Conformational Transitions in Flagellins

I. HYDROGEN ION DEPENDENCY*

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SUMMARY

Optical rotation and optical rotatory dispersion were used to study the structural conformation of flagellin, the constituent protein of flagellar filaments of bacteria. Flagellins from four mesophilic bacteria undergo a reversible transition in the pH range of 2 to 4, whereas flagellins from three thermophiles do not. Flagellin from a bacterium with an intermediate maximum growth temperature undergoes an intermediate transition. The relative differences in the acid stability of the flagellins apparently reflect the differences in heat stability observed for the respective flagellins and flagellar filaments studied. The relative resistance of bacterial flagella to disruptive agents depends at least in part on the inherent stability of the flagellin subunits.

The bacterial flagellum, an organelle of locomotion, consists of three parts, a basal region that is tightly associated with the cytoplasmic membrane, a proximal hook, and a spiral filament. The filament is the main portion of the flagellum and is constructed of globular subunits of the protein flagellin. In the case of flagella from cells of Bacillus pumilus, the subunits probably are arranged in the form of six coiled fibers that form the wall of a tube (1–3). Different models have been proposed for flagella of other organisms (4–10).

Upon treatment with acid or a variety of other agents, detached flagellar filaments disintegrate into monomeric or oligomeric flagellin molecules (11, 12). Such filaments from thermophilic bacteria, which are capable of growing at temperatures as high as 75°, are much more heat stable than comparable organelles from mesophiles (12–14). Similar differences in stability are also observed when flagellar filaments are treated with acid or other disruptive agents. All efforts to demonstrate

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stabilising or labilising materials in thermophile and mesophile flagella were unsuccessful (13, 15, 16). It seems reasonable to expect the structural integrity of the filamentous polymers to depend on the inherent stability of their constituent flagellin molecules. In a preliminary report, Yaguchi, Foster, and Koffler (17) observed that the relative stability of flagellar filaments to acid was accompanied by differences in the structural behavior of the flagellins as well. Abram and Koffler (18) have shown that flagellin from a thermophile is capable of forming filaments by self-assembly at much higher temperatures than that of B. pumilus, a mesophile. In spite of these striking differences in stability, there is only one consistent difference in the amino acid composition of mesophile and thermophile flagellins. In flagellins from thermophilic strains of Bacillus, threonine to serine ratios are significantly higher than in mesophilic strains (19). It remains to be seen whether this observation eventually can be related to conformational properties.

The present report, an extension of the work by Yaguchi et al. (17), describes experimental results that reveal the relative differences in the stability of flagellins to acid, the correlation of these differences to the thermostability of flagellar filaments, and the types of structural conformations likely to be assumed by the flagellin molecule in response to changes in pH. Determinations of optical rotation and optical rotatory dispersion were used in this study as methods for estimating the helix content of the flagellin molecules and to detect the presence, if any, of structures other than α-helix.

MATERIALS AND METHODS

Organisms—The mesophilic bacteria used were Proteus vulgaris, Bacillus pumilus 236, Bacilluslicheniformis NRS 243, and Bacillus species X1. The following thermophilic strains of Bacillus steatorrhophilus were used: 2184, FJW, 10, and 194.

Isolation and Purification of Flagella—All the mesophiles were grown at 37° for 11 to 13 hours, and the thermophiles at 55° for 8 to 10 hours except cells of B. steatorrhophilus 194 which were grown for 6 hours. Flagella were isolated and purified by the method described by Abram and Koffler (18). The purified flagella were used immediately for the preparation of flagellin.

Preparation of Flagellin Stock and Working Solutions—Aqueous suspensions of purified flagella were acidified with 0.1 N HCl to pH 2.0, kept 1 hour at that pH and room temperature, and then
centrifuged at 100,000 × g for 60 min at 5°C in a Spinco model L centrifuge. The acid-insoluble sediments were discarded, and the clear supernatant liquid was dialyzed overnight at 4°C against 0.01 M HCl and filtered through Whatman No. 1 filter paper. Transparent solutions thus prepared constituted the stock solutions of flagellin and were stored at 4°C in tightly sealed bottles. Each solution was used within 2 weeks.

In preliminary experiments, the concentration of the flagellin in the stock solution was determined by the micro-Kjeldahl procedure (20), and the standard conversion factor of 6.25 was used. In later experiments, protein concentration was determined by the biuret method (21) with standard curves based on the dry weights of flagellins from several bacterial species.

Two flagellin solutions at pH 1.8 to 2.0 and pH 10 to 11 were prepared daily from a single stock solution. Portions of the stock solution were diluted with deionized water, the pH of which was adjusted with HCl or KOH, and were finally made up to volume in volumetric flasks. The flagellin concentration in solution was estimated from the flagellin concentration in the stock solution and the dilution was made. These two solutions were then mixed to obtain solutions with pH values from 1.8 to 4.2. Usually, the flagellin solution of pH 10 to 11 was added gradually to the solution of pH 1.8 to 2.0 at room temperature. Many flagellins tend to aggregate, and solutions between pH 4.2 and 9.0 become turbid on standing at room temperature, thereby making reliable measurements within this pH range impossible.

The protein concentration in the flagellin solutions used was 0.13 to 1.1 mg per ml for rotational measurements above 250 μ and 0.03 to 0.06 mg per ml for measurements below 250 μ.

**Optical Rotation and Rotatory Dispersion**—Optical rotatory dispersion measurements in the region of 578 to 265 μ were made with a Rudolph model 20 spectropolarimeter equipped with a rocking polarizer and both mercury and xenon arc lamps as radiation sources. Measurements were made at ambient room temperatures (24 ± 2°C) with polarimeter cells having path lengths of 1.0 and 20 cm. Slit widths were adjusted to 0.5 mm or less. With the aid of a Royal RPC-4000 digital computer, rotatory dispersion parameters, α0, b0, and λ0, were determined and evaluated by the statistical procedure described by Sogami, Leonard, and Foster (22). For flagellins having helical conformation, the indicated λ0 is near or at 216 μ. Therefore, a value for b0 of –535 was used as the parameter for 100% helicity (23). A limited number of optical rotatory dispersion measurements were made with a Bendix polarimetric 62 recording spectropolarimeter, and the dispersion parameters were calculated by the statistical procedure (22) programmed for an IBM 7094 computer.

Measurements in the region of 250 to 205 μ were made with the Bendix recording spectropolarimeter with a 200-watt xenon arc lamp as a radiation source. Slit width and cell path length were 1.8 mm and 1.0 cm, respectively. Resnik and Yamaoka (24) cautioned that light-absorbing, optically inactive solutions in the Bendix instrument give rise to false rotation when the absorbance exceeds the value of 1 optical density unit. Our Bendix instrument was modified to eliminate such rotational artifacts. Nevertheless, as a further precaution, dispersion measurements were terminated when the optical density of the flagellin solution rose above unity or when the light transmission in the instrument dropped below 30%.

The optical rotation of flagellin as a function of pH was recorded at the wave length of 233 μ. The helix content at this wave length was calculated according to the procedure of Simons et al. (25), in which the reduced mean residue rotation [m]233 was reported to be –12,700° for proteins having 100% helix and –1,800° for a random coil.

**RESULTS AND DISCUSSION**

Optical rotation and its dispersion have received increasing attention as methods for estimating conformation and for detecting conformational change of proteins in solution (26). Erlander, Koffler, and Foster (29) reported that the optical rotation by flagellin from *Proteus vulgaris* at the sodium D line undergoes a pronounced change below pH 3.8 and assumed that the change is due to some unfolding of the helical conformation. The optical rotational behavior of flagellins from four mesophiles and four thermophiles as a function of hydrogen ion concentration was investigated, and the results are shown in Figs. 1 through 3 and in Table I.

A change in optical rotation is observed between pH 2 and 4 for each of the flagellins from the mesophiles, *P. vulgaris*, *B. pyruvatus*, *B. licheniformis*, and *B. thailandicus* species X1, and for the flagellin from one of the thermophiles, *B. stearothermophilus* 194. No change in rotation is observed for the flagellins from the thermophile strains, 2184, 10, and FJW. As the pH is raised above 4, rotational values for all the flagellins remain constant, even when the pH is raised to 11. Each transition is reversible, and the curves are generally asymmetric about the midpoint, the "high pH half" being somewhat broader than the "low pH half."

The pH value at the midpoint of each transition (pHt) was found to be a convenient and reproducible parameter for estimating qualitatively the relative stability of flagellins in acid solution. Table II lists these parameters together with the Td (temperature of disintegration) values obtained from viscometry measurements for the corresponding flagellar filaments, and the relative acid resistance of filaments, as determined by the amount of centrifugable material still remaining after exposure to 0.001 N HCl for 4 hours at 25°C (14). These data show that flagellins from thermophile flagella are more stable to acid than flagellins from mesophiles. Even when the pH was lowered to 1.4, no transition occurred and hence no pH transition value could be obtained for the thermophile flagellins, whereas the flagellins undergoing transitions had pHt values between 2.8 and 3.2. Flagellin from the thermophile strain 194, which behaves as an intermediate with regard to its maximum growth temperature as well as thermostability of its flagellar filaments, has a pHt of 2.6. There is an apparent order of acid stability among the flagellins from mesophilic bacteria which correlates with the relative heat and acid stabilities of corresponding flagellar filaments (Table II). In a subsequent paper, data will be presented to show that the flagellin subunits from thermophile flagella are also relatively more stable to heat than flagellin subunits from mesophile flagella. Thus, the differences in behavior of various bacterial flagella in response to disruptive agents apparently depend at least in part on the intrinsic 1 Conflicting magnitudes of [m]233 have been reported for various model compounds having a helix content of 100%. (25–27). We chose to use the value of Simons et al. (25) because it is based on naturally occurring proteins and not on homogeneous synthetic polypeptides. Further, since we were concerned more with relative differences of helix contents than with absolute values, using a magnitude other than [m]233 = –12,700° would seriously affect neither the data nor their interpretation.
stability of the constituent flagellin subunits. The question remains as to what extent the forces between subunits contribute to the stabilization of the flagellar filaments. This problem is currently being investigated.

In earlier experiments, the optical rotation of the flagellin solutions was measured at the wavelength of 313 μm with a manually operated Rudolph spectropolarimeter (Figs. 1 and 2). Later, these experiments were repeated with the Bendix recording spectropolarimeter at 233 μm (Fig. 3). When the pH profile curves in Figs. 1 and 2 are compared with the respective curves in Fig. 3, it can be seen that the pHγ parameters for each pair of respective curves are similar in that they differ by no more than 0.1 pH unit. On the other hand, the transitions observed at wavelength of 233 μm with an average span of approximately 0.6 pH unit are consistently sharper than those at 313 μm that span nearly 1.4 pH units. While this difference cannot be accounted for, it might suggest the existence of intermediate states in which the optical rotatory dispersion properties are modified in different ways. If the transition were entirely cooperative, i.e. without intermediates, one would anticipate

![Figure 1](http://www.jbc.org/)

**Figure 1.** The effect of pH on the reduced mean residue rotation at 313 μm by flagellins from mesophiles. To obtain solutions with pH values from 1.8 to 4.2, flagellin solutions at pH 10 to 11 were mixed at room temperature in varying proportions with solutions at pH 1.8 to 2.0. △, P. vulgaris; ○, Bacillus species X1; ●, B. pumilus.

![Figure 2](http://www.jbc.org/)

**Figure 2.** The effect of pH on the reduced mean residue rotation at 313 μm by flagellins from thermophiles. See legend to Fig. 1 for experimental details. ■, P. vulgaris; ○, Bacillus species X1; △, B. pumilus; ○, B. licheniformis; ●, B. stearothermophilus 194.

**Table I**

<table>
<thead>
<tr>
<th>Flagella from strains of B. stearothermophilus</th>
<th>Reduced mean residue rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2</td>
<td>pH 11</td>
</tr>
<tr>
<td>194</td>
<td>-2720°</td>
</tr>
<tr>
<td>10</td>
<td>-3200</td>
</tr>
<tr>
<td>FJW</td>
<td>-5550</td>
</tr>
<tr>
<td>2184</td>
<td>-3505</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Maximum temperature of growth*</th>
<th>Disintegration after exposure to 0.001 N HCl for 4 hours</th>
<th>pHγ, flagella</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. vulgaris</td>
<td>45</td>
<td>56.1°</td>
<td>3.2</td>
</tr>
<tr>
<td>Bacillus species X1</td>
<td>55</td>
<td>53.1°</td>
<td>75</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>57</td>
<td>58.6°</td>
<td>2.8</td>
</tr>
<tr>
<td>B. pumilus</td>
<td>51</td>
<td>59.1°</td>
<td>82</td>
</tr>
<tr>
<td>B. stearothermophilus 194</td>
<td>66</td>
<td>58.7°</td>
<td>23</td>
</tr>
<tr>
<td>B. stearothermophilus 10</td>
<td>75</td>
<td>71.8°</td>
<td>9</td>
</tr>
<tr>
<td>B. stearothermophilus FJW</td>
<td>71</td>
<td>76.3°</td>
<td>12</td>
</tr>
<tr>
<td>B. stearothermophilus 2184</td>
<td>78</td>
<td>77.1°</td>
<td>0</td>
</tr>
</tbody>
</table>

* These values are from Stenesh and Koffler (14).

* No transitions observed.
the pH transition curves to have identical midpoints and spans, irrespective of the wave length employed.

It is assumed that changes in optical rotational properties of a protein generally result from conformational transitions. To ascertain the nature and extent of conformational change that may occur in flagellin molecules, the Moffitt-Yang parameter, \( b_2 \), and the 233 mu trough method of Simmons et al. (25) were employed for the estimation of helix content. The data listed in Table III show that the flagellins from four mesophiles and the moderately thermophilic strain 194 undergo an apparent helix-coil transition in which the helix content changes by as much as 24%, whereas no significant change occurs among the flagellins of the other three thermophiles. Of course, it is possible that conformational changes also occur in these thermophilic flagellins, but were not detectable by the methods used in this study. The helix content of the soluble flagellin cannot be correlated with the differences in thermostability of the flagellan filaments. Flagellin from B. licheniformis, for example, has as much if not more helix than any of the flagellins that make up the thermophile flagella, yet the thermostability of B. licheniformis flagella is considerably less than that of the thermophile flagella. It therefore seems reasonable to conclude that factors other than helix content play a dominant role in governing the stability of the flagellins.

X-ray diffraction patterns of flagella from P. vulgaris, B. subtilis, Salmonella typhimurium, Vibrio nactchiniikovii, and a strain of Sarcina have been shown to be almost identical (4, 5), and have provided information that suggests the presence of \( \alpha \)-helix as well as cross \( \beta \) structure. Burge (6) reported, however, that preliminary measurements of the infrared absorption spectra of cast films of flagella from P. vulgaris show only the presence of the \( \alpha \) component.

With the increasing improvement of polarimetric instrumentation, investigators have been able to scan into the far ultraviolet region where Cotton effects for proteins may be observed. Consequently, many reports have been published which described the characterization of Cotton effects with respect to protein structure from model synthetic polypeptides of known structure and composition (30-32).

At pH 2 and 10, Cotton effects were observed for each of the flagellins from both mesophiles and thermophiles. The posi-

### Table III

<table>
<thead>
<tr>
<th>Organism</th>
<th>Helix content at pH 2</th>
<th>Helix content at pH 4 to 11</th>
<th>Crossover position at pH 2</th>
<th>Crossover position at pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. vulgaris</td>
<td>5</td>
<td>12</td>
<td>12</td>
<td>207</td>
</tr>
<tr>
<td>Bacillus species X1</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>207</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>16</td>
<td>13</td>
<td>13</td>
<td>210</td>
</tr>
<tr>
<td>B. pumilus</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>206</td>
</tr>
<tr>
<td>B. steathermophilus 194</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>216</td>
</tr>
<tr>
<td>B. steathermophilus 10</td>
<td>98</td>
<td>94</td>
<td>94</td>
<td>223</td>
</tr>
<tr>
<td>B. steathermophilus F. J.</td>
<td>39</td>
<td>35</td>
<td>35</td>
<td>222</td>
</tr>
<tr>
<td>B. steathermophilus 2184</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>221</td>
</tr>
</tbody>
</table>

Fig. 4. The effect of pH on the Cotton effects of flagellins from (a) B. licheniformis, (b) B. steathermophilus 194, and (c) B. steathermophilus 2184. Protein concentration of flagellin solutions was 0.05 to 0.06 mg per ml. Silt width and cell path length in Bendix spectropolarimeter were 5.8 mm and 1.0 cm, respectively; determinations were made at room temperature.
(Fig. 4a) are different from those of the Cotton effects that are computed from model compounds of similar helix-coil composition (30-32). The observed data on denatured flagellins show that the magnitude of the second minimum is less than that of the trough at 233 nm, whereas the relationship between the two minima is inverse for computed curves representing 10% helix and 90% coil. There is also less of a blue shift of the crossover than is predicted, but this shift may be dependent in part on the magnitude and position of the second minimum. Apparently, Cotton effects cannot be properly represented by curves computed exclusively from helix-coil models. Moreover, structures other than helix and coil may be present in the flagellin molecule which may be contributing to the rotational properties.

The foregoing data provide partial insight into the types of conformation that a flagellin molecule may assume as it solubilizes or dissociates from the flagellum or reassociates to form filamentous polymers. Abram and Koffler (18) described the reaggregation of B. pumilus flagellin into flagella-like filaments. This self-assembly could be initiated only above pH 4.9 and was optimal at pH 5.4. Since present data show that the conformational transition of B. pumilus flagellin occurs below pH 4 (Fig. 1), it appears that the reaggregation process requires the flagellin molecule to have a conformation characteristic of neutral pH values. On the other hand, the acid disintegration of flagellar filaments occurs well within the pH range in which conformational transitions of flagellins are observed. At pH 3.4, the disintegration of P. vulgaris flagella requires 3 to 4 days for completion, but only 20 min at pH 2.5 (34). The difference between acid stability and lability of flagella may depend upon whether or not conformational changes occur in the flagellins. Furthermore, the inherent acid stability of the constituent subunits of flagellin may be an additional factor involved in the stability of the flagellar filament. An important aspect of the stability of flagellar filaments to acid that must be considered is the nature and strength of the forces that hold the flagellins together within the flagellum. While this problem regarding other than helix and coil may be present in the flagellin molecule the absence of salts. Limited studies were made on the effect of ions on the stability of flagellin molecules and their subsequent reaggregation of B. pumilus flagellin into flagella-like filaments.

Thus far, flagellins in aqueous solutions have been studied in the absence of salts. Limited studies were made on the effect of 0.1 M KCl on B. pumilus flagellin in acid solutions. The results indicate that the subunits acquired greater acid stability. While these results cannot be interpreted in detail, they at least warrant the suggestion that electrostatic repulsion plays a role in the breakdown of the helical structure of flagellins in acid solution. The screening effect of the added ions could conceivably stabilize the helical structure of the monomer per se. Alternatively, the effect could be indirect through stabilization of dimeric or oligomeric forms of the flagellin. Erlander et al. (29) found increasing ionic strength to exert a stabilizing effect on the dimeric form of P. vulgaris flagellin. Further clarification of the effect of ions on the stability of flagellin must await additional studies which combine optical rotatory dispersion and molecular weight measurements.

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