J|A|C|S ARTICLES

Published on Web 04/12/2010

Detection of the Sulfhydryl Groups in Proteins with Slow Hydrogen Exchange Rates and Determination of Their Proton/Deuteron Fractionation Factors Using the Deuterium-Induced Effects on the $^{13}C_{\beta}$ NMR Signals

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Abstract: A method for identifying cysteine (Cys) residues with sulfhydryl (SH) groups exhibiting slow hydrogen exchange rates has been developed for proteins in aqueous media. The method utilizes the isotope shifts of the C_{β} chemical shifts induced by the deuteration of the SH groups. The 18.2 kDa *E. coli* peptidyl prolyl *cis*−*trans* isomerase b (EPPIb), which was selectively labeled with [3-^{13}C;3,3-$^{2}H_2$]Cys, showed much narrower line widths for the $^{13}C_{\beta}$ NMR signals, as compared to those of the proteins labeled with either [3-^{13}C]Cys or (3*R*)-[3-^{13}C;3-^{2}H]Cys. The $^{13}C_{\beta}$ signals of the two Cys residues of EPPIb, i.e. Cys-31 and Cys-121, labeled with [3-^{13}C;3,3-$^{2}H_2$]Cys, split into four signals in H_2O/D_2O (1:1) at 40 °C and pH 7.5, indicating that the exchange rates of the side-chain SH's and the backbone amides are too slow to average the chemical shift differences of the $^{13}C_{\beta}$ signals, due to the two- and three-bond isotope shifts. By virtue of the well-separated signals, the proton/deuteron fractional factors for both the SH and amide groups of the two Cys residues in EPPIb could be directly determined, as approximately 0.4−0.5 for [SD]/[SH] and 0.9−1.0 for [ND]/[NH], by the relative intensities of the NMR signals for the isotopomers. The proton NOE's of the two slowly exchanging SH's were clearly identified in the NOESY spectra and were useful for the determining the local structure of EPPIb around the Cys residues.

Introduction

It is well-known that the SH groups of Cys residues in proteins often play crucial roles in their biological functions.[1,2] The SH groups form hydrogen bonds and bind to various metal ions, thereby stabilizing the secondary and tertiary structures of proteins.[3,4] Another unique feature of SH groups, which distinguishes them from the other polar side-chain functional groups, is their low proton/deuteron fractionation factors ([SD]/[SH] $\approx$ 0.5).[5,6] Therefore, the SH groups of Cys residues preferentially bind to protons, rather than deuterons, in a mixture of H_2O/D_2O.[5,6] Since detailed enzymatic mechanisms are often studied by measuring the accurate kinetic isotope effects in D_2O-containing solutions, the fractionation factors of the catalytic SH groups in proteins are crucial parameters to be considered.[7]

To investigate protein structures in solution at atomic resolution, NMR is undoubtedly the most powerful method. However, the acquisition of structural information for various polar side-chain groups such as hydroxyl or amino groups, which are biologically and structurally important, has been hampered due to their intrinsically fast exchange rates with the hydrogens of the surrounding solvent molecules.[8−11] This is also the case for the SH groups of Cys residues, which are rarely observed, except for those deeply embedded in the interior of proteins.[5,6] Therefore, this important polar side-chain functional group has drawn little attention from the biological NMR community, and the number of assigned SH proton NMR signals of Cys residues deposited in the Biological Magnetic Resonance Data Bank (BMRB)[12] (http://www.bmrb.wisc.edu/) is currently less than 60. Moreover, there is presently no direct method to determine the fractionation factors, even for Cys residues in proteins with very slow hydrogen exchange rates.

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10.1021/ja101205j
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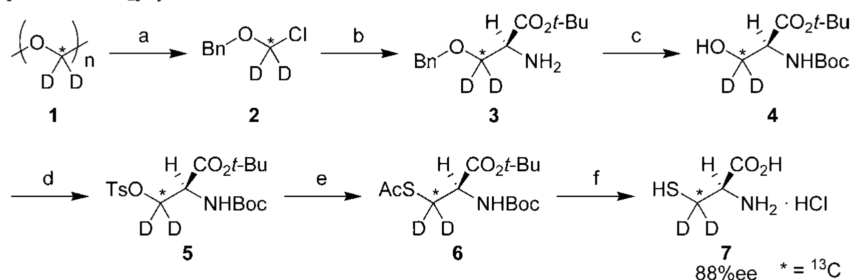
Scheme 1. Synthesis of L-[3-^{13}C;3,3-^{2}H$_2$]Cysteine[a]



[a] Reagents and conditions: (a) BnOH, HCl gas, 97%; (b) (i) *N*-(diphenylmethylene)glycine *tert*-butyl ester, chiral-PTC, 50% aqueous KOH, toluene; (ii) 0.5 M citric acid, dioxane, 76%; (c) (i) Boc$_2$O, DMAP, DMF, (ii) Pd/C, H$_2$ (4 atm), 50 °C, 76%; (d) TsCl, pyridine, 75%; (e) AcSK, DMF, 87%; (f) TFA, room temperature, then HCl, Δ, 73%.

Recently, we developed a unique method for identifying and assigning the hydroxyl groups of tyrosine (Tyr) residues that slowly exchange with the surrounding solvent hydrogens.[13] The most crucial prerequisite for observing and assigning the $^{13}C_\zeta$ peak Tyr in a high-resolution manner was to use the stereoarray isotope labeled (SAIL) Tyr, (2*S*,3*R*)-[α,β,ζ-^{13}C;β$_2$,ε$_{1,2}$-$\sim^{2}$H;^{15}N]Tyr, which has an optimized labeling pattern for such experiments.[13,14] A similar strategy could also be applied to observe the deuterium isotope shifts for the C_β signals of Cys residues in a protein: for example, the 18.2 kDa *E. coli* peptidyl *cis*−*trans* isomerase b (EPPIb). One might expect to observe the isotope shifts of the C_β signals for the Cys residues in EPPIb labeled with either uniformly ^{13}C,^{15}N-labeled Cys or SAIL Cys, i.e. (2*R*,3*R*)-[1,2,3-^{13}C;3-^{2}H;^{15}N]Cys.[15] However, this was actually not the case, since the NMR line widths of the side-chain C_β atoms with directly bonded proton(s) were too broad to observe small isotope shifts. Therefore, we optimized the isotope labeling pattern of Cys in order to obtain narrower line widths for the C_β signals, which allowed us to identify the SH groups of Cys residues in proteins with slow hydrogen exchange rates and accurately determine their fractionation factors.

Materials and Methods

Synthesis of Isotope-Labeled Cysteines. L-[3-^{13}C]Cys and L-[3-^{13}C;3,3-^{2}H$_2$]Cys were synthesized from [^{13}C]paraformaldehyde and [^{13}C;^{2}H]paraformaldehyde, respectively, via the stereoselective alkylation of the glycine−benzophenone Schiff base, using a chiral phase-transfer catalyst ((11b*R*)-(−)-4,4-dibutyl-4,5-dihydro-2,6-bis(3,4,5-trifluorophenyl)-3*H*-dinaphth[2,1-*c*:1′,2′-*e*]azepinium bromide), as shown in Scheme 1 for L-[3-^{13}C;3,3-^{2}H$_2$]Cys.[16,17] After protection of the amino group, the obtained serine derivative **3** was converted to the cysteine derivative **6** by transforming the side-chain moiety.[15] The deprotection of compound **6** was achieved by refluxing with 2 M HCl to give L-[3-^{13}C;3,3-^{2}H$_2$]cysteine **7** as the hydrochloride salt. The optical purity for the α-position was determined to be 88% ee, by an HPLC analysis on a chiral stationary column (DAICEL CROWNPAK CR+).

L-(2*R*,3*R*)-[3-^{13}C;3-^{2}H]Cys was synthesized by the previously described method for preparing SAIL Cys, i.e. L-(2*R*,3*R*)-[1,2,3-^{13}C;3-^{2}H;^{15}N]Cys, using the unlabeled ethyl hippurate in lieu of ethyl [1,2-^{13}C;3-^{2}H;3-^{15}N]hippurate.[15]

Preparation of *E. coli* Peptidyl−Prolyl *cis*−*trans* Isomerase b, EPPIb, Selectively Labeled with Isotope-Labeled Amino Acids. The EPPIb's selectively labeled with each of the isotope-labeled cysteines or [1-^{13}C]tyrosine were prepared by the *E. coli* cell-free protein expression system, as described previously.[18−20] A 5 mL reaction mixture, consisting of the cell extract from *E. coli* BL21 Star (DE3) (Invitrogen) and all other components, was placed in a dialysis tube and dialyzed against 20 mL of outer medium for 8 h at 37 °C. A total of 75 mg of the amino acid mixture, consisting of about 2 mg each of the isotope-labeled cysteines or tyrosine and 19 other unlabeled amino acids, was used for each reaction, which yielded 3−4 mg each of selectively labeled EPPIb's. The obtained labeled EPPIb's were dissolved in 50 mM sodium phosphate buffer, prepared from 100% H$_2$O, 100% D$_2$O, or H$_2$O/D$_2$O (1:1), respectively, containing 100 mM NaCl and 0.1 mM NaN$_3$, pH 7.5 (meter reading).

The sample concentrations varied from 0.2 to 0.7 mM, depending on the available labeled proteins, but there was no concentration dependence for the NMR spectra in this concentration range. In the case of the 100% H$_2$O solution, a 4.1 mm o.d. Shigemi tube containing the protein solution was inserted into a 5 mm o.d. outer tube containing pure D$_2$O for the ^{2}H lock signal.

NMR Measurements. All 1D-^{13}C NMR spectra were measured at 40 °C on a DRX600 spectrometer (Bruker Biospin; 150.9 MHz for ^{13}C), equipped with a 5 mm TCI cryogenic triple-resonance probe. The WALTZ16 decoupling scheme was used for ^{1}H and/or ^{2}H during acquisition.[21] The carrier frequency was set to 30 ppm with a sweep width of 4500 Hz, and free induction decays were acquired for 226 ms at a repetition time of 3 s. 1D-^{13}C NMR spectra with adequate signal-to-noise ratios were obtained within 6−24 h, depending on the concentrations of the samples. In order to ensure complete hydrogen−deuterium equilibration, we measured the time course of the deuterium exchange of the sulfhydryl and amide groups of the two Cys residues in D$_2$O, as shown in the Supporting Information. On the basis of this experiment, all of the protein samples dissolved in H$_2$O/D$_2$O (1:1) were incubated for at least 1 day at 40 °C to ensure that the hydrogen−deuterium equilibrium was completed before the NMR measurements.

The ^{13}C NMR exchange spectroscopy (EXSY) experiment was performed for a 0.7 mM H$_2$O/D$_2$O (1:1) solution of the EPPIb selectively labeled with [3-^{13}C;3,3-^{2}H$_2$]Cys, with the same pulse scheme used previously.[13] During the chemical shift encoding (t_1) and the acquisition of free induction decay (t_2), decoupling on ^{1}H and ^{2}H by the WALTZ16 scheme was applied.[21] The data points and the spectral widths were 512 (t_1) × 4096 (t_2) points and 1200

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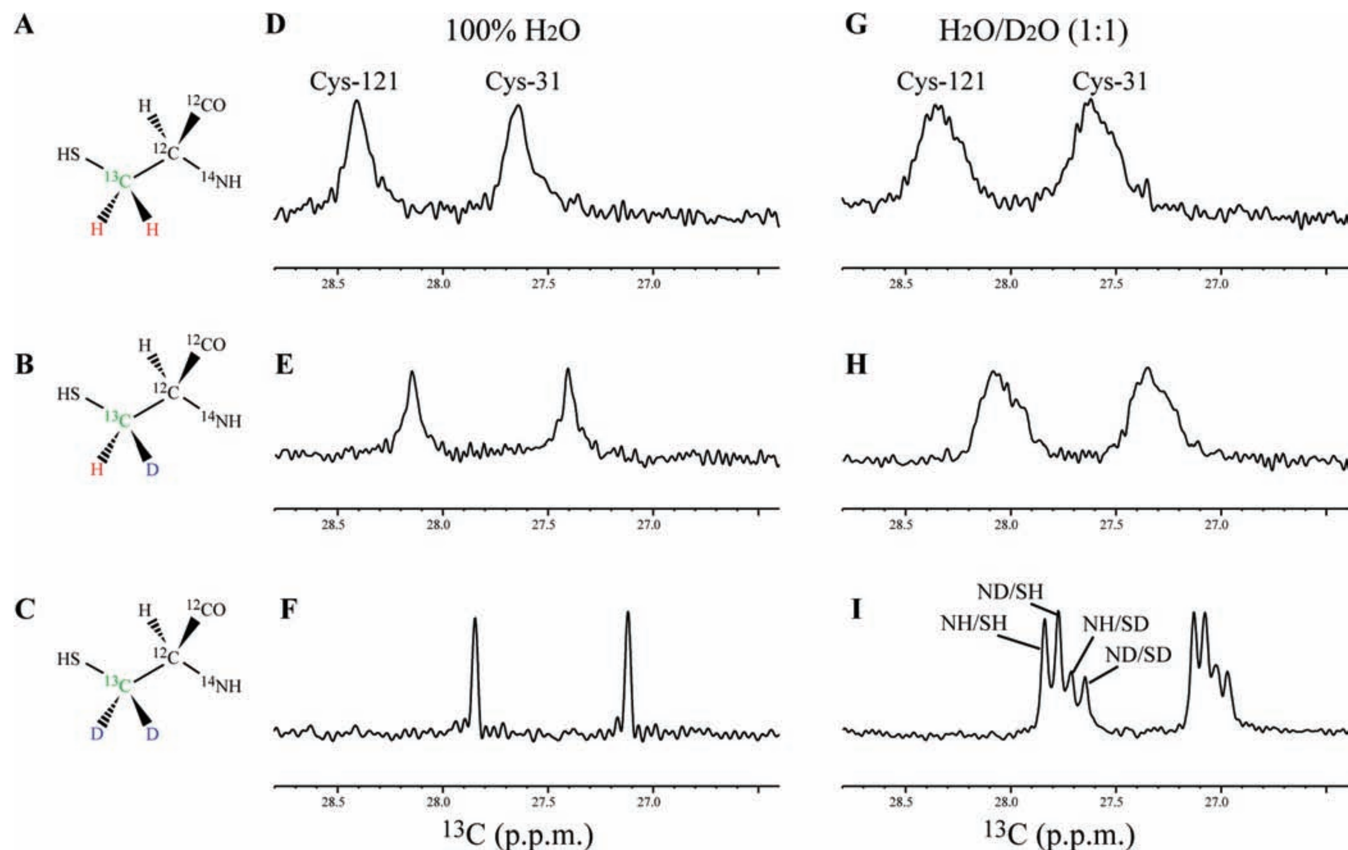

Figure 1. Comparison of the Cys $^{13}C_{\beta}$ signals of EPPIb's selectively labeled with [3-^{13}C]cysteine (A), (3*R*)-[3-^{13}C;3-$^{2}H_2$]cysteine (B), and [3-^{13}C;3,3-$^{2}H_2$]cysteine (C). The 150.9 MHz [^{2}H,^{1}H]-decoupled 1D-^{13}C NMR spectra of EPPIb's were obtained at 40 °C, pH 7.5, for 0.2−0.4 mM solutions. The spectra in D−F were obtained in H_2O, and those in G−H were in a H_2O/D_2O (1:1) solution.

Hz (ω_1, ^{13}C) × 9100 Hz (ω_2, ^{13}C), respectively, and the number of scans/FID was 240. The carrier frequency of carbon was set to 25.5 ppm. The mixing time was 600 ms, and the repetition time was 3.1 s.

We searched for NOE peaks involving the SH protons of Cys-31 and Cys-121 in ^{15}N- and ^{13}C-edited 3D-NOESY-HSQC spectra obtained from 0.3−0.5 mM EPPIb samples composed of 20 SAIL amino acids with different isotope labeling patterns of the phenylalanine and tyrosine residues.[22] The ^{15}N-edited 3D-NOESY-HSQC spectra were measured with a Bruker DRX600 spectrometer equipped with a TXI cryogenic probe. The data points and the spectral width of the ^{15}N-edited NOESY-HSQC were 256 (t_1) × 48 (t_2) × 1024 (t_3) points and 8400 Hz (ω_1, ^{1}H) × 1800 Hz (ω_2, ^{15}N) × 8400 Hz (ω_3, ^{1}H), respectively. The number of scans/FID was 32, and the NOE mixing time was 200 ms. The repetition time was 1 s. The ^{13}C-edited 3D-NOESY-HSQC experiments were performed with a DRX800 spectrometer equipped with a normal TXI probe, and the experiments were acquired for two different regions: the aliphatic and aromatic regions. In the first set for the aliphatic region, the data points and the spectral width were 210 (t_1) × 58 (t_2) × 1024 (t_3) points and 12 000 Hz (ω_1, ^{1}H) × 6700 Hz (ω_2, ^{13}C) × 10 000 Hz (ω_3, ^{1}H), respectively. The number of scans/FID was 32. The repetition time was 1 s. The carrier frequency of carbon was set to 40 ppm. In the second set for the aromatic region, the data points and the spectral width were 200 (t_1) × 40 (t_2) × 1024 (t_3) points and 12 000 Hz (ω_1, ^{1}H) × 5800 Hz (ω_2, ^{13}C) × 10 000 Hz (ω_3, ^{1}H), respectively. The number of scans/FID was 48. The repetition time was 1 s. The carrier frequency of carbon was set to 125.5 ppm. The NOE mixing time for both of the ^{13}C-edited NOESY-HSQC spectra was 145 ms.

Results and Discussion

^{13}C NMR Line-Narrowing Effect Caused by the Deuterium Substitution of the Methylene Protons on the Cys $^{13}C_{\beta}$ NMR Signals of EPPIb. Since the line widths of the $^{13}C_{\beta}$ Cys residues in EPPIb, even for the SAIL EPPIb, were too broad to resolve the isotope-shifted $^{13}C_{\beta}$ signals, it was necessary to optimize the isotope-labeling pattern of Cys in order to make the $^{13}C_{\beta}$ line width narrower than the signal separation due to the isotope shift. In doing so, the effect of the proton(s) attached to the C_{β} on the line width was examined by synthesizing [3-^{13}C]Cys, (3*R*)-[3-^{13}C;3-^{2}H]Cys, and [3-^{13}C;3,3-$^{2}H_2$]Cys, which were then incorporated within EPPIb by *E. coli* cell-free expression (Figure 1A−C).[18–20] In these labeled cysteines, only C_{β} was selectively labeled with ^{13}C, to eliminate the scalar and dipolar effects caused by adjacent ^{13}C nuclei, and thus the line-broadening effect due to the hydrogen atoms directly bonded to the $^{13}C_{\beta}$ atom could be precisely examined. The 1D-^{13}C NMR spectra of these three samples in 100% H_2O showed significantly different line widths for the ^{13}C signals by the replacement of either or both of the two methylene protons with a deuteron (Figure 1D−F): namely, ∼18 Hz ($^{13}C_{\beta}{}^1H_2$), ∼12 Hz ($^{13}C_{\beta}{}^1H^2H$), and ≤5 Hz ($^{13}C_{\beta}{}^2H_2$) (Table 1). Note that the $^{13}C_{\beta}$ showed upfield chemical shifts due to the deuterium substitutions, with an increment of 0.27 ± 0.02 ppm per deuterium. The longitudinal relaxation times (T_1) of the $^{13}C_{\beta}$ signals may become slightly longer by the replacement of one or two of the attached protons with a deuteron, thus resulting in the sensitivity loss

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Table 1. Chemical Shifts (ppm) and Line Widths ($\Delta\nu$, Hz) of the Cys $^{13}C_\beta$ Peaks in EPPIb Samples[a]

	100% H_2O		H_2O/D_2O (1:1)	
	Cys-31 $^{13}C_\beta$	Cys-121 $^{13}C_\beta$	Cys-31 $^{13}C_\beta$	Cys-121 $^{13}C_\beta$
[3-^{13}C]cysteine	28.41 ($\Delta\nu = 18 \pm 2$)	27.65 ($\Delta\nu = 18 \pm 2$)	n.d.	n.d.
(3*R*)-[3-^{13}C;3-$^{2}H_2$]cysteine	28.14 ($\Delta\nu = 12 \pm 2$)	27.40 ($\Delta\nu = 12 \pm 2$)	n.d.	n.d.
[3-^{13}C;3,3-$^{2}H_2$]cysteine	27.85 ($\Delta\nu \leq 5$)	27.12 ($\Delta\nu \leq 5$)	27.84 (NH/SH)	27.13 (NH/SH)
			27.77 (ND/SH)	27.07 (ND/SH)
			27.71 (NH/SD)	27.02 (NH/SD)
			27.65 (ND/SD)	26.97 (ND/SD)

[a] The values in the table were obtained from the NMR spectra shown in Figure 1. n.d. indicates that the C_β signals due to various isotopomers were not resolved in H_2O/D_2O (1:1).

within a fixed duration. However, this was not problematic in practice, since the significant line-narrowing effect by the deuteration compensated for such adverse effects. The ^{13}C NMR spectra of these three samples were also measured in H_2O/D_2O (1:1), which revealed additional line broadening due to the isotope shifts caused by the deuteration of the exchangeable hydrogens of the Cys residues, as discussed below. Since well-resolved $^{13}C_\beta$ signals for Cys-31 and Cys-121 were only observed with the EPPIb labeled with [3-^{13}C; 3,3-$^{2}H_2$]Cys in H_2O/D_2O (1:1) (Figure 1G−I), it was obvious that both protons on the $^{13}C_\beta$ carbon should be deuterated to accurately observe the isotope shifts induced by the deuterium substitution[23,24] of the Cys SH groups.

Identification of the Slowly Exchanging SH Protons and the Fractionation Factors for the SH's and Backbone Amides of Cys Residues in EPPIb. The ^{13}C NMR spectra of the EPPIb labeled with [3-^{13}C;3,3-$^{2}H_2$]Cys were also obtained in 100% D_2O, and were compared to those obtained in 100% H_2O and H_2O/D_2O (1:1) (Figure 2A). A comparison between these spectra revealed that the lowest and highest field peaks of the four well-resolved peaks in H_2O/D_2O (1:1), for each of the $^{13}C_\beta$ signals for Cys-31 and Cys-121, are identical with those observed in 100% H_2O and 100% D_2O, respectively (Figure 2A). Since there are two exchangeable protons for Cys, the sulfhydryl and backbone amide groups, which may affect the $^{13}C_\beta$ chemical shifts by the deuterium substitution, the observed four-line signals in the H_2O/D_2O (1:1) buffer were caused by the isotope shifts induced by the deuterium substitutions for both the SH's and backbone amides of the respective Cys residue (Figure 2B). The slow hydrogen exchange rates observed for both the SH and amide groups of Cys-31 and Cys-121 are compatible with the relative solvent accessibilities of these residues estimated from the crystal structure:[25] Cys-31 H_N, 0%; Cys-31 S, 0%; Cys-121 H_N, 9.1%; Cys-121 S, 0%. The two pairwise peaks, separated by smaller isotope shifts of 0.05−0.06 ppm, were intuitively attributed to the three-bond deuterium isotope effects on the backbone amides of the Cys residues.

To further confirm the origins of the deuterium substitution sites that induced larger isotope shifts (0.12 ± 0.02 ppm) with smaller fractionation factors ([D]/[H] ≈ 0.4), and smaller isotope shifts (0.06 ± 0.01 ppm) with larger fractionation factors ([D]/[H] ≈ 1.0), the fractionation factors of the backbone amides were independently determined by the DEALS method. The DEALS (*d*euterium−hydrogen *e*xchange rates of *a*mide on *l*ine *s*hapes) method[26−29] utilizes the line shapes of the carbonyl ^{13}C NMR signal for the (i − 1)th residue to monitor the deuterium−hydrogen exchange rate of the amide of the ith residue. The H/D exchange at the amide group causes a change in the chemical shift of the carbonyl carbon.[27,28] Given that the

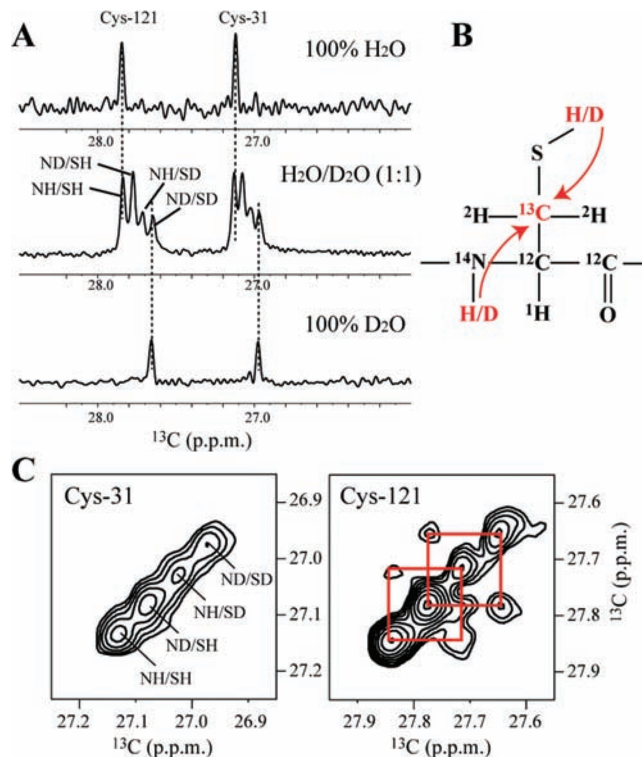

Figure 2. (A) 150.9 MHz [^{2}H,^{1}H]-decoupled 1D ^{13}C NMR spectra of EPPIb, selectively labeled with [3-^{13}C;3,3-$^{2}H_2$]Cys. The labeled EPPIb was dissolved in H_2O, D_2O, and H_2O/D_2O (1:1) buffers at concentrations ranging from 0.4 to 0.7 mM for each solution at pH 7.5 and was subjected to NMR experiments at 40 °C. The $^{13}C_\beta$ resonances for both Cys-31 and Cys-121 were observed as the four-line signals in H_2O/D_2O (1:1), due to the dual isotope effect from the backbone amide and side-chain sulfhydryl groups. (B) Structure of the [3-^{13}C;3,3-$^{2}H_2$]cysteine residue. The deuterium isotope effects from the side-chain sulfhydryl and backbone amide groups on the $^{13}C_\beta$ nuclei are shown by red arrows. (C) ^{13}C-EXSY spectra for the $^{13}C_\beta$ signals of Cys-31 and Cys-121 observed for the EPPIb selectively labeled with [3-^{13}C;3,3-$^{2}H_2$]Cys, at a mixing time of 600 ms. A 0.7 mM solution in H_2O/D_2O (1:1), pH 7.5, was used for the measurement at 40 °C. The two pairs of cross peaks for Cys-121, due to the SH/SD chemical exchange, are correlated with the diagonal peaks by red lines.

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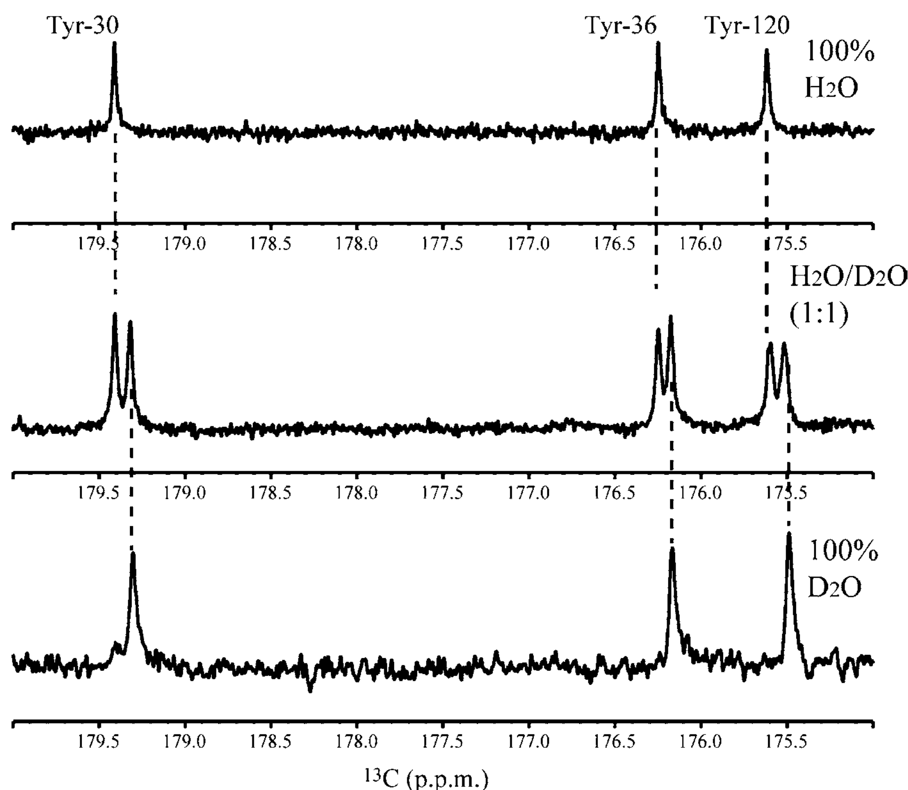

Figure 3. DEALS experiment for the carbonyl carbon ^{13}C NMR signals of the Tyr residues of EPPIb to monitor the amide deuterium−hydrogen exchange rates of the Cys residues. In order to monitor the amide exchange rates for Cys-31 and Cys-121, the carbonyl carbon NMR signals of the N-terminal neighbors of the two residues, namely Tyr-30 and Tyr-120, should be observed. For the DEALS experiment, EPPIb was dissolved in H_2O/D_2O (1:1) to observe the two-bond deuterium isotope shift effect. The fractionation factors ([D]/[H]) for the amides of Cys-31 and Cys-121 were estimated by the relative peak heights of the two isotope-shifted signals to be 0.9 and 1.0, respectively, which were identical with those obtained by the C_β signals (Table 2).

Table 2. Summary of the Deuterium-Induced Isotope Shifts for the C_β Signals of Cys-31 and Cys-121 and for the C′ signals of Tyr-30 and Tyr-120 and the Fractionation Factors for the Sulfhydryl and Amide Groups of Cys-31 and Cys-121

exchangeable group	affected C signal	isotope shift (Hz)	fractionation factor
Cys-31 SH	Cys-31 $^{13}C_\beta$	19.2 ± 3	0.5 ± 0.1
Cys-31 NH	Cys-31 $^{13}C_\beta$	9.9 ± 2	0.9 ± 0.1
Cys-31 NH	Tyr −30 $^{13}C'$	13.5 ± 1	0.9 ± 0.1
Cys-121 SH	Cys-121 $^{13}C_\beta$	16.0 ± 3	0.4 ± 0.1
Cys-121 NH	Cys-121 $^{13}C_\beta$	8.0 ± 2	1.0 ± 0.1
Cys-121 NH	Tyr −120 $^{13}C'$	13.8 ± 1	1.0 ± 0.1

H/D exchange is slow, as compared to the size of the isotope shift effect, the carbonyl signal appears as a doubled peak. In contrast, when the H/D exchange is rapid, as compared to the size of the isotope shift effect, the carbonyl signal gives one averaged signal. In the case of the backbone amides of the two Cys residues in EPPIb, i.e. Cys-31 and Cys-121, their amide exchange rates can be monitored by the carbonyl ^{13}C NMR signals of their N-terminal neighbors, i.e. Tyr-30 and Tyr-120, respectively, in an H_2O/D_2O (1:1) solution (Figure 3). Obviously, both of the amides of the two Cys residues exhibited slow exchange rates, and the fractionation factors were almost unity ([D]/[H] $\approx$ 1.0), as shown by the relative intensities of the isotope-shifted carbonyl carbon signals of Tyr-30 and Tyr-120, respectively. Therefore, the larger splitting, namely 0.11 and 0.13 ppm, is due to the two-bond isotope effect on the slowly exchanging SH groups for Cys-31 and Cys-121, respectively, which can be rationalized by their burial in the EPPIb structure. Note that the two-bond SH/SD isotope shift effect on the $^{13}C_\beta$ chemical shifts is almost identical with that for the

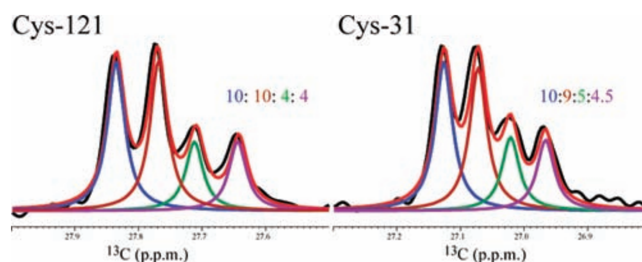

Figure 4. Fractionation factors for the sulfhydryl groups ([SD]/[SH]) and the backbone amides ([ND]/[NH]) of Cys-31 and Cys-121 in EPPIb dissolved in the H_2O/D_2O (1:1) mixture. Line-shape analyses were performed with the DNMR software (Bruker Biospin). For each analysis, the line width of each isotopomer peak was set to 5 Hz. The observed $^{13}C_\beta$ signals (black) are shown, along with the four components resolved into each of the four isotopomer peaks: SH/NH (blue), SH/ND (brown), SD/NH (green), and SD/ND (magenta), respectively. The relative intensity of each signal component was adjusted to minimize the difference between the observed (black) and simulated spectra (red).

$^{13}C_\zeta$ of the Tyr residue induced by the deuteration of OH, which was ~0.13 ppm.[13] We also performed a preliminary EXSY experiment for the sample dissolved in an H_2O/D_2O (1:1) solution. Since the $^{13}C_\beta$ signals for both Cys-31 and Cys-121 split into four peaks due to the dual isotope effect, the experiments were very time-consuming, and thus we only measured the EXSY spectrum at a mixing time of 600 ms, in order to identify the exchangeable NH/ND and SH/SD. On the basis of the data shown in Figure 2C, we could estimate the approximate hydrogen exchange rate for the SH group of Cys-121 to be $k_{ex} \approx 0.9 \pm 0.3$ s^{-1}. In addition, the fastest limit for the hydrogen exchange rates for the SH of Cys-31 was estimated

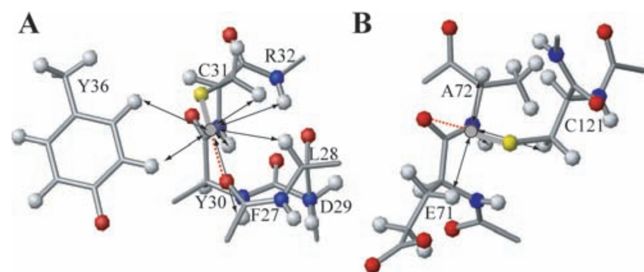

Figure 5. Hydrogen bonds involving the sulfhydryl groups of Cys-31 (A) and Cys-121 (B). Hydrogen, oxygen, nitrogen, and sulfur atoms are shown by white, red, blue, and yellow balls, respectively. The deduced position of the sulfhydryl hydrogen for each of the two Cys residues is shown by a gray circle, and the experimentally observed NOE's between the sulfhydryl groups and the nearby protons are indicated by arrows. This figure was produced by using MolMol software.[32]

to be $k_{ex} < 0.3\ s^{-1}$, and that for the amide groups of Cys-31 and Cys-121 was $k_{ex} < 0.2\ s^{-1}$.[13] The absence of SH/SD exchange cross peaks for Cys-31 implies that the hydrogen bond was formed between the sulfhydryl group of Cys-31 (*i*th residue) and the backbone carbonyl oxygen of Phe-27 ((*i* − 4)th residue). Since the amide hydrogen of Cys-31 also forms a hydrogen bond to the carbonyl oxygen of Phe-27 in the α-helix, this extra hydrogen bond results in the bifurcated hydrogen bond between Cys-31 and Phe-27. Similar bifurcated hydrogen bonds are frequently found in the Cys residues involved in α-helices, and the auxiliary hydrogen bond between the side-chain sulfhydryl group and the backbone carbonyl oxygen seems to contribute toward stabilizing the α-helices.[4] This bifurcated hydrogen bond might be responsible for the slow hydrogen exchange rates for the sulfhydryl group of Cys-31, as shown by the lack of cross peaks in the EXSY experiment, even at a 600 ms mixing time (Figure 2C).

The proton/deuteron fractionation factors for the backbone amides and side chains of the sulfhydryl groups were quantitatively determined for the Cys residues, by measuring the peak areas by a line-shape analysis of the four-line ^{13}C signals observed for the EPPIb labeled with [3-^{13}C;3,3-$^{2}H_2$]Cys dissolved in the 1:1 H_2O/D_2O (1:1) solution (Figure 4). The isotope shift values and the fractionation factors obtained by the line-shape analyses are summarized in Table 2. The SH groups of the Cys residues in EPPIb exhibited fractionation factors around ∼0.4−0.5, indicating that their SH groups have stronger affinity toward a proton over a deuteron, relative to its content in a solution.[5,6] Further accumulation of such data for SH groups will be essential to correlate the fractionation factors to their microenvironments.

NOE's Involving the SH Protons of Cysteine Residues in EPPIb. Intuitively, a discrete SH proton signal could be observable in H_2O for the Cys residues with SH groups that have a slow hydrogen exchange rate, thereby yielding two separate $^{13}C_\beta$ signals in the H_2O/D_2O (1:1) mixture due to the

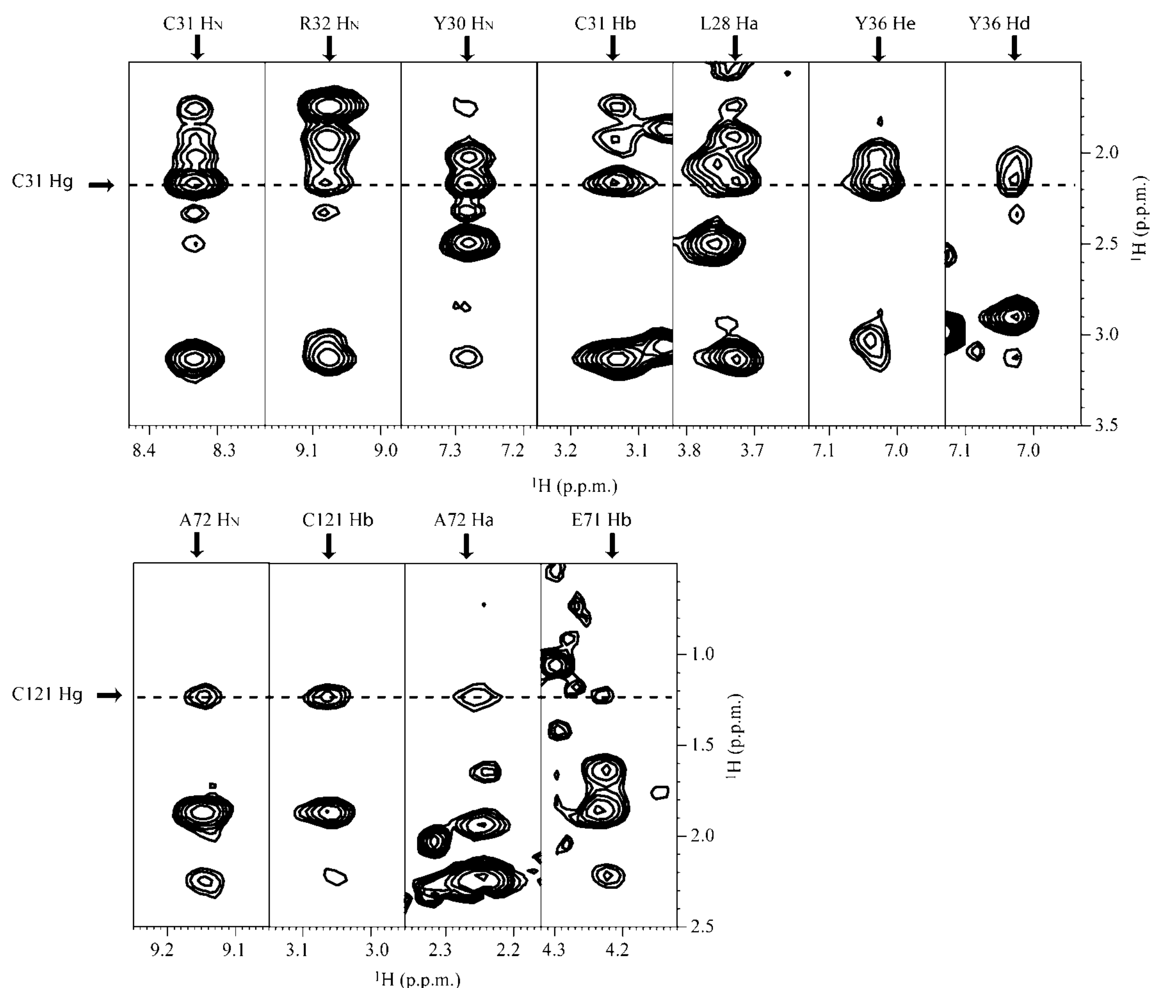

Figure 6. Strips of the ^{15}N- and ^{13}C-edited 3D-NOESY-HSQC spectra containing NOE's associated with the side-chain sulfhydryl protons of Cys-31 and Cys-121.

SH and SD species.[9] This was actually the case for the two Cys residues in EPPIb. The two signals at 2.17 and 1.24 ppm were identified by NOESY data obtained for the EPPIb fully labeled with SAIL amino acids, including (3*R*)-[ul-^{13}C;3-^{2}H;^{15}N]Cys, SAIL Cys,[15] which were assigned to the SH groups of Cys-31 and Cys-121, respectively (Figure 5). These two peaks could also be identified by the HSQC-TOCSY experiment for the EPPIb selectively labeled with (3*R*)-[3-^{3}C;3-^{2}H]Cys, which utilizes the vicinal ^{1}H−^{1}H spin couplings between SH and H_β (data not shown). The atomic coordinates of the EPPIb crystal structure (PDB #: 2NUL)[15] revealed that the carbonyl oxygen atoms of Phe-27 and Glu-71 are in close proximity to the sulfur atoms of Cys-31 and Cys-121, respectively, and seem to form hydrogen bonds with the SH groups. In solution, the locations of the SH protons were unambiguously identified from a number of observed NOEs and supported the existence of these hydrogen bonds (Figure 6). It should be noted that a hydrogen bond between the SH group of Cys and the backbone carbonyl oxygen of the residue four residues behind in the sequence thus forms the bifurcated hydrogen bonds which stabilize the α-helix as described above. It should also be noted that, in the crystal structure of EPPIb, the hydrogen bond lengths involving Cys-31 and Cys-121 are 3.3 and 3.7 Å, respectively, which are considerably longer than the typical hydrogen bond lengths observed for amide and hydroxyl hydrogen bonds, which are usually less than 3 Å. Due to the longer hydrogen bond lengths involving sulfur, which are attributed to the larger atomic size of sulfur as compared to those of nitrogen and oxygen atoms,[4] it is expected that relatively larger distance constraints should be imposed for the hydrogen bonds involving SH groups. Therefore, such hydrogen bonds are useful for refining the local structures around the SH's (Figure 6).

Conclusion

We have developed a method to detect the slowly exchanging SH groups of Cys residues in proteins. The method utilizes a protein labeled with [3-^{13}C;3,3-$^{2}H_2$]Cys, which gives very narrow line widths for the $^{13}C_\beta$ signals, thus allowing us to observe the isotope shifts due to the deuterium substitution on the side-chain sulfhydryl and backbone amide groups simultaneously. The two Cys residues in EPPIb, Cys-31 and Cys-121, are both located in the interior of the protein, and their sulfhydryl and amide groups are involved in hydrogen bonds. As a consequence, the lifetimes of the H/D atoms on the SH/SD and NH/ND groups were long enough to give discrete $^{13}C_\beta$ signals, and thus the fractionation factors of both the amide and sulfhydryl hydrogen atoms could be directly determined by the relative peak intensities of the discrete $^{13}C_\beta$ signals. However, the present method can also be applied to the surface SH groups, which generally have much faster hydrogen exchange rates and thus the signals split by the isotope shifts tend to be coalesced. In such cases, the fractionation factors could be estimated by measuring the averaged chemical shifts in solvents with different H/D ratios, as often used for small organic molecules.[5] Since the hydrogen exchange rate and the fractionation factor are presumably correlated with the solvent accessibility and the hydrogen bond strength,[6,30,31] respectively, this approach will provide various new possibilities to study the microenvironments of Cys residues in proteins. Note that the proteins labeled with isotope-labeled cysteines[33] can be prepared by either cell-free protein synthesis or the standard *E. coli* cellular expression system.[34]

Acknowledgment. This work was supported by the Targeted Protein Research Program (MEXT) to M.K. It was also supported in part by a Grant-in-Aid for Young Scientists (B) (21770110) to M.T. and a Grant-in-Aid on Innovative Areas (4104) to J.J.

Supporting Information Available: A figure detailing the time course of the deuterium exchange for the backbone amide and sulfhydryl groups of Cys-31 and Cys-121 in EPPIb, as observed by the [^{2}H,^{1}H]-decoupled $^{13}C_\beta$ NMR signals at 150.9 MHz. This material is available free of charge via the Internet at http://pubs.acs.org.

JA101205J

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