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Vibrational Spectroscopy 48 (2008) 65–68
VIBRATIONAL SPECTROSCOPY
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Raman optical activity of flagellar filaments of *Salmonella*: Unusually intense ROA from L-type self-assembled protein filaments and their possible higher level chiral organization

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Received 18 July 2007; received in revised form 16 December 2007; accepted 17 December 2007
Available online 24 December 2007

Abstract

The flagellar filament of *Salmonella* is an assembly of a single protein, flagellin. There are different helical and straight shape filaments from wild type and mutants. Raman and Raman optical activity (ROA) spectroscopies were employed to characterize the structure of flagellar filaments. The Raman spectra of normal and two straight filaments were almost same, but in the ROA spectra, intensive ROA peaks at amide I, amide III and skeletal vibrational regions were observed from only one of two straight filaments, L-type straight filaments. When the filaments were depolymerized or fragmented, the ROA bands disappeared on the scale of the large ROA intensities. It is thought that the ROA bands of L-type flagellar filaments are related to their shape, length and possible higher level chiral organization.

Keywords: Raman optical activity; Bacterial flagellum; Molecular assembly; Protein

1. Introduction

Bacteria such as *Salmonella* swim by rotating long, helical flagellar filaments as a bundle. Flagella are attached to a rotary motor that is embedded in the cell wall. The rotating flagellar filaments generate thrust during swimming [1,2]. When the cell changes the swimming direction, the filament bundle comes apart and the filament shape is changed, which is called polymorphic transformation. Twisting force associated with the reversal rotation of the motor is the cause of polymorphic transformation [3]. There are some left-handed helical, some right-handed helical, and some straight shaped filaments. Besides the twisting force generated by the motor, polymorphic transformations from one shape to another can be caused by pH, temperature or salt concentration [4,5].

The flagellar filament is a tubular structure composed of eleven protofilaments. Each protofilament is a helical assembly of a single protein with 494 amino acid residues called flagellin [6–8]. The change of the filament shape is a result of a conformation change of the flagellin protein molecules. In addition, mutations in flagellin can change the basic polymorphic form, for example straight filaments [8]. The supercoiled forms of filaments are thought to be constructed from a mixture of two types of protofilament conformations with distinct helical symmetry; the protofilaments are twisted into left-handed helices in one and right-handed in the other, and therefore they are called the L-type and R-type [6]. Two types of straight filaments are constructed only from L-type or R-type of protofilaments [9]. Structure of the some helical flagellar filaments are poorly characterized with XRD and NMR techniques due to deficiency in the symmetric property of the molecular assembly, while structural models of

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0924-2031/$ – see front matter
doi:10.1016/j.vibspec.2007.12.008

straight flagellar filaments have been proposed with electron microscope (EM) and XRD [10–13].

Structures of flagellin in monomeric and assembled forms were studied and it was determined from NMR [14] that both termini were disordered in the monomer, and that the α-helical content of flagellin was much higher in the assembly than that in monomer state from circular dichroism (CD) in far UV region [15] and with Raman spectroscopy [16]. It was indicated that both termini play important roles in formation of the flagellin assembled structure as filament.

Raman optical activity (ROA) spectroscopy, which is measured as a small difference in right- and left-circularly polarized Raman scattering light, is a promising technique for studies of solution structure of biomolecules. XRD and NMR methods are preeminent in structural biology due to their ability to reveal the details of molecular structure at atomic resolution, however, there are limitations to their applicability. In contrast to these methods, vibrational optical activity can be applied to a wide range of biological systems and can provide information regarding the structure, dynamics, local environments and the filament assembly processes [17–19]. ROA spectra of proteins contain information about the secondary and tertiary structure of polypeptide backbones, side-chain conformation changes, and interactions among molecules. There are some reports of protein, nucleic acid, and virus studies with ROA spectroscopy which imply that ROA spectroscopy is well suited to the analysis of the molecular assembly and conformational changes in molecule [20–22]. Therefore, Raman and ROA spectra are used here as a structural probe of the shapes and possible higher level chiral organization of flagellar filaments.

2. Experimental

2.1. Preparation of flagellar filaments

Bacterial strains used in this study are a wild-type strain of *Salmonella typhimurium* that contains helical flagellar filaments, SJW1103, and mutants that contains L-type and R-type straight flagellar filaments, HFG195 [23] and SJW1655 [8,9], respectively. Flagellins were purified essentially as described elsewhere [7,24]. One milliliter of flagellin solution at 7.5 mg/mL in 20 mM Tris–HCl (pH 8.0) and 1.0 mL of 3 M ammonium sulfate solution were mixed well to reconstitute flagellar filaments, and the reconstituted filaments were precipitated with centrifugation at 560,000 × *g* for 30 min at 4 °C. Precipitates were washed in 20 mM Tris–HCl (pH 8.0) twice. Monomeric samples were prepared by heating the filaments, thus prepared, at 65 °C for 5 min.

Sample protein concentrations were 15–10 mg/mL for L-, R-type straight, and normal helical flagellar solution, and 30 mg/mL for concentrated L-type straight flagellar solution.

2.2. Measurement of Raman and Raman optical activity spectra

The backscattered scattered circular polarization (SCP) Raman and ROA spectra were measured at room temperature with a BioTools Chiral*RAMAN*™ spectrometer equipped with a CCD detector. For each spectrum, 1024 scans were collected, and an exposure time is 1.029 s.

2.3. Fragmentation of flagellar filaments

Shorter flagellar filaments were prepared by ultrasonic treatment using a probe-type sonicator, US-50 (NIHONSEIKI). The treatment was carried out 200 times each for 1 s with 1 s interval on ice.

2.4. Measurement of filaments length

The lengths of the filaments were measured in negatively stained filaments by transmission electron microscopy, 1200EX (JEOL).

3. Results and discussion

The top panels of Fig. 1 show the Raman spectra of L-type flagellar filaments (assembly) (a) and flagellin monomer (b). The Raman spectra of R-type straight, and normal helical filaments are almost same as that of L-type filaments and are not shown. The amide I and the amide III bands for the flagellin assembly that contribute to α-helical content are indicated.

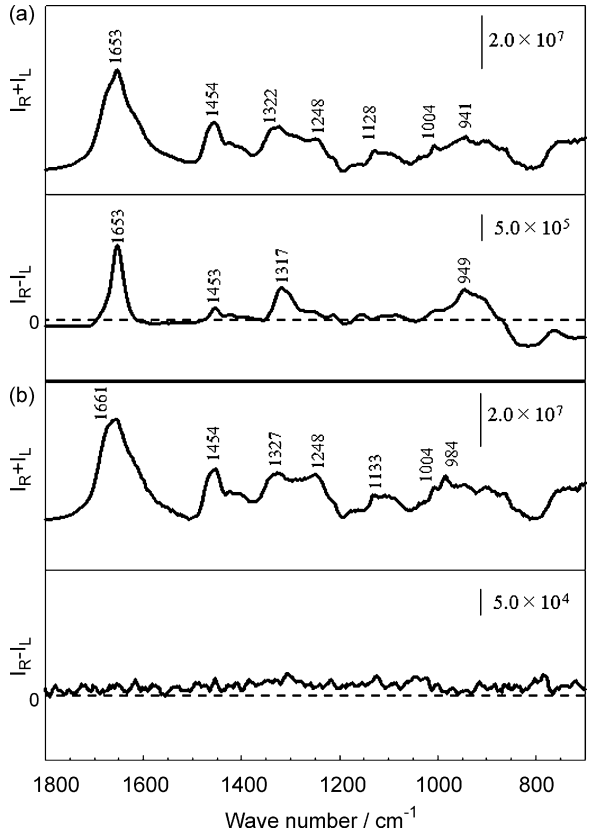

Fig. 1. Raman ($I_R + I_L$) and ROA ($I_R - I_L$) spectra of (a) L-type straight filaments and (b) flagellin monomer. The monomeric sample was prepared by heating flagellar filament. Protein concentrations of L-type filaments solution and flagellin monomer solution were 13 mg/mL. Raman spectra were spectra of sample solution subtracted by spectra of buffer.

Upon depolymerization of filaments to flagellin monomer by heating, the peaks contributing to α-helix are decreased. It is reconfirmed that α-helical structure in flagellin is reduced upon monomerization of the flagellin assembly as described previously [16].

In the ROA spectra of L-type filaments (bottom panels of Fig. 1a), three positive major bands at 1653 cm^{-1} in the amide I region, 1317 cm^{-1} in the amide III region, and 949 cm^{-1} in the skeletal vibrational region were observed. It should be stressed that the three peaks are the contributions from the amide groups in the main chain of the protein. Interestingly, strengths of these peaks are much higher, by more that one order of magnitude relative to the parent Raman intensities, than those of monomeric α-helical proteins previously reported [22]. These unusually intense ROA peaks were observed only from the L-type straight filaments, and not observed from R-type straight or normal helical filaments (data not shown). When the flagellin assembly was depolymerized into flagellin monomer by heating, the ROA peaks disappeared on the scale of the ROA of the assembled flagellin filaments (bottom panels of Fig. 1b). Even if the monomeric sample concentrations were much higher (39 mg/mL) or the accumulated scans were increased (15008 scans), there were only weak ROA signals (data not shown). It is noted that the ordered structure formed by the main chain of the protein in the L-type filament is one of the essential factors for producing the intensive ROA signals.

If the ordered structure in the L-type filament contributing to the intense ROA signals is derived from a specific alignment of monomers along the filament, the filament length will be one of important factors. Under the condition for preparation of flagellar filaments in this experiment, however, length of the filaments was not controlled. To examine effects of the filament length on ROA signal strength, the L-type flagellar filaments were shortened by sonication. The average filament lengths before and after ultrasonic treatment are 182 nm and 84 nm, respectively. Fig. 2 shows the spectra of L-type original and fragmented filaments. The Raman spectrum of the fragmented filaments is almost identical with that of the original flagellar filament, indicating no secondary structural changes in the protein by ultrasonic treatment, while there were no significant ROA signals in contrast to the intense signals from unfragmented sample (Fig. 2a and b). These results indicate that the observed very intense ROA signals vanish upon shortening the filament.

From these results, it can be seen that the intense ROA spectrum observed for intact L-type straight filaments, and not the wild-type or R-type straight filaments, is very delicate and can easily be disrupted by heating or sonication. These perturbations of the original filament structure lead to two different states of filament disassembly, heating to complete disassembly down to protein subunits and sonication to partial disassembly to shorter filament lengths. One possible interpretation of all these results is that the intense ROA spectra arise from a higher level of chiral filament organization of the whole L-type straight filaments. This higher level organization may not occur for the wild-type or the R-type filaments due to unfavorable overall filament shape. The higher level of chiral

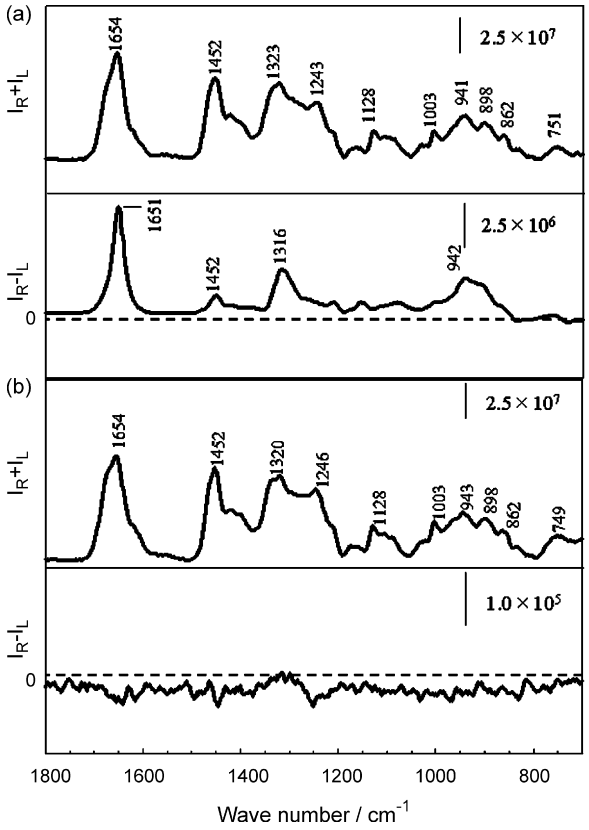

Fig. 2. Raman ($I_R + I_L$) and ROA ($I_R - I_L$) spectra of (a) non-fragmented filaments and (b) fragmented filaments. Fragmented filaments were prepared by using probe-type sonicator. Protein concentrations of L-type filaments solutions were about 30 mg/mL. Raman spectra were spectra of sample solution subtracted by spectra of buffer.

organization of the L-type filaments would be disrupted by sonication to shorter filaments and less favorable inter-filament interaction and alignment. Heating results in isolated monomer proteins and loss of flagellin alignment into protofilament and with it any chance of higher chiral organization of filaments. At this stage the idea of higher chiral organization is only a hypothesis and further experiments to elucidate the physical properties of the filaments structures that give rise to very intense ROA intensities are required in order to determine whether such further aggregation actual occurs for whole L-type straight filaments.

Because the parent Raman spectra of the all three filament types are essentially the same, it can be further concluded that nothing unusual happens to any of these samples as a whole. For example if a different phase of matter occurred for the L-type straight filament, such as liquid crystal formation or very large scale aggregation, significant changes would be seen in the parent Raman spectra. If very large filament-aggregates formed on the order of the wavelength of the laser radiation, the solution would become turbid, high levels of Rayleigh scattering would be encountered and the original Raman spectrum, which very close to that of the individual protein spectrum, would be obscured or otherwise lost. One possibility consistent with the data, as described above, is some kind of further inter-filament association or higher level chiral

organization which is sufficient in extent to amplify the ROA spectrum but not so long range that a different state of sample with a different Raman spectrum is reached. The possibility of higher level chiral organization is intriguing because it would provide yet a further level of structural chirality, available only to the L-type straight filament that would explain, at least qualitatively at this point, the origin of the unusually amplified ROA spectrum for this particular flagellar filament.

Although the R-type and normal type filaments show no significant ROA for the intensity scale and collection times used to measure the large ROA of the L-type filament, we have successfully measured preliminary Raman and ROA of the isolated monomer flagellin proteins for longer collection times. These spectra show virtually all the expected frequencies, signs and intensity patterns characteristic of proteins with a significant level of α-helical secondary structure. Spectral collection times needed for the monomer were approximately five hours versus the 20 min of detector exposure time needed to see ROA for L-type filaments. Additional ROA measurements of monomer flagellin samples are planned for the near future to more carefully delineate their spectral features.

Finally, we note that very large vibrational circular dichroism (VCD), the corresponding infrared form of vibrational optical activity, has been reported for the formation and subsequent development of protein amyloid-type fibrils in lysozyme and insulin where long-range structural chirality associated with developing fibrils may be responsible for the observation the usually large and growing VCD spectra [25]. In addition, preliminary measurements indicate that very large VCD, compared to the wild type, is observed for the same L-type straight filament samples for which unusually large ROA is reported in this paper.

4. Conclusion

Intense ROA bands were observed only from the L-type straight flagellar filaments. Upon disassembly of the flagella to the flagellin monomer by heating and fragmentation of the flagellar filament by ultrasonic treatment, the intense ROA bands were vanished. To investigate further the origin of the intensive ROA signals of the L-type filaments, and to test the hypothesis of higher level chiral organization of these filaments as the source of very intense ROA, effects of concentrations and lengths of the filaments on strength of ROA bands are now under investigation.

Acknowledgements

We thank Prof. Hideyuki Yoshimura of Meiji University for using transmission electron microscope.

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