

Sulfur Shuffle: Modulating Enzymatic Activity by Thiol-Disulfide Interchange

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The facile modulation of biological processes is an important goal of biological chemists. Here, a general strategy is presented for controlling the catalytic activity of an enzyme. This strategy is demonstrated with ribonuclease A (RNase A), which catalyzes the cleavage of RNA. The side-chain amino group of Lys41 donates a hydrogen bond to a nonbridging oxygen in the transition state for RNA cleavage. Replacing Lys41 with a cysteine residue is known to decrease the value of k_{cat}/K_m by 10^5 -fold. Forming a mixed disulfide between the side chain of Cys41 of K41C RNase A and cysteamine replaces the amino group and increases k_{cat}/K_m by 10^3 -fold. This enzyme, which contains a mixed disulfide, is readily deactivated by dithiothreitol. Forming a mixed disulfide between the side chain of Cys41 and mercaptopropyl phosphate, which is designed to place a phosphoryl group in the active site, decreases activity by an additional 25-fold. This enzyme, which also contains a mixed disulfide, is reactivated in the presence of dithiothreitol and inorganic phosphate (which displaces the pendant phosphoryl group from the active site). An analogous control mechanism could be installed into the active site of virtually any enzyme by replacing an essential residue with a cysteine and elaborating the side chain of that cysteine into appropriate mixed disulfides.

INTRODUCTION

The activities of enzymes *in vivo* are exquisitely controlled by layers of regulatory mechanisms. These modalities are lost *in vitro*. Yet, the technological and commercial roles of enzymatic processes *in vitro* are of greater significance than ever before, with applications ranging from synthetic chemistry (Wong and Whitesides, 1994) to biotechnology (Eun, 1996) to food science (Suckling, 1990). Many practical uses of enzymes are likely to benefit from added degrees of control.

Ribonucleases are enzymes that act at a crossroads of transcription and translation by catalyzing the cleavage of RNA (D'Alessio and Riordan, 1997). Controlling ribonucleolytic activity *in vitro* is especially important in manipulations in which the activity is required in some steps but detrimental in others. The ribonuclease protection assay (RPA),¹ a method for detecting and quantitating RNA transcripts, is a prime example of this sort of manipulation (Zinn et al., 1983; Ausubel et al., 1994).²

Available methods for controlling ribonucleolytic activity tend to suffer from a lack of specificity, efficacy, or permanence. For example, diethyl pyrocarbonate effectively acylates the active-site histidine residues of ribonuclease A [RNase A; EC 3.1.27.5 (Raines, 1998)], destroying its enzymatic activity, but also covalently modifies nucleic acids and other proteins. Ribonucleoside complexes with vanadium(IV) and technetium(V) are rather weak inhibitors of catalysis by RNase A (Lindquist et al., 1973; Chen and Janda, 1992), and the vanadium(IV) complex is prone to air oxidation. In contrast, the complexes of RNase A with 3',5'-pyrophosphate-linked deoxynucleotides have K_d values as low as 27 nM, but are likewise noncovalent (Leonidas et al., 1999; Russo and Shapiro, 1999). Although we and others have synthesized affinity labels and mechanism-based inactivators, the affinity labels bind weakly (Hummel et al., 1987) and the mechanism-based inactivators apparently modify nonessential residues (Stowell et al., 1995). Both types of compounds leave uninhibited a significant proportion of the ribonucleolytic activity.

We have engineered a mechanism of *in vitro* control into RNase A. This method of modulating activity is similar to many natural regulatory mechanisms in that it employs reversible covalent modification. Specifically,

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¹ Abbreviations: CSA, cysteamine; DEAE, diethylaminoethyl; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FAB, fast-atom bombardment; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; MPP, 3-mercaptopropyl-1-phosphate; MS, mass spectrometry; NTB, 2-nitro-5-thiobenzoic acid; NMR, nuclear magnetic resonance; poly(C), poly(cytidylic acid); RNase A, bovine pancreatic ribonuclease A; RPA, ribonuclease protection assay; TEA, triethylamine; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

² In an RPA, a labeled piece of anti-sense RNA is used to probe for an RNA transcript of interest. First, the labeled probe is added to a sample solution and allowed to hybridize with its cognate target RNA. Then, excess single-stranded probe is degraded by treatment with a ribonuclease, such as RNase A. Probe that has hybridized to target RNA is protected from degradation by the ribonuclease by virtue of being in a double-stranded complex with its cognate target RNA. The protected probe is then separated by electrophoresis in a polyacrylamide gel and visualized by autoradiography. If ribonucleolytic activity is not adequately controlled after the degradation step, both prior to and during electrophoresis, the signal resulting from the protected probe will be lost.

we have replaced a Lys41 with a cysteine. Lys41 is known to donate a hydrogen bond to a nonbridging oxygen in the transition state for RNA cleavage (Messmore et al., 1995). The cysteine residue can be used as a "hook" on which to hang activating or deactivating entities. The K41C RNase A variant can be activated by chemical modification with cysteamine (2-aminoethanethiol), thus replacing the primary amine of the wild-type enzyme. Cysteamine can be removed later by gentle treatment with dithiothreitol (DTT) or another reducing agent. An inhibitory phosphoryl group at the end of a thiol-reactive linker can be added to achieve more complete inactivation. A few other enzymes (Pease et al., 1987; Gloss and Kirsch, 1995) and a lectin (Hollenbaugh et al., 1995) have been activated and deactivated by reversible modification of a newly introduced cysteine residue, but further inactivation via additional covalent changes to the cysteine residue is a feature unique to the present work. Similar regimens of transient activation and inactivation may be applicable to other enzymes with critical active-site lysine residues, including other nucleases and phosphotransferases.

EXPERIMENTAL PROCEDURES

Materials. The oligonucleotide used for site-directed mutagenesis was synthesized bearing a trityl group and was purified using an Oligo Purification Cartridge from Applied Biosystems (Foster City, CA). The *Escherichia coli* strain used for protein production, BL21(DE3), was from Novagen (Madison, WI). Ellman's reagent, DTT, Hepes-free acid, Mes-free acid, and cysteamine·HCl were from Sigma Chemical (St. Louis, MO). Poly(cytidylic acid) [poly(C)] was from either Midland Certified Reagents (Austin, TX) or Sigma Chemical. All starting materials for the synthesis of 2,4-dinitrophenyl 3-phosphoryl disulfide were from Aldrich Chemical (Milwaukee, WI), and the purity of these reagents was assessed by ¹H NMR spectroscopy or thin-layer chromatography (TLC) (or both) prior to use.

Spectroscopy and Protein Quantitation. Measurements of ultraviolet and visible absorption were made with a Cary model 3 spectrophotometer from Varian (Sugar Land, TX). Protein concentrations were determined based on the wild-type value of $\epsilon = 0.72 \text{ mL mg}^{-1} \text{ cm}^{-1}$ at 277.5 nm (Sela et al., 1957). This relationship was assumed to be valid for all forms of K41C RNase A studied herein. The veracity of this assumption was supported by DTT treatment of K41C-ntb RNase A (vide infra) yielding the expected value of 1.0 ± 0.2 equiv of 2-nitro-5-thiobenzoic acid (NTB).

Preparation of K41C-(2-nitro-5-thiobenzoic acid) RNase A. A cDNA encoding K41C RNase A (Messmore et al., 1995) was expressed in *E. coli* strain BL21(DE3) under the control of the T7 RNA polymerase promoter. The resulting protein was purified as described previously (delCardayré et al., 1995; Messmore et al., 1995). After purification, the new sulfhydryl group at position 41 was protected from inadvertent reaction by treatment with 5,5'-dithiobis(2-nitrobenzoic acid), Ellman's reagent or DTNB (Ellman, 1959). The protected protein, K41C-ntb RNase A, was separated from any unprotected protein by cation exchange FPLC (MonoS column; Pharmacia, Piscataway, NJ) using a linear gradient of NaCl (0 to 200 mM) in 50 mM Hepes-NaOH buffer (pH 7.7). The protected protein eluted from the column at 60 mM NaCl, and the unprotected K41C protein eluted at 80 mM. The protected protein was stable for several months when stored at 4 °C.

Preparation of K41C-Cysteamine RNase A. The thiol-disulfide interchange reaction between K41C-ntb RNase A and cysteamine (CSA) was initiated by adding cysteamine·HCl (2–10 μL of a 0.010 M solution in 0.10 M Tris-HCl buffer, pH 7.7) to a solution (1.0 mL) of 50 mM Hepes-NaOH buffer (pH 7.7) containing K41C-ntb RNase A (0.3–0.7 mg/mL). The reaction mixture was incubated at 25 °C, and its progress was monitored by following the increase in absorbance at 412 nm, resulting from the release of a stoichiometric quantity of NTB [$\epsilon = 13\,600 \text{ M}^{-1} \text{ cm}^{-1}$ (Ellman, 1959)]. Excess cysteamine and released NTB were removed by subsequent dialysis.

Assay of Poly(cytidylic acid) Cleavage. Ribonucleolytic activity was assessed by using poly(C) as a substrate. Prior to an assay, poly(C) was precipitated from aqueous ethanol (70% v/v), solubilized in assay buffer, and quantitated for total cytidyl concentration by absorbance at 268 nm using $\epsilon = 6200 \text{ M}^{-1} \text{ cm}^{-1}$ (Yakovlev et al., 1992). Assays of poly(C) cleavage were performed at 25 °C in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M). Cleavage of poly(C) was monitored by the increase in absorbance at 250 nm using $\Delta\epsilon = 2380 \text{ M}^{-1} \text{ cm}^{-1}$ (delCardayré and Raines, 1994). Concentrations of poly(C) in assays ranged from 0.02 to 1.2 mM, in terms of individual cytidyl units. Initial rates of cleavage were fitted to the Michaelis-Menten equation with the program HYPERO (Cleland, 1979).

Deactivation of K41C-Cysteamine RNase A with Dithiothreitol. The reduction of the mixed disulfide in K41C-csa RNase A (20 μM) by DTT (20 μM or 0.32 mM) was performed in 50 mM Hepes-NaOH buffer (pH 7.7). The reaction was allowed to proceed at 25 °C, and aliquots were removed periodically to assay for ribonucleolytic activity.

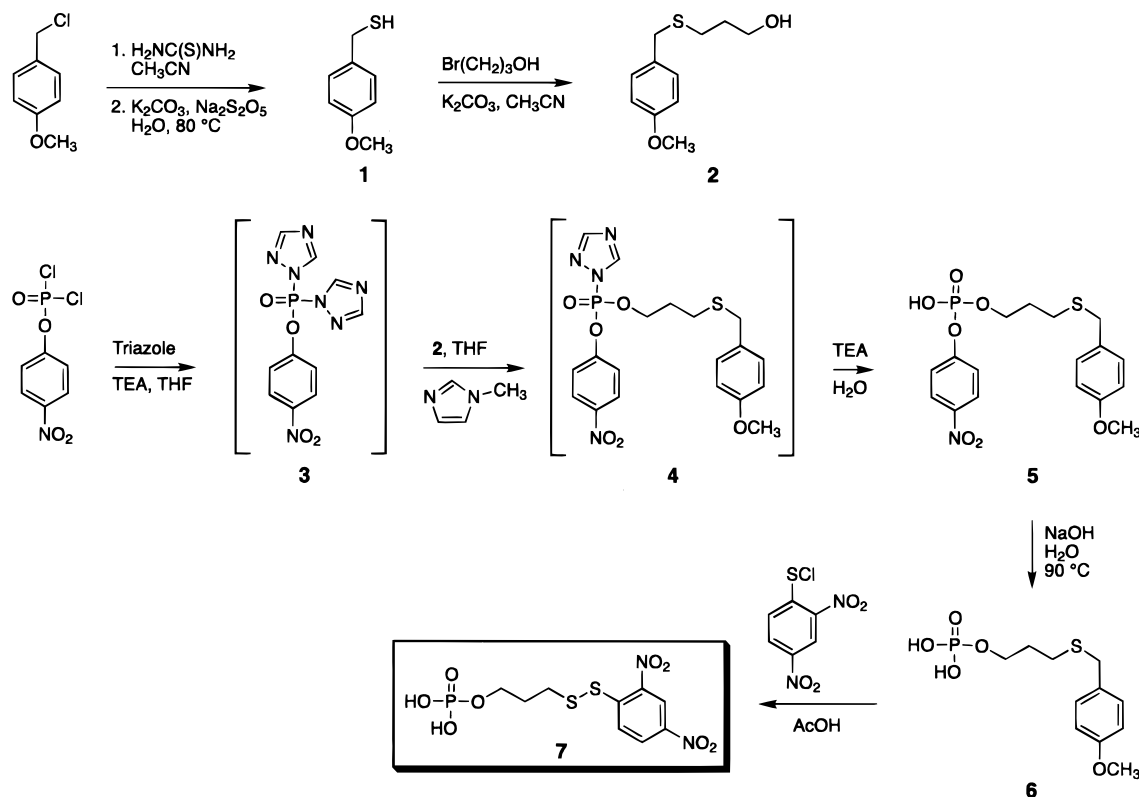
Synthesis of 2,4-Dinitrophenyl 3-Phosphopropyl Disulfide (7). Disulfide 7 was synthesized by the route shown in Scheme 1.

p-Methoxybenzylmercaptan (1). *p*-Methoxybenzylmercaptan was prepared from *p*-methoxybenzyl chloride and thiourea. A 1:1 ratio of the reactants was boiled under reflux and N₂(g) in acetonitrile for 1 h, and then stirred at room temperature. After 12 h, the reaction mixture was concentrated under reduced pressure to give a white solid. To hydrolyze the amidine groups, the solid was then dissolved in an aqueous solution of potassium carbonate and sodium bisulfite, and this solution was heated to 80 °C. The solution was subsequently acidified, and the product was extracted into chloroform, dried over MgSO₄, and evaporated to give a slightly yellow oil. ¹H NMR (CDCl₃, ppm): 7.22–7.25 and 6.86–6.83 (two m, 2H each, -C₆H₄-), 3.79 (s, 3H, -OCH₃), 3.70 (d, $J = 7.4 \text{ Hz}$, 2H, -CH₂SH), 1.73 (t, $J = 7.4 \text{ Hz}$, 1H, -SH).

3-Hydroxypropyl *p*-Methoxybenzyl Thioether (2). Equimolar amounts of *p*-methoxybenzylmercaptan and 3-bromo-1-propanol in acetonitrile were stirred under N₂(g) in the presence of potassium carbonate. The reaction was allowed to proceed, with stirring, overnight at room temperature. Compound 3 was isolated by silica chromatography, with 1:1 ethyl acetate:hexane as the mobile phase, and then evaporated under reduced pressure to give a colorless oil. ¹H NMR (CDCl₃, ppm): 7.21–7.26 and 6.84–6.87 (two m, 2H each, MeOC₆H₄-), 3.80 (s, 3H, -OCH₃), 3.71 (t, $J = 6.1 \text{ Hz}$, 2H, -CH₂OH), 3.68 (s, 2H, -C₆H₄CH₂S-), 2.53 (t, $J = 7.0 \text{ Hz}$, 2H, -SCH₂CH₂-), 1.81 (m, 2H, -CH₂CH₂CH₂-), 1.61 (br s, 1H, CH₂OH).

3-Phosphopropyl *p*-Methoxybenzyl Thioether (6). Compound 6 was synthesized by a method analogous to that of (Kraszewski and Stawinski, 1980). Bis-triazole *p*-nitrophenyl phosphate (3) was generated by the reaction

Scheme 1



of *p*-nitrophenyl-phosphorodichloridate plus triazole and triethylamine (TEA) in tetrahydrofuran under $\text{N}_2(\text{g})$, first at 0°C and then at room temperature, for a total of 30 min. Compound **2** (500 mg; 2.36 mmol) was then added in a 1:3 molar ratio relative to the dichloridate, along with 1.3 equiv of 1-methylimidazole relative to **3**. The reaction was allowed to proceed, still under $\text{N}_2(\text{g})$, for another 30 min. H_2O and TEA were then added to hydrolyze the triazole groups. After 2 h, the reaction mixture was concentrated under reduced pressure to give a yellow solid. The yellow solid was dissolved in chloroform and washed with aqueous NaHCO_3 , then concentrated to give a yellow oil. The yellow oil was dissolved in H_2O and purified by two chromatographic steps, ion exchange (Sephadex SP C-25) and RP-C18 (isocratic; 1:1 MeOH: H_2O mobile phase), to give the phosphodiester **5** in 62% yield from alcohol **2**. MS of **5** (FAB, m/e): 412.1 (calcd for $\text{C}_{17}\text{H}_{19}\text{NO}_7\text{PS}$, 412.06). Phosphodiester **5** was hydrolyzed in 1 N NaOH at 90°C for 16 h, and the resulting phosphomonoester (**6**) was neutralized and purified by anion-exchange chromatographically on DEAE Sephadex resin. Elution from the resin was effected by a linear gradient (0.1 to 1.0 M) of TEA- H_2CO_3 , which was removed as the product, was dried under reduced pressure, yielding **6** in 75% yield from **5**. ^1H NMR (D_2O , ppm): 7.06–7.10 and 6.72–6.76 (two m, 2H each, MeOC_6H_4 -), 3.73 (quartet, $J = 6.4$ Hz, 2H, $-\text{CH}_2\text{OPO}_3\text{H}_2$), 3.61 (s, 3H, $-\text{OCH}_3$), 3.50 (s, 2H, $-\text{C}_6\text{H}_4\text{CH}_2\text{S}-$), 2.37 (t, $J = 7.5$ Hz, 2H, $-\text{SCH}_2\text{CH}_2-$), 1.70 (quintet, $J = 6.6$ Hz, 2H, $-\text{CH}_2\text{CH}_2\text{CH}_2-$). ^{31}P NMR (D_2O , ppm relative to 85% w/v phosphoric acid): 1.36 (s, decoupled).

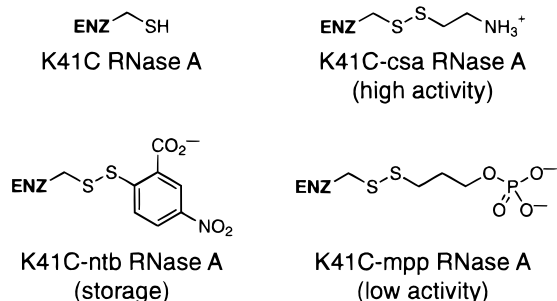
Dinitrophenyl 3-Phosphopropyl Disulfide (7). The simultaneous removal of the *p*-methoxybenzyl group from the sulfur and creation of the disulfide was accomplished by the method of (Johnson et al., 1994). Thioether **6** was stirred at room temperature for 30 min with an equimolar amount of 2,4-dinitrophenylsulfenyl chloride in acetic acid. The reaction mixture was then concentrated by

evaporation and dissolved in methanol. The product was crystallized with the addition of diethyl ether and isolated by filtration to yield disulfide **7** as the free acid in 35% yield from **6**. ^1H NMR (CD_3OD , ppm): 9.04 and 8.58–8.62 [s and m, respectively, 1H and 2H, respectively, $(\text{NO}_2)_2\text{C}_6\text{H}_3-$], 4.06 (quartet, $J = 6.2$ Hz, 2H, $-\text{CH}_2\text{CH}_2-\text{OPO}_3\text{H}_2$), 2.96 (t, $J = 7.2$ Hz, 2H, $-\text{SCH}_2\text{CH}_2-$), 2.05 (quintet, $J = 6.5$ Hz, 2H, $-\text{CH}_2\text{CH}_2\text{CH}_2-$). ^{31}P NMR (D_2O , ppm relative to phosphoric acid): 0.8 (s, decoupled).

K41C-(3-Mercaptopropyl-1-phosphate) RNase A. K41C RNase A was isolated freshly from K41C-ntb RNase A by DTT treatment and cation-exchange chromatography on a mono S column (vide supra). The protein (at $6\ \mu\text{M}$) was then reacted with a 10-fold molar excess of **7**, and then reisolated by cation-exchange chromatography. The addition of the MPP moiety caused the protein to elute from the Mono S column at 45 mM NaCl , rather than at 80 mM NaCl .

Zymogram Assay. Extremely small amounts of ribonucleolytic activity can be detected in Laemmli gels impregnated with RNA or poly(C) (Blank et al., 1982; Kim and Raines, 1993; Bravo et al., 1994). Here, such zymogram assays were performed in a polyacrylamide (18% w/v) gel containing poly(C) (0.5 mg/mL). K41C RNase A (0.4 μg) or K41C-mpp RNase A (0.4 μg) were loaded onto the gel in a nonreducing glycerol loading buffer (<20 μL). The gel was run at 50 mV until the sample had entered the resolving gel, and then the voltage was increased to 200 mV. After electrophoresis, the gel was washed for 5 min per wash, twice with 20% (v/v) 2-propanol, and twice with 0.050 M Tris-HCl buffer (pH 7.0). The gel was then incubated for an additional 30 min in the 0.050 M Tris-HCl buffer. Poly(C) remaining within the gel was stained with Toluidine Blue O (0.2% w/v), which binds only to polymeric nucleic acid, and destained with H_2O . Bands from ribonucleolytic activity where poly(C) is absent show up as clear against a deep blue background.

Scheme 2



RESULTS AND DISCUSSION

RNase A has eight cysteine residues in its amino acid sequence, which produce four disulfide bonds in the native protein. Site-directed mutagenesis has introduced a ninth cysteine at several different positions, including residues 1, 19, and 88 (R.T.R. and co-workers, unpublished results) as well as residue 41 (Messmore et al., 1995). These protein variants all fold properly and in high yield to proteins with a single free sulfhydryl group at the desired position along with the eight native disulfides. The new cysteine residues are available to react with a variety of sulfhydryl-specific reagents. We had shown previously that the replacing Lys41 of RNase A with a cysteine residue reduces k_{cat} by 2×10^4 -fold and k_{cat}/K_m by nearly 10^5 -fold (Messmore et al., 1995). We had also shown that alkylation of Cys41 with haloalkylamines can compensate for much of the lost catalytic activity.

Another type of reaction in which a newly introduced cysteine can participate is thiol-disulfide interchange, which leaves the protein involved in an intermolecular disulfide bond (Faulstich and Heintz, 1995). Thiol-disulfide interchange reactions are among the most facile chemical reactions in biology (Raines, 1997). We have exploited this versatility, vis-à-vis position 41, to modulate the ribonucleolytic ability of RNase A. The various forms of K41C RNase A that have been prepared and studied herein are shown in Scheme 2.

K41C RNase A is unstable if stored for long periods of time, presumably due to oxidation of the Cys41 sulfhydryl group by air. Consequently, we stored the K41C variant only after protecting the Cys41 sulfhydryl group by reaction with DTNB. The resultant NTB-protected protein (K41C-ntb RNase A) contains the first disulfide in which Cys41 will participate. This protein reacts rapidly with cysteamine, releasing 1 equiv of NTB and creating K41C-csa RNase A. This reaction can be monitored by the accompanying increase in absorbance at 412 nm. The reaction of enzyme (25 μM) and a 2.5-fold molar excess of cysteamine was more than half complete after 2 min and more than 80% complete after 6 min. The value of k_{cat}/K_m for poly(C) cleavage for K41C-csa RNase A is 1100-fold greater than that of K41C RNase A. Because all steps prior to cleavage of the P-O^{5'} bond are reversible, the increase in k_{cat}/K_m corresponds to an increase of 4.2 kcal/mol in the free energy of binding to the rate-limiting transition state (Table 1) (Cleland and Northrop, 1999). This rate increase is almost identical to that obtained upon alkylation of Cys41 to form K41[S-(aminopropyl)cysteine] RNase A (Messmore et al., 1995), which has a side chain at position 41 isologous to that of K41C-csa RNase A.

The stability of the intermolecular disulfide bond with cysteamine contrasts sharply with that of the native, intramolecular ones. Approximately 80% of RNase A molecules retain their native disulfide bonds after expo-

Table 1. Steady-State Kinetic Parameters for Catalysis of Poly(cytidylic acid) Cleavage by Wild-Type Ribonuclease A and the K41C and K41C-Cysteamine Variants^a

RNase A	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (M ⁻¹ s ⁻¹)	$\Delta\Delta G^\ddagger$ (kcal/mol) ^b
wild-type	604 ± 47	0.091 ± 0.022	(6.5 ± 1.2) × 10 ⁶	0.0
K41C	0.026 ± 0.004	0.36 ± 0.12	(7.3 ± 1.5) × 10	6.8
K41C-csa	3.6 ± 0.1	0.043 ± 0.009	(8.5 ± 1.5) × 10 ⁴	2.6

^a Data were obtained at 25 °C in 0.10 M Mes-NaOH buffer (pH 6.0) containing NaCl (0.10 M). Data for wild-type RNase A and the K41C variant are from Messmore et al. (1995). ^b $\Delta\Delta G^\ddagger = RT \ln[(k_{\text{cat}}/K_m)_{\text{wild-type}}/(k_{\text{cat}}/K_m)_{\text{variant}}]$.

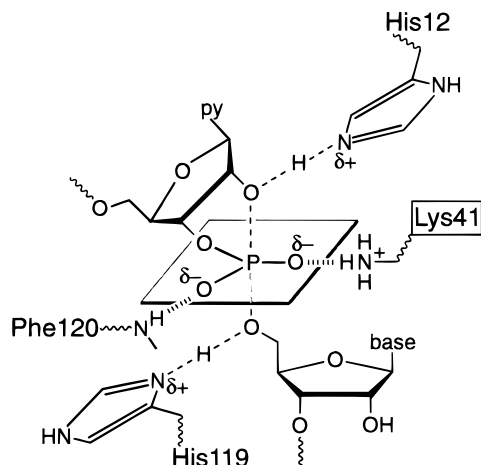


Figure 1. Putative structure of the transition state for catalysis of RNA cleavage by ribonuclease A (Raines, 1998).

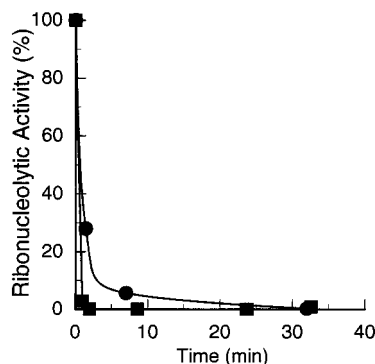


Figure 2. Deactivation of K41C-csa ribonuclease A by dithiothreitol. The reaction was performed at 25 °C in 50 mM HEPES-NaOH buffer (pH 7.7) containing K41C-csa RNase A (20 μM) and DTT [20 mM (●) or 0.32 M (■)].

sure to 0.10 M DTT (1200-fold molar excess) for 4 h at pH 8.0 and 25 °C (Li et al., 1995). We have observed that exposure to ≤ 10 mM DTT (700-fold molar excess) for 45 min at pH 7.8 and 25 °C has little or no effect on the intramolecular disulfide bonds of K41C RNase A, as monitored by enzymatic activity. In contrast, the disulfide bond to cysteamine can be reduced completely with as little as 20 μM DTT (1 M equiv) in less than 30 min. At higher levels of DTT, deactivation is even faster (Figure 2).

The unique reactivity of the sulfhydryl group at position 41 of K41C RNase A can be used as a target for additional modifications leading to more complete inactivation. We took advantage of this reactivity by synthesizing a modification reagent that creates another mixed



Figure 3. Zymogram assay of K41C ribonuclease A and K41C–(3-mercaptopropyl-1-phosphate) ribonuclease A. Lane 1: K41C RNase A (0.4 μ g). Lane 2: 10 μ L of eluent from a Mono S column (negative control). Lane 3: K41C-mpp RNase A (0.4 μ g). Samples were subjected to electrophoresis in a nonreducing SDS–polyacrylamide gel containing poly(C). Ribonucleolytic activity is indicated by a clear band after staining with Toluidine Blue O.

disulfide, altering the protein again in a reversible fashion. [Irreversible alkylation of the cysteine residue comprises another useful option (Lundblad, 1991; Viola and Wright, 1999), which was not explored here.] We chose to investigate the attachment of 3-mercaptopropyl-1-phosphate (MPP) to the sulfur of Cys41. We selected MPP because the propyl linkage would allow the phosphoryl group of MPP to assume a position similar to that of inorganic phosphate or inorganic sulfate observed in crystallographic studies (Wlodawer, 1985) and of the pentavalent phosphoryl group in the transition state for RNA cleavage (Figure 1). To attach MPP, we synthesized 2,4-dinitrophenyl 3-phosphopropyl disulfide (**7**). The synthesis of **7** proceeded in three phases (Scheme 1). In the first two phases, the MPP moiety was made by modifying 3-bromo-1-propanol, first with an appropriately protected thiol and then with a triazolephosphate. After the triazole groups had been removed, the desired disulfide was created by using 2,4-dinitrophenyl-sulfonyl chloride to replace the protecting group on the sulfur of MPP.

Compound **7** reacts readily with Cys41 of K41C RNase A. Reaction with a 10-fold M excess of **7** was 90% complete within 25 min. K41C-mpp RNase A was isolated from any traces of unreacted protein by additional cation-exchange chromatography. Zymogram analysis of K41C-mpp RNase A indicates that this protein has significantly less ribonucleolytic activity than does K41C RNase A (Figure 3).

Spectrophotometric assays of poly(C) cleavage were used in an attempt to quantify the residual catalytic activity. Changes in absorbance were followed over 5 h. The measured rates averaged (4 ± 1)% of the rates for the K41C enzyme under identical conditions and were too slow to obtain the values of steady-state kinetic parameters. Still, if the effect of adding MPP to K41C RNase A is assumed to be only on K_m , the effective molarity of inorganic phosphate can be computed by the equation describing competitive inhibition. Specifically,

$$v = \frac{k_{\text{cat}}[E][S]}{K_m(1 + [I]/K_p)} \quad (1)$$

can be rearranged to give

$$[I] = K_p \left[\left(\frac{k_{\text{cat}}[E]}{v} \right) \left(\frac{[S]}{K_m} \right) - 1 \right] \quad (2)$$

where K_p is the inhibition constant of inorganic phosphate and $[I]$ is the effective molarity of inorganic phosphate at the active site. If the value of K_p is taken to be 4.6 mM (Anderson et al., 1968) and the K41C RNase A kinetic parameters are used (Table 1), the average effective molarity associated with the measured rates of poly(C) cleavage is 0.3 M. This value is $>10^5$ -fold greater

than the actual molarity of the phosphoryl group of MPP in our assays ($[K41C\text{-mpp RNase A}] \leq 3.5 \mu\text{M}$).

Once K41C RNase A has been modified with MPP, noncovalent interactions with the pendant phosphoryl group increased markedly the stability of the intermolecular disulfide bond. DTT and β -mercaptoethanol were used over a range of conditions that are benign to the K41C enzyme in attempts to remove the MPP group from K41C-mpp RNase A. At low and moderate ionic strengths, no increase in activity was observed by zymogram analysis after incubation with up to 2 mM DTT for up to 24 h. Even in the presence of 1 M inorganic phosphate and 0.10 mM DTT, no measurable gain in activity was observable. At 2.5 M inorganic phosphate and 2 mM DTT, a gain of activity indicative of a 20% reversal to K41C RNase A was observed after 90 min.

Conclusion. The unique reactivity of the sulfhydryl group allows for rapid and specific control of enzymatic activity. The activity of K41C RNase A has been increased 1100-fold by modification with cysteamine, and decreased 25-fold by modification with 3-mercaptopropyl-1-phosphate. Enzymatic activity can thus be readily modulated over a range spanning a factor of $10^{4.4}$ -fold. An analogous control mechanism could conceivably be installed into the active site of any enzyme if an essential functional group is available for replacement with a cysteine.

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