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Reverse Transphosphorylation by Ribonuclease A Needs an Intact p_2-binding Site

POINT MUTATIONS AT LYS-7 AND ARG-10 ALTER THE CATALYTIC PROPERTIES OF THE ENZYME*

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Bovine pancreatic ribonuclease A interacts with RNA along multiple binding subsites that essentially recognize the negatively charged phosphates of the substrate. This work gives additional strong support to the existence of the postulated phosphate-binding subsite p_2 (Parés, X., Llorens, R., Arús, C., and Cuchillo, C. M. (1980) *Eur. J. Biochem.* 105, 571–579) and confirms the central role of Lys-7 and Arg-10 in establishing an electrostatic interaction with a phosphate group of the substrate.

The effects of charge elimination by Lys-7 → Gln (K7Q) and/or Arg-10 → Gln (R10Q) substitutions in catalytic and ligand-binding properties of ribonuclease A have been studied. The values of K_m for cytidine 2′,3′-cyclic phosphate and cytidylyl-3′,5′-adenosine are not altered but are significantly increased for poly(C). In all cases, k_{cat} values are lower. Synthetic activity, *i.e.* the reversion of the transphosphorylation reaction, is reduced for K7Q and R10Q mutants and is practically abolished in the double mutant. Finally, the extent of the reaction of the mutants with 6-chloropurine-9-β-D-ribofuranosyl 5′-monophosphate indicates that the phosphate ionic interaction in p_2 is weakened. Thus, p_2 modification alters both the catalytic efficiency and the extent of the processes in which an interaction of the phosphate group of the substrate or ligand with the p_2-binding subsite is involved.

Bovine pancreatic ribonuclease (RNase)[1] A (EC 3.1.27.5.) is an endonuclease that cleaves 3′,5′-phosphodiester linkages of single-stranded RNA when the base of the nucleotide in the 3′ position is a pyrimidine. Research work during the last two decades indicates the existence in RNase A of several binding subsites that recognize the negatively charged phosphates of RNA (Richards and Wyckoff, 1971; Parés *et al.*, 1980, 1991; Blackburn and Moore, 1982). A model for the interaction of RNA with RNase A was postulated and binding subsites for nitrogenated bases, riboses and phosphates, and the amino acid residues involved were indicated (Fig. 1). The existence of binding subsites, in addition to the main catalytic center, that interact with RNA has also been described for other RNases, including barnase (Mossakowska *et al.*, 1989; Day *et al.*, 1992) and RNase T_1 (Osterman and Walz, 1979; Georgalis *et al.*, 1992).

An additional phosphate-binding subsite (p_2), adjacent to the phosphate in the active center, was postulated to account for the reaction of RNase A with the halogenated nucleotide 6-chloropurine-9-β-D-ribofuranosyl 5′-monophosphate

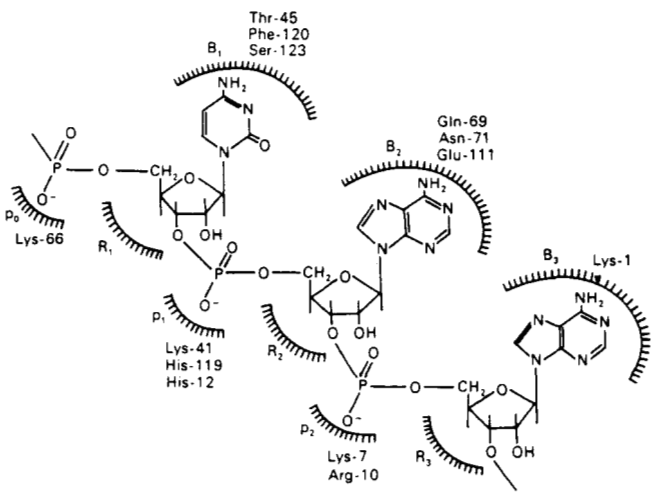

FIG. 1. **Schematic diagram of the enzyme-substrate complex for RNase A.** *B*, *R*, and *p* indicate binding subsites for base, ribose, and phosphate, respectively. B_1 is specific for pyrimidines and B_2 "prefers" purines. The phosphate group of the phosphodiester bond hydrolyzed by the enzyme binds to p_1. The residues known to be involved in each site are indicated (Parés *et al.*, 1991).

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[1] The abbreviations used are: RNase, ribonuclease; RNase A, bovine pancreatic RNase A; RNase S, ribonuclease A derivative obtained by specific cleavage with subtilisin of the peptide bond between amino acid residues 20 and 21; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; K7Q, R10Q, and K7Q plus R10Q, ribonuclease A in which Lys-7 and/or Arg-10 are replaced by Gln; 2′-CMP, cytidine 2′-monophosphate; C>p, cytidine 2′,3′-cyclic phosphate; CpC>p, cytidylyl-3′,5′-cytidine 2′,3′-cyclic phosphate; poly(C), poly(cytidylic acid); CpA, cytidylyl-3′,5′-adenosine; cl^{6}RMP, 6-chloropurine-9-β-D-ribofuranosyl 5′-monophosphate; PMSF, phenylmethanesulfonyl fluoride.

(cl^{6}RMP) (Parés *et al.*, 1980). The reaction yielded a major derivative (Derivative II) with the nucleotide label attached to the α-amino group of Lys-1. The specificity of the reaction suggested that before the covalent labeling takes place, the phosphate of the cl^{6}RMP binds in a specific phosphate-binding subsite, p_2, where the reagent is orientated to allow the nucleophilic attack by the α-NH_2 of Lys-1. The cl^{6}RMP-ribonuclease A reaction has been studied using different purine and pyrimidine nucleotides as protecting agents. The efficiency of protection followed the order 3′-AMP > 5′-AMP = 5′-CMP > 3′-CMP, which corresponds to the extent of p_2 occupancy (Richardson *et al.*, 1988). Proton nuclear magnetic resonance studies on Derivative II and on mononucleotide-RNase A complexes as well as molecular modeling and the conservation of Lys-7 and Arg-10 in all known pancreatic RNases (Beintema *et al.*, 1988) suggested that these two amino acids could belong to p_2 (Arús *et al.*, 1981; de Llorens *et al.*, 1989). Irie *et al.* (1984, 1986), using ^{31}P proton nuclear magnetic resonance and kinetic studies, also found evidence on the existence of the p_2-binding subsite. Chemical modification of Lys-7 with pyridoxal 5′-phosphate and of Arg-10 with cyclohexane-1,2-dione (Richardson *et al.*, 1990) further supported the hypothesis that these positive residues were involved in the p_2 phosphate-binding subsite.

To confirm the contribution of the positive charged residues Lys-7 and Arg-10 to the p_2 phosphate-binding subsite, we used site-directed mutagenesis to construct recombinant RNase A mutants with the following substitutions: Lys-7 → Gln, Arg-10 → Gln and both Lys-7 → Gln plus Arg-10 → Gln. Wild-type and RNase A mutants were expressed in *Escherichia coli* using a secretion system (Schein *et al.*, 1992). Kinetic parameters for the forward reaction of a variety of substrates as well as the reverse transphosphorylating activity were determined for wild-type and mutant proteins. Chemical modification of recombinant proteins with cl^{6}RMP was also carried out. All results could be interpreted in terms of the subsites model for RNase A (Parés *et al.*, 1991), confirming that both Lys-7 and Arg-10 are essential for the function of p_2.

EXPERIMENTAL PROCEDURES

Materials—T4 DNA ligase and restriction endonucleases were from New England Biolabs, Inc. (Beverly, MA). Plasmid-derived DNA fragments were isolated from agarose gels with Geneclean glass milk from U. S. Biochemical Corp. For site-directed mutagenesis the Muta-Gene kit from Bio-Rad, which includes CJ236 and MV1190 *E. coli* strains, was used. DNA sequencing was performed with a sequencing kit (Pharmacia LKB Biotechnology Inc.). Bovine pancreatic RNase (5 × crystallized), bakers yeast RNA (type XI), poly(C), C>p, CpA, cl^{6}RMP, pepsin from porcine stomach mucosa, and dialysis tubing were purchased from Sigma. Bovine pancreatic RNase was purified by a modification of the Taborsky method to obtain the RNase A fraction (Alonso *et al.*, 1986). Bio-Gel P60 and alkaline phosphatase-labeled goat anti-rabbit IgG were purchased from Bio-Rad. Poly(C) for activity-staining gels and Sephadex G-25M (PD-10), CM-Sepharose CL-6B, DEAE-Sepharose CL-6B, and Mono S HR 5/5 columns were from Pharmacia. HPLC columns were as follows: Nucleosil 10SB column was purchased from Macherey, Nagel and Co. (Düren, Germany), Protein Pak 125 column was from Millipore-Waters (Mildford, MA), and Vydac 214-TP C_4 column was from Vydac (Hesperia, CA). Immobilon P transfer membranes were from Millipore-Waters.

Expression and Site-directed Mutagenesis—Wild-type RNase A was expressed and secreted from *E. coli lon-htpr-* as previously described (Schein *et al.*, 1992). A synthetic gene for bovine pancreatic RNase A (Nambiar *et al.*, 1987) was cloned behind the murine spleen RNase signal sequence into plasmid pHR148, which includes the *trp* promoter (Rink *et al.*, 1984), to yield a plasmid designated as pTrpmuSSboRN (Schein *et al.*, 1992). Mutants K7Q, R10Q, and K7Q plus R10Q were obtained by site-directed mutagenesis; for mutagenesis, the RNase A gene fragment was cloned between *Eco*RI and *Bam*HI restriction sites of M13 mp19. Mutants were constructed according to Kunkel's method (Kunkel *et al.*, 1987). Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and purified by electrophoresis on a 12% polyacrylamide gel. The mutagenic primers were as follows: 5′-ACCGCGGCAGCACAATTT-GAACGTCAG-3′ (K7Q), 5′-GCAGCAAAATTTGAACAGCAGCA-TATGGACAGTTCC-3′ (R10Q), and 5′-ACCGCGGCAGCACAAT TTGAACAGCAGCATATGGACAGT-3′ (K7Q plus R10Q). Mutants were identified by dideoxy sequencing. Constructions were inserted at the *Nco*I-*Bam*HI site of the same expression plasmid, pTrpmuSSboRN. The entire sequence of each mutant was checked. Growth conditions for fermentor cultures, cell harvesting, and isolation of the soluble cellular fraction were performed as previously described (Schein *et al.*, 1992).

Expression rates of wild-type RNase and mutants in the supernatant and cellular fraction were assessed by immunoblotting of proteins separated by 15% SDS-PAGE and transferred to Immobilon membranes. Detection was carried out with rabbit polyclonal anti-RNase A antibodies and alkaline phosphatase-labeled goat anti-rabbit IgG.

Assay of RNase Activity—Activity of expressed proteins and chromatographic fractions from purification procedures was determined by assessing RNA hydrolysis rates by a modification of the procedure described by Isobe and Uchiyama (1986). Reaction was stopped with 25% perchloric acid containing 0.75% phosphotungstic acid and, after RNA precipitation and centrifugation at 5,000 × *g* of the precipitate, the concentration of acid-soluble nucleotides was measured at 260 nm.

Activity Staining Gels—Wild-type and mutant activity toward poly(C) was first assessed by activity-staining gels. 15% SDS-PAGE gels containing 0.6 mg/ml poly(C) as substrate were prepared as previously described (Ribó *et al.*, 1991). After electrophoresis, SDS was removed, and the gel was incubated for 40 min at pH 7.4 to allow RNase-catalyzed hydrolysis of poly(C). Gels were stained with 0.2% toluidine blue followed by destaining and silver nitrate staining. Band areas were determined at 550 nm with a CS-9000 Shimadzu densitometer.

Purification of Expressed Wild-type and Mutants—Expressed proteins were isolated both from the supernatant culture medium and from the cellular soluble fraction. Fermentor culture medium was centrifuged at 7,000 × *g* for 15 min. RNase A from the soluble fraction of the cells was isolated by the following modification of the procedure of Schein *et al.* (1992). The cell pellet was suspended in 10 mM Tris/HCl, pH 7.5, containing 20 μM PMSF, 10 mM EDTA, and lysis was performed by repeated freeze-thawing cycles from −70 to 25 °C between liquid N_2 and room temperature water. The lysate was centrifuged at 16,000 × *g* for 30 min. The supernatant was dialyzed against 15 mM Tris/HCl, pH 8, 20 μM PMSF, and loaded onto an anion-exchange column (DEAE-Sepharose CL-6B, 2.5 × 30 cm) equilibrated with the same buffer. The nonretained fractions, which contained RNase activity, were pooled and loaded onto a cation-exchange column (CM-Sepharose CL-6B, 2.1 × 30 cm) equilibrated with 15 mM Tris/HCl, pH 8. RNase was eluted using a linear salt gradient from 0 to 0.25 M NaCl, in the same buffer. One major active peak was eluted at 12 mS for RNase A, and 8 mS for K7Q and R10Q mutants. Active fractions were pooled, dialyzed against distilled water, and freeze-dried. As a final step, enzymes were purified by an HPLC gel filtration chromatography (Protein Pak 125 column) in 0.1 M Tris acetate, pH 7, at a flow rate of 0.5 ml/min. The culture supernatant fraction was dialyzed against 15 mM Tris/HCl, pH 8, 20 μM PMSF, and was applied to a DEAE-Sepharose column (2.5 × 30 cm) equilibrated with the same buffer. The subsequent steps were performed as described above for the soluble fraction of the cells. Before N-terminal sequencing, proteins were further purified by a reversed-phase chromatography (Vydac C_4 column). Samples were applied to the column equilibrated with 10% acetonitrile, 0.1% trifluoroacetic acid. For the elution, the organic phase of the solvent was increased with a 15-min linear gradient from 10% acetonitrile, 0.1% trifluoroacetic acid to 20% acetonitrile, 0.1% trifluoroacetic acid, and a 1-h linear gradient from 20 to 35% acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min.

For the isolation of the double mutant, slight modifications were introduced. From supernatant or cellular soluble fraction, RNase activity was eluted with the void volume from a DEAE-Sepharose column equilibrated with 15 mM sodium acetate, pH 5. Active fractions were pooled, freeze-dried, dissolved in 2.5 ml of 50 mM Tris/HCl, pH 7, buffer and chromatographed through a Sephadex G-25 M column (PD-10). The protein fractions from the exclusion limit were pooled, concentrated, and applied to a gel filtration column (Bio-Gel

P60, 1 × 90 cm) equilibrated with 50 mM Tris/HCl, pH 7. A major fraction with RNase activity was pooled, dialyzed, and freeze-dried. Further purification was achieved with a Mono S cation-exchange column equilibrated with 20 mM sodium acetate, pH 5. Elution was performed by applying a 30-min linear gradient from 20 mM to 0.25 M sodium acetate, pH 5, double mutant elutes at 10 mS.

The purity of enzymes was checked by 15% SDS-PAGE followed by silver nitrate staining. Protein concentrations were determined either by Lowry's method (Lowry *et al.*, 1951) or, for purified protein, spectrophotometrically at 278 nm (ϵ_{278} = 9800 M^{-1} cm^{-1}) (Sela and Anfinsen, 1957).

N-terminal Sequence—The N-terminal sequence (first 11 cycles) from isolated wild-type and mutant RNases was checked to verify that the signal sequence had been correctly cleaved. Amino acid sequence determination was performed on an Applied Biosystems 477 A protein sequencer.

Circular Dichroism Spectroscopy—Circular dichroism spectra from 200 to 320 nm were recorded at 25 °C in a nitrogen atmosphere on a Jasco J720 spectropolarimeter. The far UV CD spectra (200–230 nm) were obtained at 50 μg/ml enzyme concentration in 0.1 M Tris acetate, pH 7, with 0.1-cm optical path length cells. For the near UV CD spectra (230–320 nm), the enzyme concentration and optical path of the cell were increased to 0.15 mg/ml and 0.5 cm, respectively.

Kinetic Studies—C>p, CpA, and poly(C) were used as substrates, and the kinetic parameters were determined by the following spectrophotometric method. For C>p, the substrate concentration range was from 0.1 to 3 mM, and the activity was measured by recording the increase in absorbance at 296 nm ($\Delta\epsilon_{296}$ = 516.4 M^{-1} cm^{-1}). For the CpA assay, the concentration range was from 0.1 to 2 mM and the 286 nm absorbance decrease was measured ($\Delta\epsilon_{286}$ = 521 M^{-1} cm^{-1}). For poly(C), the substrate concentration range was 0.1–2.5 mg/ml, and the decrease in absorbance at 294 nm was monitored. Final enzyme concentrations were in the range of 0.1–0.25 μM for C>p and 5–50 nM for CpA and poly(C), depending on the activity of the enzyme assayed. All assays were carried out in 0.2 M sodium acetate, pH 5.5, at 25 °C, using 1-cm path length cells for C>p and 0.2 cm path length cells for CpA and poly(C). Kinetic parameters were obtained by the nonlinear regression data analysis program ENZFITTER (Leatherbarrow, 1987).

Kinetic Analysis by HPLC of Synthetic and Hydrolytic Activities using C>p as Substrate—The simultaneous synthetic activity from C>p to cytidylyl-3′,5′-cytidine-2′,3′-cyclic phosphate (CpC>p) and the hydrolytic activity to 3′-CMP, using very high concentrations of substrate, were analyzed by HPLC according to the method of Guasch *et al.* (1989).

Chemical Modification—The chemical modification of RNase A by cl^6RMP was performed according to Parés *et al.* (1980). The procedure was adapted for small scale reaction. 200 μg of cl^6RMP (0.4 μmols) and 100 μg of enzyme (7 nmols) were dissolved in 10 μl of 10 mM Hepes, pH 7.4, 0.1 M NaCl. The mixture was kept at 40 °C for 48 h. The reaction mixture was then dissolved in 1 ml of 15 mM Hepes, pH 8, and the reaction products were chromatographed on a Mono S HR 5/5 fast protein liquid chromatography cation-exchange column, equilibrated with the same buffer. Reaction products were eluted at a flow rate of 1 ml/min using a 60-min linear salt gradient from 0 to 0.15 M sodium acetate in 15 mM Hepes, pH 8. For the K7Q plus R10Q double mutant, which does not bind to Mono S cation-exchange resin at pH 8, separation was performed with 20 mM sodium acetate, pH 5, applying a 30-min linear gradient from 20 mM to 0.25 M sodium acetate, pH 5.

RESULTS

Expression and Purification of Wild-type and Mutant Enzymes—Wild-type RNase A and RNase mutants were expressed in *E. coli lon-htpr-* transformed with a synthetic gene (Nambiar *et al.*, 1987) in a secretion vector (pTrpmuSSboRN) as previously described (Schein *et al.*, 1992). Immunoblotting of supernatant culture medium or soluble cellular fraction of wild-type or mutant RNases with antibodies to RNase A showed a single band with the same mobility as commercial RNase A (Fig. 2*A*). Culture medium from *E. coli lon-htpr-* control, without the pTrpmuSSboRN plasmid, gave no detectable signal. Activity staining after 15% SDS-PAGE of culture medium supernatant and soluble cellular fractions also showed a single activity band at the same position as commercial RNase A (Fig. 2*B*). The overall yields ranged between 2 and 6 mg of enzyme/liter of culture medium. Table I shows the recovery yields and specific RNase purification at each purification step for wild-type RNase A from the soluble fraction of the cells. Mutants expression yields were lower than wild-type yield. Purified recombinant RNase A and mutants displayed one single band by 15% SDS-PAGE and silver staining (Fig. 3). Although amino acid substitutions at the N-terminal level can prevent the proper cleavage and

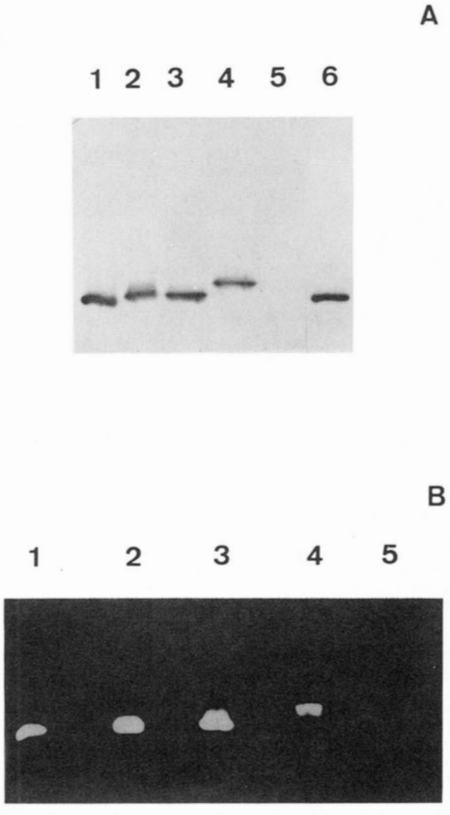

FIG. 2. **Analysis of expressed proteins by (*A*) immunoblotting and (*B*) poly(C) activity staining on 15% SDS-PAGE.** *A*, 20 μl of soluble cellular fraction of *E. coli lon-htpr-*/pTrpmuSSboRN of wild-type RNase A (*lane 1*), R10Q (*lane 2*), K7Q (*lane 3*), K7Q plus R10Q (*lane 4*), nontransformed *E. coli lon-htpr-* control (*lane 5*), 100 ng of commercial RNase A (*lane 6*). *B*, 400 pg of commercial RNase A (*lane 1*), 3 μl of supernatant fraction of wild-type RNase A (*lane 2*), K7Q (*lane 3*), K7Q plus R10Q (*lane 4*), *E. coli lon-htpr-* control (*lane 5*).

TABLE I

Purification of wild-type RNase A from cellular soluble fraction of 1 liter fermentor culture of E. coli lon-htpr-/pTrpmuSSboRN

Step[a]	Total protein[b]	Total RNase A equivalents[c]	Specific activity[d]	Yield
	mg	*mg*		%
Soluble fraction of the cells	80.0	5.0	0.06	100
DEAE-Sepharose	16.0	3.5	0.22	70
CM-Sepharose	4.0	2.7	0.67	55
Protein Pak (HPLC)	2.4	2.2	0.92	44

[a] Details of purification steps are described under "Materials and Methods."

[b] Determined by the method of Lowry.

[c] RNase A equivalents refers to milligrams of enzyme that would degrade as much RNA as the equivalent amount of standard RNase A by the RNase activity assay described under "Materials and Methods."

[d] Milligrams of RNase A equivalents/mg of total protein.

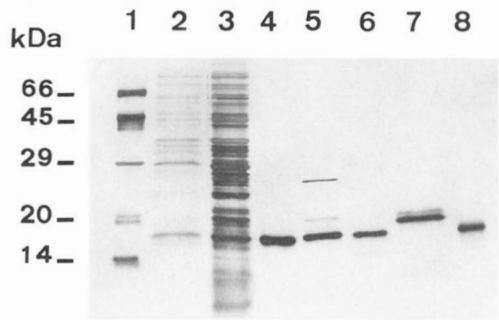

FIG. 3. **Silver stained 15% SDS-PAGE of purified wild-type and mutant RNases.** Molecular mass standards (bovine serum albumin, ovalbumin, carbonic anhydrase, trypsinogen, trypsin inhibitor, and lysozyme) in decreasing order of molecular mass (*lane 1*), 15 μl of supernatant fraction (*lane 2*), 15 μl of soluble cellular fraction (*lane 3*), 0.4 μg of purified wild-type RNase A (*lane 4*), 0.4 μg of purified R10Q (*lane 5*), 0.4 μg of purified K7Q (*lane 6*), 0.4 μg of purified K7Q plus R10Q (*lane 7*), and 0.4 μg of commercial RNase A (*lane 8*).

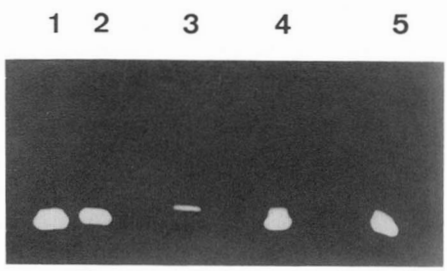

FIG. 4. **15% SDS-PAGE activity staining gel containing poly(C) as substrate.** Commercial RNase A (*lane 1*); K7Q mutant (*lane 2*); K7Q plus R10Q mutant (*lane 3*); R10Q mutant (*lane 4*); wild-type RNase A (*lane 5*). All *lanes* contained 380 pg of purified enzyme.

secretion of the protein (Yamane and Mizushima, 1988; Summers *et al.*, 1989), this was not our case as N-terminal protein sequencing (first 11 cycles) confirmed that the expressed proteins were in all cases correctly cleaved; the correctness of mutant substitutions was also checked.

Circular Dichroism—The CD spectra of commercial RNase A, expressed wild-type, K7Q, R10Q, and K7Q plus R10Q mutant enzymes are essentially identical. Comparison of the RNase A spectrum with those of the studied RNase A mutants indicates that all spectra follow a very similar pattern consistent with the data reported in the literature (Pflumm and Beychok, 1969; Strickland, 1972) and that these proteins have not undergone any major change in their overall secondary structure (results not shown).

Kinetic Studies—Activity of expressed proteins was assessed by activity-staining gels containing 0.6 mg/ml poly(C). By incubating equal amounts of each enzyme the relative activity toward poly(C) was compared (Fig. 4). A similar band width for commercial and recombinant RNase A was observed. Under the same conditions R10Q and K7Q displayed only a slightly reduced activity whereas the double mutant showed a clearly impaired activity.

Kinetic Parameters for C>p, CpA, and poly(C)—Table II summarizes the kinetic parameters obtained by the spectrophotometric method of wild-type and mutant proteins for these substrates. Under the experimental conditions used, recombinant wild-type RNase A displayed the same kinetic parameters for all tested substrates as commercial RNase A. Values of K_m for C>p hydrolysis and for CpA transphosphorylation were similar for all studied proteins, indicating that the binding of these substrates is not affected by the loss of the positive residues at positions 7 and 10. However, the K_m values for a long-chain substrate, such as poly(C), are clearly increased in the studied mutants, indicating that residues Lys-7 and Arg-10 are involved in the binding of long substrates. The catalytic activity (k_{cat}) for C>p, CpA, and poly(C) is significantly reduced for K7Q and R10Q mutants and even further for K7Q R10Q double mutant.

Synthetic Activity using C>p as Substrate—Fig. 5 shows the rate of CpC>p formation by RNase A and the expressed mutants. The synthetic activity is reduced for the two single mutants studied. In the case of the double mutant K7Q plus R10Q, practically no synthetic activity was detected, even when the enzyme concentration was increased 5-fold.

Chemical Modification by cl⁶RMP—Reaction between cl⁶RMP and K7Q, R10Q and K7Q plus R10Q produced in each case a major derivative analogous to Derivative II. The yields of derivative formation with each protein are indicated in Table III.

DISCUSSION

The work reported here gives additional support to the existence of the postulated phosphate-binding subsite p_2 (Parés *et al.*, 1980). Moreover, a central role for both Lys-7 and Arg-10 in establishing an electrostatic interaction between the enzyme and a phosphate group of the substrate is confirmed. This support comes from an analysis of two types of experiments where the effect of charge elimination from the enzyme by site-directed mutagenesis is assessed. On one hand, kinetic parameters of the mutated enzymes using both high and low molecular mass substrates are measured; on the other, the effect of the mutations on the extent of Derivative II formation is studied. Glutamines were chosen to replace Lys-7 and/or Arg-10 to maintain the overall volume of the side chain.

The kinetic properties of the mutated enzymes can be explained in terms of the subsite structure of the enzyme, assuming that Lys-7 and Arg-10 are the groups that drive the interaction with the phosphate in p_2. Except for the poly(C) substrate, where the K_m value is significantly increased, the K_m values for the mutants using C>p and CpA as substrates are unchanged. This implies that binding of these latter substrates is not affected by the loss of the positive charges of either Lys-7 and/or Arg-10, as expected from their normal specificity for $B_1R_1p_1$ (in the case of C>p) and $B_1R_1p_1B_2R_2$ (for CpA). Only the binding of poly(C) should include salt bridge interactions at p_2. However, important differences are found in the k_{cat} values of both single and double mutants with all substrates employed. This is, in principle, surprising at least for the low molecular mass substrates, which do not interact in p_2. A similar but slighter effect was described in the kinetic studies with Derivative II (Parés *et al.*, 1980); in this case a partial reduction of net positive charge in p_2 occurs as a consequence of electrostatic interactions with the label.

Different studies have suggested a role for Lys-7 in the catalytic mechanism of RNase A. Karplus' group (Brünger *et al.*, 1985), from molecular dynamics calculations, indicated that Lys-7 is displaced toward the ligand on CpA and uridine vanadate (a transition state analog of C>p) binding relative to the free enzyme, although it has no direct interaction with the ligands; they indicated a possible role for this residue in stabilizing the transition state through an intervening water network. Kinetic determinations with C>p as substrate using an S-peptide analog bearing the Lys-7/diaminobutyric acid replacement showed that the K_m value is unaffected by the substitution whereas the k_{cat} value for the Dab-7 replacement is lower than that for RNase S (Filippi *et al.*, 1987).

TABLE II

Kinetic parameters of commercial RNase A and mutants of RNAse A for C > p, CpA, and poly(C)[a]

Enzyme	C > p			CpA			poly(C)		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	Relative V_{max}/(Eo)[b]	Relative V_{max}/(Eo)/K_m
	mM	min^{-1}	$M^{-1}\ s^{-1}$	*mM*	s^{-1}	$M^{-1}\ s^{-1} \times 10^5$	*mg/ml*	%	
RNase A	0.88 ± 0.06	180 ± 20	3409.0	0.68 ± 0.07	213 ± 13	3.14	0.35 ± 0.10	100	285.7
R10Q	0.90 ± 0.05	40 ± 5	740.7	0.70 ± 0.07	123 ± 8	1.75	0.65 ± 0.03	35	53.8
K7Q	0.93 ± 0.09	27 ± 2	483.9	0.75 ± 0.06	122 ± 15	1.63	0.97 ± 0.05	36	37.1
K7Q plus R10Q	0.95 ± 0.10	11 ± 1	193.0	0.85 ± 0.10	70 ± 4	0.83	1.26 ± 0.10	6	4.8

[a] Measurements were carried out at 25 °C in 0.2 M sodium acetate, pH 5.5 (see text for experimental details).
[b] (Eo) is the final enzyme concentration. The values for commercial RNase A are taken as 100.

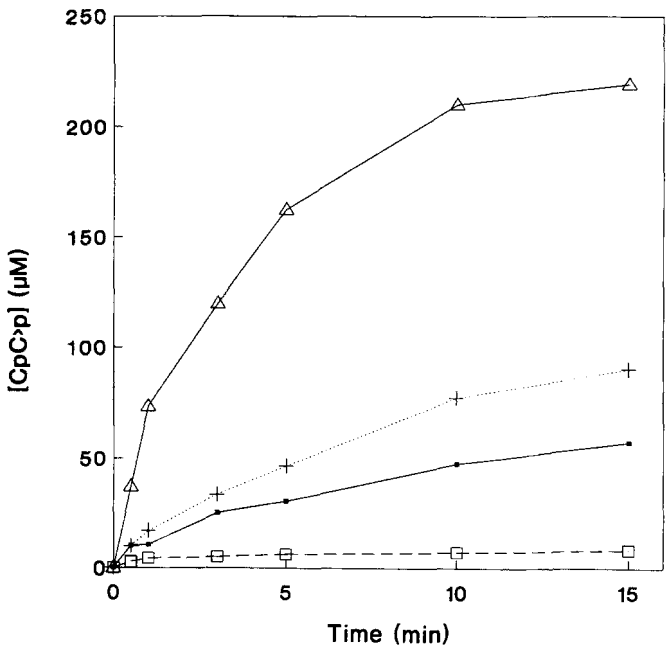

FIG. 5. **Progress curves for CpC>p formation catalyzed by RNase A (△), R10Q (+), K7Q (■), and K7Q plus R10Q (□).** No significant difference was found between the progress curves corresponding to commercial and expressed recombinant RNase A. Experimental conditions are as described under "Materials and Methods."

TABLE III

Yields of Derivative II for commercial and wild-type RNase A and of the major derivative formation for RNase A mutants. Derivative II was obtained by reaction of RNase A with cl⁶RMP as described under "Materials and Methods"

Enzyme	Reaction yield[a]
	%
RNase A	80
Wild-type	76
R10Q	44
K7Q	52
K7Q plus R10Q	39

[a] Yield of derivative formation in relation to initial enzyme.

X-ray crystallographic studies of the complexes between either RNase A or RNase S with mononucleotides and dinucleoside substrate analogs only give information about the active site of the enzyme (Wlodawer, 1984), although Borkakoti (1983) observed that in the complex between 2′-CMP and RNase A the Lys-7 side chain is repositioned. Important support for the existence of multiple binding subsites in RNase A, and specifically about the role of Lys-7, was provided by the crystallographic analysis of the complexes between the protein and deoxyadenylic acid tetramer (McPherson *et al.*, 1986); in such complex the phosphate adjacent to that occupying p_1, *i.e.* p_2, is at 4.76 Å from Lys-7 with which it is likely to form a salt bridge. Recently, the crystal structure of RNase A with bound thymidylic acid tetramer was reported (Birdsall and McPherson, 1992); in this case two pyrimidine nucleotides are bound to the enzyme's active site. No interaction of the phosphate group adjacent in the 3′ direction was observed.

The role of Arg-10 in the p_2-binding subsite is less clear. On one hand, protection studies by 3′-AMP of the reaction between cyclohexane-1,2-dione and Arg-10 suggested a role for this residue on the interaction in p_2 of the phosphate group of the nucleotide (Richardson *et al.*, 1990). On the other hand, crystallographic and ^1H proton nuclear magnetic resonance studies indicated that Arg-10 forms a salt bridge with Glu-2 and that this interaction contributes to the stability of the α-helix extending through His-12 (Strehlow and Baldwin, 1989; Osterhout *et al.*, 1989). This salt bridge is maintained in the crystallographic complex between RNase A and a pentadeoxyoligonucleotide.[2]

The analysis of the synthetic activity, the reversion of the transphosphorylation reaction, indicates that this process is absolutely dependent on an intact p_2 site. Both single mutants retained a partial synthetic activity. However, formation of CpC>p was hardly detectable with the double mutant (Fig. 5). The relative orientation of the two substrate molecules of C>p needed for synthetic activity has not yet been elucidated, but the results strongly suggest that two molecules of C>p must sit in adjacent sites, *i.e.* $B_1R_1p_1$ and $B_2R_2p_2$. The destruction of p_2 by removal of the two positive charges precludes the binding of the second substrate molecule and, hence, abolishes synthetic activity almost totally.

Previous studies (Parés *et al.*, 1980, 1991) indicated that the reaction between cl⁶RMP and RNase A produces Derivative II. It was hypothesized that before the covalent reaction takes place, the phosphate of the label binds in p_2. The corresponding halogenated nucleoside (6-chloropurine riboside) also reacts with the α-NH_2 of Lys-1 of RNase A producing a derivative analogous to Derivative II (Derivative E) but with a much lower yield than the nucleotide (Alonso *et al.*, 1986). The extent of the reaction of RNase A with the nucleoside is similar to that obtained with the nucleotide and the mutated proteins (Table III). Both in the double mutant nucleotide and native RNase A nucleoside systems no electrostatic interaction between the enzyme and the label can be established at p_2. Thus, the extent of formation of either Derivative E (Alonso *et al.*, 1986) or a derivative analogous to Derivative II with the double mutant is a reflection of the rather unspecific reaction of the 6-chloropurine reagent with the α-amino group of Lys-1, the amino group with the lowest p*K* and, therefore, the best nucleophile in RNase A under the

[2] J. Fontecilla-Camps, personal communication.

reaction conditions used. In the single mutants the remaining positive group in p_2 can still anchor the label for reaction with the α-NH_2 of Lys-1.

In conclusion, the substitution of Lys-7 and Arg-10, or both, by Gln residues produces changes in the catalytic and ligand-binding properties of RNase A that are consistent with a role for Lys-7 and Arg-10 as constituents of a cationic cluster that forms the phosphate-binding subsite p_2 adjacent to the catalytic center. Clearly, the modification of this binding subsite does not alter the interaction of the phosphate group of the substrate in the active center (at p_1), although it affects the catalytic efficiency. However, all processes in which an interaction of the phosphate group of the substrate or ligand with the p_2-binding subsite is involved, *i.e.* kinetic parameters for poly(C), synthetic activity, or reaction with cl^6RMP, are significantly altered by this modification. Further studies on the fine structural effects of these substitutions will be carried out to assess the actual role of p_2 in the catalytic mechanism both with low and high molecular mass substrates.

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