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Proposed molten globule intermediates in fd phage penetration and assembly

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The fd filamentous phage can be contracted to short rods called I-forms and to spheroidal particles called S-forms. The conversions from fd→I-forms→S-forms were previously suggested to mimic steps in fd penetration. The same conversions, in reverse order, were suggested to mimic steps in fd assembly. The I-forms and S-forms bind the hydrophobic probe, 1-anilino-napthalene-8-sulfonate (ANS); under the same conditions, fd binds this probe very poorly. Rigidly packed side chains in fd and nonrigidly packed side chains in I-forms and S-forms would explain the differences in ANS binding. A compilation of the properties of I-forms and S-forms indicate that: (i) they have compact structures; (ii) they have secondary structures of the same type as native phage; (iii) they have non-native morphologies; and (iv) they may have nonrigid side chain packing. These are the properties of molten globules.

Filamentous phage assembly; Molten globule intermediate

1. INTRODUCTION

The fd filamentous phage deposits its coat protein, pVIII, in the cell membrane [1] and exits from the host through the cell membrane without causing cell lysis; infected cells grow and divide [2]. Data suggest that phage penetration and phage assembly involve the same basic steps, but in reverse order [3–6].

At lower temperatures, chloroform causes the phage to contract to rod-like structures, called I-forms. At higher temperatures, chloroform causes the I-forms to contract to spheroidal structures [7–9], called S-forms by us [10]. Since the structure of the coat protein in I-forms is the same as the structure of the coat protein in the native phage, and since the structure of the coat protein in S-forms is the same as the structure of the coat protein in lipid bilayers, we recently proposed a model for phage penetration involving the following sequential steps for the pVIII protein; fd→I-forms→S-forms→bilayer-associated-p VIII and the reverse pathway was suggested for fd assembly [10].

In this communication we show that I-forms and S-forms may have many of the properties of molten globules, which are compact, non-native protein forms with native-like secondary structure, initially characterized by Tanford and co-workers [11] and named molten globules by Ohgushi and Wada [12]. Recent reviews stress the role of molten globules in protein folding [13,14]. A finding that putative intermediates in phage assembly are possibly molten globules would indicate that the molten globule state may also be involved in the assembly of supramolecular structures.

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2. MATERIALS AND METHODS

2.1. *Preparation of fd, I-forms and S-forms*

Filamentous phage were grown on *E. coli* JM101 kindly provided by Dr Gerald Hazelbauer and purified by polyethylene glycol precipitation followed by banding on KBr density gradients. The fd phage was contracted to I-forms and I-forms were contracted to S-forms by rapidly swirling a chloroform/water two-phase system with a Dremel tool equipped with a bullet-shaped teflon tip as described previously [10].

2.2. *Fluorescence*

Fluorescence spectra were obtained using a Perkin-Elmer MPF-3L Fluorescence Spectrometer.

2.3. *Electron microscopy*

Samples were mixed with 2% phosphotungstate, pH 6.8, overlayed on carbon-coated 300 mesh copper grids, touched with a filter paper to remove excess liquid and air dried. The samples were not fixed. A Hitachi H-300 electron microscope was used to visualize the fd phage, I-forms and S-forms.

3. RESULTS

3.1. *Conversion of fd phage to I-forms and S-forms*

The fd filamentous phage was treated with chloroform as described at about 2°C or I-forms were treated at room temperature. The untreated phage and the 2 chloroform-treated samples show that the chloroform-

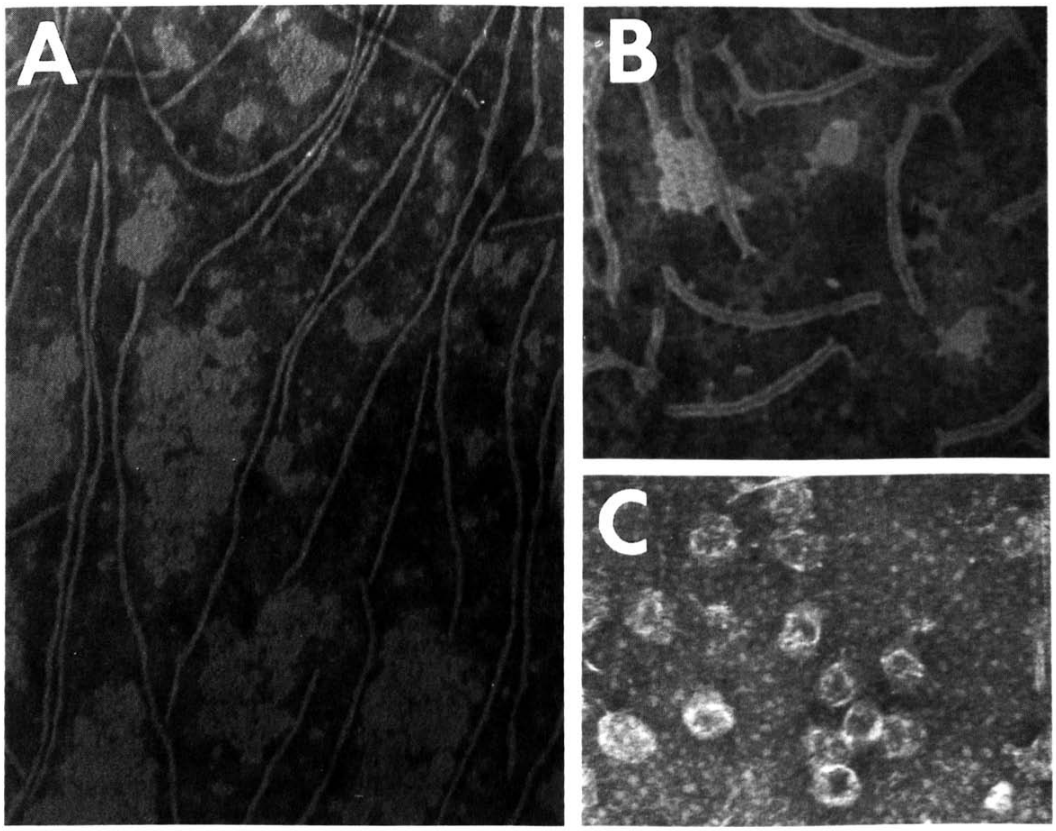

Fig. 1. Morphology of fd, I-forms and S-forms. The fd phage was treated with chloroform as described in section 2. The different panels contain typical examples of: fd (A), I-forms (B) and S-forms (C).

induced contraction occurs for fd (Fig. 1) as was described previously for the closely related f1 and M13 filamentous phages [7–9].

3.2. *ANS binding by I-forms and S-forms*

During the course of these studies, we noticed that solutions of I-forms spontaneously become cloudy if raised to room temperature. Precipitation with increasing temperature is a well-known characteristic of entropy-driven protein/protein aggregation [15] and suggested that the surface of I-forms becomes hydrophobic. The binding of the fluorescent probe 1-anilino- naphthalene-8-sulfonate (ANS) has long been used to demonstrate accessibility of hydrophobic regions of proteins; ANS is an ideal probe for this purpose because of the large increase in fluorescence as ANS moves from a polar to a nonpolar environment [16,17]. Figure 2 shows that ANS reports accessible hydrophobic regions for I-forms and S-forms but not for the intact fd phage.

4. DISCUSSION

4.1. *Are I-forms and S-forms molten globules?*

Figures 1 and 2 and other data give an interesting inventory of properties for I-forms and S-forms: (i) they are compact (Fig. 1); (ii) they are rich in α-helix, like native phage [10]; (iii) but they have non-native morphologies (Fig. 1); and (iv) they aggregate with increasing temperature and have increased accessibility of their hydrophobic regions to the hydrophobic probe, ANS (Fig. 2). These 4 properties of I-forms and S-forms correspond to characteristic properties of molten globules [11–14]; in fact it has recently been suggested that ANS binding is a useful diagnostic test for the conversion from the native state (low ANS binding) to the molten globule state (high ANS binding). It was proposed that the increased binding of ANS is due to the conversion from rigid side chain packing in the native structure to nonrigid side chain packing in the molten globule state [18].

Do I-forms and S-forms actually have nonrigid side chain packing, which is the cardinal feature of the molten globule state, or is ANS binding merely the result of exposed, but rigidly packed, hydrophobic groups? The quenching of the intrinsic tryptophan fluorescence of fd, I-forms and S-forms by 2,2,2-trichloroethanol (TCE) provides additional information on this matter. TCE quenching studies suggest that the tryptophans in fd resemble those in typical, native proteins. In contrast, the tryptophans in I-forms and S-forms are more like

indoles associated with SDS micelles (Roberts and Dunker, work in progress). These TCE quenching data are difficult to explain except in terms of nonrigid side chain packing, and so provide additional support for the molten globule nature of I-forms and S-forms.

Additional evidence supporting the molten globule nature of I-forms comes from electron microscopy. If fixatives such as glutaraldehyde are omitted, I-forms frequently have an irregular appearance, with regions of breakage that suggest a structure too weak to withstand the well-known disruptive forces that occur upon drying (Fig. 1B). Although molten globules have not previously been studied by electron microscopy, such a lack of strength is clearly the expected result from the nonrigid nature of the side chain interactions. The spheroidal shape of S-forms may serve to protect them from the forces encountered during drying, so disruption of these structures is not so prevalent.

Temperature-induced aggregation, the binding of hydrophobic probes and the obvious breakage of I-forms all suggest that the contracted forms are molten globules. However, in the final analysis, these are all indirect indicators of nonrigid side chain packing. More direct tests would be desirable.

Another property of molten globules is that they are expanded compared to the native protein. Since I-forms are nominally $1/3$ in length and 3-times fatter than fd [8], the volume of I-forms is nominally 3-times greater than that of fd (e.g. $\pi\ (3R)^2\ L/3 = 3\pi\ R^2\ L$). However, I-forms may be flattened, which would lead to an apparent diameter that is larger than the actual one. Also, there may be internalization of bulk water. Because of these complications, changes in the volume occupied by the protein would be very difficult to determine. Similar problems apply to S-forms.

4.2. *Implications of the putative molten globule nature of I-forms and S-forms*

Molten globules are receiving increasing attention as intermediates in protein folding [13–14,18]. The finding that I-forms and S-forms are likely to be molten globules leads us to propose that intermediates in filamentous phage penetration and assembly are molten globules too. Many of the characteristics of phage proheads and various viral assembly intermediates suggest that these structures may have molten globule properties as well.

Based on a number of indirect observations, Ptitsyn and co-workers raised a question about the possible involvement of molten globules in the translocation of proteins across membrane bilayers [19]. Griffith and co-workers, the discoverers of I-forms and spheroid shaped S-forms, suggested that the conversion of fd to I-forms "activates" the phage particle for membrane insertion and further provided suggestive evidence that I-forms can spontaneously fuse with pre-formed lipid vesicles if the lipids are in the fluid phase [20]. (Note that the 1982 report of the I-form/membrane fusion experiments was after the initial characterization of molten globules but before they received their name.) The finding that I-forms may be molten globules links 2 lines of research together and provides support for the hypothesis that molten globules are involved in the translocation of proteins across membranes.

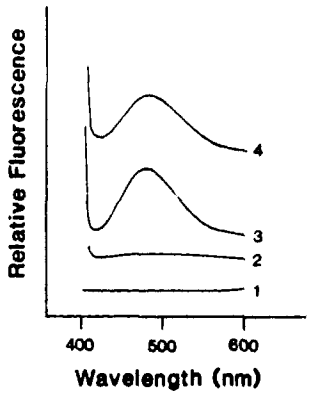

Fig. 2. Probing fd, I-forms and S-forms with ANS. ANS was added to dialysate buffer (as a control), to fd phage, to I-forms, and to S-forms. The last three all contained close to 0.1 mg/ml of total phage material, which is about 88% protein and 12% DNA. Final ANS concentration in all cases was 250 μM. Excitation was at 395 nm as described previously in studies of molten globules by ANS binding [18] and fluorescence spectra were collected over the 400–600 nm range at about 4°C. The resulting curves are from: control buffer (curve 1); fd phage (curve 2); I-forms (curve 3), and S-forms (curve 4).

Phage structure and assembly are studied because they serve as models for other supramolecular structures. The finding of a molten globular nature for 2 putative intermediates in fd phage assembly suggests that the molten globule state may be involved in more processes than 'just' protein folding and the translocation of membrane proteins. The molten globule state may also be involved in macromolecular assembly processes such as in the assembly of ribosomes, microtubules, nucleosomes and so on.

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