Journal of Biomolecular NMR, 2 (1992) 137–147
ESCOM

J-Bio NMR 044

^{1}H NMR studies of deuterated ribonuclease HI selectively labeled with protonated amino acids

Yasushi Oda[a], Haruki Nakamura[a,*], Toshio Yamazaki[b], Kuniaki Nagayama[b], Mayumi Yoshida[c], Shigenori Kanaya[a] and Morio Ikehara[a]

[a] *Protein Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan*
[b] *Biometrology Laboratory, JEOL Ltd., Akishima, Tokyo 196, Japan*
[c] *Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Machida, Tokyo 194, Japan*

Received 27 June 1991
Accepted 31 August 1991

Keywords: Ribonuclease H; Selective proton labeling; Two-dimensional NMR

SUMMARY

Two-dimensional (2D) ^{1}H NMR experiments using deuterium labeling have been carried out to investigate the solution structure of ribonuclease HI (RNase HI) from *Escherichia coli* (*E. coli*), which consists of 155 amino acids. To simplify the ^{1}H NMR spectra, two fully deuterated enzymes bearing several protonated amino acids were prepared from an RNase HI overproducing strain of *E. coli* grown in an almost fully deuterated medium. One enzyme was selectively labeled by protonated His, Ile, Val, and Leu. The other was labeled by only protonated His and Ile. The 2D ^{1}H NMR spectra of these deuterated RNase HI proteins, selectively labeled with protonated amino acids, were much more simple than those of the normally protonated enzyme. The simplified spectra allowed unambiguous assignments of the resonance peaks and connectivities in COSY and NOESY for the side-chain protons. The spin-lattice relaxation times of the side-chain protons of the buried His residue of the deuterated enzyme became remarkably longer than that of the protonated enzyme. In contrast, the relaxation times of the side-chain protons of exposed His residues remained essentially unchanged.

INTRODUCTION

For small proteins with molecular masses less than 10 kDa, a method to assign proton resonance peaks has been well established (Wüthrich, 1986). For large proteins with molecular masses exceeding 10 kDa, two different approaches using stable isotopes have been developed to resolve the enormously degenerate chemical shifts. One is a recently developed technique of heteronuclear

* To whom correspondence should be addressed.

0925-2738/$ 5.00

multi-dimensional NMR, using mostly ^{13}C and ^{15}N uniform labeling (Fesik and Zuiderweg, 1990; Ikura et al., 1990; Kay et al., 1990; Nagayama et al., 1990). The other procedure uses specific isotope labeling (LeMaster, 1990; McIntosh and Dahlquist, 1990).

Deuterium labeling is a powerful technique to simplify ^{1}H NMR spectra. Several techniques using deuterium labeling methods have been developed, such as random fractional deuteration for resolution and sensitivity enhancement, and complete deuteration for spectral editing (reviewed by LeMaster, 1990). For complete deuteration, there are two types of labeling methods. One is selective deuterium labeling in a protonated background, and the other is selective proton labeling in a deuterated background. The fully deuterated proteins, with a few proton-labeled amino acid(s), have long been known to successfully simplify 1D NMR spectra (Crespi et al., 1968; Markley et al., 1968; Brodin et al., 1989). In the present study, this selective proton labeling method was revived for 2D NMR analysis and applied to ribonuclease HI (RNase HI).

RNase H is an endonuclease, which has the specific function to cleave 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). The importance of this enzyme is suggested by its existence in organisms varying from *Escherichia coli (E. coli)* to humans. In addition, the C-terminal domain of retroviral reverse transcriptase is now considered to be essentially the same as the entire RNase H protein (Johnson et al., 1986; Doolittle et al., 1989). In the present study, RNase HI from *E. coli* was investigated. In *E. coli*, a second RNase H (RNase HII) has been described recently (Itaya, 1990), therefore the enzyme analyzed in this study is designated as RNase HI. Recently, the structure and function of this enzyme have been intensively studied. It consists of 155 amino acids and has a molecular mass of 17.6 kDa (Kanaya and Crouch, 1983). The 3D structure has been determined almost simultaneously by two independent X-ray crystallographic studies (Katayanagi et al., 1990; Yang et al., 1990).

In order to investigate the solution structure of RNase HI, we have analyzed the enzyme with the aid of heteronuclear 3D NMR techniques, using ^{15}N-labeled and ^{13}C-labeled enzymes (Nagayama et al., 1990). The resonance peaks of the backbone nuclei have already been assigned, and the secondary structure has been elucidated (Nagayama et al., 1991; Yamazaki et al., 1991). The NMR-determined secondary structure agreed well with that obtained from the X-ray crystallographic study. To obtain a detailed solution structure of RNase HI, we should assign the side-chain proton resonances, and identify the interresidue NOE cross peaks between them. To achieve this resolution, 2D ^{1}H NMR experiments, using fully deuterium-labeled enzymes with a few protonated amino acids, were carried out.

MATERIALS AND METHODS

Preparation of deuterated sodium succinate

For bacterial growth, fully deuterated glucose and sodium succinate were used as carbon sources. The deuterated glucose (96–98 atom%) was purchased from CIL, MA. The deuterated succinate was prepared following the method of Atkinson et al. (1968), which refluxes a concentrated solution of protonated sodium succinate in basic D_2O. 0.5 Mol sodium succinate (Wako Pure Chemical Ind., Ltd., Osaka) was dissolved in 200 ml 99.9% D_2O (Isotec Inc., OH) containing 0.05 mol NaOH. If the sodium succinate is hydrated, it should be dehydrated by heat at 140 C for 24 h before this procedure. After heating the solution in a stainless-steel bomb at 140 C for 24 h, the

spent D_2O was removed by evaporation or lyophilization, and fresh D_2O was added. After two cycles of the above procedure and recrystallization, a total of 0.4 mol purified deuterated sodium succinate was obtained. More than 97% of the protons were exchanged with deuterions as judged by ^1H NMR.

Expression and purification of deuterated RNase HI

Normal protonated *E. coli* RNase HI was obtained from *E. coli* strain N4830-1, harboring plasmid pPL801, and grown on LB medium, as described previously (Kanaya et al., 1989).

Deuterated enzymes were obtained from the same bacteria grown in M9 minimal culture medium prepared in 90–99.9% D_2O, including deuterated glucose or sodium succinate as a carbon source. Fifty mg *d*-biotin, 50 mg thiamine, and 100 mg ampicillin were added per liter culture. Since this strain requires three amino acids, His, Ile, and Val, 40 mg of each of these protonated amino acids (Wako Pure Chemical Ind., Ltd., Osaka) were added per liter culture to obtain the deuterated enzyme, with the specifically protonated amino acids (D-1). In order to obtain a more deuterated enzyme (D-2), labeled only by protonated His and Ile, 40 mg deuterated L-Val (99 atom% deuterated; CIL, MA) was added to the culture media with the protonated His and Ile.

After 50–80 h cultivation at 32°C (the absorbance at 550 nm of the culture reached about 0.6), overproduction of the enzyme was induced by increasing the temperature to 42°C. At the end of an additional 8 h of incubation, the cells were harvested by centrifugation and subjected to purification procedures, as described previously (Kanaya et al., 1989). The spent D_2O in the culture medium was recovered by distillation and recycled for the next cultivation. The deuterium content in the recovered D_2O was roughly 90%, as judged by ^1H NMR.

NMR analysis

All the NMR experiments were carried out on a 600 MHz spectrometer (Bruker AM-600) at 27°C. The purified enzyme was dissolved in 99.9% D_2O buffered with 0.1 M sodium acetate, pH 5.5, to a final concentration of 1–2 mM. A total of 0.27 ml of each sample was stored in the 5-mm NMR microtubes (BMS005 Shigemi Standard Joint Ind. Co., Ltd., Tokyo) (Takahashi and Nagayama, 1988). Proton chemical shifts were measured relative to the water signal [4.78 ppm relative to sodium 3-(trimethylsilyl)propionate].

NOESY and DQF-COSY spectra were recorded in the pure-phase absorption mode with the time-proportional phase incrementation method (Marion and Wüthrich, 1983). In the NOESY experiments, a mixing time of 75 ms was used. The resonance of residual H_2O was suppressed by preirradiation. 2D NMR spectra were acquired with 512 increments in the t_1 direction and 4096 data points in the t_2 direction. The time domain data were multiplied by a phase-shifted sine bell squared window function in both the t_1 and t_2 directions, and zerofilled to 4096 in the t_1 dimension before Fourier transformation. The spectral widths were 8333 Hz in both the ω_1 and ω_2 directions.

Spin-lattice relaxation times were measured by a conventional inversion-recovery method with a recycle time of 20 s on the normally protonated enzyme and the D-1 enzyme. The time courses of recovery of magnetizations were also analyzed by using least-squares fitting to single exponential functions of time.

RESULTS

Deuterated RNase HI selectively labeled with protonated amino acids

The host strain, *E. coli* N4830-1, is auxotrophic for His, Ile, and Val. *E. coli* RNase HI has 5 His, 7 Ile, and 9 Val residues. Therefore, when the bacteria is grown in a minimal culture medium in D_2O with these 3 protonated amino acids, the enzyme should be fully deuterated, and selectively labeled with protonated amino acids. The growth rate of the bacteria in the deuterated medium was about 6 times slower than that in the normally protonated minimal medium. At the final phase of the cultivation, the bacterial growth slightly decreased at 42 C, as monitored by the absorbance at 550 nm. Apparently an inhibition of the bacterial growth occurred, which has never been observed for the cultivations in the normally protonated medium. The yield of the deuterated enzyme was typically 0.5–1 mg per liter culture, about 10-fold less than that of the protonated enzyme.

As described in the preceding section, two kinds of deuterated RNase HI (D-1 and D-2) selec-

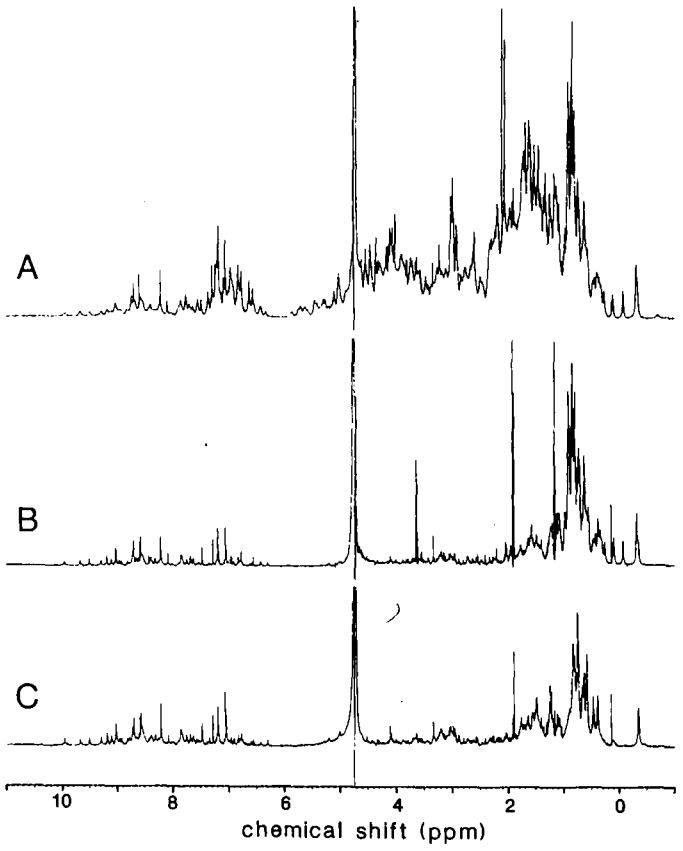

Fig. 1. ^1H NMR spectrum at 27 C of the normally protonated RNase HI (A), that of the deuterated RNase HI selectively labeled with protonated His, Ile, and Val residues, D-1 (B), and that of the deuterated RNase HI selectively labeled with protonated His and Ile residues, D-2 (C). The protein concentration was 1–2 mM in D_2O buffered with 0.1 M sodium acetate, pH 5.5.

tively labeled with protonated amino acids were prepared. Figure 1 shows the 1D ^{1}H NMR spectra of the protonated enzyme and the selectively proton-labeled enzymes, D-1 and D-2. The spectra of D-1 and D-2 are much more simple than the spectrum of the protonated enzyme. This spectral refinement is especially evident in the aromatic region from 6 ppm to 10 ppm of the D-1 and D-2 spectra, where the H2 and H4 proton resonance peaks for the His side chains are easily identified. Other resonance peaks in this region are from amide protons, which were partially exchanged with H_2O during the purification. It should be noted that the resonance peaks of the amide protons in the D-1 and D-2 spectra were sharper than those of the normally protonated enzyme. Such remarkable reduction of the line width was not observed for aliphatic protons.

In the aliphatic region, especially between 2 and 6 ppm, most of the resonances disappeared in the D-1 and D-2 spectra. In Fig. 1C, several resonance peaks of the upfield shifted methyl protons disappeared, indicating an additional labeling with deuterated Val.

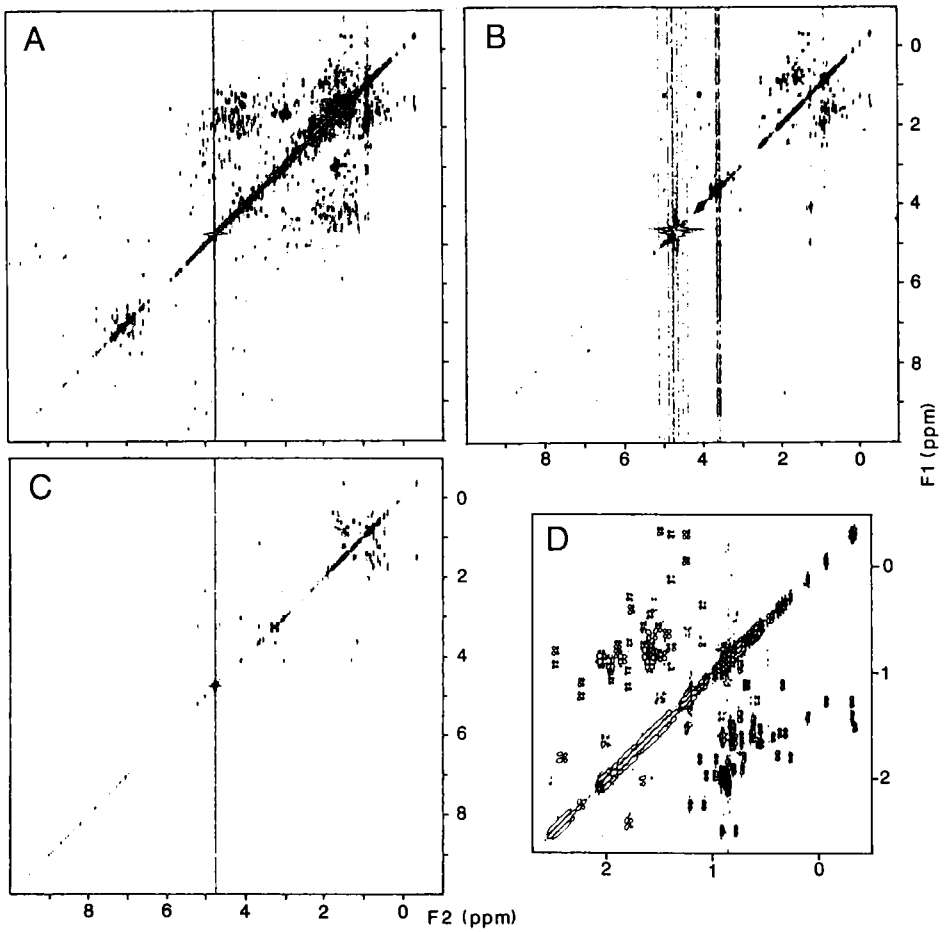

Fig. 2. DQF-COSY spectra of the normally protonated enzyme (A), and the selectively proton-labeled enzymes, D-1 (B), and D-2 (C), at 27 C. (D) The expanded DQF-COSY spectrum for the aliphatic region of D-1 enzyme. The protein concentration was 1–2 mM in D_2O buffered with 0.1 M sodium acetate, pH 5.5.

Two-dimensional ^1H NMR spectra

Figure 2 compares the DQF-COSY spectrum of D-1 (Fig. 2B) with that of the normally protonated RNase HI (Fig. 2A). The 2D spectrum of D-1 is dramatically simplified by the decreased number of cross peaks, as a result of the full deuterium labeling, except for the protonated amino acids. The cross peaks between the aromatic protons of Phe, Tyr, and Trp residues completely disappeared, as shown in Fig. 2B. The D-1 enzyme was sufficiently labeled with deuterium for the analysis of specific amino acids. In the D-1 spectrum, the resonances of only 5 His, 7 Ile, and 9 Val residues were expected to be observed. However, more than 9 characteristic connectivities for Val and Leu side chains, in which methine proton resonances are coupled to two methyl protons, were observed in the DQF-COSY spectrum of D-1 (Fig. 2D). As discussed in the next section, these unexpected resonances indicate the presence of protonated Leu residues, produced through a normal metabolic pathway from the protonated Val.

Since the methyl proton resonances were still complex in the 2D spectra of D-1, the auxotrophic *E. coli* strain was provided with deuterated valine to diminish the proton resonances of Val and Leu. Figure 2C shows the DQF-COSY spectrum of this deuterated enzyme, D-2. An expanded NOESY spectrum in the aliphatic proton region of D-2 is shown in Fig. 3B. When the NOESY spectrum of D-2 is compared with that of the normally protonated enzyme in Fig. 3A, the spin identifications for the reduced numbers of resonances in the aliphatic region are straightforward.

The DQF-COSY and NOESY spectra of D-1, D-2, and the normally protonated enzyme allowed many of the proton chemical shifts of the His, Ile, Val, and Leu side chains to be assigned. Since the chemical shifts of the backbone protons have already been completely assigned by 3D NMR techniques, using ^{13}C-labeled and ^{15}N-labeled enzymes (Nagayama et al., 1991; Yamazaki

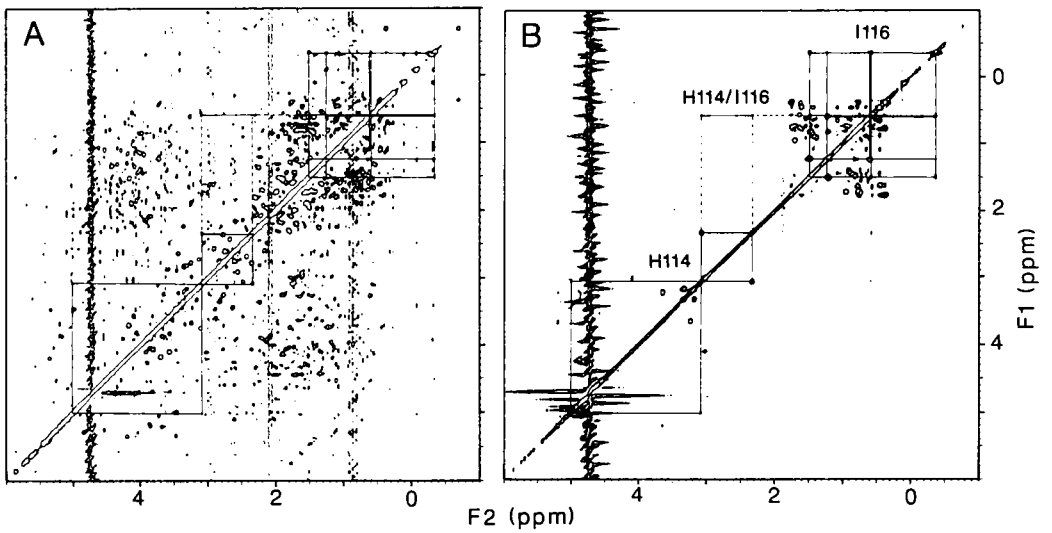

Fig. 3. The aliphatic region of NOESY spectra of the normally protonated enzyme (A) and the selectively proton-labeled enzyme, D-2 (B), at 27°C. Intraresidue NOE connectivities for His^{114} and Ile^{116} are shown by solid lines. A pair of interresidue NOEs are shown by dotted lines. The mixing time was 75 ms. The protein concentration was 1–2 mM in D_2O buffered with 0.1 M sodium acetate, pH 5.5.

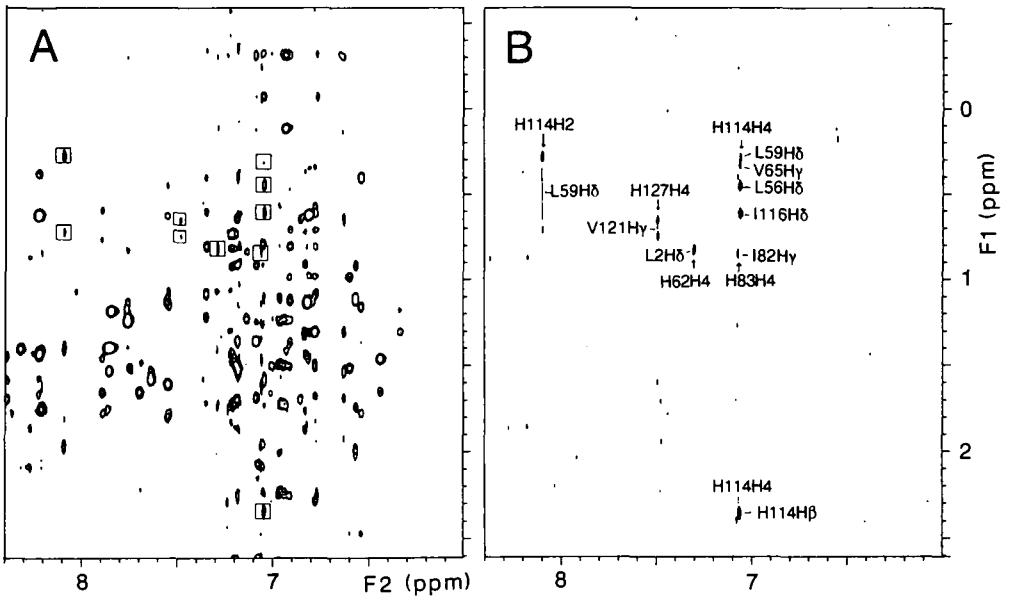

Fig. 4. The expanded NOESY spectra of the normally protonated enzyme (A) and the selectively proton-labeled enzyme, D-1 (B), at 27 C. Intraresidue NOE cross peaks between H2/H4 of His residues and methyl protons of Val, Ile, and Leu residues are labeled in (B). An intraresidue NOE cross peak between H4 and Hβ of His^{114} is also labeled in (B). In (A), the NOE cross peaks identified in (B) are framed. Conditions are described in Fig. 3.

et al., 1991), the residue numbers of those side chains were definitively assigned. All the proton chemical shifts of the His residues were easily and unambiguously assigned from the analysis of the D-1 and D-2 spectra. For instance, the NOE connectivities between Hα and Hβ, and between Hβ and H4 of His^{114} are shown in Fig. 3B and Fig. 4B, respectively. The proton resonances of 4 of the 7 Ile side chains were assigned from the D-2 spectra. In Fig. 3B, the NOE connectivities between the side-chain protons of Ile^{116} are indicated. However, due to unresolved overlapping, the proton resonances of the other Ile side chains could not be unambiguously assigned using the current experimental data. The proton resonances of 8 of the 9 Val side chains and 6 of the 12 Leu residues were also definitively assigned. As shown in Fig. 2B, the cross peaks between Hα and Hβ became more vague in the deuterated enzyme than in the normally protonated enzyme. The Hα is thought to be replaced by deuterons due to amino transformation. The assignments of the side-chain proton resonances are now in progress with parallel experiments using 3D NMR techniques. The chemical shifts of the assigned protons will be published elsewhere, together with the side-chain proton chemical shifts of other residues.

In addition to the resonance assignments, several medium- and long-range NOE cross peaks between the side-chain protons were identified from the NOESY spectra. For example, a pair of interresidue NOEs between Hβ of His^{114} and Hδ of Ile^{116} are indicated in Fig. 3B. In Fig. 4, several long-range NOEs between the imidazole protons of His residues and aliphatic protons are shown.

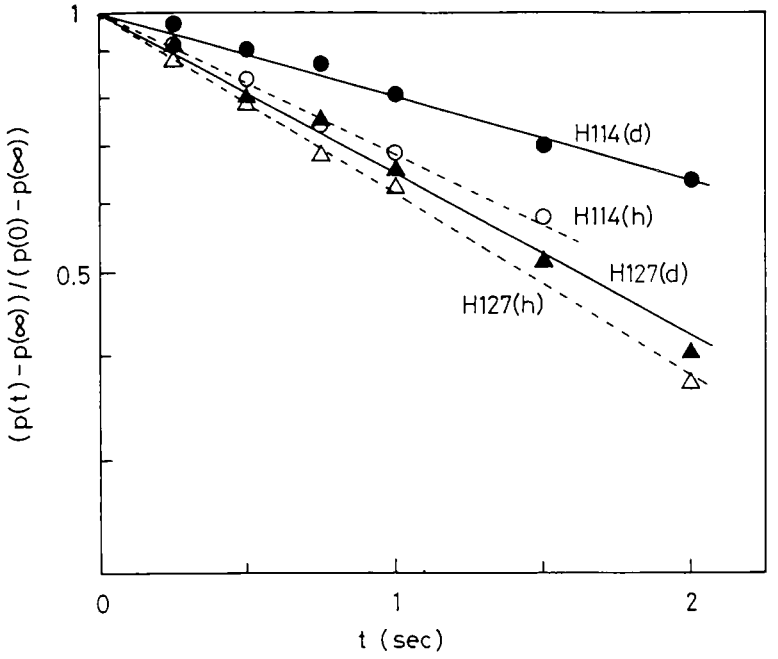

Fig. 5. Spin-lattice relaxation measurements in the normally protonated enzyme and the selectively proton-labeled enzyme, D-1, at 27 C. Conditions are described in Fig. 1. The peak height at the delay time, t, is represented by p(t). The data were obtained by inversion-recovery experiments in the normally protonated enzyme for H2 of His^{114} (open circles) and His^{127} (open triangles), and in D-1 enzyme for H2 of His^{114} (closed circles) and His^{127} (closed triangles).

Spin-lattice relaxation times

The results of spin-lattice relaxation measurements of D-1 and the normally protonated enzyme are shown in Fig. 5. All the recovery rates shown in Table 1 were obtained by fitting single exponential functions to the curves in Fig. 5. The spin-lattice relaxation time of each imidazole proton of the His residues was differently affected by the deuteration. For His^{114}, the relaxation times of the imidazole protons were significantly long, and became even longer in D-1 as compared to the protonated enzyme. The relaxation times of the imidazole protons of His^{124} were also considerably affected by the deuteration. Those of His^{89} and His^{127} showed small changes between the deuterated and protonated enzymes.

DISCUSSION

Although the host *E. coli* strain for the RNase HI overproduction system was auxotrophic for His, Ile, and Val, not only these amino acids, but also Leu residues were protonated when the 3 protonated amino acids were added to the fully deuterated culture medium. Methine and methyl protons in the Val side chains are transferred to those in Leu through the normal biochemical pathways of the amino acid synthesis (Rodwell, 1969). In fact, the proton resonances of Hγ and Hδ of 12 Leu side chains were observed, but no proton resonances of Hα nor Hβ were found in

the 2D spectra. The protons of Hα and Hβ must be replaced with deuterons through the normal biosynthetic pathway.

The full deuterium labeling, which leaves only specific protonated residues, simplifies the complex NMR spectra of a large protein by reducing the number of protons and sharpening the resonance peaks. It is very useful for the resonance assignments of the side-chain protons. However, it is occasionally still difficult to completely solve the problem of degeneracy in the 2D spectra, especially for the aliphatic protons. In the present study, even in the spectra of D-2, the protons of Ile side chains were too overlapped to be completely resolved. Moreover, their resonances did not become sharper. Therefore, the proton resonances of 3 of the 7 Ile side chains could not be unambiguously assigned. With larger proteins, this problem may become more severe. Studies such as this one, in combination with multidimensional NMR experiments using a ^{13}C-labeled protein, may solve this degeneracy of proton resonances.

The present labeling method is useful not only for the assignment of proton resonances of side chains, but also for the identification of interresidue NOE signals. We could identify 14 such NOE cross peaks from the NOESY spectra of the deuterated enzymes. For instance, a pair of NOE cross peaks between Hγ of Val^{121} and H4 of His^{127} indicates a close contact of Val^{121} in the 5th β-strand (β-E by the X-ray nomenclature; Katayanagi et al., 1990) with His^{127} in the following loop just before the 5th α-helix (α-V) (Fig. 4).

As mentioned above, the resonance peaks of the amide protons in the D-1 and D-2 spectra became sharper than those of the normally protonated enzyme. This implies that the spin-spin relaxation rates become slower in the deuterated enzyme than in the protonated enzyme, as a result of the conversion of the neighboring protons to deuterons. Since the observed backbone amide protons were slowly exchanging protons with the solvent D_2O, these protons are buried in the en-

TABLE 1
SPIN-LATTICE RELAXATION TIMES (T_1)[a] FOR IMIDAZOLE RING PROTONS OF His RESIDUES IN *E. coli* RNase HI[b]

Residue	Proton	T_1(d) (s)	T_1(h) (s)	Ratio T_1(d) T_1(h)	ASA[d] (Å^2)
His^{62}	H2	0.25 ± 0.02	N.D.[c]		129.2
His^{62}	H4	0.70 ± 0.04	N.D.[c]		
His^{83}	H2	1.7 ± 0.2	1.5 ± 0.2	1.2	128.6
His^{83}	H4	1.8 ± 0.5	N.D.[c]		
His^{114}	H2	4.5 ± 1.1	2.7 ± 0.2	1.7	23.7
His^{114}	H4	2.8 ± 0.3	1.9 ± 0.2	1.5	
His^{124}	H2	2.7 ± 0.5	1.9 ± 0.04	1.5	156.5
His^{124}	H4	2.5 ± 0.6	2.1 ± 0.2	1.2	
His^{127}	H2	2.3 ± 0.2	2.1 ± 0.1	1.1	66.5
His^{127}	H4	1.9 ± 0.3	1.9 ± 0.2	1.0	

[a] Those in the normally protonated enzyme (designated by T_1(h)) are compared with those in the deuterated enzyme, D-1 (designated by T_1(d)).

[b] The data were analyzed by fitting single exponential functions to the curves as shown in Fig. 5.

[c] Due to overlap with another signal, this value could not be determined.

[d] The values of accessible surface area (ASA) of individual residues were calculated from the X-ray structure by Katayanagi et al. (1990) following the calculation method of Shrake and Rupley (1973) with the radius 1.4 Å of the water molecule.

zyme. This means that the dipole–dipole interactions are most effective for those spins which are buried in the protonated enzyme. In contrast, the line widths for the aliphatic protons did not significantly change. In this case, the intraresidual interactions are considered to be dominant for the aliphatic protons. For the observed apparent sharpenings, the alternative explanation is the potential loss of unresolved or poorly resolved Cα proton-amide proton *J* couplings as a result of the deuteration of Cα protons.

In Table 1, we show the ratio of the spin-lattice relaxation times of imidazole protons of the deuterated enzyme D-1 to the normally protonated enzyme. The values for His^{114} were significantly affected by the deuteration. It is considered that the dipole–dipole interactions with the neighboring protons were reduced as a result of the deuteration. This suggests that the imidazole ring of His^{114} is buried in the enzyme. In fact, the His^{114} residue is buried in the detailed crystal structure (Katayanagi et al., submitted). The spin-lattice relaxation times for the His^{83} and His^{127} residues underwent only small changes, suggesting that the dipolar relaxation with protons of other residues is not likely to be important. This is consistent with the crystal structure determination that these imidazole rings are exposed to the solvent. The crystal structure analysis revealed that His^{124} is also exposed to the solvent. However, the spin-lattice relaxation times of His^{124} were considerably affected by the deuteration. The present result suggests that, in the normal, uniformly protonated enzyme, protons of other residues may exist in the neighborhood of His^{124} in solution.

The observed spin-lattice relaxation times for His residues, except for His^{124}, correlate with the values of the accessible surface area (ASA) of individual residues calculated from the crystal structure by Katayanagi et al. (1990) (see Table 1). The spin-lattice relaxation times of the imidazole protons of His^{114} were significantly long, suggesting that the imidazole ring of His^{114} is immobile in the enzyme. In fact, data from the detailed X-ray crystallographic analysis (Katayanagi et al., submitted) suggest that the imidazole protons of His^{114} form hydrogen bonds within the crystallized enzyme. The imidazole protons of His^{62} had short spin-lattice relaxation times, suggesting that the imidazole ring of His^{62} is mobile within the enzyme. This is consistent with its position in the crystal; His^{62} lies in the loop following the first helix (α-I) and points to the solvent. For His^{83} in the third helix (α-III), and His^{127} at the amino end of the 5th helix (α-V), the relaxation times are in the average range. In the crystal structure, these residues are located on the surface of the enzyme. Since a salt bridge is formed between the imidazole of His^{127} and Glu^{119}, the ASA of His^{127} is apparently small. For His^{124}, which is a constituent of a reverse turn, the spin-lattice relaxation times were longer than those expected from the ASA value. This suggests that the imidazole ring of His^{124} is less mobile in solution than in the crystal. A site-directed mutagenesis study by Kanaya et al. (1990) has revealed that His^{124} affects the catalytic activity. Moreover, this His residue is well conserved among RNase H proteins from other species and in many reverse transcriptases (Johnson et al., 1986; Doolittle et al., 1989). In solution, the imidazole ring of His^{124} could have a different conformation than in the crystal structure, and hydrogen bond with a residue (residues) in the active site of RNase HI. Further NMR studies are necessary to reveal the solution structure around His^{124} and the role of His^{124} on the enzymatic activity.

Seeholzer et al. (1986) have demonstrated the advantage of using a fully deuterated protein for the 1D ^1H NMR analysis of a complex of deuterated calmodulin with melittin. For 2D NMR experiments, this advantage was confirmed by our present study. More detailed structural information about such macromolecular complexes will be revealed with this deuterium labeling method.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. T. Ohkubo (PERI) for help in NMR measurements, Dr. M. Oobatake (PERI) for help in the calculation of ASA values, and K. Katayanagi and Dr. K. Morikawa (PERI) for discussion.

REFERENCES

Atkinson, J.G., Csakvary, J.J., Herbert, G.T. and Stuart, R.S. (1968) *J. Am. Chem. Soc.*, **90**, 498–499.

Brodin, P., Drakenberg, T., Thulin, E., Forsén, S. and Grundström, T. (1989) *Prot. Eng.*, **2**, 353–358.

Crespi, H.L., Rosenberg, R.M. and Katz, J.J. (1968) *Science*, **161**, 795–796.

Crouch, R.J. and Dirksen, M.-L. (1982) In *Nuclease* (Eds, Linn, S.M. and Roberts, R.J.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 211–241.

Doolittle, R.F., Feng, D.-F., Johnson, M.S. and McClure, M.A. (1989) *Q. Rev. Biol.*, **64**, 1–30.

Fesik, S.W. and Zuiderweg, E.R.P. (1990) *Q. Rev. Biophys.*, **23**, 97–131.

Ikura, M., Kay, L.E. and Bax, A. (1990) *Biochemistry*, **29**, 4659–4667.

Itaya, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 8587–8591.

Johnson, M.S., McClure, M.A., Feng, D.-F., Gray, J. and Doolittle, R.F. (1986) *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7648–7652.

Kanaya, S. and Crouch, R.J. (1983) *J. Biol. Chem.*, **258**, 1276–1281.

Kanaya, S., Kohara, A., Miyagawa, M., Matsuzaki, T., Morikawa, K. and Ikehara, M. (1989) *J. Biol. Chem.*, **264**, 11546–11549.

Kanaya, S., Kohara, A., Miura, Y., Sekiguchi, A., Iwai, S., Inoue, H., Ohtsuka, E. and Ikehara, M. (1990) *J. Biol. Chem.*, **264**, 4615–4621.

Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Ikehara, M., Matsuzaki, T. and Morikawa, K. (1990) *Nature*, **347**, 306–309.

Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Nakamura, H., Ikehara, M., Matsuzaki, T. and Morikawa, K. (1991) *J. Mol. Biol.*, submitted.

Kay, L.E., Clore, G.M., Bax, A. and Gronenborn, A.M. (1990) *Science*, **249**, 411–414.

LeMaster, D.M. (1990) *Q. Rev. Biophys.*, **23**, 133–174.

Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **113**, 967–974.

Markley, J.L., Putter, I. and Jardetzky, O. (1968) *Science*, **161**, 1249–1251.

McIntosh, L.P. and Dahlquist, F.W. (1990) *Q. Rev. Biophys.*, **23**, 1–38.

Nagayama, K., Yamazaki, T., Yoshida, M., Kanaya, S. and Nakamura, H. (1990) *J. Biochem.*, **108**, 149–152.

Nagayama, K., Yamazaki, T., Yoshida, M., Kanaya, S. and Nakamura, H. (1991) In *Computational Aspects of the Study of the Biological Macromolecules by NMR* (NATO ASI series) (Ed, Hoch, J.C.), in press.

Rodwell, V.W. (1969) In *Metabolic Pathways, Vol. 3* (Ed, Greenberg, D.M.) Academic Press, New York, p. 351.

Seeholzer, S.H., Cohn, M., Putkey, J.A., Means, A.R. and Crespi, H.L. (1986) *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 3634–3638.

Shrake, A. and Rupley, J.A. (1973) *J. Mol. Biol.*, **79**, 351–371.

Takahashi, S. and Nagayama, K. (1988) *J. Magn. Reson.*, **76**, 347–351.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley and Sons, New York.

Yamazaki, T., Yoshida, M., Kanaya, S., Nakamura, H. and Nagayama, K. (1991) *Biochemistry*, **30**, 6036–6047.

Yang, W., Hendrickson, W.A., Crouch, R.J. and Satow, Y. (1990) *Science*, **249**, 1398–1405.