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Binding of metal ions to *E. coli* RNase HI observed by ^{1}H-^{15}N heteronuclear 2D NMR

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SUMMARY

The divalent metal ion binding site and binding constant of ribonuclease HI from *Escherichia coli* were investigated by observing chemical shift changes using ^{1}H-^{15}N heteronuclear NMR. Chemical shift changes were monitored during the titration of the enzyme with salts of the divalent cations. The enzyme was uniformly labeled by ^{15}N, which facilitated the monitoring of the chemical shift change of each cross peak between the backbone amide proton and the amide ^{15}N. The chemical shifts of several amide groups were affected upon the addition of a divalent metal ion: Mg^{2+}, Ca^{2+}, or Ba^{2+}. These amide groups resided close to the active site, consistent with the previous X-ray crystallographic studies. From the titration analysis, a single divalent ion binding site was observed with a weak binding constant ($K_D = 2-4$ mM for the current divalent ions).

INTRODUCTION

A unique endo-ribonuclease named ribonuclease H (RNase H) specifically cleaves the RNA strand of a DNA-RNA hybrid, yielding a 3'-hydroxyl and a 5'-phosphate at the hydrolysis site (reviewed by Crouch and Dirksen, 1982). This enzyme requires specific divalent cations, Mg^{2+} or Mn^{2+}, for its enzymatic activity. In the assay for the enzyme, Mg^{2+} is routinely added (Kanaya and Crouch, 1983). It has been reported that Mg^{2+} can be replaced only by Mn^{2+} to retain enzymatic activity (Berkower et al., 1973).

A series of site-directed mutagenesis experiments of RNase HI from *Escherichia coli* (*E. coli* RNase HI) revealed that Asp^{10}, Glu^{48}, and Asp^{70} are crucial for the enzymatic activity (Kanaya et al., 1990). Recently, the 3D structure of *E. coli* RNase HI has been determined by X-ray crystallography almost simultaneously by two independent groups (Katayanagi et al., 1990; Yang et al.,

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1990). The positions of the bound divalent cation, Mg^{2+}, Ca^{2+}, Ba^{2+}, and Co^{2+}, have been revealed by analyses of crystals soaked in solutions of those metal ions (Katayanagi et al., 1990). A single metal binding site was found for each divalent ion. Each cation is positioned very close to the active site, forming strong ionic interactions with the carboxyls of Asp^{10}, Glu^{48}, Asp^{70}, and Asp^{134}. Note that the enzyme should be designated as RNase HI since a second RNase H (RNase HII) has recently been isolated from *E. coli* (Itaya, 1990).

Metal ions have crucial roles in the activities of many nucleases, including ribozymes. Some nucleases are dependent on metal ions for both binding and cleavage of the substrate. However, the cleavage mechanism, and the role of magnesium ions in the activity of RNase H, remain unknown.

In order to examine the role of metal ions in *E. coli* RNase HI, we performed titrations of RNase HI with divalent cations, and observed changes in the ^{1}H-^{15}N heteronuclear 2D NMR spectra. The complete chemical shifts of the backbone protons and ^{15}N atoms have been previously assigned to the corresponding residues (Nagayama et al., in press; Yamazaki et al., 1991), with the aid of heteronuclear 3D NMR techniques using ^{15}N- and ^{13}C-labeled enzymes (Nagayama et al., 1990). Therefore, by observing changes in position of the cross peaks in the 2D NMR spectra upon titration of the enzyme with metal ions, amino acids involved in metal binding were mapped. Binding constants of the individual residues were also obtained.

MATERIALS AND METHODS

In order to uniformly label the enzyme with ^{15}N, *E. coli* strain N4830-1, in which plasmid pPL801 was cloned (Kanaya et al., 1989), was grown in M9 minimum culture medium including $^{15}NH_4Cl$ (99.8 atom %, Shoko Co. Ltd., Tokyo). Overproduction of the enzyme was induced by increasing the cultivation temperature from 32°C to 42°C. After an additional 4 h cultivation, cells were harvested by centrifugation and subjected to purification procedures, as described previously (Kanaya et al., 1989). About 15 mg purified protein can be obtained from a 1 L culture by this method. Since the strain N4830-1 requires His, Val, and Ile, and these unlabeled amino acids were added to the culture medium, the nitrogens of His in the enzyme were not labeled by ^{15}N, and those of Val and Ile were labeled partially (Yamazaki et al., 1991).

The enzyme and the salts of divalent cations, $MgCl_2$, $BaCl_2$, and $CaCl_2$ (Wako Pure Chem. Ind. Ltd., Osaka), were solubilized in 0.1 M acetate buffer (pH 5.5) of 90% H_2O/10% D_2O (Isotec Inc.). The concentration of the enzyme was 1 mM, and those of the divalent cations were 2–100 mM for $MgCl_2$, and 0.5–50 mM for $CaCl_2$ and $BaCl_2$. In the course of titration, 1–100 μl divalent cation solution was added to 280 μl enzyme solution. All the titration measurements were performed at 27°C.

Direct couplings between amide protons and amide ^{15}N atoms of the enzyme were observed using ^{1}H-detected ^{15}N heteronuclear single-quantum coherence (^{1}H-^{15}N-HSQC) developed by Bodenhausen and Ruben (1980). The pulse sequence proposed by Bax et al. (1990) was employed. The resonance of residual H_2O was suppressed by preirradiation. 2D NMR spectra were acquired with 512 increments in the t_1 direction, and 2048 data points in the t_2 direction. The time domain data were multiplied by a phase shift sine-bell squared window function in both the t_1 and t_2 directions, and zerofilled to 2048 in the t_1 dimension and to 4096 in the t_2 dimension before Fourier transformation. The spectral width of ω_1 (^{15}N resonances) was 2315 Hz, and that of ω_2 (^{1}H resonances) was 7042 Hz.

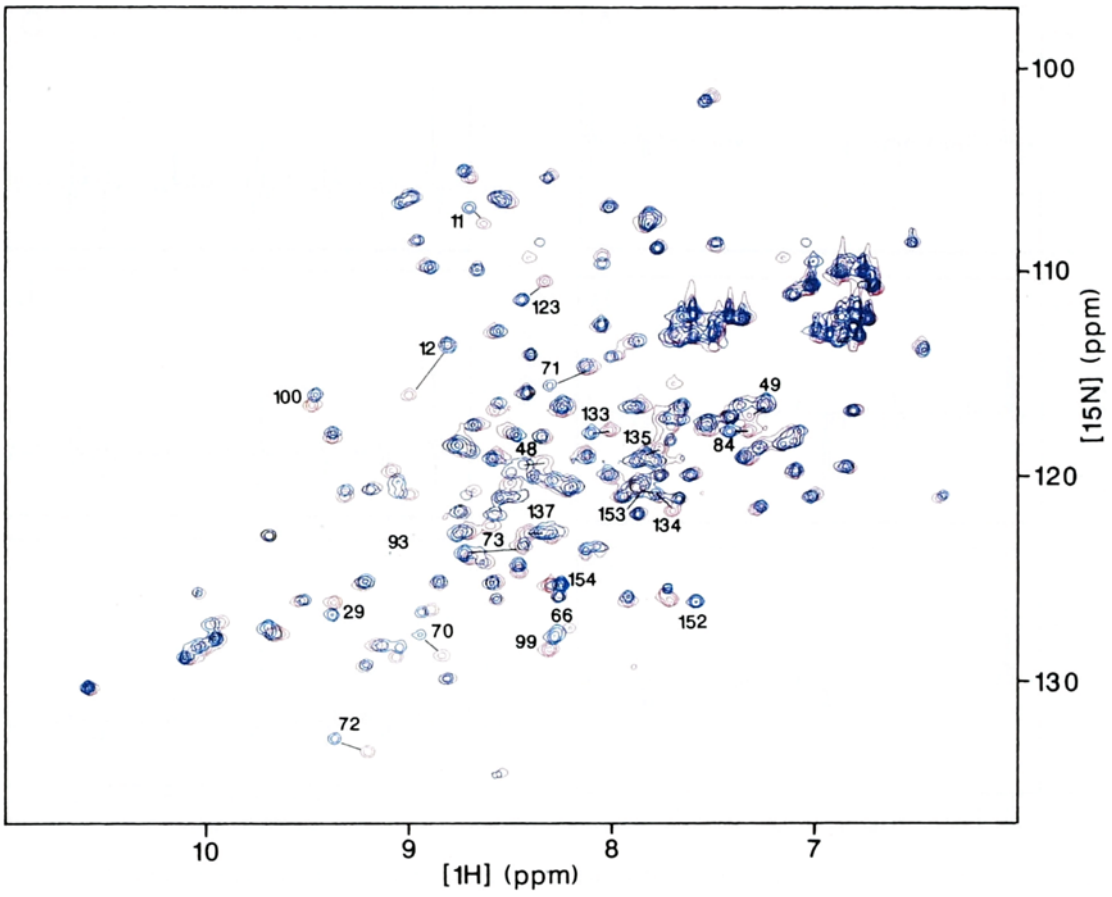

Fig. 1. Backbone amide region in the ^{1}H-^{15}N-HSQC spectrum of uniformly ^{15}N-labeled *E. coli* RNase HI at 27°C (blue). The protein concentration was 1 mM, buffered in sodium acetate, pH 5.5. Red indicates the spectrum in the presence of 100 mM $MgCl_2$. Numbers indicate residues whose cross peaks moved significantly.

All the NMR spectra were measured on a 500-MHz spectrometer (Bruker AM-500). Proton chemical shifts are relative to the water signal [4.78 ppm relative to sodium 3-(trimethylsilyl) propionate]. ^{15}N chemical shifts are relative to the ^{15}N signal of formamide (113.3 ppm).

RESULTS

^{1}H-^{15}N-HSQC spectra of the uniformly ^{15}N-labeled *E. coli* RNase HI solution are shown in Fig. 1, in the course of titration with $MgCl_2$. The cross peaks whose chemical shifts moved more than 0.05 ppm on the ^{1}H axis and/or 0.5 ppm on the ^{15}N axis when 100 mM $MgCl_2$ was added, are labeled in Fig. 1. In all the titrations, only movements of cross peaks were observed, but no cross peaks were broadened nor newly appeared. This indicates the fast exchange between the metal-free enzyme and the metal-bound enzyme.

Figure 2 shows the maximal change ($|\Delta\delta_{max}|$) of each cross peak between the backbone amide

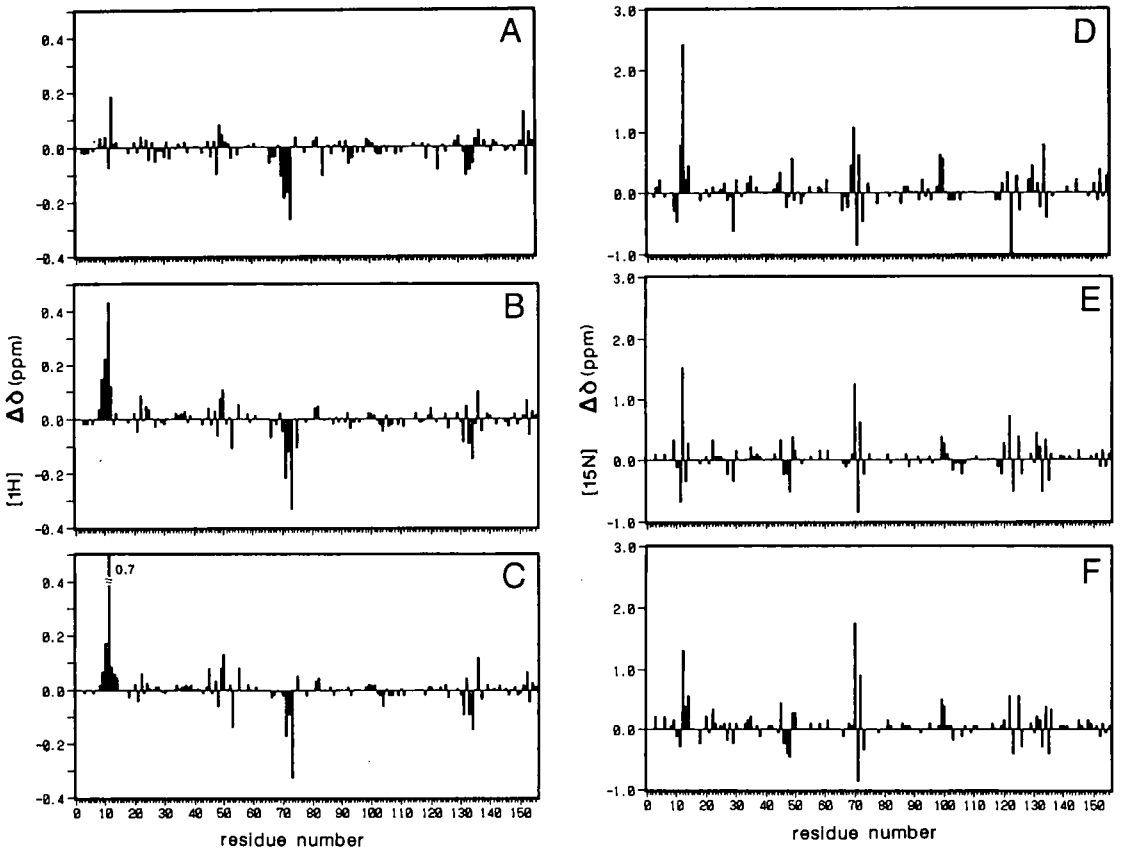

Fig. 2. The maximal backbone amide chemical-shift changes ($\Delta\delta_{max}$) between the absence and presence (100 mM for $MgCl_2$, and 50 mM for $CaCl_2$ and $BaCl_2$) of the metal ions, $MgCl_2$ (A), $CaCl_2$ (B), and $BaCl_2$ (C) on the ^1H axis, and $MgCl_2$ (D), $CaCl_2$ (E), and $BaCl_2$ (F) on the ^{15}N axis, at 27°C. The protein concentration was 1 mM, buffered in sodium acetate, pH 5.5. Positive values are downfield shifts and negative values upfield shifts.

proton and the amide ^{15}N atom in the titration with $MgCl_2$, $CaCl_2$, and $BaCl_2$. As described above, each amide proton and amide ^{15}N signal have been assigned to each residue completely, so it is clearly seen which residues are affected by the addition of divalent ions. The residues whose chemical shifts moved more than 0.05 ppm on the ^1H axis for all the titrations with $MgCl_2$, $BaCl_2$, and $CaCl_2$, or 0.5 ppm on the ^{15}N axis for all the metal titrations, are shown in Table 1. Only the chemical shifts of residues in the neighborhood of Asp^{10}, Glu^{48}, Asp^{70}, and Asp^{134} changed significantly. On the ^{15}N axis (Fig. 2, D–F), chemical shift changes were also observed in a loop region between Lys^{122} and Gly^{126}.

Figure 3 shows the normalized shifts of several cross peaks in 2D NMR spectra in the course of the titration with $MgCl_2$, $BaCl_2$, and $CaCl_2$, and the averaged values for the cross peaks listed in Table 1. From the Hill plots shown in Fig. 4, the numbers of bound metal ions per enzyme, n, and their dissociation constant, K_D, were calculated by least-square fits. The parameters are shown in Table 1. Values of n for the individual amino acid residues are almost the same ($n \cong 1$). The individual K_D values are in the millimolar range. $|\Delta\delta|/|\Delta\delta_{max.}|$ of the individual amino acid

TABLE I
METAL BINDING TO *E. coli* RNASE HI[a]

Residue[b]	Nuclei	$MgCl_2$			$CaCl_2$			$BaCl_2$		
		$\lvert\Delta\delta_{max}\rvert$[c] (ppm)	n[d]	K_D[d] (mM)	$\lvert\Delta\delta_{max}\rvert$ (ppm)	n	K_D (mM)	$\lvert\Delta\delta_{max}\rvert$ (ppm)	n	K_D (mM)
Gly^{11}	^1H	0.07	1.08±0.11	9.6±4.8	0.43	1.08±0.05	1.4±0.4	0.70	1.15±0.07	1.7±0.7
Ser^{12}	^1H	0.19	1.35±0.03	2.1±0.3	0.12	1.14±0.08	1.1±0.5	0.09	1.36±0.07	0.7±0.3
Ser^{12}	^{15}N	2.42	1.19±0.04	4.6±0.9	1.52	0.83±0.08	5.0±2.6	1.30	1.13±0.11	1.4±0.9
Glu^{48}	^1H	0.10	1.07±0.08	8.5±3.1	0.06	0.72±0.07	13.8±5.6	0.06	0.91±0.11	6.4±3.9
Asp^{70}	^{15}N	1.07	1.18±0.06	4.9±1.4	1.24	1.10±0.10	1.5±0.9	1.75	1.00±0.07	3.5±1.3
Ser^{71}	^1H	0.19	1.32±0.08	2.6±1.0	0.22	1.02±0.06	1.9±0.6	0.17	0.97±0.09	4.8±2.3
Ser^{71}	^{15}N	0.85	1.36±0.07	1.6±0.5	0.85	1.12±0.04	0.9±0.2	0.85	0.85±0.13	8.0±6.2
Gln^{72}	^1H	0.17	1.06±0.06	10.7±2.8	0.12	1.14±0.04	1.1±0.3	0.09	0.83±0.09	10.0±5.2
Gln^{72}	^{15}N	0.62	1.16±0.06	5.4±1.6	0.62	0.94±0.08	3.3±1.6	0.90	0.92±0.06	4.7±1.6
Tyr^{73}	^1H	0.26	1.17±0.06	6.6±1.8	0.33	0.91±0.09	5.7±3.0	0.33	1.15±0.07	1.8±0.7
Cys^{133}	^1H	0.10	1.07±0.06	9.5±2.7	0.09	1.23±0.07	0.8±0.3	0.09	1.15±0.09	1.7±0.8
Asp^{134}	^1H	0.09	1.30±0.09	2.2±0.9	0.15	1.01±0.10	2.4±1.4	0.15	1.07±0.05	2.0±0.5
Average[e]	$^1H/^{15}N$	-	1.19±0.05	4.8±1.0	-	1.04±0.03	1.9±0.4	-	1.04±0.05	2.9±0.8

[a] All measurements were performed at 27°C in 0.1 M sodium acetate buffer, pH 5.5.

[b] The residues, whose $\lvert\Delta\delta_{max}\rvert$ are above 0.05 ppm on the ^1H axis or 0.5 ppm on the ^{15}N axis for all three metal ions, are listed.

[c] Absolute values of the maximal chemical-shift changes at a concentration of 100 mM for $MgCl_2$, and 50 mM for $CaCl_2$ and $BaCl_2$.

[d] The numbers of bound metal ions per enzyme, n, and their dissociation constant, K_D, were obtained from Hill plots by least-square fits.

[e] The average values were calculated from the average values of $\lvert\Delta\delta\rvert/\lvert\Delta\delta_{max}\rvert$ for each metal ion.

residues changed similarly for each cation as shown in Fig. 3. From the averaged values of $\lvert\Delta\delta\rvert/\lvert\Delta\delta_{max.}\rvert$ for each divalent ion, n and K_D were calculated in the same manner, and they are also listed in Table 1.

DISCUSSION

The current results about the divalent ion binding to *E. coli* RNase HI in solution are consistent with the previous X-ray crystallographic study (Katayanagi et al., 1990), in which the position of each bound divalent ion in the enzyme crystals was determined. One metal ion binds very close to the active site, and is surrounded by Asp^{10}, Glu^{48}, Asp^{70}, and Asp^{134}. In the present NMR study, the addition of salts to the enzyme solution caused significant shifts for the cross peaks corresponding to the amide protons and ^{15}N atoms of the amino acid residues around the active site, suggesting the binding of each divalent ion (Fig. 5). The significant shifts are also observed for the cross peaks of the C-terminal residues of Gln^{152} and Val^{153} (Fig. 5). However, the amide cross peaks of Gln^{152} and Val^{153} changed in a different manner from the other cross peaks, as shown in Fig. 6. From the X-ray structural analysis, Gln^{152} and Val^{153} are located far from the active site.

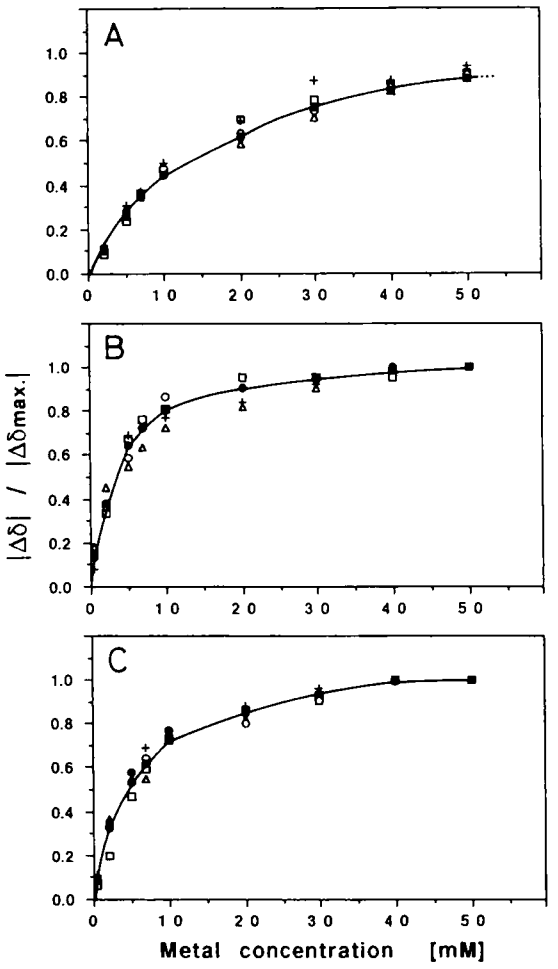

Fig. 3. Normalized shifts ($|\Delta\delta|/|\Delta\delta_{max}|$) in the course of the titration with $MgCl_2$ (A), $CaCl_2$ (B), and $BaCl_2$ (C) at 27°C. The data are obtained from the ^1H chemical shifts for Ser^{12} (open square), Glu^{48} (open triangle), and Asp^{134} (cross), and the ^{15}N chemical shift for Asp^{70} (open circle). The average values (closed circle) were obtained from the data for the residues listed in Table 1, and are connected by a solid line. $|\Delta\delta|$ represents the absolute value of the chemical-shift change, and $|\Delta\delta_{max}|$ represents the absolute value of the maximal chemical-shift change at 100 mM for $MgCl_2$, and 50 mM for $CaCl_2$ and $BaCl_2$. The protein concentration was 1 mM, buffered in sodium acetate, pH 5.5.

Therefore, these chemical shift changes are probably not due to the binding of the specific divalent cations. In fact, when KCl was added to the salt-free enzyme solution, only the ^1H chemical shifts of the cross peaks corresponding to Gln^{152} and Val^{153} changed significantly (data not shown). The Cl^- ion, bound to the enzyme near Gln^{152} and Val^{153}, is proposed to cause these changes in the chemical shifts. Another cation weak binding site near Gln^{152} and Val^{153} is also considered.

The metal binding constants of the enzyme are not very different for three divalent cations. However, the averaged values for cross peaks with significant changes, listed in Table 1, are slightly different among them. The binding constant of Mg^{2+} is the weakest, K_D = 4.6 mM, Ba^{2+} is the middle, K_D = 2.9 mM, and Ca^{2+} is the most tight, K_D = 2.2 mM. It should be noted that

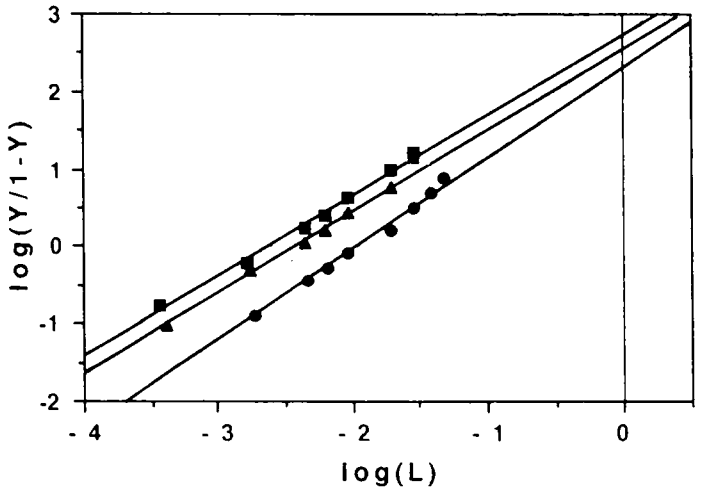

Fig. 4. Hill plots for the average values of metal ion binding to RNase H, as shown in Fig. 3. Conditions are described in Fig. 3. The data for $MgCl_2$ (closed circles), $CaCl_2$ (closed squares), and $BaCl_2$ (closed triangles) were analyzed by least-square fits. Y represents the fraction determined from $|\Delta\delta|/|\Delta\delta_{max}|$, and L represents the concentration of the free metal ions (M).

these metal bindings are rather weak, as compared to other nucleases. For instance, deoxyribonuclease I (DNase I) from bovine pancreas has two strong Ca^{2+} binding sites with K_D = 0.014 mM (pH 7.5) (Price, 1972). At pH 5.5, one was still a tight binding site (K_D = 0.022 mM), but the other became weaker (K_D = 0.5 mM). Staphylococcal nuclease has a single tight Ca^{2+} binding

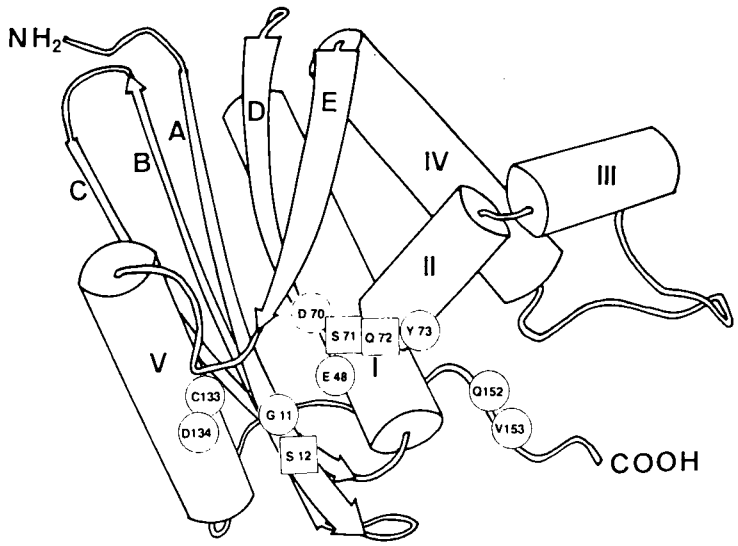

Fig. 5. Structure of *E. coli* RNase HI. The chemical shifts of the residues labeled with solid squares moved significantly, both on the ^1H and ^{15}N axes, by addition of the metal ions. Those residues with solid circles moved only in the ^1H axis. The residues with dotted circles moved only on the ^{15}N axis.

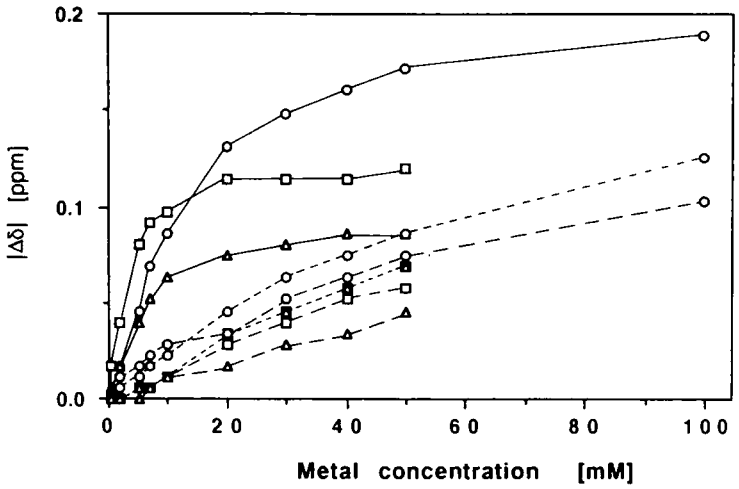

Fig. 6. ^1H chemical-shift changes ($|\Delta\delta|$) in the course of the titration with $MgCl_2$ (open circles), $CaCl_2$ (open squares), and $BaCl_2$ (open triangles), at 27°C. The protein concentration was 1 mM, buffered in sodium acetate, pH 5.5. The data points are connected by solid lines for Ser^{12}, by dotted lines for Gln^{152}, and by dashed lines for Val^{153}.

site with K_D = 0.51 mM (Serpersu et al., 1986). In the active site of *E. coli* DNA polymerase I large fragment (Klenow fragment), two metal binding sites have been found. One is a tight binding site with K_D = 2.5 μM, and the other is a weak site (K_D = 0.6 mM) which is increased 100-fold by TMP (Mullen et al., 1990). The present experiments were carried out at pH 5.5, so the dissociation constants might be larger than those at pH 7. When the enzyme forms a complex with its substrate, a DNA/RNA hybrid, it may bind the metal ions much more tightly, similar to *E. coli* DNA polymerase I.

The resulting n values ($n \cong 1$) indicate that a single divalent ion binds to the individual residues. They do not directly reveal the total number of the binding ions to the active site. However, the titration curves for the residues residing close to the active site are very similar within experimental error (Fig. 3). The X-ray crystallographic studies have shown that these residues are located very close to each other (Katayanagi et al., 1990; Yang et al., 1990). Therefore, it is considered that a single divalent ion binds the active site of the substrate-free enzyme. Yang et al. (1990) considered the possible binding of two Mg^{2+} ions to RNase H, from an analogy to the active-site structure of *E. coli* DNA polymerase I, which requires two metal ions and four carboxylates for the 3′,5′-exonuclease activity (Derbyshire et al., 1988; Beese and Steitz, 1991). For further discussion of the catalytic mechanism, the number of the bound metal ions in the presence of the substrate should be determined.

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