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THERMAL PERTURBATION DIFFERENCE SPECTROSCOPY OF SALMONELLA FLAGELLIN

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SUMMARY

The appropriateness of a two-state model for the previously reported thermal transition of Salmonella flagellin and the reversibility of this process recently has been questioned by others. Spectrophotometric evidence is presented here that reveals two separate thermally-induced structural transitions in flagellin which may resolve apparent controversy. A low temperature transition (I) appears between 28 and 35°C with a midtransition temperature of 30.7°C. With increasing temperature in this transition region a progressive shift to the red of the absorbance band at 284 nm occurs. The latter, probably due to an infolding of tyrosyl residues, is paralleled by a decrease in the rate of polymerization of flagellin. The temperature profile for the spectral behavior of flagellin in transition I fulfills criteria for a two-state process and is fully reversible. A second transition, also reversible, is observed between 40 and 60°C with a midtransition temperature near 50°C. Transition II,observed as a blue spectral shift of the absorbance band at 277 nm,is better described as a multistate process. Tyrosyl residues appear to maintain the conformational integrity of the polymerizable state of flagellin.

A decade ago two anomalous effects of temperature were reported on the kinetics and magnitude of the volume change (ΔV) accompanying the in vitro self-assembly of Salmonella flagellin to form flagellar filaments (1). In the first observation, the rate of the assembly was seen to increase with temperature until 28°C. Above 28°C the rate decreased and approached zero near 43°C. This finding was also noted in viscometric studies on B. subtilis flagellin (2) and flagellins from various Salmonella flagella mutants (3,4). Second, at temperatures between 22-28°C the value of ΔV was 157 ml/mole of flagellin polymerized; above 28°C ΔV increased with temperature to a limiting value of 306 ml/mole at 35°C (Fig. 5 in ref. 1).

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Polymerization of a monomer to the end of a growing polymer is a bimolecular reaction. Therefore, the foregoing observations could be attributed to changes occurring with temperature in either one or both of these components. Although alternative interpretations could have been presented, the results then were interpreted by assuming that the monomer (M) was in equilibrium with an inactive (not directly polymerizable) more compact form (M_i). At that time there was no other evidence for the existence of the postulated low temperature conformational isomer, M_i.

Results of ultraviolet absorbance studies on flagellin under conditions where it is not polymerized (i.e., free of ends) are presented below as direct evidence for a reversible thermally-induced monomer state in the temperature region of 28-36°C. Although functionally relevant, this conformational transition has been difficult to observe by others (5,6) due to the more extensive unfolding of the protein that occurs during a second transition at temperatures above 40°C. This has led to controversy on the proposed mechanism for polymerizing flagellin and disagreement with conclusions resulting from the proposal (7,8).

EXPERIMENTAL PROCEDURES

Flagellin was isolated from Salmonella SJ 25 cells by previously published methods (9,1,3). The protein was purified further by putting it through two polymerization-depolymerization cycles (6). Flagellin was polymerized to flagellar filaments by using sonic fragments of flagella as nucleating agents. The protein was stored at 4°C as polymer in a solvent containing 0.15M KCl and 0.05M potassium phosphate (pH 7.0). Prior to use solutions of flagellar filaments were dissociated to the flagellin monomer by incubating for 5 min at 65°C and clarified by centrifugation in a Spinco Model L centrifuge at 78,000xg for 1 h. Flagellin samples were consumed within two weeks after isolation of the protein. A molecular weight of 6×10^4 for flagellin (7,8,9) was used in calculations. Protein concentrations were determined by the biuret method (10) standardized with bovine plasma albumin 2X crystallized (Armour Co., Chicago, Illinois).

Spectral measurements were made in a Cary Model 14 recording spectrophotometer. Conditions chosen for each experiment are described in figure legends. Figures represent an average of more than four spectra converted to A_M and ΔA_M values as a function of wavelength. Difference spectra were determined with a 0 to 1 or 0 to 0.1 optical density slidewire at a dynode setting of 3. Matched stoppered thermojacketed absorption cells (Hellma Cells, Inc., Jamaica, N.Y.) with a path length of 1.00 cm were used for spectral measurements. The temperature of sample and reference cuvettes were independently varied and controlled to within 0.05°C of set temperatures with two thermoregulated water circulators (Haake, Model F). The absorbance of solu-

tions used for difference spectra was less than two optical density units; slit widths did not exceed 0.5 mm at all wavelengths for which data are reported. To avoid stray light errors and other spectral artifacts slit widths were varied and Beer's Law (the linear dependence of absorbance on concentration) was verified in absorption and difference absorption maxima for all specimens used. Spectra were scanned and retraced with excellent reproducibility at 15 nm per min and a chart speed of 2 in per min.

RESULTS

The ultraviolet absorption spectrum of flagellin between 240 and 330 nm is shown in Fig. 1A. In addition to the absorption maximum at 277.5 nm typically noted in proteins, flagellin's spectrum contains a pronounced notch on the longwave side of the absorption envelope due to a minor band centered at 284 nm. In preliminary experiments to determine the influence of variations in temperature on the uv absorption spectrum of flagellin two kinds of difference spectra were observed. The first of these, Fig 1B, is the type obtained when the temperature of the sample is varied between 28 and 40°C. The second type in Fig 1C is typical of temperature difference spectra when the sample's temperature is between 40 and 75°C. Characteristic features in these difference spectra are positive maxima near 241 and 288 nm and negative maxima at 278 and 284 nm. The wavelengths at which the positive pair of difference absorption maxima occur increase slightly with temperature: One varies between 241 and 245 nm and the other between 288 and 293 nm. Furthermore, the positive difference absorbance in the short wavelength region is more than 5.4 times greater than in the longer wavelength region. The wavelengths for the negative difference absorption maxima, however, do not vary with temperature.

Figure 2 shows temperature profiles for each of the positive and negative difference absorption maxima. The positive maximum occurring above 288 nm (Figure 2A) undergoes a reversible low temperature thermal transition at a mid-transition temperature of 30.7°C. Profiles for the negative difference absorption maxima at 278 (Figure 2B) and 284 nm (Figure 2C) show parallel transitional increases with temperature. Maximum values of $-\Delta A_M$ occur at 57°C; additional increases in temperature produce lower absorption difference maxima.

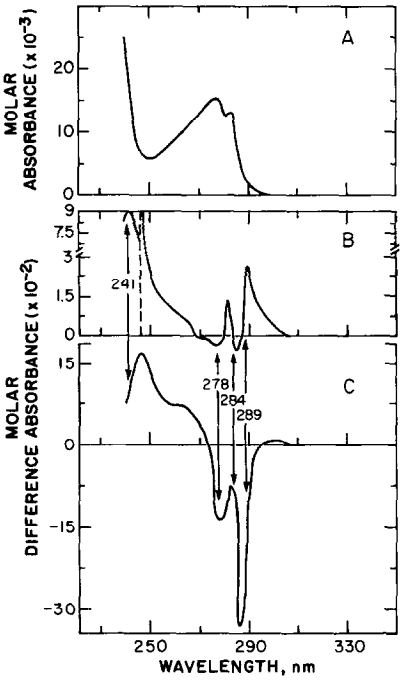

Figure 1. Comparison of absorption spectrum and thermal perturbation difference spectra of flagellin in 0.15M KCl and 0.05M potassium phosphate (pH 7.0).

A. Spectrum of flagellin vs. solvent at 25.0°C.

B. Characteristic thermal perturbation difference spectrum of flagellin in the low temperature region. Reference cell, 25.0°C; sample cell 36.0°C. The scale of the ordinate is divided to accommodate the increase in ΔA_M below 245 nm.

C. Characteristic thermal perturbation difference spectrum of flagellin in the high temperature region. Reference cell, 25.0°C; sample cell 60.5°C.

A shift in the position of the absorption maximum in the normal spectrum ordinarily will appear in the difference spectrum as a maximum located where the slope of the normal spectrum was steepest.

The midtransition temperature for the 278 and 284 nm negative maxima is about 50.3°C. The maximum occurring below 245 nm (Figure 2D) seems to have elements of both the low temperature and higher temperature transitions. The lower one occurs at a midtransitional value of 30.4°C. At temperatures above 45° this difference absorption maximum continues to increase without reaching a plateau. The thermal perturbation difference spectra in Figure 2 were fully reversible. Closed circles represent values obtained after the temperature had been lowered from the highest temperatures used.

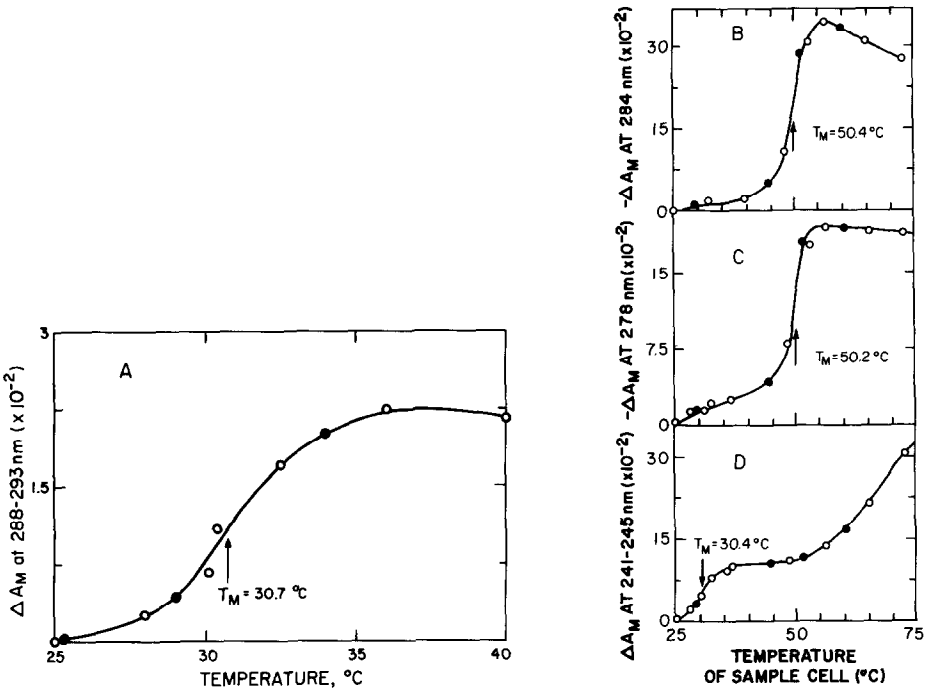

Figure 2. Thermal profiles for positive and negative difference absorption maxima of flagellin. Reference cells, 25.0°C; sample cells are at temperature listed in the abscissa.

A. Wavelength of ΔA_M increases from 288-293 nm as the temperature rises.

B. $-\Delta A_M$ at 284 nm is independent of temperature. Note small pretransitional changes between 25 and 40°C. Above 57°C there is a decrease in $-\Delta A_M$.

C. $-\Delta A_M$ at 278 nm is independent of temperature. Pre-and postransitional changes are visible.

D. Wavelength of ΔA_M increases from 241-245 nm as the temperature rises.

Open circles refer to sample which were heated to the listed temperature and filled to samples cooled from the highest temperature. Arrows indicate midtransition temperatures.

DISCUSSION

Flagellin contains no tryptophan (15,16). Therefore, absorption by the protein above 270 nm can be assigned solely to tyrosyl side chain chromophores. The notch in the absorption envelope of flagellin (Figure 1A) is due probably to the existence of (some of) these tyrosyl residues in a relatively nonaqueous environment away from the polar solvent. Such spectra have been reported for model compounds of tyrosine in apolar solvents (17,18).

The changes in absorption between 240 and 300 nm with variations in temperature undergo two reversible thermal transitions. Formally, the positive difference maximum above 288 nm in the low temperature region (Figure 1B) is due to a red shift of the 284 nm peak (Figure 1A). This is equivalent to the transfer of tyrosines from aqueous to organic phases. The protein can be regarded as a solvent for the chromophores buried within it. A transition in this difference peak occurs between 28 and 36°C, a region where flagellin also becomes more compact (1,11,19). Since the temperature profile of the transition is the same when examined using different observables, i.e., (i) localized chromophores (this work), (ii) size and shape of the protein (11,19), and (iii) polymerizability (3,4) it passes the test for a two-state process proposed by Lumry et al (20).

Less is known of the higher temperature transition. The negative difference maxima observed at 284 and 278 nm in the high temperature region is often seen in proteins. It is referred to as the "denaturation blue shift" and reflects the exposure to solvent of buried tyrosyl residues (16). It is worth emphasizing that although the difference in conformation between the polymerizable monomer (M) and inactive, nonpolymerizable monomer (M_i) appears to be small when estimated hydrodynamically (11,19), this difference clearly is crucial in determining the self-assembly of this protein.

Apparent equilibrium constants for the thermal transitions can be evaluated from the curves in Figure 2. For example, in transition region I (Figure 2A) the equilibrium $M \rightleftharpoons M_i$ is determined by

$$K_I = [Mi] / [M] = \Delta A_M/(225 + \Delta A_M) \qquad (3)$$

and in transition II (Figure 2B) the equilibrium between M_i and extensively unfolded monomer (Mu) $Mi \rightleftharpoons Mu$ is given as

$$K_{II} = [Mu] / [Mi] = (-\Delta A_M)/\Delta A_M + 3500)$$

Van't Hoff plots of the results are shown in Figure 3. Transition I occurs over a 7° temperature range and its van't Hoff plot is linear. Transition II, however, which occurs over a wider range (∿12°C), has a temperature

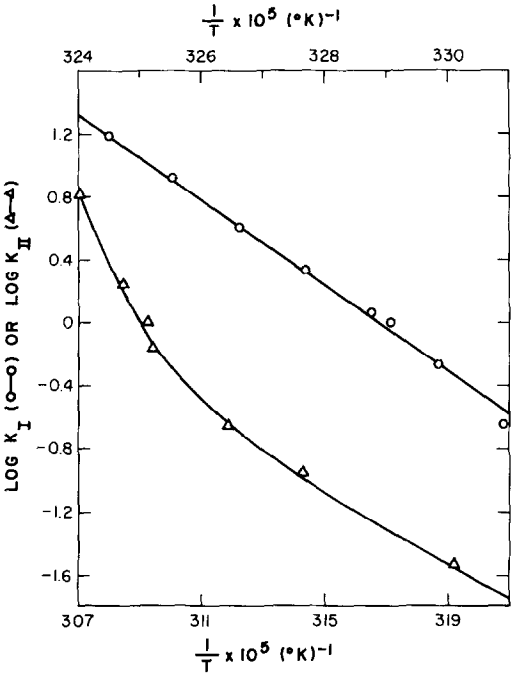

Figure 3. Semilogarithmic graphs of apparent equilibrium constants versus the reciprocal temperature observed in low (0-0) and high (Δ-Δ) temperature regions. Equilibrium constants evaluated from equations (3) and (4) as described in the text. Abscissa at top of the figure refers to low temperature data (0-0).

dependent enthalpy change since its van't Hoff plot is appreciably curved. Apparent thermodynamic parameters obtained are summarized in Table I. The large value of ΔS^{o} illustrates particularly the high degree of order that is present in the polymerizable monomer.

CONCLUSION

Tyrosyl residues in flagellin are sensitive reporters of conformational changes that occur in this protein. Alterations in their local environment are reflected as blue or red shifts in the position of the major and minor ultraviolet absorption bands at 277.5 and 284 nm. In order for flagellin to polymerize to flagellar filaments certain tyrosyl residues must remain exposed to solvent on the protein's surface. It is tempting to speculate that these

TABLE I

Thermodynamic Parameters for Low and High Temperature Equilibria of Flagellin*

Transition	Process	Temperature (°C)	ΔF^o (kcal/mole)	ΔH (kcal/mole	ΔS^o (e.u.)
I	$M \rightleftharpoons M_i$	30.7	0	117	385
II	$M_i \rightleftharpoons M_u$	50.2	0	60	185

*Calculated from data in Figures 2A (transition I) and 2B (transition II).

residues are involved in flagellin-flagellin interactions during the assembly process.

Thermal perturbation of the absorption spectrum of the tyrosine chromophores in flagellin provide evidence that the protein undergoes two separate reversible transitions. Additional studies are in progress to determine more fully the various roles of tyrosyl residues in flagellin.

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