THERMAL TRANSITIONS OF *PROTEUS MIRABILIS* FLAGELLIN AS STUDIED BY CIRCULAR DICHROISM AND ADIABATIC DIFFERENTIAL CALORIMETRY

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1. Introduction

Bacterial flagella are organelles of locomotion. In their long helical part identical units of only one protein species, flaggelin, with a mol. wt. of almost 40 000 [1] are held together via non-covalent bonds. By sonication, treatment with detergents, acid, alkali or heat the flagella can be dissociated into the protomeric flagellin. Under physiological conditions at room temperature the monomeric flagellin can be kept in a monodisperse state.

After addition of small 'seeds', in this case fragments obtained by sonic oscillation of flagella, or after adding so-called salting out-salts the flagellin can be re-polymerized into long helical filaments resembling the original flagella [2, 3]. One interesting feature of the 'seed' polymerization is its abnormal polymerization rate behaviour. While at low temperatures the polymerization rate of flagellin (of mesophilic bacteria) increases with temperature it becomes constant at about 29°C and decreases very fast with increasing temperatures [4, 5]. Gerber et al. [5] proposed an explanation for these temperature effects assuming a fast pre-equilibrium centered at about 30°C between an active and an inactive form of the monomeric flagellin with a transition enthalpy between both states of about 110 kcal/mol. Indeed these authors could present thermal transition curves of *Salmonella* flaggelins obtained by dilatometric and spectroscopic techniques in favour of their concept [5–7]. On the contrary preliminary circular dichroic (CD) measurements [1, 8] have already indicated a more complex thermal behaviour of flagellin.

Because the knowledge of thermal induced conformational transitions of the monomeric flagellin is necessary to understand the in vivo and in vitro polymerization behaviour the resolution of the discrepancies was desirable. We tried to approach this problem by combining careful CD measurements at different wavelength with the direct calorimetric determination, using a new adiabatic scanning difference calorimeter.

2. Materials and methods

The cultivation of the *P. mirabilis* bacteria, the isolation of the flagella and the preparation of purified flagellin solutions by a depolymerization–polymerization-cycling process was made as described previously [9]. Most measurements were performed in a 0.2 M NaCl, 0.05% NaN_3, 0.01 M sodium phosphate buffer at pH 7. CD spectra were recorded by a Cary 60 spectropolarimeter equipped with a Cary 6002 CD attachment (Varian, Manrovia, U.S.A.). Fused thermostated cells of path length 10,1 or 0.2 mm respectively

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(Bodenseewerk Perkin Elmer, Überlingen, and Hellma, Müllheim, Germany) were used. The exact sample temperature in the cuvette was determined by means of a small thermistor probe (Knauer, Berlin, Germany). The data are expressed as mean molar ellipticity per residue $[\theta]$ in (deg × cm^2)/decimole.

The calorimetric measurements were made in a new adiabatic differential scanning calorimeter designed and built by M. Grubert [10]. This apparatus works with a gold-plated double cell, each compartment containing 25 ml of solvent and solution respectively. The flagellin solutions measured varied from 130 to 260 mg per 25 ml corresponding to about 0.5 to 1%. The extra heat which must be supplied to the solution cell to maintain zero temperature difference between the cells was registered as a function of temperature and time respectively. Several runs were made in the temperature range between 5°C and 65°C at a heating rate of 14°C/hr.

Concentration determination was either performed spectroscopically or by means of biuret reaction based on microkjeldahl determination [4].

3. Results and discussion

Whereas at low temperatures and pH 7 *P. mirabilis* flagellin shows a CD spectrum typical for α-helix containing proteins, its spectrum at 52°C or above resembles more that of a random coiled polypeptide chain [1]. A convenient observable, for the thermal induced disordering process of flagellin, is the ellipticity at 220 nm.

In fig. 1 the mean molar ellipticity per residue at 220 nm, $[\theta]_{220}$, of *P. mirabilis* flagellin is plotted against temperature. The values were obtained from two different runs at flagellin concentrations of 0.08% and 1.5% respectively. No significant differences could be detected between both concentrations. While below 24°C the (negative) ellipticity decreases very slowly in a linear manner with increasing temperature it changes rapidly between 24 and 52°C. Assuming as a first approximation a two-state transition [11] between a

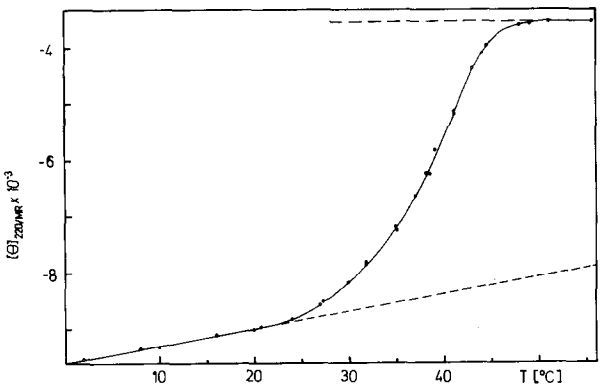

Fig. 1. Mean molar ellipticity per residue at 220 nm of *P. mirabilis* flagellin at pH 7 as function of temperature.

native (N) and a thermally denatured (TD) flagellin conformation below and above this transition temperature range, an apparent equilibrium constant:

$$K' = -\frac{[\theta](T) - [\theta]_N(T)}{[\theta]_{TD}(T) - [\theta](T)}$$

can be calculated with $[\theta](T)$ being the mean molar ellipticity at temperature T. The ellipticity values of the pure states $[\theta]_{TD}$ and $[\theta]_N$ at each temperature have been obtained by extrapolating the values from

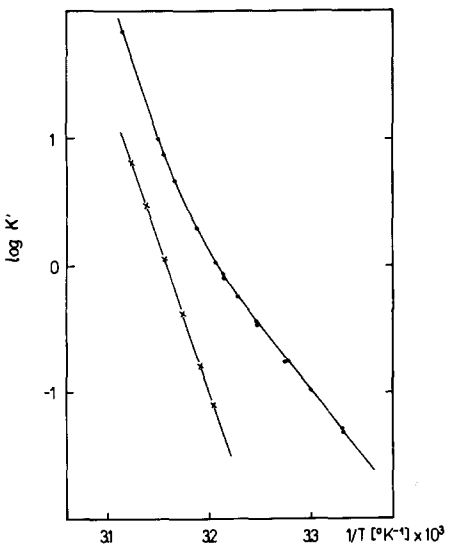

Fig. 2. Semilogarithmic plots (Van 't Hoff plots) of the apparent equilibrium constants versus reciprocal temperature obtained from ellipticity change of flagellin at 220 nm (o—o—o) and 287 nm (×—×—×) respectively.

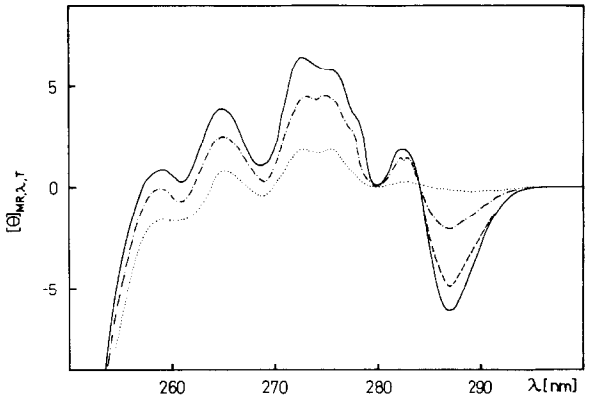

Fig. 3. Circular dichroism (aromatic region) of *P. mirabilis* flagellin at pH 7 and 35°C (——), 40°C (– – –), 45°C (–·–·–) and 50°C (·····) respectively.

the pre- and post-transition region as indicated by dashed straight lines in fig. 1.

As can be seen in fig. 2 the Van 't Hoff plot of log K' versus the reciprocal temperature yields not a straight but a strongly curved line. This indicates that the simple two state assumption may not be valid in this case and that at least two or more steps with three or more macroscopic thermodynamic states may be involved in the transition.

Fig. 3 shows the CD spectra in the aromatic region of *P. mirabilis* flagellin solutions. At temperatures below 36°C no significant spectral changes can be observed, whereas drastic spectral changes occur between 36°C and 52°C. While the decrease of the ellipticity can only be observed with low accuracy

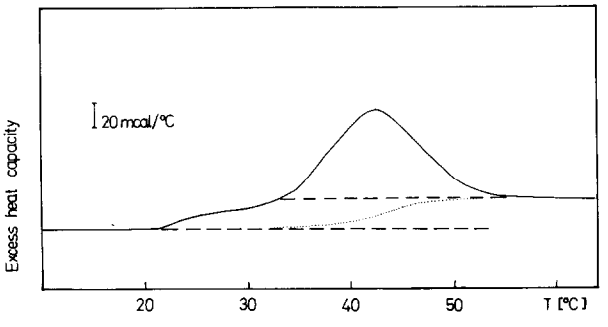

Fig. 4. Variation with temperature of the excess heat capacity of the sample solution containing 260 mg *P. mirabilis* flagellin in 25 ml neutral buffer.

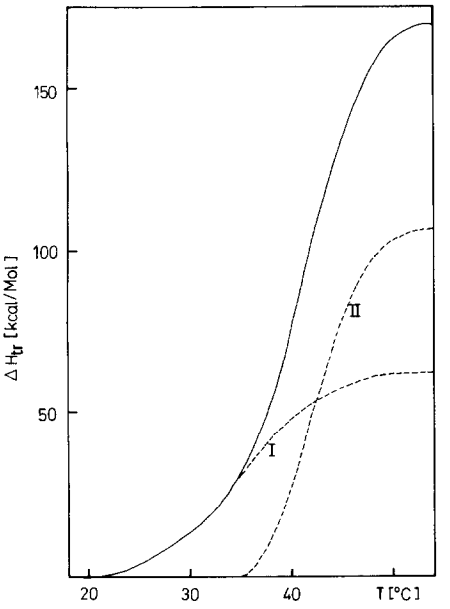

Fig. 5. Enthalpy increase due to conformational transitions with temperature for the whole denaturation process (——) and both separated stages I and II (– – – –) respectively.

between 260 and 280 nm because of the low signal–noise ratio and base line shifts in this spectral region the disappearance of the ellipticity minimum at 287 nm related to one of the tyrosine electronic transitions can be followed with relatively high accuracy.

Again assuming this ellipticity change to reflect an equilibrium transition from one state (X) to another (TD), an apparent equilibrium constant K' can be calculated. The Van 't Hoff plot (fig. 2) yields an almost straight line favouring the two state approximation. From its slope an effective enthalpy of 100 ± 10 kcal/mole is obtained for this transition centered at about 43.5 ± 1°C.

Recent ultraviolet difference spectroscopic measurements (unpublished results) agree with these results yielding almost identical values for the transition midpoint temperature and enthalpy. Thus it seems possible that this temperature-sensitive transition around 43°C is also reflected by the high temperature part of the ellipticity versus temperature curve at 220 nm (fig. 1) and that the slower ellipticity change below 36°C can mostly be attributed to another conformational transition with a lower transition enthalpy. This hypothesis

is supported by the calorimetric measurements on flagellin solutions.

Fig. 4 shows the exemplary calorigram of *P. mirabilis* flagellin. The scale corresponding to a heat capacity of 20 mcal/°C is shown. No attempt was made to determine the absolute partial heat capacity of the protein. The excess heat capacity of the sample solution does not change significantly between 5 and about 22°C, but increases slightly between 22 and 32°C. Between 34 and 52°C a large heat absorption peak with a maximum between 42 and 43°C can be observed. After recooling the excess heat capacity changes were about 25% lower in a second run indicating that the flagellin denaturation is not completely reversible at these high concentrations and long heating times applied. At 54°C and above, the excess heat capacity remains almost constant, but at a significantly higher level than at low temperatures. The displacement of the baselines obtained by extrapolation from either the pre- or the post-transition regions, indicated by dashed lines in fig. 4, corresponds to a molar heat capacity change of *P. mirabilis* flagellin during thermal transition of about 3 ± 1 kcal/mole. Such an increase of the heat capacity during protein denaturation is now a well-known phenomenon and is believed to result primarily from the exposure of the hydrophobic side chains to solvent [12–14].

The shape of the curve (fig. 4) suggests again that the thermally induced transformation of flagellin at pH 7 does not allow a simple two-state treatment but is a more complex process. The observed heat capacity increase below 34°C could be at least partially attributed to a temperature-dependent heat capacity of the native state of the flagellin [13]. But this increase can also reflect conformational transitions between two or more intermediate states separated by relatively low transition enthalpies [14, 15]. In this case the absorbed heat due to the transitions alone can be calculated approximately assuming that the actual heat capacity of the flagellin increases proportionally to the absorbed heat as indicated by the dotted curve in fig. 4. Thus the whole transition enthalpy corresponding to the area between this baseline and the measuring curve can be obtained as about 170 kcal/mole.

In fig. 5 the change of the absorbed heat due to the transition calculated in this way is plotted against temperature, yielding an asymmetrical transition curve. Under the assumption of 2 two-state transitions a resolution into two transition curves can be tried as indicated in fig. 5 by two dotted symmetrical curves. The second curve (II) centered at 42.5°C ± 1°C corresponds to a calorimetric enthalpy of 105 ± 10 kcal/mole and corresponds closely with the transition curve obtained from the ellipticity change at 287 nm. The first curve (I, fig. 5) was constructed assuming that the enthalpy increase below 35°C can be exclusively attributed to a symmetrical transition with a calorimetric and Van 't Hoff enthalpy of about 60 and 55 kcal/mole respectively. A similar separation can be made for the transition curve obtained from the ellipticity changes at 220 nm (fig. 1) yielding similar Van 't Hoff enthalpies and thus supporting this kind of view. It is interesting to note in the literature that similar complex properties have been found for thermal transitions of several enzymes at neutral pH values [15, 16]. Considering the accuracy of the measurements and the assumptions made for separation the differences between the calorimetric and Van 't Hoff enthalpies are not large enough to exclude this two-steps mechanism. At this state of investigation it can only be said that the thermal denaturation of *P. mirabilis* flagellin can be well described assuming that the molecules undergo two co-operative transitions at about 35° and 43°C with transition enthalpies of about 60 and 110 kcal/mole respectively.

It is quite evident that, at least in the case of *P. mirabilis* flagellin, no transition with a transition enthalpy of 110 kcal/mole can be observed at 30°C as described by Gerber et al. [5–7] for *Salmonella* flagellins. However, because the temperature dependence of the CD [8] and the polymerization rate behaviour of both the *Salmonella* flagellins [17] and this *P. mirabilis* flagellin are very similar, and because both flagellins are even able to co-polymerize [18] the calorimetric responses should also be very similar. Our results show that the simple two-state treatment of any observable changes is only allowed with reservation and that the formal application of the Van 't Hoff equation may lead to wrong results. This may be the reason for the differing thermodynamic values proposed by Gerber et al. [5, 17].

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