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Calorimetric Studies on the *in vitro* Polymerization of *Pr. mirabilis* Flagellin

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Abstract. The heat effects accompanying the isothermal *in vitro* polymerization of *Pr. mirabilis* flagellin on short flagella fragments (seeds) have been measured in phosphate buffer pH 7, at various temperatures employing a batch microcalorimeter. Additionally, at 20 °C, measurements have been performed in phosphate as well as Tris-HCl buffer at pH 7.5.

The rate of both heat uptake and release during the process of polymerization was shown to be proportional to the rate of molar ellipticity changes observed by parallel circular dichroism experiments.

No change in the state of protonation of flagellin occurs during the polymerization as indicated by the constancy of the enthalpy values determined in buffers with different heats of ionization. The apparent molar enthalpy of polymerization at 25 °C, pH 7, is -34.7 ± 3 kcal per mole of flagellin, the relatively large error mainly resulting from uncertainties of the determination of the percentage of unpolymerized monomers after completion of the reaction.

The most prominent feature of the results obtained in this study is the large temperature variation of the enthalpy, corresponding to a temperature independent heat capacity change of $\Delta c_p = -3039 \pm 100$ cal per degree per mole of flagellin, the error limits referring to the standard deviation in a linear regression analysis.

Key words: Calorimetry — Circular Dichroism — Flagellin — Polymerization.

Introduction

Bacterial flagella consist of a helical assembly of flagellin subunits fixed at one end in the cell membrane (for reviews see Asakura, 1970; Bode, 1973). Locomotion of the bacterium seems to be caused by a passive wave motion of the helical filament, the flexibility and stability of which is provided by strong non-covalent bonds of high specificity.

Treatment of native flagella with various dissociating agents leads to reversible splitting into flagellin monomers of 40000 molecular weight. Under certain conditions (pH 7, 0.15 M NaCl, ~ 20 °C) these subunits represent a homogeneous system which does not polymerize unless short fragments of flagella ("seeds") are added (Asakura *et al.*, 1964, 1966, 1968; Bode *et al.*, 1972). This observation

together with the stability of flagella prove native flagellin subunits under the given conditions to be in a kinetically determined non-equilibrium state; only the helical aggregate is thermodynamically stable under physiological conditions.

The slow nucleation and the relatively fast chain growth in the polymerization reaction of flagellin are characteristic properties of a cooperative process. This view is supported by recent studies of Gerber *et al.* (1973), who present evidence for "critical" monomer concentrations of flagellin, which coexist in equilibrium with flagella independent of the concentrations of polymers. These authors estimated the enthalpy change for the conversion of active monomers into polymers to be endothermic by + 40 kcal per mole of flagellin and considered this result suggestive of important contributions of hydrophobic interactions to the polymerization of flagellin.

On the other hand the same authors point out that according to the difference of the activation enthalpies calculated from the rate constants of the polymerization and depolymerization processes of flagellin and flagella, respectively, the flagellin polymerization should be exothermic with a reaction enthalpy of about — 40 kcal per mole of flagellin.

In the present study an attempt has been made to resolve this discrepancy between the two approaches. In order to circumvent the insufficient accuracy of the indirect methods of calculating the thermodynamic data, direct calorimetric determinations of the reaction enthalpy of the flagellin polymerization were performed in a broad range of temperature.

Materials and Methods

The isolation and purification of flagella from *Pr. mirabilis* were the same as described previously (Bode *et al.*, 1972; Glossmann and Bode, 1972). Fractional centrifugation and repeated depolymerization — seed-polymerization cycles were performed in 0.15 M NaCl, 0.05% NaN_3, 0.01 M sodium phosphate buffer, pH 7. The solutions were stored at 4 °C. "Seeds" (average length $\sim 0.5\,\mu m$) were prepared by sonicating purified flagella solutions $2 \times \frac{1}{2}$ min at ~ 0 °C (Asakura *et al.*, 1964; Bode, 1971). After incubation at room temperature for 2 hours the seeds were sedimented by high-speed centrifugation, dissolved to a concentration of about 15 mg/ml in 0.2 M NaCl, 0.02% NaN_3, 0.01 M sodium phosphate buffer, pH 7, and stored at 4 °C.

Flagellin monomers were prepared by acidifying saltfree suspensions of purified flagella with HCl (Abram and Koffler, 1964), spinning down the acid insoluble material and neutralizing the supernatant after a rapid change to pH 10 (Glossmann and Bode, 1972). The flagellin solutions obtained by this procedure had a concentration of approximately 20 mg/ml (0.2 M NaCl, 0.02% NaN_3, 0.01 M sodium phosphate buffer pH 7). The concentration was determined either by a modified biuret method or by UV absorption ($A_{276nm} = 2.06$ for a 1% (w/w) solution, pathlength 1 cm). The concentration of flagella and seeds were based on the biuret method only. To express data in molar quantities a subunit molecular weight of 40000 was assumed.

Circular dichroism spectra were measured in a Cary 60 spectropolarimeter equipped with a Cary 6002 CD attachment, using a fused thermostated cell of 0.2 mm path length (Hellma, Müllheim, Germany). In order to follow the progress

of the polymerization reaction the ellipticity at 220 nm (θ_{220}) was measured after mixing e.g. 0.1 ml seed solution and 0.15 ml monomer solution. The temperature was determined with a thermistor probe in the cuvette.

Sedimentation analyses were performed in an analytical ultracentrifuge (Beckman, Model E) equipped with an automatic photoelectric scanning system.

Calorimetric measurements were carried out in an LKB Model 10700-2 batch-type microcalorimeter (LKB, Bromma, Sweden). The calorimeter was equipped with two additional thermostats and enclosed by a climate box (Colora, Lorch, Germany) thus allowing measurements in a temperature range between 2 and 60 °C. A period of approximately 2 h for temperature equilibration before the start of the reaction turned out to be long enough to ensure a steady baseline. The reaction was followed for 2 to 3 h and its completeness was checked by an additional mixing procedure. Calibration runs were performed after each measurement as well as separate determinations of the heat of friction of the solutions.

Results

A typical feature of the polymerization of flagellin is the strong decrease of the molar ellipticity during the polymerization process caused by the large difference between the ellipticities of monomeric and polymeric flagellin (Klein *et al.*, 1968; Bode *et al.*, 1972; Uretani *et al.*, 1972). Since previous experiments have shown that the rate of change of ellipticity of a flagellin-seed-mixture is directly proportional to the rate of flagellin polymerization (Bode, unpublished results), the time course of $\Delta\,\theta_{220}$ was used to characterize the time course of polymerization during the calorimetric measurements, using the same seed solutions and monomer solutions at identical mixing ratios and temperatures in both experiments.

Fig. 1 shows the change of ellipticity at 220 nm of a flagellin-seed-mixture at 25 °C (solid line) as a function of time and the corresponding time course of the integral heat output of the calorimeter (dashed line). The apparent sigmoidicity of the calorimetric profile is caused by the time constant of the instrument and has no significance with respect to the kinetic mechanism of the polymerization. From the ellipticity data shown in Fig. 1 one calculates that the polymerization reaction has proceeded to about 36%, 58%, 85% and 95% after 15, 30, 60 and 90 min, resp. while the calorimetric results yield 53%, 80% and 93% for the completion of the reaction after 30, 60 and 90 min. After 2 h no further significant change could be observed. Similar measurements as demonstrated in Fig. 1 were performed at various temperatures. Numerical values of these enthalpies are listed in Table 1. The molar enthalpies given in the table are corrected with respect to the percentage of monomers polymerized, derived from sedimentation analyses with the reaction mixture after completion of the polymerization in the calorimeter. Further corrections taken into account are the heats of dilution of both flagellin monomers and seeds. While dilution of the seeds did not make any significant enthalpic contribution, heat involved in diluting the monomers amounted to about — 1 kcal per mole of flagellin at 25 °C.

The apparent molar enthalpy of a reaction can be influenced by the buffer used in the experiments, when ionization processes occur (Hinz *et al.*, 1971; Sturtevant, 1972). This possibility has been investigated and the results are included in Table 1,

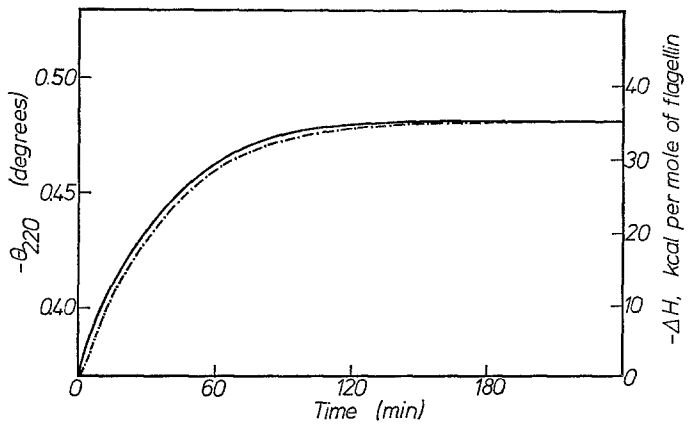

Fig. 1. Time course of ellipticity changes at 220 nm (solid line) and integral heat output (dashed line) as monitored during polymerization of flagellin monomers. Ellipticity measurements refer to a solution of 10.2 mg/ml flagellin, 6.1 mg/ml seeds, 0.01 M sodium phosphate 0.2 M NaCl, 0.02% NaN_3, at pH 7, 25 °C. Optical path length is 0.2 mm. The calorimetric experiment was performed with an identical solution

Table 1. Enthalpies of polymerization of flagellin at pH values 7.0 and 7.5 in two buffers, at various temperatures

T °C	Buffer	Monomer conc. of flagellin mg ml^{-1}	Percentage of monomers polymerized	ΔH kcal (mole of flagellin)$^{-1}$
		4.81	88	+ 14.5
10	A	3.96	85	+ 10.7
		6.59	81	+ 12.6
13	A	6.24	85	+ 0.9
17	A	7.54	78	− 10.8
20	A	6.34	92	− 16.3
	B	11.13	88	− 13.9
	B	12.27	77	− 14.3
	C	12.06	78	− 14.7
	C	12.12	76	− 15.4
25	A	10.05	85	− 33.9
		5.99	77	− 35.4
30	A	7.38	80	− 43.9
		5.10	70	− 47.8
33	A	10.40	85	− 63.3
		7.37	81.5	− 54.9

The buffer systems used were the following
A: 0.2 M NaCl, 0.01 M sodium phosphate, 0.02% NaN_3, pH 7
B: 0.15 M NaCl, 0.05 M sodium phosphate pH 7.5
C: 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5.

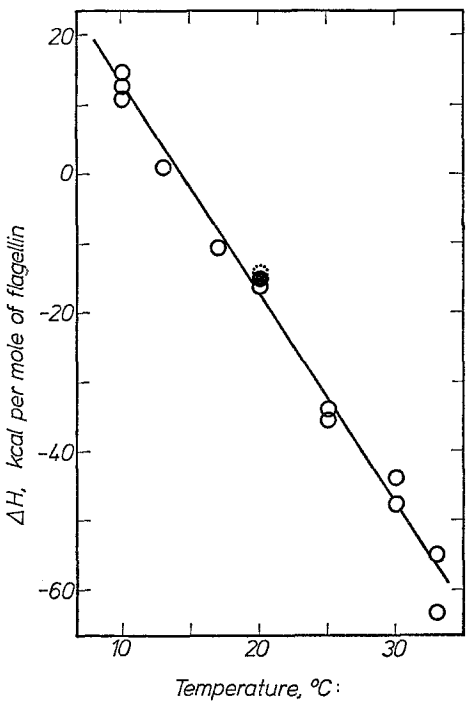

Fig. 2. Temperature dependence of the molar enthalpy of polymerization of flagellin. Open circles refer to measurements at pH 7, in 0.2 M NaCl, 0.01 M sodium phosphate, 0.02 % NaN_3 solution, while the dashed and filled circle refer to experiments at pH 7.5 in solutions containing 0.15 M NaCl, 0.05 M sodium phosphate and 0.15 M NaCl, 0.05 M Tris-HCl, respectively

buffers B and C. The constancy of the enthalpy of polymerization irrespective of the buffer system indicates that protonation or deprotonation reactions are not involved in the polymerization of flagellin.

Above 20 °C, reactions in the calorimeter went to completion within approximately 2 to 3 h when 3.5 ml of flagellin solution and 2 ml of seed solution were employed. Due to the decrease of reaction rate with decreasing temperature the ratio of seeds to monomers had to be reversed to obtain similar times for duration of the experiments under 20 °C.

Fig. 2 is a graphical representation of the enthalpy values given in Table 1 to particularly emphasize, that within the limits of experimental error the enthalpy of polymerization varies linearly with temperature in the temperature range studied. Linear regression analysis leads to the equation

$$\Delta H\ (T)\ \text{kcal/mole} = 43.4 - 3.0\ T$$

with T as temperature in °C. The standard error of the enthalpy value in this fit is $\pm$ 3 kcal/mole. Calorimetric experiments at higher temperatures than 33 °C have not been performed because of increasing errors due to unspecific aggregation and instability of the instrument. The temperature coefficient of the enthalpy is the heat capacity change at constant pressure p

$$\Delta c_p = \left(\frac{\partial \Delta H}{\partial T}\right)_p = -3.0 \pm 0.1\ \text{kcal/(degree·mole)}\ .$$

Table 2. Comparison of thermodynamic data on interaction of proteins with various types of molecules

Reaction	Molecular Weight	T °C	pH	Buffer[a]	ΔH cal/mole	Δc_p cal/deg·mole	Δh_A cal/115 g	Δc_{pA} cal/deg·115 g	References[b]
Avidin + Biotin	68300	25	5—9	T	— 80000	— 928	— 135	— 1.56	1.2
Haemoglobin + Haptoglobin	67000	25	7.4	U	— 41200	— 2300	— 70.7	— 3.95	3
Trypsin + Trypsin Inhibitor (Soybean)	23800	25	5.0	V	+ 8600	— 442	+ 41.5	— 2.13	4
Ribonuclease S-Peptid + S-Protein	13895	25	7.0	W	— 39800	— 1460	— 329.4	— 12.1	5
Aldolase + Hexitol-1,6-diphosphate	150000	25	7.5	X	+ 1300	— 1100	+ 1.0	— 0.84	6
Yeast GAPDH + NAD	140000	25	7.32	Y	— 49600	— 2080	— 40.7	— 1.7	7
Flagellin Polymerization	40000	25	7.0	ABC	— 34700	— 3039	— 99.8	— 8.7	This study

[a] The letters in column 5 refer to the following buffer systems:
T, Ammonium acetate or borate buffer, ionic strength 0.1—0.15; U, 0.15 M-phosphate; V, 0.2 M KCl, 0.05 M $CaCl_2$; W, unbuffered solution, 0.3 M NaCl; X, values are given corrected for buffer effects; Y, 0.05 M potassium phosphate, 0.05 M KCl·0.002 M EDTA; ABC, see Table 1.

[b] The numbers in column 10 refer to:
1. Green, 1966; 2. Bjurulf *et al.*, 1971; 3. Adams and Weiss, 1969; 4. Baugh and Trowbridge, 1972; 5. Hearn *et al.*, 1971; 6. Hinz *et al.*, 1971; 7. Velick *et al.*, 1971.

Discussion

The evaluation of the enthalpy data in Table 1 is based on the fact that "native" flagellin polymerizes almost completely into polymeric flagella, whereas flagella do not dissociate significantly under physiological buffer conditions (0.15 M NaCl, pH 7, room temperature). This means that under these conditions the monomer-polymer equilibrium lies on the side of the polymer and that the flagellin fraction of 10 to 20%, that remains unpolymerized at the end of the experiments, presumably has lost irreversibly its ability to polymerize. This conclusion is supported by the fact that the percentage of unreacted monomeric flagellin remains constant for the same preparation batch almost independently of dilution of the monomer-polymer mixture (Bode, unpublished result). It may be added here that Kuroda (1972) has found similar percentages (about 20%) of unreacted monomeric material after 4 h of incubation, in his polymerization studies on *Proteus*, *Salmonella* and *Bacillus* flagellins. The remarkable parallelism between the time course of polymerization as monitored by calorimetric and circular dichroic experiments (Fig. 1) suggests that heat effects are associated with

the polymerization reaction proportional to the flagellin turnover and that the solutions before and about 3 h after mixing are stable within the calorimetric vessels. Neither the sedimentation pattern nor the electronmicroscopic observations present any evidence for the occurrence of other species besides monomers and helical polymers (Asakura, 1970). Therefore the assumption seems to be valid that the heat production measured in the calorimeter after mixing originates only in the specific helical polymerization and not in other unspecific aggregation.

The most prominent result of the presented data is the strong temperature dependence of the polymerization enthalpy. Due to the heat capacity change of — 3.0 kcal per degree per mole of flagellin, the reaction enthalpy is positive below ~ 14 °C and negative above ~ 14 °C. This phenomenon of strongly temperature dependent enthalpies involved in the reaction of proteins with molecules of various size has repeatedly been observed (Table 2, column 7). On the other hand, thermal denaturation of proteins, including flagellin, is accompanied by a large increase (e.g. 3 kcal per degree per mole of flagellin) of the apparent molar heat capacities (Tanford, 1970; Bode and Blume, 1973). In accordance with current assumptions this increase of heat capacity results primarily from the increased exposure of hydrophobic groups to the aqueous environment (*cf.* the solubility studies of amino acids, Tanford, 1970).

Reversing the argument provides a means of rationalizing the experimentally determined high negative heat capacity changes accompanying the flagellin polymerization. Flagellin has a particularly high surface to volume ratio due to its highly asymmetric shape (Bode *et al.*, 1972). Incorporation into the compact flagella structure reduces the surface area in contact with water considerably. As in the case of the endothermic polymerization of tobacco mosaic virus protein (Jaenicke and Lauffer, 1969) this process may be assumed to involve a desolvation reaction, which would contribute to a decreased heat capacity of the system according to common concepts of hydrophobic bonding. This interpretation gets support from observations of Gerber *et al.*, (1967) that the flagellin polymerization is associated with a significant volume increase, as expected for hydrophobic interactions (Kauzmann, 1959). However, remembering the actual correlation times of water molecules in the vicinity of hydrophobic groups (v. Goldammer and Hertz, 1970) one should not forget the pictorial aspect of the above argument (Holtzer and Emerson, 1969). Another contribution to the large negative heat capacity change during flagellin polymerization may arise from tightening of the structures of both the associating monomer and the corresponding combining site at the growing flagellum with concomitant loss of excitable internal degrees of freedom, such as rotation and torsional vibration about valence bonds (Sturtevant, 1972). The large ellipticity decrease at 220 nm during polymerization is commonly attributed to an increase of α-helical content. In fact it may reflect a higher degree of order assumed by the tertiary structure of the flagellin molecule during association. Therefore it is possible that the isolated flagellin molecule referred to in general as active or native may in fact represent only an intermediate state between a thermally disordered and a compact structure and that the polymerizing reaction is only a continuation of a refolding process. This interpretation gains support from the remarkable agreement between the absolute value of the heat capacity change associated with thermal denaturation of flagellin [+ 3 kcal per

degree per mole (Bode and Blume, 1973)], and that involved in the seed polymerization as determined in this investigation (— 3.0 kcal per degree per mole). However, according to shape and structure determination and to the adiabatic calorimetric experiments the isolated flagellin behaves in most regards like other native protein molecules (Bode *et al.*, 1972; Bode and Blume, 1973). Kinetic results suggest at least two main association steps (Asakura, 1970; Kuroda, 1972) which may be interpreted as a desolvation step followed by a conformational change.

Inspection of column 6 in Table 2 reveals, that large positive as well as large negative enthalpy values have been determined for reactions of proteins with a variety of other molecules.

A comparison of the molar quantities, useful as they are for other purposes does not seem to be appropriate to detect possible differences or similarities of the binding reactions. Therefore enthalpy values have been listed in column 8 of Table 2 calculated on the basis of an average molecular weight per amino acid of 115.

Although this procedure has its weaknesses too in that it could cover up predominant energetic contricutions of specific reactive groups, like ionization reactions, hydrogen bond formation etc., it appears to be more apt to characterizing gross conformational changes as they are reflected in the temperature coefficient of the enthalpy.

Column 9 of Table 2 shows heat capacity changes per amino acid residue ($\overline{M} = 115$) calculated from the experimentally determined values given in column 7. It seems to be worth mentioning that in those cases where two macromolecules of comparable molecular weight are involved in the reaction, substantially higher heat capacity decreases occur, indicating major conformational rearrangements. Unfortunately without additional information it is not possible to specifically apportion the heat capacity changes to either the reversal of hydrophobic hydration or a general decrease in vibrational and rotational degrees of freedom. The large negative reaction enthalpy at 25 °C determined for the flagellin polymerization in this calorimetric investigation is evidently not of extraordinary magnitude. However, this result is incompatible with a value of + 40 kcal per mole of flagellin as reported by Gerber *et al.* (1973) for polymerization of Salmonella flagellin.

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