Mobility of the Terminal Regions of Flagellin in Solution*

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The mobility of the disordered terminal regions of flagellin was examined in detail based on 1H NMR chemical shifts and spin-lattice relaxation times in the rotating frame. Proteolytic fragments of flagellin with terminal deletions of different sizes were used to compare the dynamical properties of various N- and C-terminal segments.

We found that dynamic properties of different terminal segments were similar to each other and were close to those of the heat-denatured state of flagellin. The main chain of these terminal segments undergoes rapid motions with effective correlation times of 1.3–4.1 × 10⁻⁹ s. The terminal regions contain no large segments with well-defined structure. However, comparison with the random-coiled state of poly-L-lysine suggests significant structural constraints in the terminal regions (as well as in the heat-denatured flagellin) which may reflect the existence of some highly fluctuating secondary structure, as suggested by earlier CD studies.

Helical filaments of bacterial flagella are composed of multiple copies of a single protein, flagellin. Proteolytic experiments and calorimetric studies have indicated that the terminal regions of flagellin, residues 1–65 and 451–494 in i-type flagellin from Salmonella typhimurium, have no stable compact structure in solution (Kostyukova et al., 1988; Vonderviszt et al., 1989). 1H NMR spectroscopy gave direct evidence for disorder and mobility in the terminal regions (Aizawa et al., 1990). Surprisingly, comparison of the far-UV CD spectra of intact flagellin with those of the flagellin devoid of the terminal regions strongly suggested that the terminal regions contain a significant amount of α-helical secondary structure. This is supported also from the secondary structure prediction (Vonderviszt et al., 1990). These α-helices have been expected to exhibit marginal stability because they are exposed to the aqueous environment.

Although earlier CD and calorimetric studies indicated that polymerization of flagellin is accompanied by large conformational changes, it is not clear which part of flagellin was responsible for the inferred conformational rearrangements (Uratani et al., 1972; Bode et al., 1974; Fedorov et al., 1984).

Comparison of the NMR spectra of monomeric and polymeric flagellin shows that the mobile terminal segments of flagellin become ordered in the filaments (Aizawa et al., 1990). Recent experiments comparing the filament-forming abilities of various terminally truncated flagellin fragments have revealed that the terminal regions play an important role in stabilizing filament structure and controlling polymorphic conformation of the filaments (Vonderviszt et al., 1991). It has been suggested that the terminal regions become ordered during the self-assembly process simply by incorporating and stabilizing their marginally stable secondary structural elements into a compact structure (Vonderviszt et al., 1989, 1990).

In order to understand the structural rearrangements in the disordered parts of monomeric flagellin upon self-assembly into filaments it is important to characterize precisely the initial and final conformational states. In the present report we evaluate the disorder and essentially mobility of the N- and C-terminal regions of monomeric flagellin from the Salmonella wild type strain SJW1103 based on 1H NMR chemical shifts and spin-lattice relaxation times in the rotating frame (T₁). We chose proton T₁ for the study of local mobility because it is free from spin diffusion which hampers evaluation of local mobility in T₂ (Akasaka et al., 1990) and free from J modulation which hampers accurate measurements of relaxation rates in spin echo T₂ (Freeman and Hill, 1971). We evaluate mobility based on effective correlation times determined from T₁. Proteolytic fragments of flagellin with terminal deletions in the disordered terminal parts were used to compare the mobilities of the N- and C-terminal regions.

MATERIALS AND METHODS

Chemicals and Proteins—Carboxypeptidase A-DFP, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, and soybean trypsin inhibitor were obtained from Sigma; endoproteinase Lys-C, endoproteinase Arg-C, and endoproteinase Glu-C from Staphylococcus aureus V8 were purchased from Boehringer. Other chemicals were of analytical grade from commercial sources.

The isolation of flagellin from S. typhimurium wild type strain SJW1103 and the preparation of its F40 tryptic fragment, comprising the compact parts of flagellin, were done as described previously (Vonderviszt et al., 1989). Preparation of terminally truncated fragments of flagellin has been recently described elsewhere (Vonderviszt et al., 1991). Briefly, F(50-494) was produced by mild digestion of flagellin with endoproteinase Lys-C. This fragment has an intact C-terminal region but lacks almost all of the disordered N-terminal part. The mobile C-terminal region can be selectively removed by mild degradation with V8 protease followed by carboxypeptidase A treatment. The first step of degradation by V8 results in the removal of the C-terminal 462–494 segment. Further treatment of the 1-461 fragment with carboxypeptidase A yields the F(1-452) fragment. The F(37-450) fragment was prepared by proteolysis of flagellin with

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1 The abbreviations used are: T₁, longitudinal relaxation time in the rotating frame; T₂, effective correlation time; T₉, rotational correlational time; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; T₁, longitudinal relaxation time; T₉, transverse relaxation time.

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endoproteinase Arg-C. This fragment is deprived of the whole C-terminal and about half of the N-terminal disordered regions. Proteolytic fragments of flagellin were isolated from digestion mixtures by fast-protein liquid chromatography on a Mono-Q ion-exchange column (Pharmacia LKB Biotechnology Inc.).

Measurements of Circular Dichroism—Circular dichroic (CD) spectra in the range of 185-250 nm were recorded on a Jasco-600 spectropolarimeter using standard procedures. Measurements were made using cylindrical fused quartz cells with a path length of 0.1 cm. Samples were dissolved in 10 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. CD measurements were performed at 25 °C and at 60 °C using the same sample at a protein concentration of 0.17 mg/ml.

'H NMR Measurements—'H NMR measurements on flagellin and its fragments were carried out in 10 mM deuterated phosphate buffer/150 mM NaCl (pH 7.0, direct pH meter reading) at protein concentrations in the range of 3-5 mg/ml. The sample of poly-L-lysine was dissolved in 2H2O to a concentration of 1 mg/ml and the pH was adjusted to be pH 1.5 using 2HCl. Samples were placed into 5-mm outer diameter tubes. All 'H NMR measurements were performed at 400 MHz on a JEO-LGX400 spectrometer with quadrature detection.

Two-dimensional spectra by homonuclear Hartmann-Hahn spectroscopy (HGHAVA) (Bax and Davies, 1985) of flagellin at 27 and 58 °C were obtained in the phase sensitive mode as described by Stases et al. (1982). Free induction decay signals in the T2 domain were collected into 2048 complex points with a spectral width of 4500 Hz (SW). For each i, 64 scans were signal-averaged with a recycle time of 2.5 s. The 'H HO signal, which was placed on resonance, was saturated during the recycle time. A total of 256 i experiments were collected and the data were zero-filled into 512 complex points.

Longitudinal relaxation times (T1) in the rotating frame were measured by a (90°-τ/2 - spin-lock) - acquisition sequence for flagellin and its fragments at 25 °C and for flagellin at 58 °C. The longitudinal magnetization during spin-locking decays with the time constant T1 (Jones, 1966). The H1 rf field of 2.3 gauss was used for spin locking. Measurements were performed with a recycle time of 7 sec and a number of accumulation of 256.

Analysis of T1 Data—Since the static magnetic field (H0) was 94 K gauss and the applied rf-field (H1) for spin locking was 2.3 gauss, the conditions were established as follows

\[ T_0 \gg T_1 \]

and

\[ \gamma H_1/2\pi \gg \sqrt{2}/N \]

Under these conditions and in the absence of exchange effects, T1 of protons can be expressed as follows.

\[ T_1 = 9/32 \gamma^2 H_1^2 \left[J_0(0) + 10J_1(0) + J_2(0)\right] \]

This relationship was derived from Equation 11 of Jones (1966). \( \gamma \) is the gyromagnetic ratio of the proton, \( \hbar \) is the Planck constant divided by 2\( \pi \), and \( \omega \) is the Larmor angular frequency of the proton, equal to 2.512 \( \times \) 10\(^6\) Hz. \( J_n(\omega) \) values (\( m = 0, 1, 2 \)) are the spectral density functions and are expressed for any nonmethyl proton i by the following equation,

\[ J_n(\omega) = K_n \sum_i \left(1/r_i^n\right) \cdot \tau_{im} (1 + \omega^2 \tau_{im}^2) \]

where \( K_n \) is a constant. We have assumed that: the proton-proton dipolar interactions are the dominant sources of relaxation and the summation is, in principle, taken for all protons \( j \) interacting with protons \( i \). All these dipolar interactions are assumed to be modulated by a single effective correlation time of \( \tau_{im} \). The parameter \( \tau_{im} \) takes care of any possible internal motions (other than the methyl group rotations) in addition to the whole molecular rotation (\( \tau_{gi} \)).

For a methyl proton \( i \) in a methyl group which undergoes random three-site jumps, \( J_n(\omega) \) can be given by Equation 22 of Woessner (1983). The correlation time for the whole three-site random jumps can be assumed to be much faster than the effective correlation time, \( \tau_{im} \), then

\[ J_n(\omega) = 0.25 \cdot K_n \cdot 2 \cdot \left(1/r_{gi}^{(\text{methyl})}\right) \cdot \tau_{im} (1 + \omega^2 \tau_{im}^2) + K_n \sum_i \left(1/r_i^{(\text{methyl})}\right) \cdot \tau_{im} (1 + \omega^2 \tau_{im}^2) \]

where \( r_{gi}^{(\text{methyl})} \) denotes the interproton distance within a methyl group and \( \Sigma \) denotes the summation for all the interacting protons \( j \) except for those within the methyl group containing proton \( i \). For protons in the exposed segments where interresidue contributions are expected to be small, \( \Sigma (r_{ij}^{(\text{methyl})}) \) values are approximated by intraresidue contributions within each amino acid residue alone. For each proton of each amino acid type, a range of \( r_{ij} \) values are obtained from 10 energy-minimized conformations of each amino acid (Vasquez et al., 1983). Then, this range of \( r_{ij} \) values causes the range of \( \tau_{im} \) values obtained from experimental \( T_1 \), data using Equations 3-5.

For protons in the tightly packed core region, interresidue contributions to \( \Sigma (1/r_{ij}^{(\text{methyl})}) \) term may not be negligible. However, since no crystal structure is available for flagellin or any of its fragments, evaluation of \( \tau_{im} \) values from experimental \( T_1 \), data was not carried out for protons in the core region.

RESULTS

NMR Spectra of Flagellin and Its Fragments—In order to investigate in detail the dynamic properties of the disordered terminal regions of flagellin comprising residues 1-65 and 451-494\(^1\) (Vonderviszt et al., 1989), NMR experiments were performed on various terminally truncated fragments (Fig. 1). To explore possible differences in the dynamic features of the C- and N-terminal regions we used the F(59-494) and F(1-452) fragments. The F(69-494) fragment has an intact C-terminal region but lacks almost all of the disordered N-terminal region while the disordered C-terminal region is selectively removed in the F(1-452) fragment. To obtain information about subparts of the N-terminal disordered region the F(37-450) fragment, which lacks the whole C-terminal and about half of the N-terminal disordered regions, was prepared. NMR characteristics of these fragments were compared to those of flagellin and its F40 fragment which is composed of the protease resistant part of the molecule (Vonderviszt et al., 1989). In what follows, terminal regions of flagellin missing in the F40 fragment will be referred to as the C- and N-terminal segments.

The 'H NMR spectrum of flagellin consists of both sharp and broad signals (Fig. 2A). Comparison with the spectrum of F40 (Fig. 2E) shows that signals stemming from the N- and C-terminal segments of flagellin are sharp and are piled onto the overwhelmingly broad signals of F40 as has been described previously (Aizawa et al., 1990). In this paper the sharp signals stemming from the terminal segments are called "the sharp components" while the signals originating from the broad signals of F40 are called "the broad components." All the chemical shifts of the sharp components coming from the side chain protons are almost the same with those of the random-coiled poly-peptide (Wüthrich, 1986) as previously described (Aizawa et al., 1990, Fig. 24). These chemical shifts of the side chain protons also coincide with those of the heat-denatured flagellin (Fig. 2F) measured at 58 °C.

\(^1\)The originally published amino acid sequence of flagellin from S. typhimurium (Joys, 1985), used in our previous works (Vonderviszt et al., 1989, 1990), has been recently found to be partially incorrect in the central region (Kanto et al., 1991; Smith and Selander, 1990). The revised sequence consists of 494 residues.
well above the transition temperature (about 50 °C, Aizawa et al., 1990). Protons in the main chain also exhibit similar spectral characteristics. The two-dimensional HOHAHA spectra in Fig. 3, in which only the sharp components were observed because of their exceptionally long $T_1$ values, show the correlation between the methyl protons and the $\alpha$-protons of Ala (1.43 and 4.35 ppm), Ile (0.94 and 4.25 ppm), and Val (0.96 and 4.15 ppm), respectively, at 27 and 58 °C. Chemical shifts are essentially unchanged upon heat denaturation, i.e., the microenvironments of the $\alpha$-protons of Ala, Ile, and Val in the native peptide segments giving the sharp components of flagellin are similar to those of the corresponding $\alpha$-protons in heat-denatured flagellin.

Upon preirradiation with a radiofrequency wave at the position of aromatic protons (6.95 ppm) for 1 s, signals of the broad components of flagellin corresponding to the F40 part disappeared (Fig. 4). This phenomenon of inter-residue cross-saturation and spin diffusion occur preferentially in the compact region of the protein with strong inter-residue interactions (Akasaka, 1983). On the other hand, almost all of the signals of the sharp components originating from the N- and C-terminal segments remained in Fig. 4. These results show qualitatively that the F40 fragment forms a compact region of flagellin while the N- and C-terminal segments are disordered with little interresidue interaction.

$T_1$ Values of Flagellin and Its Fragments.—To evaluate the mobility of the C- and N-terminal segments in a more quantitative manner, the longitudinal relaxation times in the rotating frame ($T_1$ values) were measured for individual reso-

**Fig. 2.** $^1$H NMR spectra of flagellin (A), F(59-494) (B), F(1-452) (C), F(37-450) (D), and F40 (E) at 25 °C and heat-denatured flagellin (F) at 58 °C. Peaks originating from unique residues of the terminal segments, i.e. peaks of the methyl protons of Met-465 and of the aromatic protons of Phe-53 and Tyr-458 are marked with arrows.

**Fig. 3.** Two-dimensional $^1$H NMR HOHAHA spectra of the region between the methyl protons and the $\alpha$-protons of flagellin at 27 °C and 58 °C. Cross-peaks for Ala (1.43 vs. 4.35 ppm), Val (0.96 vs. 4.15 ppm), and Ile (0.94 vs. 4.25 ppm) are essentially at the same positions in the native and heat-denatured state.

**Fig. 4.** Comparison of the $^1$H NMR spectrum of flagellin (A) to that obtained after the preirradiation (B) at the indicated position of the aromatic protons of 6.95 ppm for 1 s with an rf powers of $\gamma B/2\pi = 228$ Hz (B).

**Table I**

<table>
<thead>
<tr>
<th>Proton type</th>
<th>Chemical shifts (ppm)</th>
<th>Number of protons (predicted from intensity)/calculated from amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala $\beta$</td>
<td>1.43</td>
<td>15/21</td>
</tr>
<tr>
<td>Lys $\beta$</td>
<td>0.89</td>
<td>4/19</td>
</tr>
<tr>
<td>Arg $\beta$</td>
<td>1.88</td>
<td>4/13</td>
</tr>
<tr>
<td>Thr $\gamma$</td>
<td>1.23</td>
<td>2/6</td>
</tr>
<tr>
<td>Leu $\gamma$</td>
<td>0.89</td>
<td>22/24</td>
</tr>
<tr>
<td>Ile $\delta$</td>
<td>0.95</td>
<td>11/15</td>
</tr>
<tr>
<td>Lys $\delta$</td>
<td>1.65</td>
<td>36/26</td>
</tr>
<tr>
<td>Arg $\delta$</td>
<td>3.2</td>
<td>1/2</td>
</tr>
<tr>
<td>Lys $\epsilon$</td>
<td>3.0</td>
<td>4/1</td>
</tr>
</tbody>
</table>

*The intensity is estimated from the difference spectrum between flagellin or fragments and F40.
Mobility of the Terminal Regions of Flagellin

**FIG. 5.** Time dependence of the longitudinal magnetizations in the rotating frame in the aliphatic region for flagellin (A) and F40 (B) and in the aromatic region for flagellin (C) and F40 (D).

![Graph showing time dependence of longitudinal magnetizations](image)

**FIG. 6.** Examples of logarithmic plots of the longitudinal magnetizations in the rotating frame for signals at 1.43 ppm against the spin-locking time. F40 ( ), F(37-450) ( ), F(1-452) ( ), F(59-494) ( ), and flagellin ( ). These plots are each fitted to two lines ( . . . . . ) whose slopes correspond to two \( T_{1r}^{-1} \) values.

![Graph showing logarithmic plots](image)

Observations of flagellin and its fragments. The time dependences of the longitudinal magnetization in the rotating frame for flagellin (Fig. 5, A and C) and F40 (Fig. 5, B and D) show large differences in decay times between the sharp components and the broad components. For example, at 20 ms, the broad components have already disappeared completely and only the sharp components remain. These observations indicate that the \( T_{1r} \) values of the sharp components are much longer than those of the broad components. The time dependence of the longitudinal magnetization at each position of the spectrum should therefore be composed of at least two components with different decay constants. In the analysis of the \( T_{1r} \) data of flagellin, we have assumed that the decays of the magnetization are composed of two exponential functions of time: a fast exponential decay due to the broad component and a slower one due to the sharp component (Fig. 6). Similarly, decays of the longitudinal magnetization of the F(37-450), F(59-494), and F(1-452) fragments were also approximated by two exponential functions (Fig. 6 and Table II) using nonlinear least-square fitting algorithm. \( T_{1r} \) values for the broad components were all found to be very similar for F40, F(37-450), F(59-494), F(1-452), and flagellin and only the values for F40 are shown. \( T_{1r} \) values of heat-denatured flagellin at 58 °C could be obtained by fitting to a single exponential decay curve. In order to obtain a reference \( \tau_{\text{eff}} \) values for a completely random-coiled polypeptide chain, \( T_{1p} \) values of the poly-L-lysine under the acidic condition (Hanssum and Rüthjens, 1980) were also measured (Table III).

\( T_{1p} \) values of the sharp components showed subtle differences among flagellin and its fragments (Table II). They were generally 4-10 times longer than those of the broad components of F40, but two to three times shorter than those of heat-denatured flagellin at 58 °C. The only exceptions are the \( T_{1p} \) values of the ring protons of Phe-53 at 7.35 ppm, which showed distinctly short \( T_{1p} \) values.

**Effective Correlation Times (\( \tau_{\text{eff}} \)) of the N- and C-terminal Segments**—Since the chemical shifts and spin diffusion experiments strongly suggest that the N- and C-terminal segments have little interresidue interactions (Figs. 2-4), only the intraresidue dipolar interactions may be assumed to contribute to \( T_{1p} \). Thus the effective correlation times (\( \tau_{\text{eff}} \) values) can be calculated for the sharp components of flagellin and of its terminally truncated fragments from the experimental \( T_{1p} \) values using Equation 3 (see “Materials and Methods”). The resulting \( \tau_{\text{eff}} \) values are shown as bar graphs in Fig. 7. The \( \tau_{\text{eff}} \) values for heat-denatured flagellin at 58 °C were obtained similarly and were converted to effective values at 25 °C using the following relation as follows.

\[
(\tau_{\text{eff}})_{25} = (\tau_{\text{eff}})_{58} \times (\eta_{58}/\eta_{25}) \times (273 + 58)/(273 + 25)
\]

where \( \eta_{25} \) and \( \eta_{58} \) denote the viscosities of water at 25 and 58 °C, respectively.
Table II

<table>
<thead>
<tr>
<th>Proton type</th>
<th>ppm</th>
<th>F40, F(37-450), F(1-452), F(59-494), Flagellin, and heat-denatured flagellin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experimental  $T_{1p}$ values</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured (58°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Longitudinal relaxation times in the rotating frame ($T_{1p}$ values) for the broad components of F40 and for the sharp components of F(37-450), F(1-452), F(59-494), flagellin, and heat-denatured flagellin</td>
</tr>
<tr>
<td>Ala β</td>
<td>1.43</td>
<td>8.5  55  49  57  50  120</td>
</tr>
<tr>
<td>Lys β</td>
<td>1.88</td>
<td>10  34  38  43  55  120</td>
</tr>
<tr>
<td>Arg β</td>
<td>0.89</td>
<td>10  66  80  90  77  145</td>
</tr>
<tr>
<td>Ile β</td>
<td>0.95</td>
<td>12  66  80  92  78  135</td>
</tr>
<tr>
<td>Thr γ</td>
<td>1.23</td>
<td>22  40  37  43  43  120</td>
</tr>
<tr>
<td>Val γ</td>
<td>1.09</td>
<td>10  66  80  90  77  145</td>
</tr>
<tr>
<td>Leu γ</td>
<td>0.95</td>
<td>12  66  80  92  78  135</td>
</tr>
<tr>
<td>Ile δ</td>
<td>1.65</td>
<td>25  31  46  47  40  110</td>
</tr>
<tr>
<td>Lys δ</td>
<td>1.65</td>
<td>25  31  46  47  40  110</td>
</tr>
<tr>
<td>Arg γ</td>
<td>1.65</td>
<td>25  31  46  47  40  110</td>
</tr>
<tr>
<td>Ile γ</td>
<td>1.65</td>
<td>25  31  46  47  40  110</td>
</tr>
<tr>
<td>Met-465 ε</td>
<td>2.08</td>
<td>77  85</td>
</tr>
<tr>
<td>Pro β</td>
<td>2.10</td>
<td>10  10  10  10  10  10</td>
</tr>
<tr>
<td>Arg δ</td>
<td>3.20</td>
<td>45  50  52  55  50  100</td>
</tr>
<tr>
<td>Lys ε</td>
<td>3.00</td>
<td>40  45  47  43  57  150</td>
</tr>
<tr>
<td>Ring protons</td>
<td>6.50</td>
<td>6  6  6  6  6  6</td>
</tr>
<tr>
<td>Tyr-458 ε</td>
<td>6.83</td>
<td>100 90  200</td>
</tr>
<tr>
<td>Tyr-458 δ</td>
<td>7.12</td>
<td>80  70  175</td>
</tr>
<tr>
<td>Ring protons</td>
<td>7.35</td>
<td>8  8  8  8  8  8</td>
</tr>
<tr>
<td>Phe-53 ring protons</td>
<td>7.35</td>
<td>30  23  22  170</td>
</tr>
</tbody>
</table>

*For the broad components of F40, the described proton types are not applied and only chemical shifts are available.

Table III

<table>
<thead>
<tr>
<th>M, Da</th>
<th>$T_{1p}$ 15,000-30,000 ms</th>
<th>$T_{1p}$ 15,000-30,000 ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Protons</td>
<td>260 1.0-1.4</td>
<td>1.0-1.4</td>
</tr>
<tr>
<td>β-Protons</td>
<td>140 0.66</td>
<td>0.66</td>
</tr>
<tr>
<td>γ-Protons</td>
<td>150 0.46</td>
<td>0.46</td>
</tr>
<tr>
<td>δ-Protons</td>
<td>170 0.46</td>
<td>0.46</td>
</tr>
<tr>
<td>ε-Protons</td>
<td>250 0.30</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Although low resolution x-ray fiber diffraction (Namba et al., 1989) and electron microscopic studies (Trachtenberg and DeRosier, 1987) as well as hydrodynamic experiments (Bode et al., 1972) suggest that flagellin has an elongated shape, a rough estimation of its rotational correlation time $(r_{rot})$ can be obtained from the Stokes-Einstein Equation (Tanford, 1985) under the assumption that it is a rigid spherical molecule. The calculation yields a value of $1.8 \times 10^{-9}$ s for $T_{rot}$ of flagellin and $1.3 \times 10^{-9}$ s for $T_{rot}$ of the F40 fragment.

In comparison, $r_{eff}$ values obtained from the Ala methyl resonances of the sharp components at 1.43 ppm were $1.3-4.1 \times 10^{-9}$ s for flagellin and for any of its fragments (Fig. 7). Since $r_{eff}$ value of Ala methyl group reflects the mobility of the peptide backbone (Akasaka, 1985), the result indicates that the main chain of the N- and C-terminal segments undergoes rapid segmental motions comparable to the heat-denatured flagellin whose $r_{eff}$ value for Ala methyl groups is $1.3-2.5 \times 10^{-9}$ s but slower than that of the random-coiled poly-lysine $(r_{eff} = 1.0-1.4 \times 10^{-9}$ s, Table III).

If the signals at 1.43 ppm for the broad components of F40 were to stem also from Ala methyl groups only and their $T_{1p}$ values were determined solely by intramolecular interactions,
their \( \tau_{\text{eff}} \) values could also be calculated from Equations 3 and 5 to be \( 1.3 \times 10^{-8} \) s. Although the above assumptions do not strictly hold, the obtained \( \tau_{\text{eff}} \) value is in good agreement with the \( \tau_d \) value calculated above from the Stokes-Einstein Equation for F40.

The \( \tau_{\text{eff}} \) values of methyl or methylene protons in the sharp components of flagellin and all of its fragments at 1.88, 0.89 or 0.95, 1.65, 3.20, and 3.00 ppm were estimated to be nearly equal to or smaller than those of Ala methyl groups at 1.43 ppm (Fig. 7). This indicates that these side chains also undergo rapid segmental motions. The ring protons of Phe-53 at 7.35 ppm exhibit exceptionally large correlation times. This means either that the Phe ring is somewhat immobilized or that the short \( T_1 \) value resulted from the neglect of the exchange term in Equation 3. In either case, the results suggest that the free mobility of the Phe-53 ring is restricted. Tyr-458 and Thr residues of sharp components also showed \( \tau_{\text{eff}} \) values slightly larger than the \( \tau_{\text{eff}} \) value of Ala methyl groups.

**CD Spectra of Heat-denatured Flagellin**—To characterize the structural properties of heat-denatured flagellin, far-UV CD spectra of the native and heat-denatured states were measured and compared. The CD spectrum of flagellin at 25 °C (Fig. 8, solid line) was essentially equivalent to those reported earlier (Urata et al., 1972; Vonderviszt et al., 1990) showing typical helical features. Proteins in the fully denatured (random coil) state exhibit monotonically decreasing, featureless CD spectra in the range of 250–200 nm. However, the CD spectrum at 60 °C for heat-denatured flagellin (Fig. 8, dotted line) clearly shows a shoulder at around 220 nm. This observation indicates that the heat-denatured flagellin, whose NMR spectral features are similar to those of the sharp components of native flagellin, is not in a random-coiled state and contains some (probably \( \alpha \)-helical) secondary structure.

**DISCUSSION**

The aim of the present study was to characterize in detail the structural and dynamic properties of the disordered terminal regions of monomeric flagellin. We found that the NMR spectra of flagellin and its terminally truncated fragments exhibited very similar features. Many sharp components originating from the terminal regions pile onto the broad components originating from the compact ordered part largely corresponding to F40. Comparison of the chemical shifts of the sharp components in the one-dimensional NMR spectra of flagellin and its terminally truncated fragments to the chemical shifts of heat-denatured flagellin (Figs. 2 and 3) suggests that the microenvironments of protons in various segments of the terminal regions are similar to those of the protons of heat-denatured flagellin. Spin-diffusion experiments (Fig. 4) also suggest that the C- and N-terminal segments of flagellin are disordered while the F40 fragment forms a compact core region.

Mobility of the terminal segments was examined in detail by comparing the spin-lattice relaxation times in the rotating frame \( (T_1) \). \( T_1 \) values of the sharp components of flagellin and its terminally truncated fragments were much longer than those of the broad components of F40. Because from the results of Figs. 2–4 as described above the terminal regions of flagellin appeared to be disordered in their entirety, this enabled the analysis of the \( T_1 \) data to obtain effective correlation times, \( \tau_{\text{eff}} \) which were used to evaluate the mobilities of these terminal regions more quantitatively.

The \( \tau_{\text{eff}} \) values of Ala methyl groups of the C- and N-terminal segments (1.3–4.1 \( \times 10^{-8} \) s), reflecting the mobility of the main chain, were found to be obviously larger than \( \tau_{\text{eff}} \) values of poly-L-lysine (1.0–1.4 \( \times 10^{-9} \) s). The molecular rotations are expected to be much slower than the segmental motions for our poly-L-lysine sample with a molecular weight distribution of 15,000–30,000 so that \( \tau_{\text{eff}} \) values will not be dependent on the molecular size. From \( 1^1 \)C NMR \( T_1 \) measurements (Hansum and Rüthjens, 1980; Saito and Smith, 1973) we estimated the \( \tau_{\text{eff}} \) value of poly-L-lysine to be 0.3–0.5 \( \times 10^{-6} \) s at 25 °C. Assuming that the \( \tau_{\text{eff}} \) value of poly-L-lysine represents the intrinsic mobility of a fully random-coiled polypeptide chain, the mobility of the very flexible terminal segments of flagellin can be considered as restricted. Comparative analysis of the far-UV CD spectra of flagellin and its F40 fragment indicated \( \alpha \)-helical secondary structure; this \( \alpha \)-helical structure has been hypothesized to be highly fluctuating and unstable because of its contact with the aqueous environment (Vonderviszt et al., 1990). The restricted mobility of the disordered terminal regions may reflect the existence of this rapidly fluctuating \( \alpha \)-helical structure.

Comparison of the \( \tau_{\text{eff}} \) values for the sharp components of various terminally truncated fragments of flagellin does not reveal significant differences in the dynamic properties of different terminal segments. On average, the dynamic properties of the disordered terminal regions are rather close to those of the heat-denatured state whose CD spectrum is shown in Fig. 8. It should be emphasized that the obtained \( \tau_{\text{eff}} \) values are those averaged over the protons found at individual chemical shifts, and that a few protons with especially low mobility could not be detected if they existed. It is important to note that even the heat-denatured state of flagellin is suggested by CD analysis to contain some (probably \( \alpha \)-helical) secondary structure. There is a wide variety of evidence suggesting that unfolded proteins are usually not true random coils at extremes of pH or temperature in the absence of denaturants (Creighton, 1990, and references therein). A few residues in the terminal regions, such as Phe-53 and Tyr-458, showed restricted mobility. Actually, prediction of the secondary structure (Vonderviszt et al., 1990) has suggested that both Phe-53 and Tyr-458 are contained in \( \alpha \)-helical regions.

In conclusion, there are no significant differences in the

![Fig. 8. Circular dichroic spectra of flagellin (solid line) and heat denatured flagellin (dotted line) in the far-UV region. The spectra were recorded at 25 and 60 °C in 10 mM phosphate buffer (pH 7.0), containing 150 mM KF, using the same sample.](image-url)
mobility of different segments in the disordered terminal regions of flagellin. They are exceptionally mobile having no larger segments with well defined tertiary structure. However, their structure cannot be considered completely disordered as shown by comparison to the putative random-coiled state of poly-L-lysine, but is rather similar to that of the heat-denatured state of flagellin which contains a certain amount of helical structure. The present NMR results complement earlier CD observations (Vonderviszt et al., 1990) showing that the terminal regions contain some unstable α-helical structure. In a liquid environment, the terminal regions of flagellin cannot form stable tertiary structures. They can fold only under particular conditions, e.g. during polymerization onto a special binding surface at the end of flagellar filaments.

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