 Thermal Stability of *Escherichia coli* Ribonuclease HI and Its Active Site Mutants in the Presence and Absence of the Mg$^{2+}$ Ion

PROPOSAL OF A NOVEL CATALYTIC ROLE FOR Glu$^{48}$

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*Escherichia coli* ribonuclease HI, which requires divalent cations (Mg$^{2+}$ or Mn$^{2+}$) for activity, was thermostabilized by 2.6–3.0 kcal/mol in the presence of the Mg$^{2+}$, Mn$^{2+}$, or Ca$^{2+}$ ion, probably because the negative charge repulsion around the active site was canceled upon the binding of these metal ions. The dissociation constants were determined to be 0.71 mM for Mg$^{2+}$, 0.035 mM for Mn$^{2+}$, and 0.16 mM for Ca$^{2+}$. Likewise, various active site mutants at Asp10, Glu48, Asp70, or Asp134 were thermostabilized by 0.4–3.0 kcal/mol in the presence of the Mg$^{2+}$ ion, suggesting that this ion binds to these mutant proteins as well. The dissociation constants of Mg$^{2+}$ were determined to be 9.8 mM for D10N, 1.1 mM for E48Q, 18.8 mM for D70N, and 1.8 mM for D134N. Thus, the mutation of Asp10 or Asp70 to Asn considerably impairs the Mg$^{2+}$ binding, whereas the mutation of Glu48 to Gln or Asp134 to Asn does not. Comparison of the thermal stability of the mutant proteins with that of the wild-type protein in the absence of the Mg$^{2+}$ ion suggests that the negative charge repulsion between Asp10 and Asp70 is responsible for the binding of the metal cofactor. Glu48 may be required to anchor a water molecule, which functions as a general acid.

Ribonuclease HI from *Escherichia coli* endonucleolytically hydrolyzes the RNA strand of a DNA/RNA hybrid at the P-O3' bond in the presence of an Mg$^{2+}$ ion, which is the most preferable metal cofactor for the enzyme and can be replaced only by an Mn$^{2+}$ ion (Berkower et al., 1973). The optimum pH for the enzymatic activity is around pH 8–9. The enzyme is structurally homologous to the RNase H domain of human immunodeficiency virus 1 (HIV-1) reverse transcriptase (Hostomsky et al., 1993). A similar folding topology has been observed in the structures of other enzymes as well, such as the RuvC Holliday junction resolvase (Ariyoshi et al., 1994), the catalytic domains of the integrases from HIV-1 (Dyda et al., 1994), avian sarcoma virus (Bujacz et al., 1996), and the bacteriophage Mu transposase core (Rice and Mizuuchi, 1995), which are functionally unrelated and have poor sequence similarity. A characteristic common to all of these enzymes is that they require an Mg$^{2+}$ or Mn$^{2+}$ ion for activity and that a cluster of the acidic amino acid residues, typically 3–4 in number, provides the metal ion binding sites. However, none of the catalytic mechanisms of these enzymes is fully understood.

For the catalytic mechanism of *E. coli* RNase HI (EC 3.1.26.4), two alternative mechanisms have been proposed. They are a general acid-base mechanism (Nakamura et al., 1991, Oda et al., 1993, Katayanagi et al., 1993b, Kaishiwaki et al., 1996), which had previously been proposed as a carboxylate-hydroxyl relay mechanism, and a two-metal ion mechanism (Yang et al., 1990). These mechanisms differ from each other in the number of metal ions required for activity and in the activation mechanism of the hydroxyl ion, which attacks the phosphate group for the RNA cleavage. According to the general acid-base mechanism, one metal ion, instead of two, is required for activity, and the attacking hydroxyl ion is activated by an amino acid residue, instead of the metal ion. This discrepancy in the catalytic mechanism mainly results from the observations of different numbers of enzyme-bound metal ions in the crystallographic studies. The RNase H domain of HIV-1 contains two metal ions (Davies et al., 1991), whereas those of *E. coli* RNase HI (Katayanagi et al., 1993b) and the catalytic domain of avian sarcoma virus integrase (Bujacz et al., 1996) contain only one metal ion. Although further studies will be required to determine the catalytic mechanism of the enzyme, the data accumulated thus far suggest that the general acid-base mechanism is more likely than the two-metal ion mechanism. For example, crystallographic (Katayanagi et al., 1993b), NMR (Oda et al., 1991), and kinetic (Huang & Cowan, 1994) studies suggest that only one metal ion binds to the substrate-free enzyme. A kinetic study using inert transition-metal complexes suggests that the RNA hydrolysis does not proceed through a nucleophilic attack by a hydroxyl ion activated by the metal cofactor, but rather through the stabilization of a transient intermediate of an outer sphere complex with the metal cofactor (Jou & Cowan, 1991). A kinetic study using a synthetic substrate modified at the cleavage site also suggests that a metal ion interacts with the 2'-hydroxyl group of the RNA, instead of the phosphate group, to form an outer sphere complex (Uchiyama et al., 1994). Recently, bacteriophage T4 RNase H was shown to have two metal ion binding sites (Mueser et al., 1996). However, this protein is structurally and functionally greatly diverged from *E. coli* RNase HI.

Site-directed mutagenesis experiments revealed that Asp$^{10}$, Glu$^{48}$, Asp$^{70}$, His$^{124}$, and Asp$^{134}$ are involved in the catalytic function (Kanaya et al., 1990a; Oda et al., 1993; Harukuni et al., 1994). The mutation of Asp$^{10}$ to Asn or Ala, Glu$^{48}$ to Gln or Ala, Asp$^{70}$ to Asn or Ala, or Asp$^{134}$ to Ala almost completely abolishes the enzymatic activity. In contrast, the mutation of Asp$^{124}$ to Asn does not seriously affect the enzymatic activity. The mutation of Asp$^{10}$ to Glu, Glu$^{48}$ to Asp, or Asp$^{70}$ to Glu does not completely abolish the enzymatic activity, but dramatically
reduces it. Structural determinations of the mutant proteins with the Asp9→Asn, Glu48→Gln, or Asp70→Asn mutation suggested that the formation of hydrogen bond networks freezes the catalytic residue or prevents the binding of the Mg2+ ion and thereby inactivates the enzyme (Katayanagi et al., 1993a). It was later shown that such hydrogen bond networks are not formed in the Asp9→Asn or Asp70→Asn mutation. Based on these observations, as well as the result that Asp9 and Glu48 are involved in the metal binding (Katayanagi et al., 1993b), a carboxylate-hydroxyl relay mechanism was proposed (Nakamura et al., 1993, Oda et al., 1993, Katayanagi et al., 1993b). In this mechanism, two water molecules act as a general acid and base. Asp70 functions as a proton acceptor and facilitates the attack of the water molecule, which acts as a general base, on the phosphodiester substrate. His124 functions as a proton pump and enhances the catalytic efficiency by removing a proton from Asp70 (Oda et al., 1993). However, the recent determination of the crystal structures of the mutant proteins, D134H, D134N, and D134A, in which Asp134 is replaced by His, Asn, and Ala, respectively, allowed us to revise this mechanism so that His134, instead of Asp70, acts as the proton acceptor (Kashiwagi et al., 1996). The mutant proteins D134H and D134N retain high enzymatic activity (60–90% of the wild-type enzyme), whereas D134A is almost fully inactive (Haruki et al., 1994). Interestingly, the overall structures of these mutant proteins are identical to that of the wild-type protein, and the positions of the δ-polar atoms at residue 134 in the wild-type, D134H, and D134N proteins coincide well with one another. It is therefore likely that this δ-polar atom is required to hold the attacking water molecule. The basis for the proposal of this new catalytic mechanism is the location of the imidazole group of His124. It is located near the δ-polar atom at residue 134 so that they share the attacking water molecule. The mutations of His124 dramatically reduce the catalytic activity, but do not abolish it, because Asp134 can act as a proton acceptor and can substitute for His124 (Kashiwagi et al., 1996).

Like the active site residues of *E. coli* RNase HI, charged residues are often clustered in the active sites of enzymes. Therefore, it seems likely that electrostatic strain caused by the unfavorable interactions causes a local instability in the active sites of the enzymes. In fact, many mutations that alleviate such electrostatic repulsion have been shown to increase the conformational stabilities of proteins such as staphylococcal nuclease (Hibler et al., 1987), pig citrate synthase (Zhi et al., 1991), cellular retinoic acid-binding protein (Zhang et al., 1992), barnase (Meiering et al., 1992), and T4 lysozyme (Shoichet et al., 1985). In addition, a good correlation between an increase in the conformational stability and a decrease in the enzymatic activity has been observed for these active site mutants. These results suggest that the local instability caused by the electrostatic strain makes the active site conformationally flexible, and thereby makes the enzyme functional. This hypothesis is supported by observations that the active site mutants of retinoic acid-binding protein are more thermostable than the wild-type protein in the absence of ligand (retinoic acid), but are not thermostable as the protein-ligand complex (Zhang et al., 1992), and that some of the crystal structures of the active site mutants of T4 lysozyme are similar to that of the enzyme in an enzyme-product complex (Shoichet et al., 1995).

NMR determination of the pKα values of all of the carboxyl groups in *E. coli* RNase HI, in the absence and presence of the Mg2+ ion, indicated that there are strong electrostatic interactions among the four carboxylates in the active site, especially between Asp9 and Asp70, which can be canceled when the Mg2+ ion binds to the protein (Oda et al., 1994). Therefore, it would be informative to analyze the conformational stability of this protein and its active site mutants in the presence and absence of the metal cofactor at an alkaline pH at which the enzyme is functional. If these proteins were stabilized in the presence of the metal cofactor, it would be possible to determine the number of metal ions bound to the protein and the dissociation constant by the method that was used to analyze the binding of anions or cations to RNase T1 (Pace and Grimsley, 1988). Since the role of each active site residue in the catalytic function of the enzyme is not fully understood, such studies would provide important information.

We report that binding of the metal ion or elimination of the carboxyl ion at residue 10, 70, or 134 by mutation, or both, increased the thermal stability of *E. coli* RNase HI by up to ~3 kcal/mol at pH 9, probably due to the cancellation of electrostatic charge repulsion around the active site. We also report that, in contrast to the corresponding mutations of the other three acidic active site residues, the mutation of Glu48 to Gln does not seriously affect either the protein stability or the binding of the Mg2+ ion. Nevertheless, this mutation almost fully inactivates the enzyme. We therefore propose an alternative catalytic mechanism for the enzyme in which Glu48 anchors a water molecule that functions as a general acid.

**EXPERIMENTAL PROCEDURES**

**Materials**—The wild-type *E. coli* RNase HI protein and its active site mutants (D10N, D10A, D10E, E48Q, E48A, E48D, D70N, D70A, D70E, D134H, D134N, and D134A) were previously overproduced and purified (Kanaya et al., 1990a, 1990b). These proteins were stabilized in the presence and absence of 2.0 mg/ml bovine serum albumin by measuring the CD value at 220 nm, using a J-600 spectropolarimeter from Japan Spectroscopic Co. Protein concentrations were determined by UV absorption, using the absorption coefficient 0.55 cm–1 mg–1 ml–1 (Kanaya et al., 1990b). The RNase H activity was determined at 30 °C, in 10 mM Tris-HCl (pH 8.0) containing 10 mM MgCl2, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 10 μg/ml bovine serum albumin by measuring the radioactivity of the acid-soluble digestion product from the 3H-labeled M13 DNA/RNA hybrid (Kanaya et al., 1991).

**Enzymatic Activity**—The RNase H activity was determined at 30 °C, in 10 mM Tris-HCl (pH 8.0) containing 10 mM MgCl2, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 10 μg/ml bovine serum albumin by measuring the radioactivity of the acid-soluble digestion product from the 3H-labeled M13 DNA/RNA hybrid (Kanaya et al., 1991).

**Protein Concentration**—The protein concentrations were determined by UV absorption, using the absorption coefficient A280 of 2.0 (Kanaya et al., 1990b).

**Thermal Denaturation**—Thermal denaturation curves and the temperature of the midpoint of the transition (Tm) were determined as described previously (Katayanagi et al., 1993) by monitoring the change in the CD value at 220 nm, using a J-600 spectropolarimeter from Japan Spectroscopic Co. Proteins were dissolved in 10 mM glycine HCl buffer (pH 3.0) or in 50 mM glycine NaOH buffer (pH 9.0) containing 20% glycerin and 2.8 M urea. The difference in the free energy change for unfolding between the mutant and wild-type proteins (∆G) was calculated as ∆Gmutant – ∆G(wild-type). Since the ∆G(wild-type) value is
FIG. 1. Thermal denaturation curves of E. coli RNase HI. The apparent fraction of unfolded protein is shown as a function of temperature. Thermal denaturation curves were determined in the presence of MgCl2 (A) or NaCl (B) at pH 9.0 by monitoring the change in the CD value at 220 nm, as described under “Experimental Procedures.” Salt concentrations were 0 mM (●), 2 mM (●), 10 mM (△), 20 mM (▲), 50 mM (○), and 0.2 M (□) for MgCl2 and 0 mM (●), 0.2 M (○), 0.5 M (△), 1.0 M (▲), and 1.5 M (□) for NaCl.

RESULTS



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However, they also show that the protein stability did not increase beyond a constant level, at which the protein is stabilized by approximately 10 °C in Tm or 3 kcal/mol in ΔGm, in the presence of low concentrations of these salts except for NaCl. The protein stability was not considerably increased in the presence of a low concentration of NaCl but was increased beyond this level in the presence of a high concentration of NaCl. These results strongly suggest that the mechanism by which the protein is stabilized in the presence of MgCl2, MnCl2, or CaCl2 is different from that in the presence of NaCl and that the protein stability increased in the presence of these salts due to the binding of the divalent cations to the active site of the protein.

The additivity of the stabilization effects of MgCl2 and NaCl was analyzed by measuring the thermal denaturation curve of the protein in the presence of both of these salts. The protein was stabilized by either 8.6 or 16.6 °C in Tm in the presence of either 50 mM MgCl2 or 2.5 mM NaCl, respectively. If the stabilization effect of NaCl resulted from the binding of the Na+ ion to the Mg2+ binding site of the protein, no additivity would be observed. However, the protein stability in the presence of both of these salts was much higher than that in the presence of either one of them (Fig. 3). The stabilization effect of the Mg2+ ion in the presence of 2.5 mM NaCl (5.8 °C in Tm) was slightly lower than, but comparable with, that in the absence of NaCl (8.6 °C in Tm). These results strongly suggest that the stabilization of the protein in the presence of the high concentration of NaCl is not primarily due to the binding of the Na+ ion to the Mg2+ binding site but mainly results from the weak binding of the chloride ion to the protein and/or an increase in the internal hydrophobic interactions of the protein. It seems unlikely that there is an additional cation binding site in the protein because E. coli RNase HI is a basic protein with a pI value of 9.0 (Kanaya et al., 1989) and is rich in basic amino acid residues.

The number of metal ions bound to the protein (Δn) was calculated from the least-squares line of the plot of ΔG versus aL (activity of the metal ion) data (Fig. 4) as 0.84 ± 0.04 for Mg2+, 1.10 ± 0.10 for Mn2+, and 0.95 ± 0.32 for Ca2+. These results support the previous observation (Katayanagi et al., 1993b; Oda et al., 1991) that only one metal ion binds to the active site of the substrate-free enzyme. The dissociation constants were also calculated by using the equation of Schellman (1975) by assuming the Δn value as 1.0 so that the calculated data fit the experimental data well, as shown in Fig. 5. They were 0.71 mM for Mg2+, 0.265 mM for Mn2+, and 0.16 mM for Ca2+ in the units of the ion activities of these salts. Thus, among these divalent cations, the Mn2+ ion binds most strongly to the protein.

Thermal Stabilities of the Mutant Proteins—The pK values of the carboxyl groups in the active site of E. coli RNase HI have been reported to be 6.1 for Asp10, 4.4 for Glu48, 2.8 for Asp70, and 4.1 for Asp314 (Oda et al., 1994). Therefore, the repulsive forces among these carboxyl groups must be the strongest at pH values above 6.1, whereas they must be weakened at pH values lower than 6.1 and almost completely abolished at a pH below 2.6. To determine whether the acidic active site residues contribute equally to the negative charge repulsion in the active site at alkaline pHs, the thermal stabilities of 13 active site mutants (five for Asp10, three for Glu48 and Asp70, and two for Asp314) were analyzed at both pH 3.0 and 9.0 in the absence of the metal cofactor. It has previously been reported that the thermal unfolding of the protein is fully reversible at pH 3.0 in the absence of denaturants (Kanaya et al., 1993). If the mutation increased the protein stability at pH 9.0, without seriously affecting it at pH 3.0, the stabilization of the protein would mainly result from the elimination of the electrostatic...
repulsion around the active site. Alternatively, if the mutation equally increased the protein stability at either pH, the stabilization of the protein would occur for other reasons. The results are summarized in Table I. All the Asp\textsuperscript{10} mutant proteins, except for D10N, were more stable than the wild-type protein at both pH 9.0 and 3.0. However, the increment in the protein stability at pH 9.0 (1.96–4.05 kcal/mol in ΔG) was much larger than that at pH 3.0 (0.44–2.42 kcal/mol in ΔG) for these mutant proteins, except for D10E, in which the number of carboxyl groups in the active site is unchanged. Thus, the ionization of Asp\textsuperscript{10} was shown to be responsible for the negative charge repulsion around the active site. Likewise, the ionizations of Asp\textsuperscript{70} and Asp\textsuperscript{134} were each shown to be responsible for the negative charge repulsion around the active site.

**Binding of Mg\textsuperscript{2+} Ion to Active Site Mutants**—To examine whether the elimination of the negative charge at position 10, 48, 70, or 134 affects the binding of the metal cofactor, the thermal stabilities of the mutant proteins were analyzed at pH 9.0 in the presence of 0.2 mM MgCl\textsubscript{2}. If the elimination of the negative charge impaired the binding of the Mg\textsuperscript{2+} ion, the thermal stability of the mutant protein would not be significantly changed in the presence of 0.2 mM MgCl\textsubscript{2}. However, all of these mutant proteins were stabilized by 0.39–3.02 kcal/mol in ΔG in the presence of 0.2 mM MgCl\textsubscript{2} (Table I), indicating that the Mg\textsuperscript{2+} ion also binds to these mutant proteins. It is noted that all of the mutant proteins, except for D10A, D134N, and D134A, were nearly as stable as the wild-type protein under these conditions. As typical examples, in Fig. 6 the thermal denaturation curves of the mutant proteins, D10N, E48Q, D70N, and D134N, are compared with that of the wild-type protein in the absence or presence of the Mg\textsuperscript{2+} ion. These results suggest that the negative charge repulsion in the active site contributes to the destabilization of the protein by approximately 3 kcal/mol in ΔG, and can be canceled by the binding of the metal cofactor, the elimination of the carboxyl groups in the active site by mutation, or by both. Both Asp\textsuperscript{134} mutant proteins were more stable than the wild-type protein even in the presence of the Mg\textsuperscript{2+} ion, probably because this residue is affected by long-range, nonspecific negative charge repulsion, rather than the specific repulsion in the active site. In the vicinity of Asp\textsuperscript{134}, Gln\textsuperscript{131} and Gln\textsuperscript{135} are clustered in addition to other active site residues.

The thermal stabilities of the mutant proteins D10N, E48Q, D70N, and D134N were analyzed in the presence of various concentrations of Mg\textsuperscript{2+} ion to determine the Mg\textsuperscript{2+} ion dissociation constants. These were calculated by employing the Δn value of 1.0 as 9.8 mm for D10N, 1.07 mm for E48Q, 18.8 mm for D70N, and 1.82 mm for D134N in the units of the ion activity of
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Parameters characterizing the thermal unfolding of E. coli RNase HI and its active site mutants

Thermal denaturation curves of the proteins were determined in 50 mM glycine NaOH buffer (pH 9.0) containing 2.8 M urea and 20% glycerol, either in the absence or presence of 0.2 M MgCl₂ as well as in 10 mM glycine HCl buffer (pH 3.0) in the absence of MgCl₂. Tₘ is the temperature of the midpoint of the thermal denaturation transition. ΔTₘ is the change of the Tₘ of the mutant proteins relative to that of the wild-type protein. ΔΔGₘ is the change of the free energy change of unfolding of the mutant proteins relative to that of the wild-type protein at the Tₘ of the wild-type protein, which was calculated as described under “Experimental Procedures.” ΔHₘ is the enthalpy change of unfolding at Tₘ which was calculated by van’t Hoff analysis. ΔTₛ (Mg) and ΔΔGₛ (Mg) are the changes of the Tₛ and ΔGₛ of the proteins, which were determined at pH 9.0 in the presence of MgCl₂ relative to those determined at pH 9.0 in the absence of MgCl₂. Errors, which represent the 67% confidence limits, are within ±0.8 °C in Tₛ.

| Protein  | pH 9.0 | pH 9.0 (+0.2 M MgCl₂) | pH 3.0 |
|----------|--------|-----------------------|--------|
|          | Tₘ     | ΔTₘ                  | ΔΔGₘ         | Tₘ     | ΔTₘ                  | ΔΔGₘ         | Tₘ     | ΔTₘ                  | ΔΔGₘ         |
| Wild-type| 47.0   | 0                    | 57.3 ± 0.0   | 10.3   | 3.01                 | 59.0 ± 0.1  | 95.1   | 0                    | 99.1 ± 0.0  |
| D10N     | 53.8   | 6.8                  | 59.2 ± 0.9   | 4.4    | 1.33                 | 47.4 ± 2.6  | 90.5   | -0.7                 | 94.0 ± 0.7  |
| D10A     | 50.7   | 13.7                 | 64.8 ± 7.5   | 2.34   | 1.30                 | 58.1 ± 8.1  | 105.3  | 2.42                 | 107.7 ± 2.4 |
| D10E     | 50.4   | 3.4                  | 57.3 ± 0.3   | 0.09   | 1.4                  | 51.5 ± 1.5  | 114.6  | 1.12                 | 115.7 ± 1.2 |
| D10S     | 56.2   | 9.2                  | 57.6 ± 0.7   | -0.24  | 1.3                  | 53.8 ± 1.5  | 97.7   | 0.44                 | 98.1 ± 0.4  |
| D10H     | 55.2   | 8.2                  | 56.5 ± 0.8   | -0.42  | 1.3                  | 53.8 ± 1.5  | 97.7   | 1.12                 | 98.8 ± 1.2  |
| D70E     | 48.0   | 1.0                  | 56.6 ± 0.7   | -0.21  | 8.6                  | 49.8 ± 0.2  | 99.1   | -0.06                | 100.1 ± 0.06|
| E48Q     | 46.1   | -1.0                 | 56.1 ± 1.2   | -0.36  | 10.1                 | 49.8 ± 0.2  | 91.6   | -0.06                | 92.6 ± 0.06 |
| E48D     | 46.2   | -0.8                 | 56.6 ± 0.7   | -0.21  | 10.4                 | 50.8 ± 0.8  | 103.1  | 0.23                 | 104.3 ± 0.23|
| D70N     | 52.5   | 5.5                  | 56.1 ± 1.2   | 0.36   | 3.7                  | 48.8 ± 1.2  | 110.1  | -0.35                | 111.4 ± 0.35|
| D70A     | 50.8   | 3.8                  | 56.4 ± 0.9   | -0.27  | 5.6                  | 49.7 ± 0.3  | 85.5   | -0.09                | 86.5 ± 0.09 |
| D70E     | 47.4   | 0.4                  | 56.5 ± 0.8   | -0.24  | 9.1                  | 51.8 ± 1.8  | 90.4   | 0.53                 | 91.9 ± 0.53 |
| D134N    | 53.4   | 6.4                  | 60.8 ± 3.5   | 1.08   | 7.4                  | 49.7 ± 0.3  | 110.5  | -0.09                | 111.6 ± 0.09|
| D134A    | 53.8   | 6.8                  | 61.0 ± 3.7   | 1.14   | 7.2                  | 52.9 ± 2.9  | 104.8  | 0.85                 | 106.6 ± 0.85|

Fig. 6. Thermal denaturation curves of the wild-type and mutant proteins. Thermal denaturation curves were determined at pH 9.0 in the absence (A) and presence (B) of 0.2 M MgCl₂, as described in the legend for Fig. 1. ○, wild-type; △, D10N; ●, E48Q; ◊, D70N; ▲, D134N.

Fig. 7. Plot of ΔΔG as a function of the concentration of added MgCl₂. The ΔΔG values were calculated from the ΔTₛ values, which were determined from the thermal denaturation curves such as those shown in Fig. 6, as described under “Experimental Procedures.” Symbols are the same as in Fig. 6. The lines represent the best fit to the experimental data calculated according to the equation given by Schellman (1975) by using the Δn value of 1.0 and Mg²⁺ dissociation constants of 9.8 mM for D10N, 1.07 mM for E48Q, 18.8 mM for D70N, and 1.82 mM for D134N. The ΔΔG value is the difference between the ΔG value of the wild-type or the mutant protein either in the presence or absence of the Mg²⁺ ion and the ΔG value of the wild-type protein in the absence of the Mg²⁺ ion.
Stability-Activity Relationship—Amino acid residues that are involved in catalytic function or substrate binding are not necessarily optimized for stability. In fact, in a number of enzymes that have been examined, catalytic residue mutations that almost fully abolish the enzymatic activity frequently increase the protein stability, probably due to the elimination of unfavorable electrostatic interactions. Examples of such mutations are Glu11 to Phe, Met, and Ala and Asp220 to Asn, Thr, and Ser for T4 lysozyme (Shoichet et al., 1995); Lys77 to Ala and His102 to Ala for barnase (Meiering et al., 1992); and His274 to Gly and Arg and Asp375 to Gly, Asn, and Gln for pig citrate synthase (Zhi et al., 1991). When E. coli RNase HI was examined for this type of a stability-activity relationship by site-directed mutagenesis experiments, among the mutations at the four acidic active site residues, those at Asp10, Asp70, and Asp134, which almost fully inactivate the enzyme, increase the protein stability by 1.08–4.05 kcal/mol in the absence of the Mg2+ ion. In contrast, the mutation of Glu48 to Gln or Ala, which almost fully inactivates the enzyme, does not seriously affect the protein stability. Since these mutant proteins with different types of amino acid substitutions could not be distinguished from each other in terms of the protein stability, it seems unlikely that the increase in the stability, due to the elimination of the carboxyl group at this position, is fully canceled by the decrease in the stability, due to the introduction of a given residue. In fact, crystallographic studies have previously shown that the conformation of the protein is not seriously changed by the mutation of Glu48 to Gln (Katayanagi et al., 1993a). Furthermore, the observation that the carboxyl group of Glu48 has the normal pKₐ value of 4.4 (Oda et al., 1994), suggests that this carboxylate is not affected either by specific electrostatic interactions in the active site or by long-range nonspecific electrostatic interactions.

Metal Binding Site—It has been reported that, unlike the Ca²⁺ binding site, the Mg²⁺ binding site is partially buried in a shallow cleft of the protein molecule and uses solvent oxygens to coordinate one hemisphere of the bound metal ion (Needham et al., 1993). This site, therefore, can easily vary its size to accommodate various sizes of metal ions and, thereby, exhibits less selectivity for metal ions. In the current study, we showed that Mg²⁺, Ca²⁺, and Mn²⁺ ions bind to E. coli RNase HI with dissociation constants of 0.7 mM, 0.16 mM, and 0.035 mM, respectively. This indicates that the Mg²⁺ ion binds most weakly and the Mn²⁺ ion binds most tightly to the protein. These differences reflect the differences in the concentrations of these ions required for optimal enzymatic activity. The optimal concentrations of these ions for enzymatic activity were previously reported to be 2 to 4 mM for Mg²⁺ and <0.2 mM for Mn²⁺ (Berkower et al., 1973). However, the enzymatic activity of E. coli RNase HI in the presence of 0.2 mM MnCl₂ is much less (7.5%) than that in the presence of 4 mM MgCl₂ (Berkower et al., 1973). In addition, little enzymatic activity is observed in the presence of 0.1–10 mM CaCl₂. Thus, the metal binding site of E. coli RNase HI is highly selective with respect to the enzymatic function, although it does not provide a constrained ion cavity. It remains to be determined why the enzyme prefers Mg²⁺ for activity.

Conformational Change of Glu48 upon Mg²⁺ Binding.—The determination of the crystal structure of E. coli RNase HI complexed with the Mg²⁺ ion previously showed that the carboxyl group of Glu48 directly coordinates with the Mg²⁺ ion (Katayanagi et al., 1993b). The conformations of the active site residues in E. coli RNase HI complexed with the Mg²⁺ ion are shown superimposed upon those in the Mg²⁺-free enzyme in Fig. 8. Upon the binding of the Mg²⁺ ion, the side chain of Glu48 moves by 1.87 Å so that the distance between the carboxyl group and the Mg²⁺ ion is reduced to 2.4 Å. In contrast, the distances between the other carboxyl groups and the Mg²⁺ ion remain almost constant. The distances from the Mg²⁺ ion are 2.1 Å to Asp10, 4.4 Å to Asp70, and 5.4 Å to Asp134. Based on these results, it has been proposed that Glu48 is not involved in the catalytic function but is responsible for the Mg²⁺ binding. However, the present studies of the thermal stabilities of the E. coli RNase HI active site mutants do not support this proposal because they suggest that the binding of the Mg²⁺ ion is not seriously affected by the elimination of the carboxyl group at residue 48. It seems likely, therefore, that the carboxyl group of Glu48 is not responsible for Mg²⁺ binding but moves toward the bound Mg²⁺ ion to coordinate with it, probably by an electrostatic attraction. The observations that the pKₐ values of Asp10 and Asp70 shift from 6.1 to 4.2 and from 2.6 to 3.4, respectively, upon the binding of the Mg²⁺ ion to the protein, whereas those of Glu48 and Asp134 do not (Oda et al., 1994) support our proposal that Glu48 is not involved in Mg²⁺ binding. In addition, the findings that Asp64 and Asp221, in the catalytic domain of avian sarcoma virus integrase (Bujacz et al., 1996), and Asp443 and Asp408, in the RNase HI domain of HIV-1 reverse transcriptase (Davies et al., 1991) coordinate a metal cofactor support our proposal because these residues correspond to Asp10 and Asp70 in E. coli RNase HI, respectively. Then, the question arises as to the role of Glu48 in the catalytic function of the enzyme. Why does the mutation of this residue to Gln or Ala almost fully abolish the enzymatic activity?

Role of Glu48 in the Catalytic Function—According to the latest model for the catalytic mechanism of E. coli RNase HI, His102 accepts a proton from the water molecule (water molecule B), which acts as a general base; Asp10 assists it by correctly positioning the water molecule B; Asp70 anchors the water molecule (water molecule A), which acts as a general acid; and Asp10 and Glu48 coordinate with the Mg²⁺ ion (Kashiwagi et al., 1996). However, our results strongly suggest that repulsive forces between the negatively charged side chains of Asp10 and Asp70 are responsible for the binding of the Mg²⁺ ion to the correct position in the active site of the enzyme. Therefore, Glu48, instead of Asp70, may be required to anchor...
the water molecule A. When the water molecule A supplies a proton to the 3′-oxygen group of the RNA product, it is converted into the hydroxyl ion. This hydroxyl ion should be promptly removed to facilitate the next cleavage reaction cycle. Since the acidic nature of Glu48 is responsible for the enzymatic activity and because Glu48 is located close to the water molecule A in a model for the enzyme-substrate complex (Iwai et al., 1995), this residue may be required to eject the hydroxyl ion from the active site due to the negative charge repulsion. We propose that the negatively charged side chain of Glu48 moves to the functional position upon Mg2+ binding, in which it anchors the water molecule A, and facilitates the replacement of a negatively charged hydroxyl ion with a neutral water molecule during the catalytic cycle (Fig. 9).

Recently, the double mutant protein D10R/E48R, in which Asp10 and Glu48 are both replaced by Arg, was shown to retain 87% of the enzymatic activity of the wild-type protein in the absence of the Mg2+ ion (Casareno et al., 1995). This mutant protein was constructed to examine whether mutations of the acidic amino acid residues that are responsible for the Mg2+ binding, to Arg or Lys, provide sufficient positive charge density in the active site to mimic the role of the divalent cation. This result shows that Glu48 is not related to the catalytic function, and it does not support our proposal. However, the optimal pH of the enzymatic activity of the mutant protein greatly shifted to lower values (pH 5.5) as compared with that of the wild-type protein. At an acidic pH, the hydroxyl ion, which is produced when the 3′-oxygen group of the RNA product extracts a proton from the water molecule A, is probably easily replaced by a water molecule without the assistance of an acidic amino acid residue such as Glu48. Nevertheless, studies on the catalytic function of the mutant protein D10R/E48R would provide useful information about the catalytic mechanism of the wild-type enzyme. It would be informative to examine whether the shift in the pH optimum of the enzymatic activity of the mutant protein reflects the shift in the pKₐ value of His124.

Stability of D10A—The mutant protein D10A is exceptionally more stable than the wild-type protein at both pH 3.0 and 9.0. The Tₘ value of this mutant protein is the highest among those of the E. coli RNase HI variants with single amino acid substitutions constructed thus far (Ota et al., 1995). The near ultraviolet circular dichroism spectrum of this mutant protein is similar to that of the wild-type protein, indicating that the tertiary structure of the protein is not markedly changed by the mutation. The ratio of the aqueous surface area in the folded state to that in the unfolded state was calculated for Asp10 as 13.4% (Ou et al., 1987; Ooi and Oobatake, 1991), indicating that this residue is almost fully buried inside the protein molecule. Since other mutant proteins in which Asp10 is replaced by polar residues are less stable than D10A, the dramatic thermostability of the mutant protein D10A may be due to an increase in the hydrophobic interactions. Further studies, such as crystallographic analyses, will be necessary to understand the stabilization mechanism of this mutant protein.

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REFERENCES

Ariyoshi, M., Vassilev, D. G., Iwaski, H., Nakamura, H., Shinagawa, H., and Morikawa, K. (1994) Cell 78, 1063–1072
Berkower, I., Leis, J., and Hurwitz, J. (1973) J. Biol. Chem. 248, 5914–5921
Bujard, G., Jaskolski, M., Alexandratos, J., Wlodawer, A., Merkel, G., Katz, R. A., and Skalka, A. M. (1996) Structure 4, 89–96
Casareno, R. L. B., Li, D., and Cowan, J. A. (1995) J. Am. Chem. Soc. 117, 11011–11012
Davies, J. F., Hostokzka, Z., Hostoksky, Z., Jordan, S. R., and Matthews, D. A. (1991) Science 252, 88–95
Dyda, F., Hickman, A. B., Jenkins, T. M., Engelman, A., Craige, R., and Davies, D. R. (1994) Science 266, 1981–1986
Haruki, M., Nagushi, E., Nakai, C., Liu, Y.-Y., Oobatake, M., and Kanaya, S. (1994) Eur. J. Biochem. 220, 623–631
Hilber, D. W., Slodowich, N. J., Reynolds, M. A., and Gerli, R. G. A. (1987) Biochemistry 26, 6278–6286
Hostoksky, Z., Hostoksky, Z., and Matthews, D. A. (1993) in Nucleases (Linn, S. M., and Roberts, R. J., eds) 2nd Ed., pp. 341–376, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Huang, H.-W., and Cowan, J. A. (1994) Eur. J. Biochem. 219, 253–260
Iwai, S., Kato, S., Nakas, M., Ohtsuka, E., and Kanaya, H. (1995) FEBS Lett. 368, 315–320
Jou, R., and Cowan, J. A. (1991) J. Am. Chem. Soc. 113, 6685–6688
Kanaya, S., Kehara, A., Miyagawa, M., Matsuoka, T., Morikawa, K., and Ikehara, K. (1989) Biochemistry 28, 11456–11549
Kanaya, S., Kehara, A., Miura, Y., Sekiguchi, A., Iwai, S., Inoue, H., Ohtsuka, E., and Ikehara, M. (1996a) J. Biol. Chem. 261, 4615–4621
Kanaya, S., Kimura, S., Katsuda, C., and Ikehara, M. (1996b) Biochem. J. 327, 59–66
Kanaya, S., Katsuda, C., Kimura, S., Nakai, T., Kitakuni, E., Nakamura, H., Katayanagi, K., Morikawa, K., and Ikehara, M. (1991) J. Biol. Chem. 266, 6038–6044
Kanaya, S., Oobatake, M., Nakamura, H., and Ikehara, M. (1993) J. Biol. Chem. 268, 117–136
Kashiwagi, T., Jeanteur, D., Haruki, M., Katayanagi, M., Kanaya, S., and Morikawa, K. (1996) Protein Eng., in press
Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Ikeda, M., Matsuzaki, T., and Morikawa, K. (1990) Nature 347, 306–309
Katayanagi, K., Ishikawa, M., Okumura, M., Ariyoshi, M., Kanaya, S., Kawanoy, S., Suzuki, M., Tanaka, I., and Morikawa, K. (1995a) J. Biol. Chem. 269, 22099–22099
Katayanagi, K., Okumura, M., and Morikawa, K. (1995b) Proteins Struct. Funct. Genet. 17, 337–346
Kimura, S., Nakamura, H., Hashimoto, T., Oobatake, M., and Kanaya, S. (1992) J. Biol. Chem. 267, 21538–21542
Lide, R. D. (ed) (1994) CRC Handbook of Chemistry and Physics, pp. 5–94, CRC Press Inc., London
Meiering, E. M., Serrano, L., and Fersht, A. R. (1992) J. Mol. Biol. 225, 585–589
Miller, J. H. (ed) (1972) Experiments in Molecular Genetics, p. 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Morikawa, K. (1996) Experiments in Molecular Genetics, Cold Spring Harbor, NY
Mueuer, T. C., Nosal, N. G., and Hyde, C. C. (1996) Cell 85, 1101–1112
Nakamura, H., Oda, Y., Iwai, S., Inoue, H., Ohtsuka, E., Kanaya, S., Kimura, S., Katsuda, C., Kato, S., Nakamura, H., and Ikehara, M. (1999a) J. Biol. Chem. 274, 11791–11798
Oda, Y., Yoshida, M., and Kanaya, S. (1993) J. Biol. Chem. 268, 88–92
Oda, Y., Yamazaki, T., Nagayama, K., Kanaya, S., Kuroda, Y., and Nakamura, H. (1994) Biochemistry 33, 5275–5284
Ooi, T., Oobatake, M., Nemethy, G., and Scheraga, H. A. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 3086–3090

9. The Tₘ value of this mutant protein is the highest among those of the E. coli RNase HI variants with single amino acid substitutions constructed thus far (Ota et al., 1995). The near ultraviolet circular dichroism spectrum of this mutant protein is similar to that of the wild-type protein, indicating that the tertiary structure of the protein is not markedly changed by the mutation. The ratio of the aqueous surface area in the folded state to that in the unfolded state was calculated for Asp10 as 13.4% (Oui et al., 1987; Ooi and Oobatake, 1991), indicating that this residue is almost fully buried inside the protein molecule. Since other mutant proteins in which Asp10 is replaced by polar residues are less stable than D10A, the dramatic thermostability of the mutant protein D10A may be due to an increase in the hydrophobic interactions. Further studies, such as crystallographic analyses, will be necessary to understand the stabilization mechanism of this mutant protein.

3 S. Kanaya, unpublished data.
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Ooi, T., and Oobatake, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2859–2863
Ota, M., Kanaya, S., and Nishikawa, K. (1995) J. Mol. Biol. 248, 733–738
Pace, C. N., and Grimsley, G. R. (1988) Biochemistry 27, 3242–3246
Record, M. T., Anderson, C. F., and Lohman, T. M. (1978) Q. Rev. Biophys. 11, 103–178
Rice, P., and Mizuuchi, K. (1995) Cell 82, 209–220
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
Schellman, J. A. (1975) Biopolymers 14, 999-1018
Shoichet, B. K., Baase, W. A., Kuroki, R., and Matthews, B. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 452–456
Uchiyama, Y., Miura, Y., Inoue, H., Obitsu, K., Ueno, Y., Ikehara, M., and Iwai, S. (1994) J. Mol. Biol. 243, 782–791
Yamasaki, T., Kanaya, S., and Oobatake, M. (1995) Thermochim. Acta 267, 379–388
Yang, W., Hendrickson, W. A., Crouch, R. J., and Satow, Y. (1990) Science 249, 1388–1405.
Zhang, J., Liu, Z.-P., Jones, T. A., Gierasch, L. M., and Sambrook, J. F. (1992) Proteins Struct. Funct. Genet. 13, 87–99
Zhi, W., Srere, P. A., and Evans, C. T. (1991) Biochemistry 30, 9281–9286