Conformational adaptability of the terminal regions of flagellin

Ferenc Vonderviszt,* Masashi Sonoyama,‡ Mitsuo Tasumi,‡ and Keiichi Namba*

*ERATO, Molecular Dynamic Assembly Project, 5-9-5 Tokodai, Tsukuba 300-26, Japan; and ‡Department of Chemistry, Faculty of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT Secondary structure formation in the disordered terminal regions of flagellin were studied by circular dichroic (CD) spectroscopy, Fourier transform infrared spectroscopy, and x-ray diffraction. The terminal regions of flagellin are known to form α-helical bundles upon polymerization into flagellar filaments. We found from comparative CD studies of flagellin and its F40 tryptic fragment that a highly α-helical conformation can be induced and stabilized in the terminal regions in 2,2,2-trifluoroethanol (TFE) containing solutions, which is known to promote intra-molecular hydrogen bonding.

Two oligopeptides, N(37–61) and C(470–494), each corresponding to a portion of terminal regions and predicted to have a high α-helix forming potential, were synthesized and studied. Both peptides were disordered in an aqueous environment, but they showed a strong tendency to assume α-helical structure in solutions containing TFE. On the other hand, peptides were found to form transparent gels at high concentrations (>15 mg/ml) and all three methods confirmed that the peptides become ordered into a predominantly β structure upon gel formation.

Our results show that large segments of the disordered terminal regions of flagellin can adopt α-helical as well as β structure depending on the environmental conditions. This high degree of conformational adaptability may be reflecting some unique characteristics of the flagellin termini, which are involved in self-assembly and polymorphism of flagellar filament.

INTRODUCTION

Although the terminal parts of flagellin are disordered in solution (Kostyukova et al., 1988; Vonderviszt et al., 1989), secondary structure prediction suggests that these regions have a high potential for α-helix formation (Fedorov et al., 1988; Vonderviszt et al., 1990), which might be manifested in appropriate environments, such as in the filament form. Actually, comparison of the CD spectra of flagellin in its monomeric and polymeric state shows large increase of the α-helical content upon polymerization into filaments (Uratani et al., 1972). X-ray diffraction studies also suggest that the domains at inner radius of the flagellar filament, which are constructed from the terminal regions of flagellin, consist of α-helical bundles arranged parallel to the filament axis (Namba et al., 1989). It has been recently suggested based on sequence comparison studies that the α-helical regions of the neighboring subunits interact to form a coiled-coil structure (Homma et al., 1990).

We have investigated the α-helix formation in the terminal parts of flagellin by comparing the CD spectra of flagellin and its F40 fragment under various conditions known to be conducive to α-helix formation. The F40 fragment consists of the compact ordered parts of flagellin, 66 to 450, but lacks the disordered terminal regions (Vonderviszt et al., 1989). Two 25 residue long oligopeptides corresponding to segments 37 to 61 (N(37–61)) and 470 to 494 (C(470–494)) of the disordered terminal regions, which were predicted from the sequence to be α-helical (Vonderviszt et al., 1990), were also synthesized to study their behaviors in the secondary structure formation in various environments.

Abbreviations used: CD, circular dichroism; FTIR, Fourier transform infrared; TFE, 2,2,2-trifluoroethanol.

F. Vonderviszt's current address is Institute of Enzymology, Hungarian Academy of Sciences, Budapest, H-1518, Pf. 7, Hungary.

M. Sonoyama's present address is Division of Biological and Chemical Sciences and Technology, Faculty of Technology, Tokyo University of Agriculture and Technology, 2-24-16 Nakamachi, Koganei 184, Japan.

K. Namba can currently be found at the International Institute for Advanced Research, Matsushita Electric Industrial Co., Ltd. 6-2 Sakakigatsubo, Hosono, Seika 619-02, Japan.

Address correspondence to K. Namba.

MATERIALS AND METHODS

The isolation of flagellin from *Salmonella typhimurium* wild-type strain SJW1103, and the preparation of its proteolytic fragments have been described (Vonderviszt et al., 1989). Here, we used the F40 fragment prepared by trypsin. The concentrations of flagellin and the fragment were determined from absorption measurements at 280 nm using extinction coefficients of $E^{1\%}_{280} = 3.6$ for flagellin and 4.3 for F40 (Vonderviszt et al., 1989). The two oligopeptides, each 25 residues long, corresponding to segments 37 to 61 (N(37–61)) and 470 to 494 (C(470–494)), were synthesized by Peptide Institute, Inc. (Minoh, Osaka).

CD spectra were recorded at room temperature on a JASCO J-600 spectropolarimeter (Hachioji, Tokyo) using standard procedures. For protein and peptide solutions at concentrations around 0.1 mg/ml, measurements were made using cylindrical fused quartz cells with 1 mm path length and in 10 mM phosphate buffer (pH 7.0) containing 150 mM-KF and various concentrations of TFE.

For CD measurements of peptides at very high concentrations (~30 mg/ml) the following cell was specially designed. A quartz disc of 18 mm in diameter and 1 mm thick was mounted on a grooved edge of cylindrical plastic tube with outer and inner diameters exactly the same as the cylindrical fused quartz cells that are normally used. A drop of 2.5 μl peptide solution was placed on the quartz disc and another quartz disc was gently placed on the top to make a thin layer of the solution (~10 μm) between the two discs. The edge of the discs was sealed with silicon oil to prevent the solution from drying out and the whole cell was placed in the J-600 spectropolarimeter. The thickness of

0006-3495/92/12/1672/06 $2.00

the thin layer of solutions can be controlled by adjusting the volume of the drops. Therefore, this method can be used for a wide range of concentrations.

FTIR spectra were recorded on a JEOL JIR-100 spectrometer (Akishima, Tokyo) using standard procedures. Peptides were prepared as 30 mg/ml solutions in 1 mM phosphate buffer (pD 7.0) and were put into CaF_2 cells with a path length of 15 μm adjusted by teflon spacers. Spectra were recorded at room temperature at a nominal resolution of 2 cm^{-1}. 300 scans were added for each spectra.

X-ray diffraction experiments were carried out using a RIGAKU RU-200 rotating anode x-ray generator (Shinjuku, Tokyo) with a fine focal cup, operated at 40 kV and 25 mA. The focal size was 0.1 × 1 mm. Double mirror optics (Rigaku) were used to produce a well-collimated beam of CuKα radiation (λ = 1.5418 Å). Peptide solutions were put into capirallies of 0.7 mm in diameter and exposed for 6 h. The specimen-to-film distance was 170 mm. X-ray diffraction patterns were recorded on 20 × 25 cm imaging plates (Fuji Film, Minato, Tokyo). Exposed imaging plates were scanned with a BA100 scanner (Fuji Film) using 0.1 mm rasters, the digitized images were transfered to a micro Vax II computer (Digital Equipement Corp., Toshima, Tokyo) and displayed on a D-Scan GR4416 graphics terminal (Seiko Instruments, Eto, Tokyo) to locate the center position of diffraction patterns. Diffracted intensities were circularly integrated to obtain radial intensity distributions.

RESULTS

Intra-molecular H-bonds are stabilized in organic solvents such as 2,2,2-trifluoroethanol (TFE). TFE is known to promote stable secondary structure in polypeptides at low or moderate concentrations (below 60–70% TFE (vol/vol) or 25–30 mol% TFE) (Nelson and Kallenbach, 1986, 1989; Bruch et al., 1989). It stabilizes helices only in regions that have a high intrinsic helical propensity, but regions that are helix disfavoring are not induced to become helical. However, at higher concentrations TFE strongly favors the α-helical state and may induce β structure–α-helix transtions (Stone et al., 1985). We used TFE-containing solutions to study the secondary structure formation in the disordered terminal regions of flagellin. The conformation of the polypeptide backbone was monitored by measurement of the CD in the 185–250 nm region. Helix formation was followed by measuring the ellipticity increase at 222 nm (Chen et al., 1972).

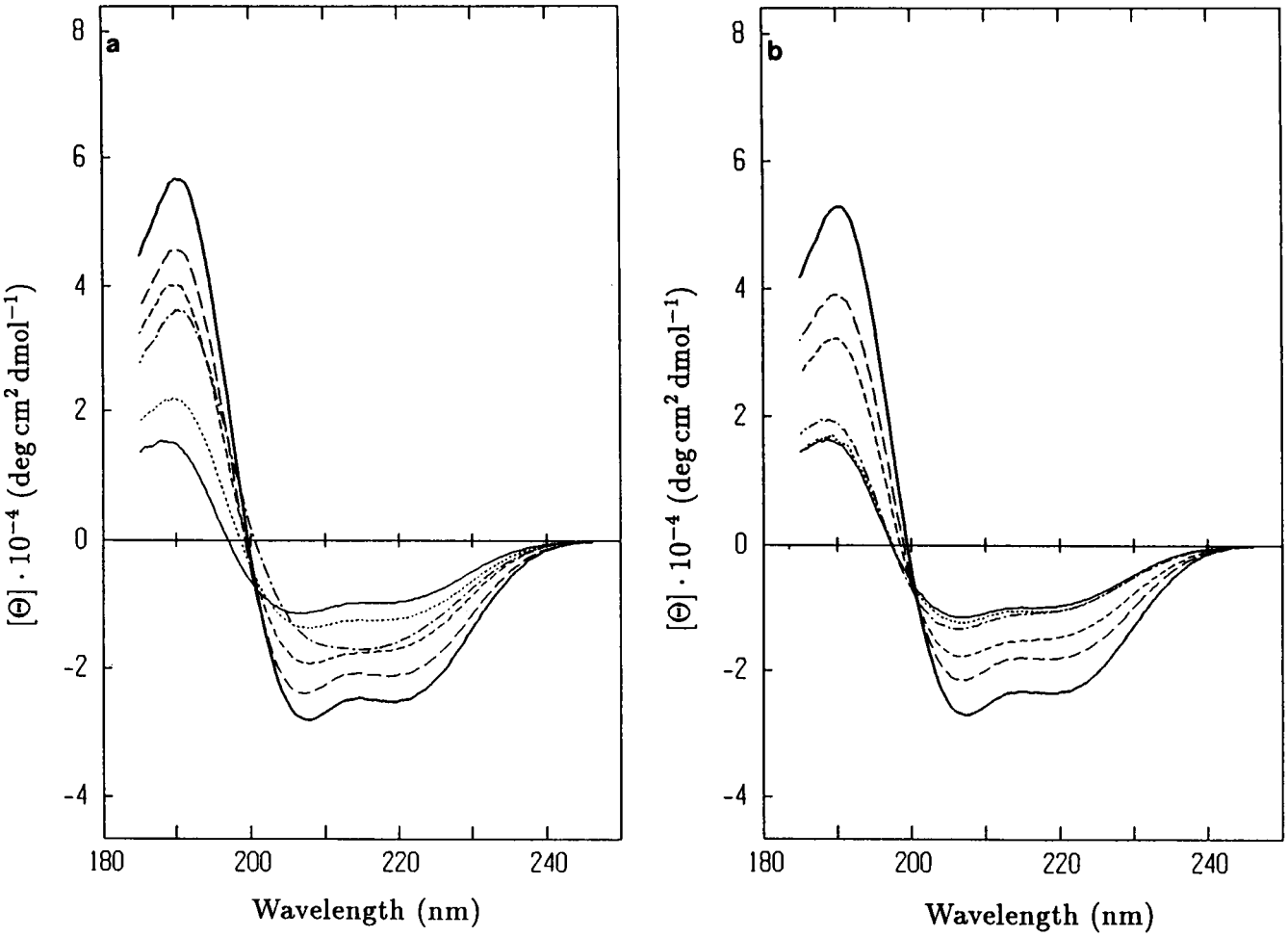

FIGURE 1 CD spectra of flagellin and F40 at various TFE concentrations. (*a*) Flagellin; (*b*) F40; (*c*) difference (flagellin – F40). Lines indicate: 0% TFE (——); 10% TFE (· · ·); 20% TFE (· – · – ·); 40% TFE (---); 60% TFE (– – –); 80% TFE (——).

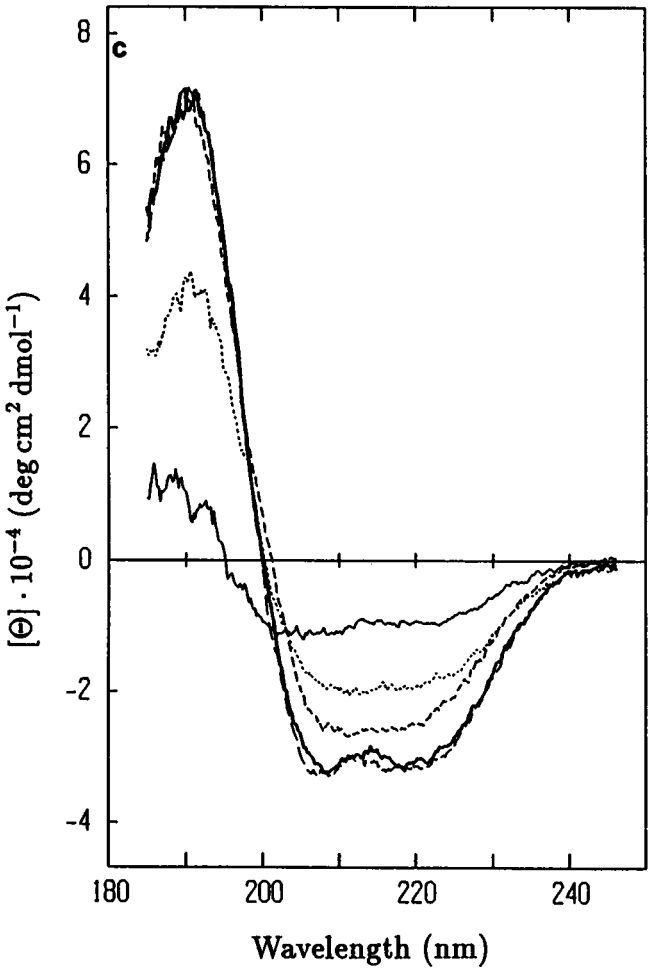

FIGURE 1 (continued)

Addition of TFE had a dramatic effect on the CD spectrum of flagellin as well as its F40 fragment (Fig. 1, *a* and *b*). Both flagellin and F40 exhibited increasing α-helical signals as the TFE concentration increased. Flagellin exhibited anomalous CD spectra in a narrow range of the TFE concentration ~ 20% probably as a result of aggregation. However, we did not find any sign of aggregation at higher or lower TFE concentrations. The difference spectrum of flagellin and F40 (Fig. 1 *c*) shows that residues in the disordered terminal parts of flagellin are also undergoing a transition to an α-helical conformation. While flagellin and F40 yields monotonically increasing CD signals with increasing TFE concentrations under the studied conditions, the mean residue ellipticity of the difference spectrum attains a limiting value at ~60% TFE. The limiting ellipticity value at 222 nm characteristic for the terminal regions was found to be 30,100 ± 1,000 deg · cm²/dmol. Taken the helix content to be directly proportional to the mean residue ellipticity at 222 nm (Chen et al., 1972), the terminal regions exhibit a maximum helical content of ~90% at 60% TFE.

These results show that the terminal regions of flagellin can assume as high as 90% helical structure under appropriate conditions. However, even this almost perfect ordering is not enough to explain the large difference between the CD spectra of flagellin in its polymeric and monomeric states. While we cannot exclude the possibility that special packing effects of α-helices in the filament form are responsible for the enhanced CD signals, these observations may also indicate that upon self-assembly conformational rearrangements occur not only in the disordered terminal regions of flagellin but also in the compact central (F40) part of the molecule. Our experiments provide indirect support for this idea because a relatively small amount of TFE was found enough to induce changes in the secondary structure of F40 (Fig. 1 *b*).

To investigate secondary structure formation in the disordered terminal regions in further details, two oligopeptides, N(37–61) and C(470–494), were synthesized and studied. Regions of flagellin corresponding to the peptides are predicted to contain long α-helical segments. Fig. 2 shows the effect on the CD spectrum of adding TFE to the peptides in 1 mM phosphate buffer, pH 7.0. In the absence of TFE, the CD spectrum has a minimum at 197 nm, showing that the peptides take no significant secondary structure. Addition of TFE results in spectra which have minimum at 206 nm and a shoulder at 222 nm, suggesting the formation of an α-helical conformation. The isodichroic point at 201 nm is consistent with the idea that each amino acid is either in an α-helical or random coil state. Under the same conditions, the helical content of the NH_2-terminal peptide is significantly higher than that of the COOH-terminal one, which may be explained by the presence of a helix breaking Pro residue at position 485 in C(470–494). The helical content of the peptides increased monotonically with increasing TFE concentrations, however even at 80% TFE the peptides showed only ~40 and 28% helicity, respectively, which is rather low as compared with the almost perfect helical ordering of the terminal regions of flagellin under similar conditions. These results clearly demonstrate that the intramolecular environment of the protein can dramatically influence the conformational properties of oligopeptide sequences.

We found that both peptides can form transparent gels at high concentrations (>15 mg/ml) under appropriate conditions. The gel formation of the peptides in the absence of salts was a slow process, especially for the N(37–61) peptide. Upon addition of salts, the rate of gel formation increased considerably. FTIR spectroscopy of the gels showed prominent bands in the region of amide I at 1,618 and 1,633 cm^{-1} (Fig. 3), suggesting the existence of a β-sheet conformation. X-ray diffraction patterns taken from the gels showed a sharp and strong reflection with a spacing of 4.68 Å (Fig. 4), which corresponds to the usual interchain distance in β-sheet structures, that commonly lies between 4.6 and 4.8 Å. We devised a method to measure CD spectra at very high protein concentrations, which enabled us to follow gel formation by

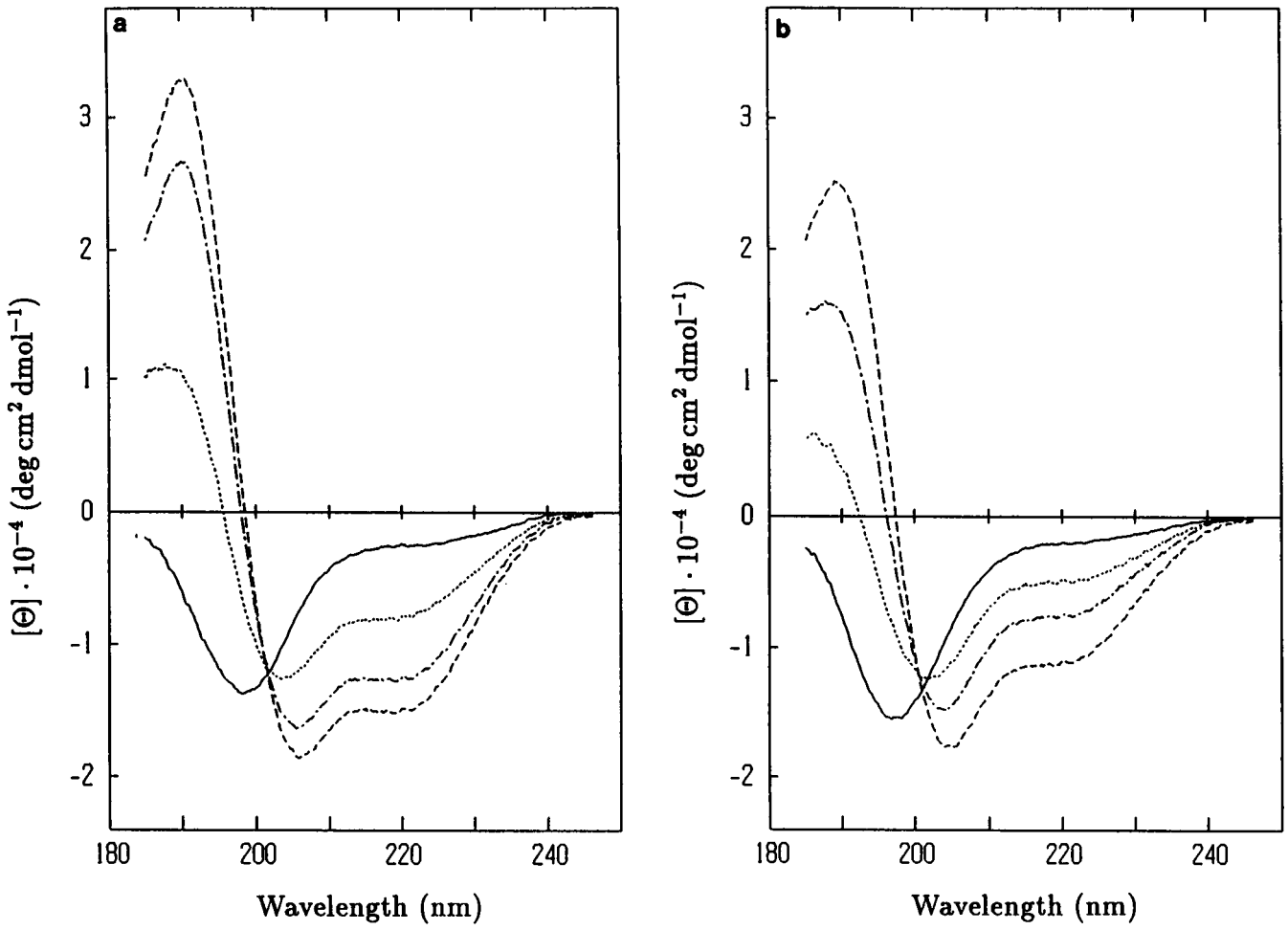

FIGURE 2 CD spectra of the peptides as a function of the TFE concentration. (*a*) N(37–61); (*b*) C(470–494). Lines indicate: 0% TFE (——); 20% TFE (· · ·); 40% TFE (·– ·– ·); 80% TFE (---).

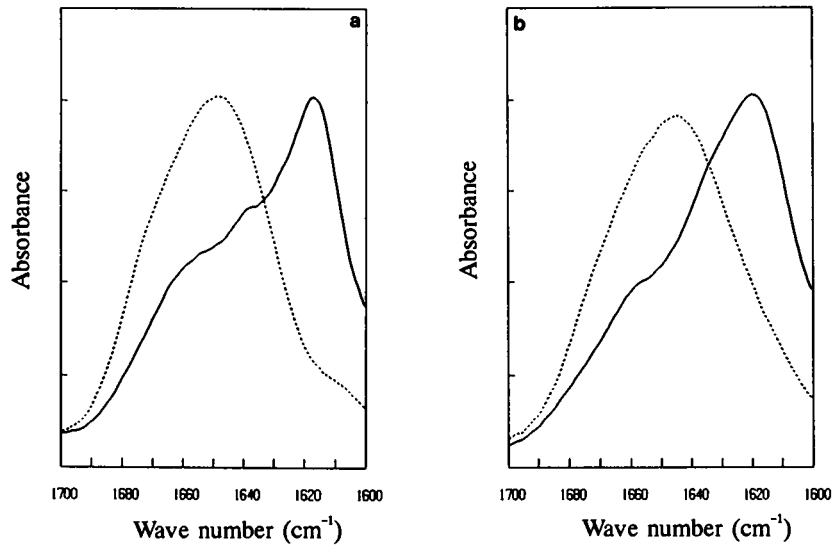

FIGURE 3 FTIR spectra of the peptides, N(37–61) and C(470–494), before (· · ·) and after (——) the gel formation. The peptides were prepared as 30 mg/ml solutions in 1 mM phosphate buffer (pD 7.0).

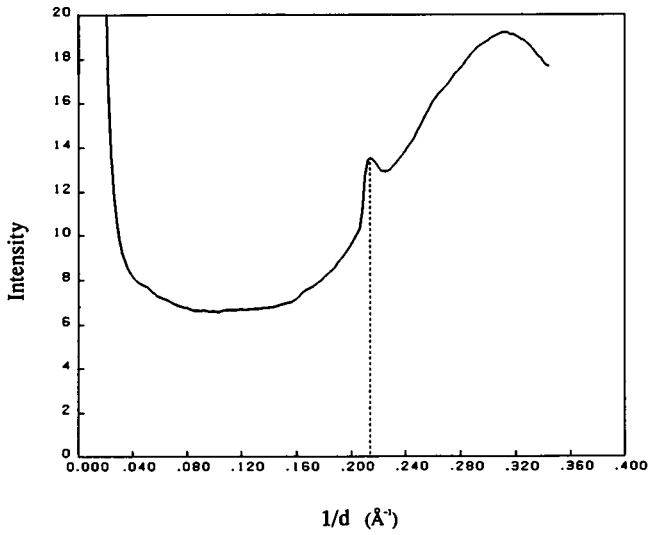

FIGURE 4 Intensity distribution profile of x-ray diffraction from a peptide gel. The x-ray diffraction patterns were mostly circular symmetric with a little tendency of orientation. Therefore, circular averaging was done to make the plot of the radial intensity distribution. The peak at 4.68 Å indicated by dotted line is on the broad background scattering from water.

CD spectroscopy. (Our method may be useful for other systems as well, e.g., to study macromolecular interactions that occur only at high concentrations. The method is described in Materials and Methods.) Although the measured spectra may be somewhat distorted by scattering effects, they also clearly indicate that gel formation is accompanied by a coil → β transition. As an example, Fig. 5 shows the evolution of CD spectra upon gel formation by C(470–494) in H_2O at a peptide concentration of about 30 mg/ml. Gel formation of the C-peptide was almost complete after 5 h in H_2O and the gel formed showed a typical CD spectrum for β structure with a single minimum ~217 nm.

DISCUSSION

As already described in the Introduction, the terminal regions of flagellin are disordered and become folded in α-helical bundles upon polymerization into flagellar filaments. It is thought that the disordered termini prevent flagellin molecules from polymerizing spontaneously until they are transported to the tip of the filament through the central channel. This kind of functional motif for regulating self-assembly, that is, folding of a partially disordered structure triggered only by some specific interactions between components, is also found in self-assembly process of tobacco mosaic virus, a rod shaped virus with a helical symmetry (Namba and Stubbs, 1986; Caspar and Namba, 1990). The structure of simian virus 40, a spherical virus with icosahedral symmetry, in which subunits are packed in a few nonidentical positions, has revealed that COOH-terminal segments of ~60 residues invade into neighboring subunits in quite different conformations depending on the way the subunits interact, and the ways the COOH-terminal segments invade into neighbors suggest that in this case, as well, the COOH-terminal segments are disordered until those subunits interact (Liddington et al., 1991). Conformational adaptability of the COOH-terminal segments is thus essential for the proper assembly of simian virus 40.

Flagellin subunits are also known to be packed in a few nonidentical positions to form supercoiled filament (Asakura, 1970; Calladine, 1975, 1976, 1978; Kamiya et al., 1979), which makes flagellar filament a functional screw. Our studies show that large segments of the disordered terminal regions of flagellin may readily adopt α-helical as well as β structure depending on the environmental conditions. Although the experimental conditions used to monitor those secondary structure formation are somewhat remote from physiological ones

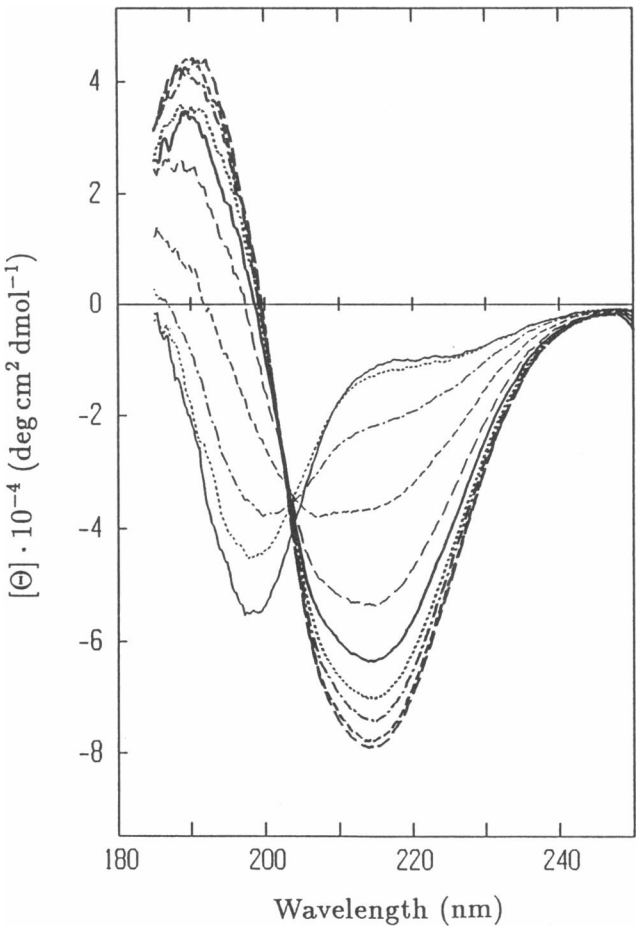

FIGURE 5 Time course of gel formation of C(470–494) as followed by CD spectroscopy. Lines indicate the time after the preparation of the peptide solutions: 25 min (——); 40 min (· · ·); 60 min (– · – · –); 80 min (– – –); 105 min (– – –); 130 min (——); 160 min (· · ·); 190 min (– · – · –); 240 min (– – –); 290 min (– – –). Almost no changes were observed until 25 min.

and the conformational variation in the flagellar filament could not be as large as the one observed for the peptides in solution, and therefore there is no reason to suppose that β to α transitions actually play a role in polymerization; the potential of this high degree conformational adaptability may play some roles in self-assembly and in supercoiling of flagellar filaments. Further studies on the detailed structure as well as the conformational changes of flagellin will unravel the function of the dynamic behavior of this protein.

We would like to thank Drs. H. Hotani and F. Oosawa for continuous support and discussions, and Dr. C. J. Jones for critical reading of the manuscript.

Received for publication 24 June 1992 and in final form 11 September 1992.

REFERENCES

Asakura, S. 1970. Polymerization of flagellin and polymorphism of flagella. *Advan. Biophys.* 1:99–155.

Bruch, M. D., C. J. McKnight, and L. M. Gierasch. 1989. Helix formation and stability in a signal sequence. *Biochemistry.* 28:8544–8561.

Calladine, C. R. 1975. Construction of bacterial flagella. *Nature (Lond.).* 225:121–124.

Calladine, C. R. 1976. Design requirements for the construction of bacterial flagella. *J. Theor. Biol.* 57:469–489.

Calladine, C. R. 1978. Change of waveform in bacterial flagella: the role of mechanics at the molecular level. *J. Mol. Biol.* 118:457–479.

Caspar, D. L. D., and K. Namba. 1990. Switching in the self-assembly of tobacco mosaic virus. *Adv. Biophys.* 26:157–185.

Chen, Y.-H., J. T. Yang, and H. M. Martinez. 1972. Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry.* 11:4120–4131.

Fedorov, O. V., A. S. Kostyukova, and M. G. Pyatibratov. 1988. Architectonics of a bacterial flagellin filament subunit. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 241:145–148.

Homma, M., D. DeRosier, and R. M. Macnab. 1990. Flagellar hook and hook-associated proteins of *Salmonella typhimurium* and their relationship to other axial components of the flagellum. *J. Mol. Biol.* 213:819–832.

Kamiya, R., S. Asakura, K. Wakabayashi, and K. Namba. 1979. Transition of bacterial flagella from helical to straight forms with different subunit arrangement. *J. Mol. Biol.* 131:725–742.

Kostyukova, A. S., M. G. Pyatibratov, V. V. Filimonov, and O. V. Fedorov. 1988. Flagellin parts acquiring a regular structure during polymerization are disposed on the molecule ends. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 241:141–144.

Liddington, R. C., Y. Yan, J. Moulai, T. L. Benjamin, R. Sahli, and S. C. Harrison. 1991. Structure of simian virus 40 at 3.8Å resolution. *Nature (Lond.).* 354:278–284.

Namba, K., and G. Stubbs. 1986. Structure of tobacco mosaic virus at 3.6A resolution: implications for assembly. *Science (Wash. DC).* 231:1401–1406.

Namba, K., I. Yamashita, and F. Vonderviszt. 1989. Structure of the core and central channel of bacterial flagella. *Nature (Lond.).* 342:648–654.

Nelson, J. W., and N. R. Kallenbach. 1986. Stabilization of the ribonuclease S-peptide a-helix by trifluoroethanol. *Proteins.* 1:211–217.

Nelson, J. W., and N. R. Kallenbach. 1989. Persistence of the a-helix stop signal in trifluoroethanol solutions. *Biochemistry.* 28:5256–5261.

Stone, A. L., J. Y. Park, and R. E. Martenson. 1985. Low-ultraviolet circular dichrosim spectroscopy of oligopeptides 1–95 and 96–168 derived from myelin basic protein of rabbit. *Biochemistry.* 24:6666–6673.

Uratani, Y., S. Asakura, and K. Imahori. 1972. A circular dichroism study of *Salmonella* Flagellin: evidence for conformational change on polymerization. *J. Mol. Biol.* 67:85–98.

Vonderviszt, F., S. Kanto, S.-I. Aizawa, and K. Namba, K. 1989. Terminal regions of flagellin are disordered in solution. *J. Mol. Biol.* 209:127–133.

Vonderviszt, F., H. Uedaira, S. Kidokoro, and K. Namba. 1990. Structural organization of flagellin. *J. Mol. Biol.* 214:97–104.