides extending from residues 111–124 [16] or residues 115–124 [15], peptides that form non-covalent complexes with the digested RNase protein.

Of course, the interpretation of results obtained from chemical modifications that introduce bulky substituents into the protein or from reconstructed non-covalent complexes between RNase A and synthetic peptides are often complicated by issues not directly

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*Abbreviations:* RNase A, bovine pancreatic ribonuclease; RNase 1–120, polypeptide composed of residues 1–120 of ribonuclease A; PAGE, polyacrylamide gel electrophoresis; C(>p, cytidine-2′,3′-cyclic phosphate; U>p, uridine-2′,3′-cyclic phosphate; UpA, uridylyl-(3′->5′)-adenosine

relating to the contribution to catalysis of the side chains themselves. This is, of course, precisely the sort of problem well suited for site-directed mutagenesis studies. We report here the behavior of mutant forms of RNase A where Lys-41 is replaced by Arg and Asp-121 is replaced by Glu.

## 2. MATERIALS AND METHODS

Genes for the mutants described here were prepared by modular mutagenesis of a synthetic gene coding for RNase A, described elsewhere [17]. Oligonucleotides were synthesized on an Applied Biosystems model 380A synthesizer using phosphoramidite chemistry and purified either by polyacrylamide gel electrophoresis (PAGE) on 16% gels, or, in the case of short oligonucleotides, by thin layer chromatography. Both native and mutant proteins were expressed in *E. coli* behind the lambda promoter, using a modification [18] of the expression system described previously [19]. All expressed RNases were purified as described in these previous papers. The protocols yielded 2-15 mg/ml of RNase A and its mutants, all homogeneous by PAGE (Fig. 1), both denaturing and native.

Kinetics were performed using either uridylyl-(3' → 5')-adenosine (UpA, Sigma) or cytidine-2',3'-cyclic phosphate (C>p, Sigma) as substrate. The rate of hydrolysis of UpA (15-200 μM) at 25°C was measured in sodium acetate buffer (pH 5.0, 100 mM) by coupling the reaction to the deamination of adenosine by adenosine deaminase (Sigma), which was followed at 275 nm according to published procedures [20]. The rate of hydrolysis of C>p at 37°C in Tris-HCl buffer (pH 7.1, 100 mM) was measured by UV spectroscopy at 294 nm according to published procedures [21]. The concentration of enzyme was measured either by measuring its absorbance at 280 nm (absorbance of a 1% solution is 7.3; alternatively, at 278 nm, the molar extinction coefficient is 9,800) or by the Bio-Rad modification of the Bradford assay [22].

## 3. RESULTS AND DISCUSSION

With UpA as a substrate, native RNase A (expressed in *E. coli*) has a $k_{cat}$ of $1455 \pm 67\ s^{-1}$, a $K_M$ of $323 \pm 52$ μM, and a $k_{cat}/K_M$ of $4.4 \times 10^6\ M^{-1} \cdot s^{-1}$ (determined from 7 independent kinetic runs). Commercial RNase A (from Boehringer-Mannheim) has a similar $k_{cat}/K_M$ of $4.8 \times 10^6$. Replacing Lys-41 by an Arg yields a mutant (K41R) of RNase A that has a $k_{cat}$ of $30 \pm 3\ s^{-1}$, a $K_M$ of $355 \pm 70$ μM, and a $k_{cat}/K_M$ of $8.5 \times 10^4\ M^{-1} \cdot s^{-1}$, measured with UpA in 3 independent kinetic runs. Thus, the $k_{cat}/K_M$ of the K41R mutant is only 1.9% that of native enzyme with UpA as the substrate.

Replacing Asp-121 by a Glu yields a mutant (D121E) of RNase A that has a $k_{cat}$ of $425 \pm 38\ s^{-1}$, a $K_M$ of $573 \pm 76$ μM, and a $k_{cat}/K_M$ of $7.3 \times 10^5\ M^{-1} \cdot s^{-1}$, measured with UpA in 4 independent kinetic runs. Thus, the $k_{cat}/K_M$ of the D121E mutant is 17% that of native enzyme with UpA as the substrate.

Replacing both residues yields a double mutant (K41R D121E) with a $k_{cat}$ of $16 \pm 6\ s^{-1}$, a $K_M$ of $218 \pm 62$ μM, and a $k_{cat}/K_M$ of $6 \times 10^4\ M^{-1} \cdot s^{-1}$, measured with UpA in two independent kinetic runs, at an enzyme concentration of 32.7 nM.

Assays were also run with C>p as substrate at two different concentrations. Data from these assays are collected in Table I, where it can be seen that results obtained were quantitatively similar to those obtained with UpA as substrate.

These results show that the side chain of Lys-41 is quite important for catalytic activity. This conclusion can be compared to that recently reported by Miranda and Petsko, who prepared a mutant of rat ribonuclease where Lys-41 was replaced by Ala [23]. This mutant is reported to have 2% of the catalytic activity of RNase A using RNA as substrate, and 3.4% the catalytic activity of RNase A using U>p as substrate [23]. These results are remarkably consistent with the results reported here for the K41R mutant, despite the fact that the new residue (Arg vs. Ala), the substrate (UpA and C>p vs. U>P), and the 'context' (bovine RNase versus rat RNase) are all different in these two studies. Further, the fact that comparable activities are observed when Lys-41 is replaced by Arg (a conservative substitution where the positive charge is retained) and by Ala (where the positive charge is lost) suggests that in our mutant, the side chain of Arg-41 does not contribute to catalysis, even though it retains a positive charge.

The smaller impact of the substitution at position 121 suggests that Asp-121 is not as intimately involved in catalysis as is Lys-41. Our kinetic results can be compared with the results obtained with the 'semi-synthetic' proteins formed by non-covalent complexation between RNase 1-120 and various synthetic peptides [15,16]. With Asn-121 in the peptide, the turnover catalyzed by the complex is 4.5% of the catalytic activity of the com-

Fig. 1. Gel of mutants of RNase used in this work: lane 1, RNase A (Boehringer-Mannheim; lane 2, K41 R; lane 3, K41R D121E; lane 4, D121E; lanes 5 and 6, RNase A (expressed in *E. coli*).

Table I

Catalytic activity of mutant RNases with 2'-3'-cyclic-CMP as substrate*

| | [C>p] | |
|---|---|---|
| Mutant | 100 μM | 400 μM |
| K41R | <1% | 2% |
| D121E | 17% | 16% |
| K41R D121E | 3% | 2% |

*Expressed as % relative to wild type.

plex reconstructed with a peptide containing Asp at position 121 (with C>p as substrate); the decrease in turnover is primarily in catalytic activity, not in the ability of enzyme to bind substrate [16]. In contrast, when the peptide complementing the truncated RNase 1-120 contains a Glu as position 121, the $V_{max}$ is 64% and the $K_M$ 150% that of the analogous reconstruction with Asp-121 (with CpA as substrate) [15].

From a structural standpoint, these results are difficult to interpret without crystallographic data on the mutants themselves. Nevertheless, the loop containing Lys-41 is known from crystallographic work to undergo a large conformational change when substrate binds [24], a conformational change that is believed to deliver the side chain of Lys-41 to the position where its protonated amine group can best interact with the negatively charged pentacoordinated phosphate group in the transition state. It is conceivable that the same conformational change does not deliver the positively charged group in the side chain of arginine to the same position, given the different geometry of an arginine side chain. It would be interesting to learn whether compensating mutations in this loop would restore some or all activity to the K41R mutant.

Interestingly, introducing the D121E mutation into a RNAse mutant in which Lys-41 has already been replaced by an Arg apparently does not lower the catalytic activity further (Table I). Indeed, although the measured rates are quite low and therefore imprecise, it appears that the double mutant has a higher activity than the K41R mutant itself under at least some assay conditions (e.g. [C>p] = 100 $\mu$M). It is, of course, possible that an alternative mechanism is operating in the double mutant and future work will be directed in part to exploring this possibility.

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