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Dynamics of the three methionyl side chains of *Streptomyces* subtilisin inhibitor. Deuterium NMR studies in solution and in the solid state

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Abstract

Streptomyces subtilisin inhibitor (SSI) contains three methionine residues in a subunit: two (at positions 73 and 70) in the crucial enzyme-recognition sites P1 and P4, respectively, and one (Met 103) in the hydrophobic core. The motions of the side chains of these three Met residues and the changes in mobility on binding with subtilisin were studied by deuterium NMR spectroscopy in solution and in crystalline and powder solids. For this purpose, the wild-type SSI was deuterium-labeled at the methyl groups of all three Met residues, and three artificial mutant proteins were labeled at only one specific Met methyl group each. In solution, for methionines 73 and 70, the effective correlation times were only $0.8–1.0 \times 10^{-10}$ s indicating that the two side chains on the surface fluctuate almost freely. On formation of a complex with subtilisin, however, these high mobilities were quenched, giving a correlation time of 1.1×10^{-8} s for the side chains of methionines 70 and 73. The correlation time of Met 103, located in the hydrophobic core, was at least 1.0×10^{-8} s in free SSI, showing that its side chain motion is highly restricted. The nature of the internal motions of the three Met side chains was examined in more detail by deuterium NMR spectroscopy of powder and crystalline samples. The spectral patterns of the powder samples depended critically on hydration: immediately after lyophilization, the side-chain motions of the three Met residues were nearly quenched. With gradual hydration to 0.20 gram of water per gram protein-water, the orientational fluctuation of the methyl axes of methionines 70 and 73 was selectively enhanced in both amplitude and frequency (to about 1 MHz) and, at nearly saturating hydration (0.60 gram of water per gram protein-water), became extremely high in amplitude and frequency (>10 MHz). In contrast, the polycrystalline wild-type SSI spectrum showed fine structures, reflecting characteristic motions of the Met side chains. The polycrystalline spectrum could be reproduced reasonably well by the same motion models and parameters used to simulate the powder spectrum at the final level of hydration, suggesting that the side-chain motions are similar in the fully hydrated powder and in crystals. Spin-lattice relaxation measurements gave evidence that, even in crystals, the methyl axes of all three Met residues undergo rapid motions with correlation times between 10^{-8} and 10^{-10} s, comparable to the correlation times in solution. Finally, in the hydrated stoichiometric complex of SSI with subtilisin BPN′ in the solid state, large-amplitude motions are absent, but the side chains of methionines 70 and/or 73 are likely to have small-amplitude motions.

Keywords: deuterium NMR; methionyl side chain dynamics; hydration of solid proteins; protein internal motion; specific deuteration; *Streptomyces* subtilisin inhibitor

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Abbreviations: SSI, *Streptomyces* subtilisin inhibitor; P*i*, P*i*′ and S*i*, S*i*′, nomenclature of Schechter and Berger for the subsites of a substrate (or an inhibitor) and of a protease; T_1, longitudinal (spin-lattice) relaxation time; T_2, transverse relaxation time; τ_c, correlation time.

A protein molecule is a dynamic entity with a variety of motions over a wide frequency range. This dynamic view of a protein molecule comes from various sources, including *B* factors in X-ray analysis, fluorescence, flush photolysis, Raman spectroscopy, molecular dynamic calculations, hydrogen exchange, and NMR spectroscopy (for examples, see Brook et al., 1987;

Frauenfelder et al., 1988). Of these methods, deuterium NMR is unique in that it allows direct observation and detailed analysis of internal motions of amino acid side chains both in the solid state (Oldfield & Rothgeb, 1980; Kinsey et al. 1981a, 1981b; Rice et al., 1981; Batchelder et al., 1982; Gall et al., 1982; Keniry et al., 1983, 1984; Baianu et al., 1984; Colnago et al., 1987; Leo et al., 1987; Davis, 1988; Shon et al., 1991) and in solution (Wooten & Cohen, 1979; Akasaka et al., 1988), provided that site-specific deuterium labeling is made. For example, in cytochrome *c* (Spooner & Watts, 1991), and in filamentous bacteriophage Pf1 coat protein (Nambudripad et al., 1991), amino acid residues with distinct internal motions have been identified with deuterium NMR in the solid state.

Despite the evidence for internal motion, however, some basic questions and details concerning protein molecular dynamics, particularly in globular proteins, are unclear. These include the following questions. (1) How related are the motions of a specific amino acid residue of a protein in the solid state and in solution? (2) How are internal motions in the solid state related to the degree of hydration? (3) How are the internal motions of a protein in powder form related to those in a crystal? (4) How do the motions of a specific type of amino acid, such as methionine, differ at different locations in a protein molecule (e.g., surface and core)? (5) How are the internal motions of a protein affected by interaction with another protein?

To gain some insight into these questions, the methyl groups of the three Met side chains in a subunit of *Streptomyces* subtilisin inhibitor (SSI), a dimeric protein of MW 23,000 (Fig. 1), were labeled with deuterium, and their motions were studied by deuterium NMR both in the solid state and in solution, using line shape analysis and spin relaxation measurements. SSI is a protease inhibitor that inhibits the activity of a wide variety of proteases, particularly those of bacterial origin, including subtilisin BPN′. The two Met residues at positions 73 and 70 are located on the surface and respectively constitute the reactive-site (P1) and P4 residues of the inhibitor (Schechter & Berger, 1967). Their side chains penetrate respectively into the S1 and S4 pockets of a serine protease and determine the specificities of binding (Kojima et al., 1990a, 1990b, 1991; Takeuchi et al., 1991a). In contrast, the methyl group of Met 103 is located far (about 15 Å) from the enzyme-binding region and is totally buried in the hydrophobic core (Mitsui et al., 1979). The dynamics of the long side chain of methionine are considered to be particularly sensitive to the microenvironment (Keniry et al., 1983). Furthermore, because in methionine the rotation of the methyl group about its threefold axis is expected to be much more rapid (10^{11} Hz or more at 20 °C) (Andrew et al., 1976; Batchelder et al., 1983) than the deuterium Larmor frequency and is unlikely to affect the spin-lattice relaxation time (T_1) of the methyl deuterons, the mobility of its side chain (which affects the orientation of the methyl group) would be the main influence on T_1. If slower internal motions (in the milli- to microsecond range) are present, they may be reflected in the line shape of the deuterium NMR spectrum for the solid-state protein, in which the overall rotation of the molecule is quenched.

Although, in wild-type SSI, the ^{1}H NMR signals of the methyl protons of methionines 70, 73, and 103 are clearly observed at discrete positions in solution (Akasaka et al., 1982; Tamura et al., 1991), the corresponding deuterium NMR signals were difficult to separate because of line broadening. To identify the deuteromethyl signal of each Met residue and to analyze its dynamics by deuterium NMR in solution and in the solid state, we prepared three artificial SSI mutants, in each of which two of the three Met residues were replaced with other amino acids. The intact methionine in each mutant (Met 70, 73, and 103, respectively) was subjected to specific deuteration by biosynthesis.

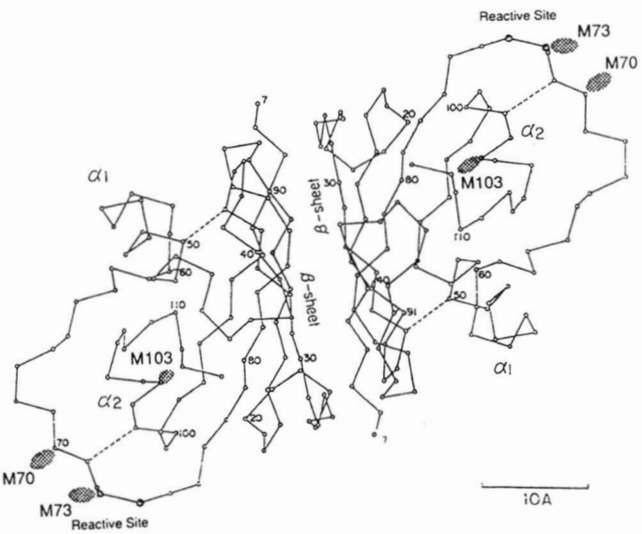

Fig. 1. Crystal structure of *Streptomyces* subtilisin inhibitor (α-carbon backbone) consisting of two identical subunits, showing the location of the three methionyl side chains (Mitsui et al., 1979), with chemical structure of [methyl-$^2H_3$]methionine.

Results

^{2}H-NMR in solution

Results with the wild-type SSI([methyl-$^2H_3$]Met70,73,103)

Figure 2 shows ^{2}H NMR spectra of SSI([methyl-$^2H_3$]Met-70,73,103) in ^{2}H-depleted water (10 mg/mL) measured at 20 °C by the inversion recovery pulse sequence (180°-τ-90°-τ-FID) with varying τ. The main peak centered at about 2 ppm is the combined resonance from intact SSI([methyl-$^2H_3$]Met-70,73,103), whereas the small peak appearing as a shoulder at about 2.7 ppm is attributable to SSI([methyl-$^2H_3$]Met-70,73,103) in which the exposed side chains of Met 70 and Met 73 have been oxidized to sulfoxides (Akasaka et al., 1982). A tiny peak at 4.8 ppm is due to residual $^2H^1HO$. A careful examination of Figure 2 indicates that the main peak itself consists of two components, narrow and broad, which differ in longitudinal relaxation times (T_1) and also slightly in chemical shift. From the semilogarithmic plot of their intensities against τ, the T_1 value of the broader component was determined to be 98 ms, whereas that of the narrower component was determined to be 180 ms (Table 1). The integral intensity of the broader component corresponds to approximately one-third of the total intensity, whereas that of the narrower component corresponds to approximately two-thirds of the total intensity. From the comparison of their chemical shift positions and line widths with those of the as-

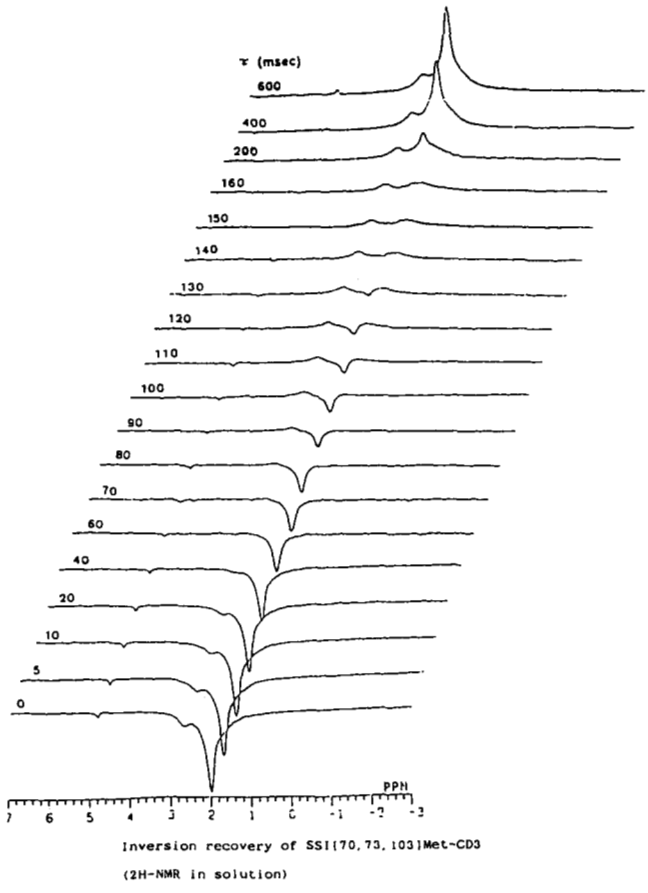

Fig. 2. Inversion-recovery ^2H NMR spectra of SSI([methyl-$^2H_3$]Met-70,73,103) in ^2H-depleted water (10 mg/mL) measured at 20 °C by the pulse sequence (180°-τ-90°-τ-FID) with varying τ (see text for explanation). The small peak appearing as a shoulder at about 2.7 ppm is attributable to SSI([methyl-$^2H_3$]Met-70,73,103) in which the side chains of Met 70 and Met 73 were oxidized to sulfoxides, and the tiny peak at 4.8 ppm is due to residual $^2H^1HO$.

signed proton signals of the three Met methyl groups (Tamura et al., 1991), it is clear that the broader component represents Met 103 (proton chemical shift $\delta = 1.78$ ppm) and the narrower component represents the combined intensities of Met 70 ($\delta = 2.078$) and Met 73 ($\delta = 2.124$).

Results with the mutants 73K103L (70MetC$^2H_3$), 70I103L (73MetC$^2H_3$) and 70I73K (103MetC$^2H_3$)

The three SSI mutants prepared by site-directed mutagenesis were 73K103L (70MetC$^2H_3$), 70I103L (73MetC$^2H_3$), and 70I73K (103MetC$^2H_3$). ^1H NMR spectra confirmed that these mutations did not appreciably alter the protein conformation. Therefore, the deuterium NMR spectra of singly labeled Met residues in these mutants were considered adequately to represent the motions of the labeled Met residues in the wild-type SSI. Figure 3 shows the inversion recovery spectra of these mutant proteins in ^2H-depleted water (5 mg/mL) at 20 °C. Correlation times from T_1 values were determined for individual Met residues, and only those that are consistent with the correlation times from T_2 are listed in Table 1.

Results with SSI([methyl-2H$_3$]Met-70,73,103) in complex with subtilisin BPN′

When SSI([methyl-$^2H_3$]Met-70,73,103) (I_2) was complexed with the protease subtilisin BPN′ (E), it formed a molecular complex with two molecules of the enzyme (E_2I_2; MW 78,000), and all the deuterium NMR signals for Met 70, 73, and 103 were broadened (Fig. 4).

^2H-NMR in the solid state

The wild-type SSI([methyl-$^2H_3$]Met-70,73,103) and 73K103L (70MetC$^2H_3$) were obtained in both powder and crystal forms. For 70I103L (73MetC$^2H_3$) and 70I73K (103MetC$^2H_3$), only powder samples were used, owing to the failure of these proteins to grow as crystals.

First, the motion of the Met 73 side chain was examined for the powder sample of 70I103L (73MetC$^2H_3$) at various degrees of hydration, as shown in Figure 5 with corresponding simulated spectra. Second, the polycrystalline sample of 73K103L (70MetC$^2H_3$) was used to investigate the motion of the Met 70 side chain, as shown in Figure 6 together with a simulated spectrum. Figures 7 and 8 show the ^2H-NMR spectra for the polycrystalline and powder forms, respectively, of wild-type SSI([methyl-$^2H_3$]Met-70,73,103) at different degrees of hydration. The computer simulation of the experimental spectra was conducted on the assumption that the spectrum for Met 103 is given by subtracting the spectra for Met 70 and 73 from the spectrum for the wild-type SSI([methyl-$^2H_3$]Met-70,73,103). Inversion recovery and τ variation experiments are shown in Figures 9 and 10, respectively, for the wild-type SSI([methyl-$^2H_3$]Met-70,73,103). Figure 11 shows the ^2H-NMR powder spectra for a complex of wild-type SSI([methyl-$^2H_3$]Met-70,73,103) with subtilisin BPN′ in the dry and hydrated states.

Discussion

Protein in solution

In analyzing the relaxation data of methyl deuterons, the first motion we must consider is the random three-site jumping of the methyl group about the S_δ-C_ϵ bond. On the grounds that the rotational barrier for a methyl group is determined primarily by local chemical bonds rather than by the environment of the methyl group (Andrew et al., 1980; Ishima et al., 1991), the correlation time for the random jumps of the Met methyl groups of SSI at 20 °C was estimated from the low-temperature proton T_1 data of the crystalline amino acid D,L-methionine (Andrew et al., 1976) by extrapolating it to 20 °C. A value even an order of magnitude smaller (10^{-12} s) was estimated from deuterium T_1 data of crystalline L-methionine (Batchelder et al., 1983) by extrapolating the correlation times at low temperature to 20 °C. Whichever of the two values is adopted, this motion is faster than the Larmor precession (6.14×10^7 Hz) by at least three orders of magnitude, and the T_1 and T_2 values are not sensitive to the frequency of this motion. The consequence of this rapid motion is simply that the quadrupolar interaction is reduced by $(3\cos^2\gamma - 1)/2$, where γ is the angle between the methyl axis and the C-^2H bond, resulting in the elongation of both T_1 and T_2 by about 10-fold. The result is that the T_1 and T_2 values of the Met methyl deuterons are sensitive only to the tumbling mo-

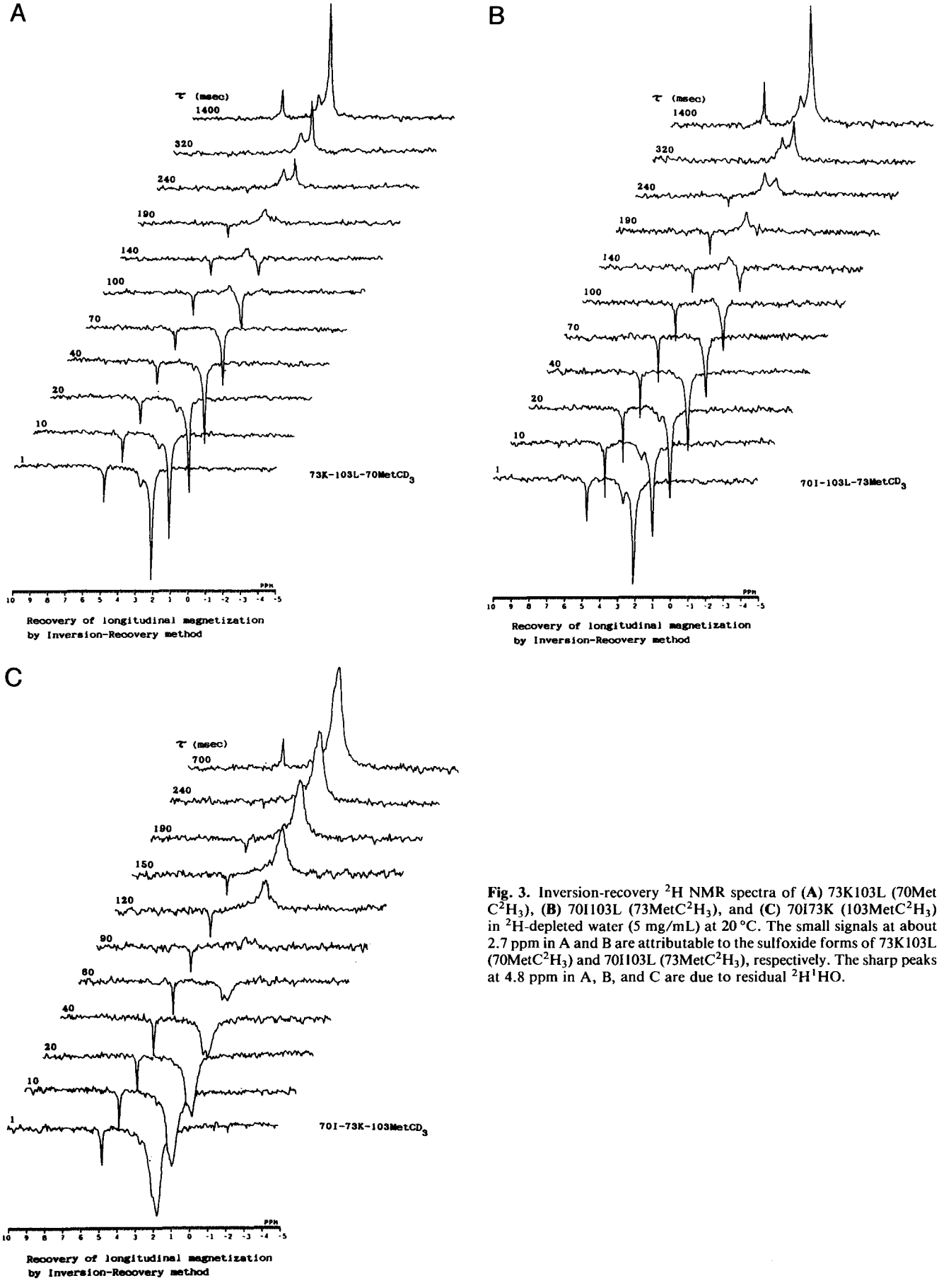

Fig. 3. Inversion-recovery ^2H NMR spectra of (**A**) 73K103L (70Met C^2H_3), (**B**) 70I103L (73MetC^2H_3), and (**C**) 70I73K (103MetC^2H_3) in ^2H-depleted water (5 mg/mL) at 20 °C. The small signals at about 2.7 ppm in A and B are attributable to the sulfoxide forms of 73K103L (70MetC^2H_3) and 70I103L (73MetC^2H_3), respectively. The sharp peaks at 4.8 ppm in A, B, and C are due to residual $^2H^1HO$.

Table 1. *Longitudinal (T_1) and transverse (T_2) relaxation times of methyl deuterons of the methionines in Streptomyces subtilisin inhibitor in solution*[a]

Type of SSI	T_1 (ms)	Line width (Hz)	Correlation time (s)	
			From T_1	From line width
Wild-type SSI([methyl-$^2H_3$]Met-70,73,103)				
Narrow component	180	10	1.4×10^{-10}	$<1.0 \times 10^{-9}$
Broad component	98	50	1.0×10^{-8}	$<1.1 \times 10^{-8}$
Wild-type SSI([methyl-$^2H_3$]Met-70, 73,103) complexed with subtilisin	102	120	1.1×10^{-8}	$<3.0 \times 10^{-8}$
73K103L (70MetC^2H_3)	250	8	9.8×10^{-11}	$<6.2 \times 10^{-10}$
70I103L (73MetC^2H_3)	330	9	7.6×10^{-11}	$<7.0 \times 10^{-10}$
70I73K (103MetC^2H_3)	120	42	1.3×10^{-8}	$<1.0 \times 10^{-8}$

[a] T_1 and T_2 relaxation times were measured at 61.4 MHz at 20 °C, and the correlation times for the reorientation of methyl axes were calculated from Equations 1 and 2.

tion of the methyl axis (i.e., of the S_δ-C_ϵ bond), which may reflect the whole rotation of the protein molecule plus internal motions of the Met side chain. When this tumbling motion of the methyl axis is analyzed with a single effective correlation time τ_c, then the T_1 and T_2 values for the methyl deuterons of a Met residue can be expressed by the following equations (Abragam, 1961):

$$\frac{1}{T_1} = \frac{(3\cos^2\gamma - 1)}{4} \cdot \frac{3}{40} \cdot \left(\frac{e^2qQ}{h}\right)^2 \cdot \left(1 + \frac{\eta^2}{3}\right) \cdot \left(\frac{\tau_c}{1 + \omega^2\tau_c^2} + \frac{4\tau_c}{1 + 4\omega^2\tau_c^2}\right), \quad (1)$$

$$\frac{1}{T_2} = \frac{(3\cos^2\gamma - 1)^2}{4} \cdot \frac{3}{80} \cdot \left(\frac{e^2qQ}{h}\right)^2 \cdot \left(1 + \frac{\eta^2}{3}\right) \cdot \left(3\tau_c + \frac{5\tau_c}{1 + \omega^2\tau_c^2} + \frac{2\tau_c}{1 + 4\omega^2\tau_c^2}\right). \quad (2)$$

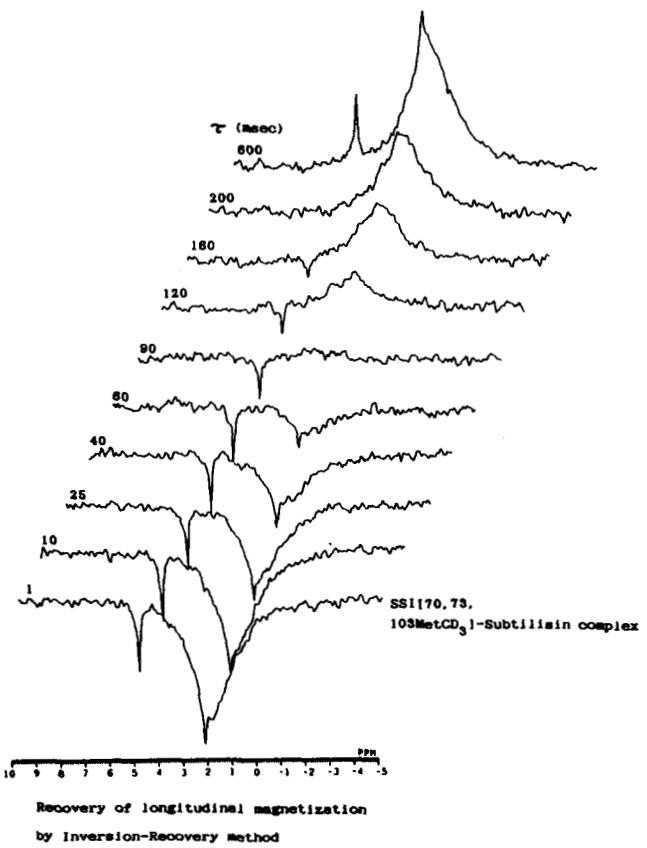

Fig. 4. Inversion-recovery ^2H NMR spectra of SSI([methyl-$^2H_3$]Met-70,73,103) complexed with subtilisin BPN′ in ^2H-depleted water at 20 °C. The tiny, sharp component at 2.1 ppm on top of the main broad signal is attributable to the residual fraction of free (uncomplexed) SSI([methyl-$^2H_3$]Met-70,73,103). The sharp singlet at 4.8 ppm is due to residual $^2H^1HO$.

We adopted $\gamma = 69.5°$, a quadrupole coupling constant $e^2qQ/h = 168$ kHz, and the asymmetry parameter $\eta = 0$ (Kinsey et al., 1981a). Generally, two correlation times are obtained from a single T_1 value, because T_1 is a bivalued function of the correlation time. Only the one that was consistent with the correlation time from T_2 was chosen as appropriate for each Met residue (see Table 1).

The correlation times for the broad component (Met 103)—1.0×10^{-8} s from T_1 and 1.1×10^{-8} s from T_2 (line width)—nearly coincide. They also coincide fairly well with the correlation time (1.0–1.4×10^{-8} s) obtained from deuterium T_1 and T_2 values for the buried Trp 86 ring of SSI (Akasaka et al., 1988). These values are also close to what is expected for the rotational correlation time of a globular protein of MW 23,000 from the Stokes–Einstein equation (Inoue & Akasaka, 1987), showing that these correlation times represent the overall rotation of the protein molecule. This conclusion does not necessarily mean that the side chain of Met 103 is totally immobilized in the protein framework; rather, it only means that the correlation time of the side-chain motion of Met 103 is, at least, not smaller than 1.0×10^{-8} s. This point will be examined in more detail later in relation to solid-state SSI.

On the other hand, for the narrower component (methionines 70 and 73), the correlation time from T_1 that is consistent with

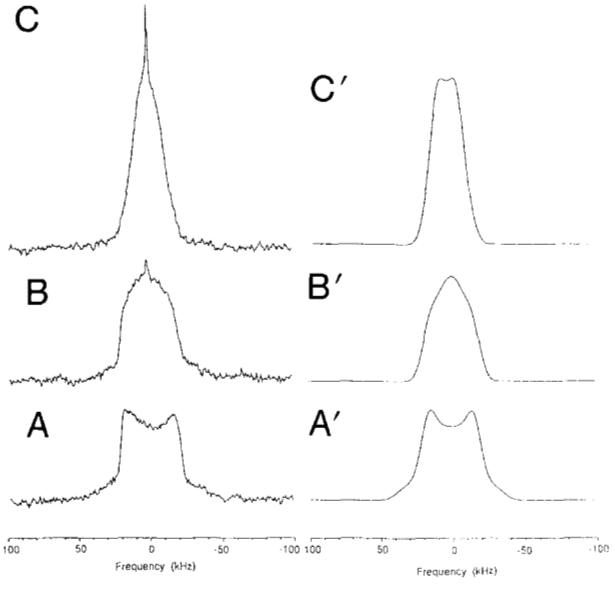

Fig. 5. Deuterium NMR spectra of powder 70I103L (73MetC^{2}H$_3$). **A:** As lyophilized (may contain 0.09 grams of water per gram protein-water; Tamura et al., 1992). **B:** At a hydration of 0.20 gram of water per gram protein-water. **C:** At a hydration near saturation (0.60 gram of water per gram protein-water). Each spectrum was obtained by accumulating about 450,000 transients with a repetition time of 0.22 s and Fourier transformation with an exponential filter of 500 Hz. The sharp singlets in the middle of the spectrum in B and C are due to the naturally abundant ^{1}H^{2}HO. **A′:** Computer simulation pattern obtained by assuming a random jump of the methyl axis between two isoenergetic sites spanning an angle of 80° at a rate of 10 kHz (see Table 1). **B′:** Computer simulation pattern obtained under the assumption of a two-site random jump of the methyl axis spanning an angle of 65° at a rate of 1.0 MHz (Table 2). **C′:** Computer simulation pattern obtained for random jumps between neighboring sites of four isoenergetic positions on a distorted cone at a rate of 10 MHz or higher (Table 2). Spectra A′, B′, and C′ were drawn after applying 0.5-kHz Lorentzian broadening and 10.0-kHz Gaussian broadening.

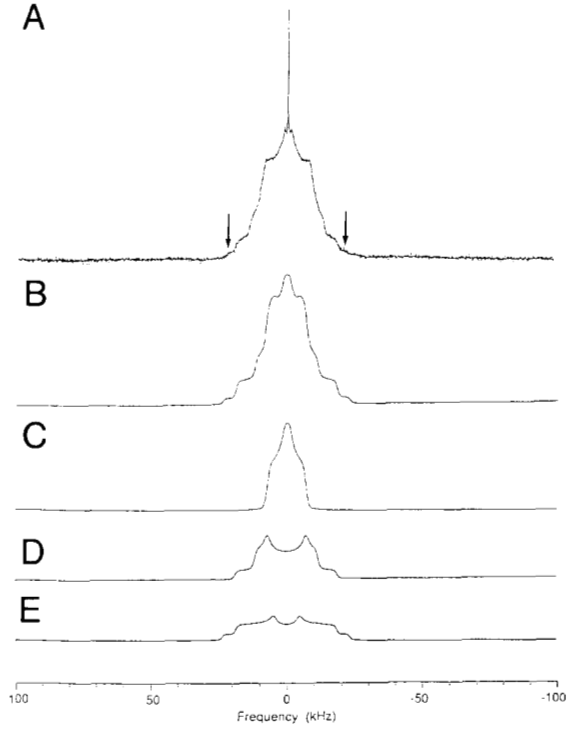

Fig. 7. A: Deuterium NMR spectra of polycrystalline SSI([methyl-^{2}H$_3$]Met-70,73,103), obtained by accumulating 963,000 transients with a repetition time of 0.22 s and Fourier transformation with an exponential filter of 10 Hz. The sharp component in the middle is due to residual ^{1}H^{2}HO in ^{2}H-depleted water. **B:** Computer simulation pattern of A, obtained by combining C, D, and E. **C:** Computer simulation pattern for Met 70 (same as Fig. 6B). **D:** Computer simulation pattern for Met 73 (same as Fig. 5C′ except that a Gaussian broadening of 5.0 kHz was applied). **E:** Computer simulation pattern for Met 103 obtained under the assumption of a two-site jump of the methyl axis spanning an angle of 60° (Table 2).

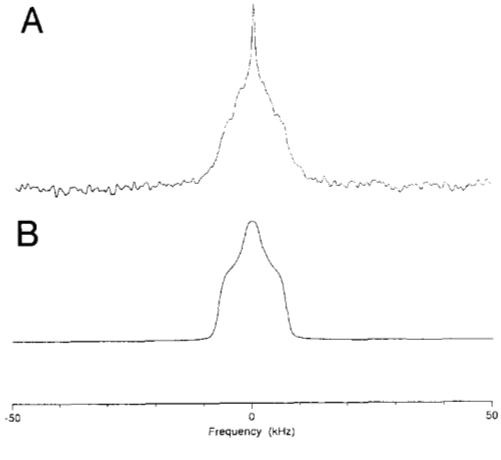

Fig. 6. A: Deuterium NMR spectrum of polycrystalline 73K103L (70MetC^{2}H$_3$), measured under conditions similar to those of Figure 5. The sharp singlet on top of the spectrum is due to naturally abundant ^{1}H^{2}HO. **B:** Computer simulation pattern under the assumption that the methyl axis jumps between neighbors of four different sites located on a large conic angle close to the magic angle (55.6°) at a rate of 10 MHz or higher (Table 2).

T_2 is 1.4×10^{-10} s. This value represents effective correlation times of the side-chain motions of methionines 70 and 73. We conclude, therefore, that the side chains of Met 70 and Met 73 undergo extensive motions at rates at least two orders of magnitude larger than that for whole-molecular rotation in solution.

The correlation time for Met 103 (1.3×10^{-8} s) obtained from the mutant 70I73K (103MetC^{2}H$_3$) agreed rather well with that obtained from the broad component of the wild-type SSI([methyl-^{2}H$_3$]Met-70,73,103). The extremely short correlation times obtained for methionines 70 and 73 (1.0×10^{-10} s and 0.8×10^{-10} s, respectively) also agreed fairly well with that from the narrow component in the wild-type SSI([methyl-^{2}H$_3$]Met-70,73,103) and confirmed that both of these side chains undergo extensive and rapid internal motions in addition to the methyl group rotations. Thus, it is clear that the rate of motion of a Met side chain depends critically on where it is located in the protein.

The small peaks at about 2.7 ppm in Figure 3A and B are due to the sulfoxide forms of 70Met-C^{2}H$_3$ and 73Met-C^{2}H$_3$, respectively. Their relaxation rates are slightly faster, and the corresponding motions consequently slower, than those of their nonsulfoxide counterparts. These differences probably reflect hindrance of rotation of the Cγ-S$_\delta$ bond in the sulfoxide form.

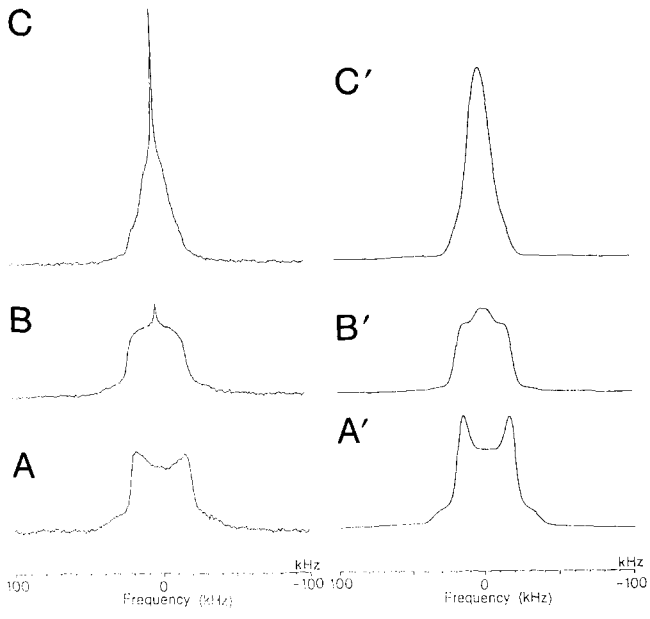

Fig. 8. Deuterium NMR spectra of powder SSI([methyl-$^2H_3$]Met-70,73,103). **A:** As lyophilized (may contain up to 0.09 gram of water per gram protein-water). **B:** At a hydration of 0.20 gram of water per gram protein-water. **C:** At a hydration near saturation (0.60 gram of water per gram protein-water). Spectra were obtained under conditions similar to those of Figure 5. The sharp singlets on top of spectra B and C are due to naturally abundant $^1H^2HO$. **A′:** Computer simulation pattern under the assumption of two-site random jumps at a common rate of 10 kHz for the methyl axes of all the three Met residues, each spanning an angle of approximately 80° (same as in Fig. 5A′). **B′:** Computer simulation pattern obtained under the assumption that the methyl axes of Met 70 and Met 73 undergo a random two-site jump spanning an angle of 65° at a rate of 1.0 MHz (as in Fig. 5B′), whereas the motion of Met 103 is unchanged (Fig. 8B′). **C′:** Computer simulation pattern by using the same motional parameters assumed for the three Met residues in crystals (Fig. 7B), but allowing extra line widths of 5 kHz for inhomogeneous broadening (Fig. 8C′).

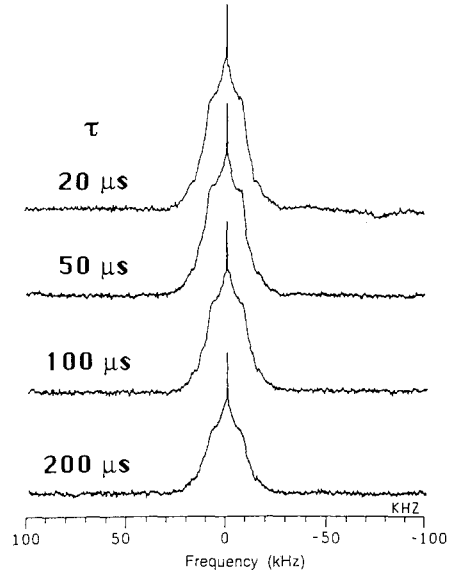

Fig. 9. Dependence of the ^2H NMR spectrum of polycrystalline SSI([methyl-$^2H_3$]Met-70,73,103) on the time interval τ in the quadrupole echo pulse sequence. The sharp singlets on top of the spectra are due to naturally abundant $^1H^2HO$.

For the complexed form, the separation of the signals observed for the free form was not detected. Only average T_1 values and therefore average correlation times for the three methyl groups could be deduced (Table 1). The correlation time obtained from the line width (3.0×10^{-8} s) is just about what is expected for the rotational correlation time of a protein complex of MW 78,000 from the Stokes-Einstein equation, apparently indicating that the extensive fluctuations of the side chains of Met 70 and Met 73 in free SSI were quenched as a result of the complex formation. However, the correlation time estimated from T_1 (1.1×10^{-8} s) is distinctly shorter than the correlation time from T_2. This result may suggest that some internal motions are present in the Met side chains of SSI even when it forms a complex with the enzyme.

Solid-phase protein

Characteristic features of the side-chain motions of methionine and the strategy for simulation of deuterium NMR spectra of SSI

For the deuteron of a fixed C-^2H group with asymmetry parameter = 0, the quadrupole splitting $\Delta\nu$ is given by

$$\Delta\nu = \frac{(3\cos^2\theta - 1)}{2} \cdot \frac{3}{4} \cdot \frac{e^2qQ}{h} \qquad (3)$$

(Abragam, 1961), where θ is the angle that the C-^2H bond makes with the magnetic field. For powder or polycrystalline

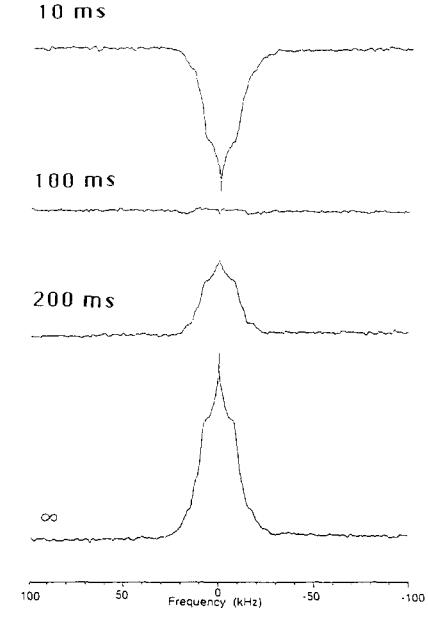

Fig. 10. Inversion-recovery ^2H NMR spectra of polycrystalline SSI([methyl-$^2H_3$]Met-70,73,103) at 20 °C, with the times indicated being the intervals between the 180° pulse and the quadrupole pulse sequence. The sharp component in the middle is due to residual $^1H^2HO$ in ^2H-depleted water.

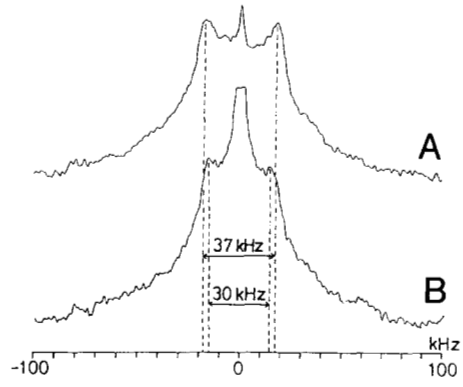

Fig. 11. Deuterium NMR spectra of powder SSI([methyl-$^2H_3$]Met-70, 73,103) when it forms a complex with subtilisin BPN'. **A:** As lyophilized. **B:** At a hydration of 0.60 gram of water per gram protein-water. Spectra were measured with a 90° pulse of 6 μs duration and, after accumulation of 30,000 transients with a repetition time of 0.52 s, Fourier transformed with an exponential filter of 1,000 Hz and displayed in the power mode. The singlets in the middle of the spectra are due to naturally abundant $^1H^2HO$.

samples, the actual patterns are given by summing up Equation 3 over all solid angles. For the C_α deuteron of polycrystalline valine, splitting of 117 kHz was observed when the field was perpendicular to the C_α-^2H bond (data not shown). For the deuterons of a methyl group that undergoes a rapid three-site random jump, the quadrupole splitting $\Delta\nu$ is reduced by $(3\cos^2\gamma - 1)/2$, where γ is the angle that the methyl axis makes with the magnetic field, and is given by

$$\Delta\upsilon = \frac{(3\cos^2\theta - 1)}{2} \cdot (3\cos^2\gamma - 1) \cdot \frac{3}{4} \cdot \frac{e^2qQ}{h}. \qquad (4)$$

For the methyl deuterons of polycrystalline valine, we determined experimentally that the splitting is 74 kHz when the field is parallel to the methyl axis and 37 kHz when the field is perpendicular to it. These values were assumed to be the same for methyl deuterons of a methionyl residue when no motion of the methyl axis is present. For the methyl deuterons of polycrystalline methionine, both the 74-kHz splitting and the 37-kHz splitting were significantly narrowed at 20 °C, indicating that some kind of motion of the methyl axis is present.

Methionine has a long side chain consisting of C_α-C_β, C_β-C_γ, and C_γ-S_δ single bonds, all of which can contribute to the orientational fluctuation of the terminal S_δ-C_ϵ bond (i.e., the methyl axis). Deuterium NMR spectra of crystalline [C_α-$^2H_1$]methionine and [C_α,C_β-$^2H_3$]methionine were obtained. The former clearly showed a splitting of about 120 kHz, indicating that the orientation of the C_α-^2H bond is nearly fixed, whereas the latter showed substantially narrowed signals, with a splitting of about 40 kHz attributable to the C_β-^2H group in addition to the approximately 120-kHz splitting attributable to the C_α-^2H group at 20 °C (Naito & Akasaka, 1988). The above result indicates that the C_α-C_β bond, located at the bottom of the long side chain, undergoes some reorientational motion at 20 °C, in agreement with the observation by Sparks et al. (1988), who analyzed this motion in detail and concluded that a rapid two-site jump with a jump angle of about 90° is involved.

In view of the above conclusion about the side-chain mobility of methionine in crystals, the motion of the S_δ-C_ϵ bond of a Met residue in a protein should, in general, be considered in terms of rotations about the C_α-C_β, C_β-C_γ, and C_γ-S_δ bonds, rather than just the rotation about the C_γ-S_δ bond (Colnago et al., 1987). Unfortunately, because we measured only the signal of the C_ϵ deuterons, we could not characterize all these rotations. We therefore chose to simulate the spectrum of the C_ϵ deuterons of the methyl groups of SSI in terms of an *effective* motion of the methyl axis, taking account of all the rotations that can affect it. Throughout the simulation, we chose the simplest possible model of methyl axis reorientation that accounts for the observed spectrum. That is, we first tried a two-site random jump model, and, if that failed, we tried random jumps (between two adjacent sites) within a minimum set of equally populated sites on a cone or a slightly distorted cone. Here, the parameters to be fitted were the number of sites, the jump angle or the conic angle, and the rate of jumps between adjacent sites. This approach can be regarded as a combination of a jump model and a diffusion-on-a-cone model.

In practice, we obtained higher-quality spectra, better suited to simulation, from crystalline samples than from powder samples. We were able to prepare deuterium-labeled crystals of the wild-type SSI([methyl-$^2H_3$]Met-70,73,103) in a sufficient quantity (40 mg) to give a deuterium NMR spectrum with a good signal-to-noise ratio (Fig. 7A). This spectrum showed a number of fine structures pertaining to different contributions from the three Met residues. To analyze this spectrum in terms of the motions of individual Met residues, we first analyzed the spectra of the three specifically labeled mutants by computer simulation. The results were used to analyze the spectrum of the wild-type SSI([methyl-$^2H_3$]Met-70,73,103) are shown in Figure 7A.

Analysis of internal motions of Met 73 in 70I103L (73MetC$^2H_3$) in the powder form

Given that we failed to crystallize 70I103L (73MetC$^2H_3$), we measured the deuterium NMR spectra in the powder form, but with careful control of hydration (Tamura & Akasaka, 1992). Figure 5A shows the deuterium NMR spectrum of 70I103L (73MetC$^2H_3$) in a lyophilized powder, which may contain 0.09 gram of water per gram of protein-water (Tamura & Akasaka, 1992). The doublet spectrum with approximately 37-kHz splitting indicates the presence of rapid methyl-group rotation, as expected (Andrew et al., 1976; Keniry et al., 1983). However, the broad nature of the spectrum, and particularly the absence of the 74-kHz component (corresponding to the quadrupole splitting when the magnetic field is parallel to the methyl axis) suggests that, in addition to methyl group rotation, some reorientational motion of the methyl axis itself may be present. The simplest model of random reorientation (jumping) of the methyl axis between two equally populated sites can reproduce the spectrum in Figure 5A (with allowance for inhomogeneous broadening due to microscopic heterogeneity), only by having the methyl axis jumping between two sites that differ in orientation by ±40–50° at a rate of about 10 kHz (Fig. 5A'; Table 2). If this motion were to correspond solely to the rotation about the C_γ-S_δ bond of the methionyl residue (Colnago et al., 1987), its angle of rotation (ϕ) should be 80–100°. A simulated spectrum for the case of $\phi = 80°$ is shown in Figure 5A'.

When the powder sample was allowed to hydrate (see the Materials and methods), the spectrum in Figure 5A underwent a

Table 2. *Equilibrium orientations (sites) and exchange matrices for methyl axes assumed for computer simulation of ^2H NMR spectra of [methyl-$^2H_3$]methionine of Streptomyces subtilisin inhibitor (Figs. 5–8)*[a]

	Equilibrium sites			Exchange matrices			
Met residue	α	β	γ	Site 1	Site 2	Site 3	Site 4
Crystals[b]							
Met 70		4-site jump			k = 10.0 MHz		
Site 1	0	55.6	0	$-2k$	k	0	k
Site 2	68	55.6	0	k	$-2k$	k	0
Site 3	180	55.6	0	0	k	$-2k$	k
Site 4	248	55.6	0	k	0	k	$-2k$
Met 73		4-site jump			k = 10.0 MHz		
Site 1	0	40	0	$-2k$	k	0	k
Site 2	90	30	0	k	$-2k$	k	0
Site 3	180	40	0	0	k	$-2k$	k
Site 4	270	30	0	k	0	k	$-2k$
Met 103		2-site jump			k = 1.0 MHz		
Site 1	0	+30	0				
Site 2	0	−30	0				
Powder[c]							
Met 70, 73, 103 (as lyophilized)							
		2-site jump			k = 10 kHz		
Site 1	0	+40	0				
Site 2	0	−40	0				
Met 70, 73 (hydrated to 0.2 g water per gram protein-water)							
		2-site jump			k = 1.0 MHz		
Site 1	0	+32.5	0				
Site 2	0	−32.5	0				
Met 103 (hydrated to 0.2 g water per gram protein-water)							
Same as in lyophilized powder							
Met 70,73,103 (hydrated to 0.6 g water per gram protein-water)							
All parameters same as in crystals							

[a] For each site, the relative orientation of the methyl axis of methionine is represented by Euler angles α, β, and γ (in degrees).

[b] Lorentzian width of 0.5 kHz (for T_2) and Gaussian width of 1.0 kHz (for inhomogeneous broadening) were applied for all simulations in crystals.

[c] Lorentzian width of 0.5 kHz (for T_2) and Gaussian width of 5.0 kHz (for inhomogeneous broadening) were applied for all simulations in powder samples.

dramatic change in two steps (Fig. 5B,C). At a hydration of 0.20 gram of water per gram protein-water, the doublet splitting shifted to a characteristic bell shape (Fig. 5B), indicating a distinct change in the motion of the methyl axis. Again, the two-site random-jump model can reproduce this spectrum reasonably well (with allowance for inhomogeneous broadening) only within a narrow range of jump rate of approximately 1.0 MHz differing in a range of orientational angle ± 30–$60°$ or ϕ = 60–120 °C. Figure 5B′ depicts an example for $\phi = 80°$ and with a jump rate of 1.0 MHz.

When the hydration approached saturation (0.60 gram of water per gram protein-water) (Tamura & Akasaka, 1992), the spectrum became narrower, as shown in Figure 5C. (Note that the central sharp singlet is due to increased $^1H^2HO$ content.) This pattern could no longer be reproduced with any of the two-site jumps nor with the three-site jumps. An adequate fit was obtained with a model of four-orientational jumps occurring at a rate higher than 10 MHz, as in the case of Met 70 in crystals, but the angle of span is much smaller than the magic angle 54.7°. In fact, a best fit was obtained with a combination of conic angles of 30 and 40° (Fig. 5C′). Such a complex motion of the methyl axis necessitates rotations about the C_α-C_β, C_β-C_γ, and C_γ-S_δ bonds, rather than just the C_γ-S_δ bond.

The dramatic spectral changes that occur on hydration and the consequent increase in the frequency of motion clearly demonstrate that hydration is almost essential for internal motion of Met 73 (located on the outside of the protein molecule). Hydration-induced mobility in a solid protein was demonstrated previously in lysozyme powder using the hydrogen exchange reaction (Schinkel et al., 1985). At the final stage of hydration, where the motion is most prominent, the degree of hydration is close to that in SSI crystals (41 vol%, Satow et al., 1973). It is expected, therefore, that the motion at the final stage of hydration of the powder sample will be close to the motion in the crystalline state. Evidence supporting this idea was obtained in the study of the wild-type SSI in which all three Met residues had been deuterated, from the close similarity of the deuterium NMR spectra of the polycrystalline and the hydrated powder samples (see below).

Analysis of internal motions of Met 70 in polycrystalline 73K103L (70MetC$^2H_3$)

Our crystals of 73K103L (70MetC$^2H_3$) gave a strikingly sharp, singlet-like deuterium NMR signal for 70MetC$^2H_3$ (Fig. 6A), which showed no hint of the 37-kHz doublet splitting typical of an immobilized methyl axis. This result indicates that the methyl axis of Met 70 in crystals undergoes an extensive motion besides the rapid three-site jumps. We attempted to simulate this motion in terms of random jumps of orientation of the methyl axis between neighbors of a minimum set of equally populated sites. No two-site or three-site jump models adequately reproduced the spectrum. A reasonable fit was obtained for an effective motion of the methyl axis jumping rapidly (10 MHz or higher) among a minimum set of four orientations on a cone with a conic angle 55.6° (close to the magic angle of 54.7°) (Table 2; Fig. 6B).

A rapid motion of the terminal methyl axis at an angle close to the magic angle represents an effective motion rather than the real motion, but it indicates that there is extensive motion of the whole side chain of Met 70. This conclusion is in qualitative agreement with the result of X-ray analysis, which finds very weak electron density for the whole side chain of Met 70, allowing no reliable determination of its orientation, and vague electron density even for the main chain from Cys 71 through Tyr 75 (Mitsui et al., 1979; Hiromi et al., 1985).

Internal motions of Met 103 based on the analysis in polycrystalline SSI([methyl-$^2H_3$]Met-70,73,103)

Unfortunately, neither as crystals nor as powder could we prepare enough 70I73K (103MetC$^2H_3$) to permit direct ^2H NMR analysis of the internal motions of Met 103 in the solid state. However, we were able to obtain this information from our well-resolved deuterium NMR spectrum for crystalline wild-type SSI (Fig. 7A). We assumed that the deuterated methyl signals of Met 70 and Met 73 in this spectrum are essentially the same as those in the spectra of polycrystalline 73K103L (70MetC$^2H_3$) (Fig. 6) and the fully hydrated powder of 70I103L (73MetC$^2H_3$)

(Fig. 5C), respectively. We then searched for an extra component that could be attributed to Met 103.

The wings of the spectrum in Figure 7A show a component with shoulders at ±23 kHz (arrows) that is not found in Figures 5C and 6. We may therefore assign this component to the methyl signal of Met 103. The presence of a 37-kHz splitting, but the lack of the 74-kHz splitting characteristic of a fully immobilized methyl axis (cf. Fig. 4A), indicates clearly that the methyl axis of Met 103 also undergoes a limited motion. Within the two-site jump model, the observed shoulders at ±23 kHz can be reproduced by a random jump of the methyl axis with a unique spanning angle of ±30° at a rate higher than 1.0 MHz (Table 1; Fig. 7E). The reason we failed to detect the side-chain motion of Met 103 in solution was not because it was absent, but because the rotational diffusion of the protein molecule as a whole is such an excellent relaxation sink that it obscures the effect of the internal motion on T_1 (Akasaka, 1991).

The whole spectral pattern of Figure 7A was then reproduced as shown in Figure 7B, which was obtained by simply adding Figure 7C (*the same* as Fig. 6B) for Met 70, Figure 7D (obtained with *the same* exchange matrix as Fig. 5C′, but with a Gaussian broadening of only 1.0 kHz) for Met 73, and Figure 7E for Met 103. The fairly good coincidence between the experimental (Fig. 7A) and simulated (Fig. 7B) spectra may be taken to indicate that the simple models assumed above for the motions of the three Met residues effectively represent the reality. The slight discrepancy between the experimental (A) and simulated (B) spectra is due in particular to a slight misfit of the simulation represented by Figure 7C to the spectrum of Met 70. Whether this discrepancy means that the side-chain motion of Met 70 in the wild-type SSI is slightly different from that in the mutant 73K103L (70MetC^{2}H$_3$) (Fig. 6) is not clear.

Analysis of internal motions in SSI([methyl-$^2H_3$]Met-70,73,103) in the powder form—similarity of motions in hydrated powder and crystals

Figure 8A shows the spectrum of SSI([methyl-^{2}H$_3$]Met-70,73,103) in lyophilized powder, which may contain up to 0.09 gram of water per gram protein-water. The presence of broad 37-kHz doublet components with a smeared 74-kHz splitting is typical for methyl deuterons with slow two-site jumps, as was the case for 70I103L (73MetC^{2}H$_3$) (Fig. 8A′, same as in Fig. 5A′). When the protein sample was hydrated to 0.20 gram of water per gram protein-water, another typical pattern (Fig. 8B), found already in Figure 5B, appeared, showing that the methyl axes of Met 70 and Met 73 undergo a random reorientation at an intermediate frequency of approximately 1.0 MHz (exactly as in Fig. 5B′), whereas the motion of Met 103 remain unchanged (Fig. 8B′). The spectrum further transformed into a final pattern as the hydration proceeded nearly to saturation (0.60 gram of water per gram protein-water; Tamura & Akasaka, 1992) (Fig. 8C). Beside its similarity to Figure 5C, Figure 8C clearly has extra shoulders attributable to a 37-kHz doublet splitting. It is to be noted that this pattern could be reproduced well by using *exactly the same geometrical and kinetic parameters used to simulate the patterns in crystals* (Table 1; Fig. 7B), provided an extra line width of 5 kHz is allowed for possible inhomogeneous broadening (Fig. 8C′).

The last result gives support to the assumption employed in the simulation of Figure 7A and leads us to conclude that the internal motions in a sufficiently hydrated powder sample of a protein are close, if not identical, to those in crystals for both external and internal residues. This conclusion appears to be consistent with the notion that the native (folded) structure of a globular protein (as revealed in crystals) can be realized in powder samples as a result of careful hydration. This notion is also in accord with the observation that the catalytic activity of a globular protein appears in a powder after proper hydration (Careri et al., 1980).

Deuterium spin relaxation

τ-dependence

Figure 9 shows the dependence of the spectrum of polycrystalline SSI([methyl-^{2}H$_3$]Met-70,73,103) on the time interval τ in the quadrupole echo pulse sequence. Apart from the decrease in intensity, the line shape remained nearly the same for different τ-values between 20 and 200 μs. This indicates that the internal motions with characteristic time constants of 20–200 μs (or 50–500 kHz) are not present in those Met side chains. This result is consistent with the results of the line-shape analysis, which predicted that the side chains of all three methionines undergo motions in the time range of at least 1 MHz or higher. In Figure 9, signals decay at nearly a common rate of 0.5 kHz, which is attributable to T_2 relaxation. This decay rate was taken into account in all the spectral simulations as Lorentzian broadening of 0.5 kHz.

Spin-lattice relaxation times (T_1)

The line shape analysis gave only the lower limits for the rates of internal motions of the Met side chains. We now turn to the results of spin-lattice relaxation time measurements in the crystalline solids, which may indicate the rates of internal motions more directly. Figure 10 shows inversion-recovery spectra of polycrystalline SSI([methyl-^{2}H$_3$]Met-70,73,103). No appreciable differences could be detected in among the decay constants of different spectral components of Figure 10. Within the limits of experimental error, they gave a common T_1 value of 160 ms, a value fairly comparable to the T_1 values in solution (Table 1). As discussed above, the rotations of the methionine methyl groups themselves are too fast to affect the T_1 values. Spin-lattice relaxation of the methyl deuterons must therefore arise from orientational fluctuations of the methyl axes, which are anisotropic in nature.

The anisotropic motion renders the methyl deuteron T_1 values dependent on magnetic field orientation with respect to the methyl axis. Theoretical expressions for anisotropic T_1 have been worked out for several simple cases by Torchia and Szabo (1982). We may apply a simple case of jumping between two equivalent positions (Equation 36 of Torchia & Szabo, 1982) to Met 103 (spanning ±30°), and calculate its correlation time. Unfortunately, because our data (Fig. 10) are not accurate enough to deduce the magnetic field dependence of T_1 experimentally, and because T_1 is a bivalued function of the jumping rate, we are allowed to determine only the range of correlation times consistent with the observed average T_1 value (160 ms). The range of correlation time we obtain for Met 103 is 1.6×10^{-10} to 1.0×10^{-8} s, which means that the motion of the side chain of Met 103 in the packed hydrophobic core region is quite rapid.

Nearly the same correlation-time ranges were obtained for Met 70 and Met 73 when the motions of their methyl axes were

analyzed as two-site random jumps spanning ±55.6° and ±40°, respectively. Thus, it is concluded that the side chains of all the three Met residues of SSI in crystals undergo rapid motions in similar time ranges of 10^{-8} to 10^{-10} s.

The effective correlation times determined in solution for the side-chain motions are 1×10^{-8} s or more for Met 103 and approximately 10^{-10} s for Met 70 and 73 (Table 1), which fall in the correlation-time range for the same residues in crystals. Our result suggests that the rate of the side-chain motion of each Met residue of SSI is at least not widely different between crystals and solution.

Significance of the internal motions of the Met side chains in free SSI and in the enzyme-inhibitor complex

Met 73 and Met 70 occupy respectively, the P1 and P4 sites of the inhibitor – that is, the primary and secondary sites important for controlling the specific binding of SSI to serine proteases (Kojima et al., 1990a,b; Takeuchi et al., 1991). The unusually high mobility of the side chains of methionines 73 and 70 would enable them to adopt the best orientations relative to the S1 and S4 pockets of the target enzyme, subtilisin BPN′.

Our results showed that methionines 70 and 73 differ in their motions. This finding is in qualitative agreement with the result of the X-ray diffraction study, in which clear electron densities were observed for the side chains of Met 73 and Met 103, but Met 70 gave a very poor electron density, which allowed no reliable determination of the side chain orientation (Satow et al., 1980). On the other hand, a naive extrapolation of the X-ray result would suggest that the side chain of Met 73 is fixed, whereas that of Met 70 has ample motions. This expectation was not borne out in our ^{2}H NMR study, which clearly proved the presence of large-amplitude motions in the side chains of both Met 70 and Met 73.

Although it is not known whether it involves reorientations about bonds other than the C_γ-S_δ bond, a relatively large-angle and rapid reorientation of the methyl axis was found for Met 103, which is buried in the densely packed, hydrophobic core region of SSI, surrounded by such residues as Phe 97, Phe 111, Val 16, Val 78, and Leu 80 (Mitsui et al., 1979). Thus, the methyl axis of Met 103 should be able to undergo reorientation only in concert with simultaneous motions of the surrounding residues. Direct NMR evidence for reorientation of amino acid side chains in densely packed protein cores has been rather limited, except for the demonstration of ring flip of a Phe or Tyr residue in cytochrome c and basic pancreatic trypsin inhibitor, as evidenced by ^{1}H NMR in solution (Wüthrich & Wagner, 1978) and by ^{2}H NMR in the solid state (Gall et al., 1981). Recently, extensive reorientation of the C_γ-C_δ axis of several Leu residues has been demonstrated in staphylococcal nuclease in solution by ^{13}C NMR spin relaxation measurements (Nicholson et al., 1992). Our result for Met 103 of SSI gives additional evidence that the structure of the compact hydrophobic core of a globular protein is indeed fluctuating.

Figure 11 shows the deuterium NMR spectra of powder SSI([methyl-$^2H_3$]Met-70,73,103) when it forms a complex with subtilisin BPN′. Because the spectra are displayed in the power mode, they are not suited for simulation analysis, but qualitative information may be obtained from the splitting. The lyophilized powder (Fig. 11A) shows a 37-kHz doublet splitting, indicating that the methyl axis lacks any significant reorientational motion. Even upon hydration to 0.60 gram of water per gram protein-water (Fig. 11B), the spectrum did not show a dramatic change, in contrast to the case of free SSI (Fig. 8C). The splitting of the doublet clearly narrowed to 30 kHz, however, suggesting that the methyl axis now has an orientational fluctuation. In a two-site jump model, this 7-kHz narrowing requires a fluctuation of the methyl axis over an angle of +30° (Soda & Chiba, 1969). Granted that hydration induces a native conformation in SSI, the above observation suggests that small-amplitude motions are present in the side chains of methionines 70 and 73 in the S1 and/or S4 pockets of the enzyme. The presence of rapid internal motions in the Met side chains of SSI in the enzyme-inhibitor complex had been inferred from T_1 measurement in solution. A recent X-ray diffraction analysis of the crystalline enzyme-inhibitor complex (Takeuchi et al., 1991b) has shown that the S1 pocket of subtilisin BPN′ contains a few water molecules, which apparently gives ground for this motion.

Materials and methods

Site-directed mutagenesis of SSI

The SSI gene from *Streptomyces albogriseolus* S-3253 had been cloned and expressed in *S. lividans* 66 (Obata et al., 1989a, 1989b). We obtained our three kinds of mutated SSI gene as follows. The plasmids pSIAX70I73K(103M) and pSIAX73K103L (70M) were obtained by replacing the Met 70 codon with an Ile codon (ATC) and the Met 103 codon with a Leu codon (CTG) in the plasmid pSIAX73K (Kojima et al., 1990b), respectively. The mutagenic primers used were 5′-CT GAC GTC ATC* TGC CCG-3′ for Met 70-Ile, and 5′-G TGC GAG C* TG AAC GCG C-3′ for Met 103-Leu, where the bases with asterisks indicate a mismatch. The plasmid pSIAX70I103L(73M) was obtained by substituting a Leu codon for the Met 103 codon in pSIAX70I, in which the Met 70 codon had been replaced by an Ile codon. Mutation was confirmed by the dideoxy sequencing. Reconstruction of mutated pSI52 from mutated pSIAX and insertion of the SSI gene into the *Streptomyces* plasmid pIJ702 followed by transformation of *S. lividans* 66 were conducted as described previously (Kojima et al., 1990b). All these mutants showed inhibitory activity against subtilisin BPN′.

Preparation of SSI([methyl-$^2H_3$]Met)

SSI([methyl-$^2H_3$]Met-70,73,103) was prepared by cultivating *S. albogriseolus* S-3253 in a culture medium containing an amino acid mixture in which normal methionine had been replaced with L-[methyl-$^2H_3$]methionine (^{2}H content > 99%, CEN, Saclay, France). The three mutants, 73K103L (70MetC^2H_3), 70I103L (73MetC^2H_3), and 70I73K (103MetC^2H_3), were similarly prepared by cultivating *S. lividans* 66 transformed by the plasmids described above. Secreted SSI was precipitated with ammonium sulfate and then purified by passing through an anion-exchange column (DE52, Whatman) and a gel filtration column (Sephacryl S-200, Pharmacia). The final solution containing SSI was dialyzed against H_2O, and the purified protein sample was lyophilized and stored in powder form.

To prepare a powder sample of SSI–subtilisin BPN′ complex, appropriate solutions of SSI and subtilisin BPN′ were mixed, and the complex was purified through gel filtration. After dialysis against H_2O, the complex was lyophilized.

Crystallization

The wild-type SSI and the mutants were crystallized according to a procedure reported previously (Sato & Murao, 1973). A 40-mg quantity of the purified protein sample was dissolved in 1.0 mL of 0.04 M phosphate buffer, pH 7.3, made up with ^2H-depleted water (^2H content = 0.5 ppm, ISOTEC), and 30% ammonium-sulfate-saturated, ^2H-depleted phosphate buffer, 0.01 M, pH 7.0, was then added until the solution was approximately saturated with the protein. After centrifuging for 10 min at 15,000 rpm, the supernatant was transferred to Eppendorf tubes, each containing 100 μL. The tubes were seeded by adding an aliquot of dilute solution containing fractured SSI crystals and were kept standing at 20 °C. The crystals grew up to 1.5 mm in a few days and were gathered into an NMR sample tube. Little of the protein remained in the supernatant, so the crystals were estimated to contain about 40 mg of SSI. Crystallization was successful for the wild-type SSI and the mutant 73K103L (70MetC^2H_3), but not for the other two mutants.

Hydration of powder samples

The purified deuterium-labeled SSI samples were dissolved in H_2O and lyophilized. Hydration was performed by exposing the lyophilized samples to an atmosphere equilibrated with water vapor of natural isotopic composition (^2H isotope content = 0.015 %), as previously reported (Tamura & Akasaka, 1992). The degree of hydration was controlled by changing the time of exposure to this atmosphere. The water content of the protein powders was estimated directly from the deuterium NMR spectrum of the solid protein, from the integral intensity of the $^1H^2HO$ signal relative to that of the [methyl-$^2H_3$]Met signal of the protein itself (Tamura & Akasaka, 1992).

Deuterium NMR spectroscopy

Deuterium NMR spectra in solution were measured at 20 °C on a JEOL GX-400 spectrometer at 61.4 MHz without proton decoupling and without a field-frequency lock. The protein samples were dissolved in the ^2H-depleted water in 10-mm O.D. tubes at 20 °C to concentrations of 5–10 mg/mL. Typically, 50,000 scans were accumulated with a repetition time of 0.52–1.0 s. T_1 was measured by the inversion-recovery method. Transverse relaxation times T_2 (in seconds) were obtained from full line widths in half-intensity $\nu_{1/2}$ (in hertz) from the relation

$$T_2 = \frac{1}{\pi \nu_{1/2}}. \quad (5)$$

These times should be taken to represent the lower limits of the intrinsic T_2 values, because of extra line width due to field drift and other possible causes, although an independent experiment indicated that the field drift should be less than a few hertz in 10 h.

Deuterium NMR spectra in solid-state samples were measured on a JEOL GX-400 spectrometer modified to permit solid-state deuterium NMR measurements (Naito & Akasaka, 1989). The radiofrequency (rf) transmitter power from the original JEOL GX-type spectrometer was amplified by about 13 db with the combined use of a narrow-band amplifier (800 W, Henry Radio, model 2006A) and an rf attenuator, and was sent to a deuterium solid-state probe through a duplexer designed for higher power at the resonance frequency of 61.4 MHz. A solenoid coil of 6-mm O.D. was used for both excitation and detection. A 12-bit digitizer was used at a sampling rate of 200 kHz.

Protein samples of 50 mg or more were used for each measurement. Because of the extremely low deuterium content of the protein samples, typically as many as 400,000 to 1 million scans were accumulated with a repetition time of 0.22 s to obtain a single spectrum with reasonable signal-to-noise ratio. The carrier frequency was set on the $^1H^2HO$ resonance. The powder samples were sealed in 5-mm O.D. tubes. The polycrystalline samples were also sealed in 5-mm O.D. tubes together with aliquots of mother liquor. A quadrupole echo pulse sequence ((90°)x-τ-(90°)y-τ-FID) (Davis et al., 1976) was used for detection, with 16 times of phase cycling, an interval τ of 50 μs, and a repetition time of 0.2 s unless otherwise specified. All measurements were done with a 90° pulse of 2.4-μs duration, except for the spectrum of SSI–subtilisin complex and the spectra of some deuteromethionines, which were obtained at an early phase of the work with a 90° pulse of 6 μs. For measurements of T_1, the inversion-recovery pulse sequence was combined with the quadrupole echo pulse sequence.

Spectral simulation

Computer simulation of deuterium NMR spectra in powder or polycrystalline solids was carried out by a program developed in our laboratory (Matsushita, 1988). The time evolution of the spin-density matrix under rf pulses and under Zeeman and quadrupolar interactions during the quadrupole echo pulse sequence was calculated explicitly by solving the Liouville von Neumann equation with the approximation of infinitely sharp rf pulses. The effect of side-chain motion was treated by introducing exchange matrices that connect the elements of the density matrix corresponding to different orientational sites during the interval τ. The final free induction decay signals were Fourier transformed to obtain the frequency domain spectra. The program was written in FORTRAN, and the calculation was performed at the Data Processing Center of Kyoto University.

A Lorentz width of 0.5 kHz was taken into account for transverse relaxation in all simulations. Furthermore, extra Gaussian broadenings of 1 and 5 kHz were applied in the final presentation of the simulations of all polycrystalline and powder spectra, respectively, to allow for possible inhomogeneous broadening.

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