Minireview

The membrane insertion of colicins

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Pore-forming toxins, such as colicin A, are water-soluble proteins that insert into lipid bilayers. The water-soluble structure of Colicin A is known at a high resolution and this review describes the kinetic and structural steps involved in its soluble-to-membrane bound transformation.

Colicin; Membrane insertion; Brominated lipid; Molten globule; Fluorescence energy transfer; Pore formation

1. INTRODUCTION

The study of the membrane insertion mechanism of pore-forming colicins has also become a study of factors influencing gross conformational change in proteins and provides data which are relevant to protein folding in general. The determination of the structure of the water-soluble form of the colicin A pore-forming fragment by Parker et al. [1] (Fig. 1) has given us the equivalent of a shop window mannequin – an assemblage that, although capable of being reset in many different postures, has an essential form which we are not yet competent to change.

The colicins kill sensitive Gram-negative bacteria by means of a three-step process [2]. Firstly, they bind to a specific outer-membrane receptor; then they are translocated across both this membrane and the periplasmic space. The third step depends on the type of colicin involved. Colicins E3 and E2 are nucleases and need to cross the inner membrane to reach their target. Colicins E1, Ia, Ib, N, B and A permeabilise the inner membrane causing cell death by K^+ loss [3]. This behaviour is analogous to that exhibited by colicins in planar lipid bilayer experiments. The normally low electrical conductance of the bilayer at 75 mV applied potential difference (PD) increases in steps after colicin is added [4]. These steps correspond to the formation of single ion-conducting channels, each one capable of causing the K^+ efflux seen in living cells. Consequently we currently believe that these pore-forming colicins

insert into the bacterial inner membrane forming transmembrane channels. The voltage dependence of the channel activity seen in vivo and in vitro indicates that the formation of a channel requires a transmembrane PD. In colicins A (ColA) and E1 (ColE1) the C-terminal domain, isolated after proteolytic digestion, carries the complete pore-forming activity [4] and it is this domain which has been used in most of the following work.

The passage of ions across the low dielectric constant interior of a lipid bilayer is currently thought to require a 'watery' channel that is wholly proteinaceous. Watery denotes that the channel may either mimic the presence of water, as in gramicidin [5], or be water filled, as in the case of porin [6]. The predominance of α-helical secondary structure in colicins [7] means that the pore may be made from transmembrane α-helices which provide polar side chains across the membrane core. By geometry it becomes clear that if the channel is lined by such helices then at least four are required to provide a channel with a diameter of that of colicin. Hence the C-terminal fragment of these colicins changes from a water-soluble globular protein of known 3D structure to a membrane-spanning ion channel whose structure can only be guessed at.

2. THE MEMBRANE INSERTION MECHANISM

It can be shown by several methods [8,9] that colicins will bind spontaneously to negatively charged lipid phases without the need for a transmembrane PD. The conversion from soluble to membrane-bound protein is therefore spontaneous and voltage independent. The structure formed at this stage is a closed state of the colicin channel and is the most amenable to biochemical and biophysical study. As yet, few data exist relating to the conformation of the voltage-opened channel. The affinity of colicins for membranes can be enhanced by
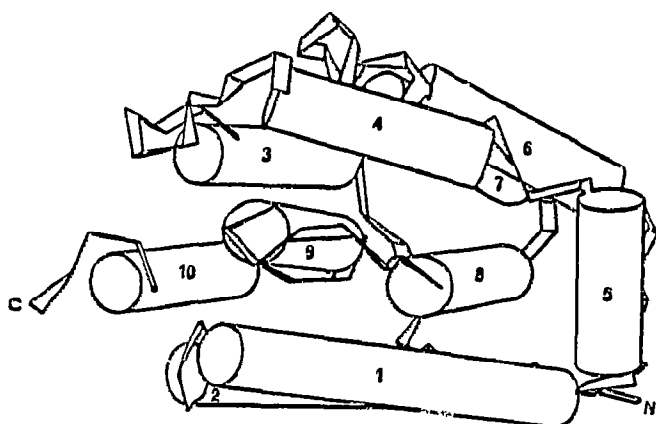
Fig. 1. The three-dimensional structure of colicin A water-soluble thermolytic fragment from Parker et al. [10]. Helices are represented as cylinders [33]. The buried helices, 8 and 9, are entirely hydrophobic. Reprinted with permission from Nature 337 (1989) 93–96.

either increasing the fraction of negatively-charged lipids or by lowering the bulk pH or ionic strength [8,9]. All of these would suggest that electrostatic effects are important at this stage. This point was raised in the proposal of the umbrella model [10] for colicin insertion which transforms the known 3D soluble structure into a membrane integral protein. After initially binding electrostatically to the membrane surface, it was suggested that the buried helical hairpin of the colicin molecule is the driving force behind the membrane insertion. The polar residues incapable of inserting into the bilayer remain on the surface and no channel is formed. The helical hairpin insertion mechanism suggested by Englemann and Steitz [11] suggests that the hairpin should span the membrane, with the connecting loop projecting into the opposite polar region.

We have recently followed the kinetics of this insertion by the use of brominated lipid quenchers of tryptophan fluorescence [12]. When vesicles of brominated dioleoylphosphatidylglycerol are added to a solution of ColA, the fluorescence decreases in an exponential manner eventually reaching a new steady state. This is because membrane insertion brings the tryptophans of the ColA C-terminal fragment close to the bromines in the membrane. Two factors controlling the insertion kinetics are important for our understanding of the insertion mechanism. Firstly, the insertion kinetics are strictly dependent on the lipid/protein ratio rather than protein or lipid concentration alone, and secondly, they depend on pH and the proportion of negatively charged lipids in the same way as does the extent of insertion into lipid monolayers. The first observation means that we are not following the bimolecular reaction expected from the initial binding of protein to lipids. We see a slow insertion mechanism which follows the fast and currently invisible initial binding step. The second means that pH and ionic strength are not simply affecting

binding but also control this slow insertion step which occurs after binding. Going from pH 5 to 4 in 50 mM buffer there is a sharp transition of insertion kinetics from about 0.015 s^{-1} to 0.120 s^{-1}. This supports the results obtained for ColE1 by Davidson et al. [13] who also postulated that the neutralisation of carboxyl groups is the prerequisite for the insertion step. Clearly, factors such as pH, ionic strength and negative charge density affect a rate-limiting step in the insertion process that is not simply binding. This step is, by definition, the formation of an insertion-competent state of ColA. The greater the proportion of bound colicin in this state the faster are the kinetics of insertion. Acidic conditions also enhance the activity of other toxins such as diptheria toxin [14].

3. THE INSERTION-COMPETENT STATE

This state was investigated for ColE1 [15] in a study of the effect of detergents and low pH on the far ultra-violet circular dichroism spectra (far UV-CD). Although the helix content was sensitive to both variables it was not clear what was happening to the structure. Detergents do, however, have specific effects on the ColE1 activity and, at levels far below the critical micelle concentration (CMC), they provoke insertion into membranes containing no negative charge [16]. Detergents and the low pH effect increased the protease sensitivity of the channel-forming fragment of ColE1 [17]. When the buried Cys residue was labelled with the fluorescent molecule, IAEDANS, the aqueous exposure of this probe was also enhanced by low pH. There was no increase in the hydrodynamic radius at pH 3.5 but a large increase on total denaturation by 6 M guanidinium chloride. The insertion-competent state is therefore not unfolded but has lost the tight packing of the native form. The sharp transition in activity around pH 4.0 for ColE1 [13] and pH 4.5 [8,12] for ColA cannot be easily ascribed to the protonation of individual charged residues.

At pH 2.5 ColA has 15% less α-helix, as judged by far UV-CD, than at pH 4.0. However, if one examines the environment of the aromatic residues by near UV-CD, the detailed spectrum at pH 4.0 becomes almost a flat line at pH 2.5 [18]. This is one of the characteristics of a protein conformation termed the 'molten-globule state' [19]. Due to reduced tertiary interactions the aromatic residues lose their asymmetric environment and adopt an isotropic conformation within the molecule without becoming exposed to the aqueous phase (as judged by the tryptophan emission wavelength). Molten globule states have been inferred as intermediates in protein folding [20] and may be involved in the translocation or insertion of proteins into membranes [21]. Nevertheless, pH 4.0, and not pH 2.5, is low enough for maximal ColA activity. In a recent paper [18] we showed that on a negatively charged vesicle the surface

potential attracts protons and provides a local pH of up to 1.62 pH units below that of the bulk. Hence, at pH 4.0, ColA attached to the surface will experience a pH value sufficient to convert it totally into the molten globule state. It was also shown by the use of brominated lipids that the *surface* pH necessary for the sharp increase in insertion kinetics is equal to *bulk* pH value that produces the ColA molten globule in aqueous solution [12,18]. Increases in ionic strength and decreases in the proportion of negative lipids both serve to reduce the *Δ*pH and thus their effect on insertion kinetics and overall activity is explicable. The molten globule state can be induced by several means, such as temperature or as a kinetic intermediate of protein renaturation ([20], unpublished data). The recent evidence that ColA unfolds during translocation through the outer membrane of the target cell [22] suggests that the molten globule may also occur in vivo without the need for an acidic compartment in the periplasmic space.

4. THE INSERTED STATE

The colicin prior to insertion is thus a looser aggregation of the helices seen in the known soluble structure. After insertion, and in the absence of potential, the molecule adopts a new steady state which is accessible to spectroscopic analysis [7]. The tryptophans still appear to be in an isotropic environment, shielded from aqueous quenchers but now accessible to membrane soluble and brominated-lipid quenching [7]. The α-helix content is similar to that of the soluble form [7,23,24].

In the umbrella model large reorientations of the helices occur which expose the hydrophobic interior of the molecule to the membrane's hydrophobic core [10]. This can be described as a three member rigid-body problem in which helices 1 and 2, helices 3, 4, 5, 6 and 7 and helices 8 and 9 are the predicted folding units. They are chosen because they form the three layers of the high resolution structure; the attempt being to produce a membrane bound conformation with the minimum disturbance to the known structure. The model thus provides a series of testable predictions, notably that helices 1 and 2 should fold out away from helices 3–7, with both groups situated on the surface, and that helices 8 and 9 should form a transmembrane hairpin. The large conformational change of helices 1 and 2 has been confirmed by fluorescence energy transfer distance measurements between the tryptophans on helices 5, 6 and 7, and fluorescent probes attached to cysteine residues introduced into helix 1 and 2 by site-directed mutagenesis [25]. These points are 10–15 Å; further apart in the membrane-bound state than in either the soluble form or the attached non-inserted state. Recent distance measurements have, however, cast doubt on whether the helical hairpin traverses the membrane as a separate unit. There was not the expected distance increase between helices 8 and 9 and the tryptophans when the

colicin inserted into lipids. This may mean that the membrane-bound conformation is that of a pen-knife (Fig. 2) rather than an umbrella, with helices 8 and 9 still running parallel to the membrane surface (unpublished data). This need not disagree with the brominated lipid quenching of tryptophans as this does not require actual collisions between fluorophore and quencher [26]. Polarised Fourier transform infra-red (FT-IR) spectroscopy should indicate the proportion of helices lying perpendicular to the membrane. Two studies on ColA and ColE1 have not reached a firm conclusion on this question but in each case their results could be explained by the presence of two helices perpendicular to the membrane [23,24]. Lipophilic labelling methods are unlikely to differentiate helices spanning the membrane from those lying at the polar/non-polar interface [27].The recently solved structure of the pore-forming insecticidal δ-toxin from *Bacillus thurigiensis* reveals a seven-helix bundle pore-forming domain similar to colicin A but containing a single buried hydrophobic helix [28]. The pen-knife model proposed for the insertion of ColA will satisfy more the structural constraints of the δ-toxin than the umbrella model by avoiding transmembrane insertion of a single helix.

5. THE OPEN CHANNEL

The transmembrane PD moves large parts of the colicin molecule from the surface into the membrane phase and some even to the opposite side [27,29,30]. These, now intramembrane, segments must form the ion channel. The transmembrane PD may even provoke a molten-globule to native-folded transition. Most evidence
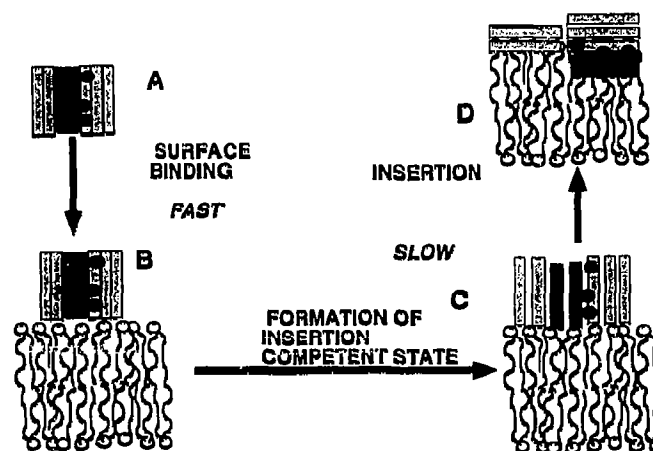

Fig. 2. The steps in colicin membrane insertion. (A) The soluble ColA has the characteristics of a normal globular protein. (B) ColA binds rapidly to negatively charged vesicles but is still in a native-like state. (C) There is a slow surface pH-dependent change to the insertion competent state with reduced tertiary interactions. (D) The molecule assumes the inserted form which is probably the pen-knife type arrangement seen here, i.e. without separate insertion of a transmembrane helical hairpin. Tryptophans are marked as black discs, and hydrophobic helices as dark shaded rectangles.

indicates that a single colicin is sufficient to make the transmembrane channel [29]. The C-terminal 136 residues of ColA alone form a wild-type channel [31] and 88 residues of ColE1 have a channel forming ability [32], so this makes it very difficult to make a canonical channel of even just four 20-residue alpha helices. In addition, the existing helices of ColA are all too short to span the bilayer completely [1]. We should consider whether we are limited to altering the posture of our original mannequin to fit the available data, or does the molten globule intermediate give us a free hand to propose fundamentally new structures?

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