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Conformation of colicin A: apparent difference between cytoplasmic and extracellular polypeptide chain

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Cytoplasmic colicin A has the ability to bind to membranes and to form stable dimers. This form remains stable even in the presence of 1% SDS at 25 °C. Both of these properties were not observed for extracellular colicin A suggesting a possible difference in the conformation between cytoplasmic and extracellular colicin A.

Colicin; Amphipathic protein; Conformational change

1. INTRODUCTION

Colicin A is a protein toxin which can kill sensitive cells by collapsing the membrane potential [1]. It can form voltage-dependent ionic channels in planar lipid bilayers and the C-terminal domain is involved in this process [2,3].

Colicin A is released from colicinogenic cells as a water-soluble, monomeric polypeptide (M_r 63 000). It is first produced in free polysomes in the cytoplasm and released long after synthesis [4] through the action of a small protein which causes an increase in the envelope permeability [5,6].

In the course of previous studies on the non-uniform elongation process of nascent chains of colicin A, we noticed that nascent colicin A can bind to membranes [7,8]. This result was at variance with the properties of extracellular colicin A previously reported [9,10]. A study was therefore undertaken to resolve this problem.

The results presented here strongly suggest a difference in conformation between cytoplasmic and extracellular colicin A. Cytoplasmic colicin A has the ability to bind to membranes and to form dimers in the presence of SDS micelles. Neither of these properties was observed for extracellular colicin A. We thus propose that a conformational change in the polypeptide chain occurs upon release from the cytoplasm to the medium.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains, growth conditions and conditions of radiolabelling*

Citrobacter freundii CA31, the natural host of pColA, and *E. coli* K12 strain W3110 pColA9 have been described [5,11]. These strains were grown in Luria broth or minimal medium and the conditions for induction of colicin A synthesis with mitomycin C and radiolabelling were exactly as described in [7].

2.2. *Gel electrophoresis, immunoblot analysis and fluorography*

Sample preparation and SDS-polyacrylamide gel electrophoresis were performed as in [7], except that the percentage of acrylamide was 7.5% in order to obtain sufficient migration of the colicin A dimer.

The samples were applied either in 200 mM Tris, 39% sucrose, 1 mM EDTA, pH 8.8, (TS buffer) or in the same buffer but containing in addition 3%

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SDS and 1% β-mercaptoethanol. This latter buffer is referred to as TS + DB (denaturation buffer).

For immunoblot analysis, after SDS gel electrophoresis, proteins were transferred to nitrocellulose paper, allowed to bind primary antibody, and visualized using horseradish peroxidase-conjugated second antibody essentially as described by Towbin et al. [12]. The mouse monoclonal anti-colicin A antibody 1C11 directed against an epitope located in the N-terminal region of colicin A has been described [13].

Gel fluorography and densitometer scannings of fluorograms were carried out as in [7].

2.3. *Membrane preparation*

The cells of W3110 pColA9 were grown in minimal medium. When the culture reached an absorbance of 0.3 at 600 nm, 0.1 μg/ml of mitomycin C was added. After 3 h of induction, 320 μCi [^{35}S]methionine was added for 20 s and a sample was removed, then 20 mM unlabelled methionine was added and another sample was removed after 2 min of chase. The cells were immediately converted into spheroplasts, and the spheroplasts were osmotically lysed as in [14]. The membrane pellet was applied, in a 60% sucrose buffer, to the bottom of the sucrose gradient (35–55%) and subjected to density fractionation by centrifugation at 50 000 rpm in an SW60 Ti rotor for 15 h. In flotation gradients of this type, membrane components move upward to seek their isopycnic positions while denser components remain in the bottom of the tube.

3. RESULTS

3.1. *Nascent colicin A and intermediates containing more than 450 amino acids can bind to membranes*

We have previously demonstrated that translation is a non-uniform process [7,8]. In these studies, we noticed that large intermediate nascent polypeptide chains of colicin A had the ability to sediment with a particulate fraction [8]. However, in this previous work we did not demonstrate binding to the membrane fraction since we did not rule out the hypothesis of protein aggregation. To this end, here the membrane fraction was rapidly isolated from [^{35}S]methionine pulse-labelled cells and from chased cells. Again we observed that nascent chains which contained more than 450 amino acids and the entire colicin A fractionated with the membrane (fig.1). However, a prominent band of 33 kDa was also immunoprecipitated by the antiserum directed against colicin A. This band was not an intermediate since in contrast to the high-M_r intermediates it was not chased. We believe that this band represents the major degradation product from the outer membrane protease Cpr (colicin A protease) previously described [15]. We noticed that during fractionation procedures, with time, shorter bands appeared which were im-

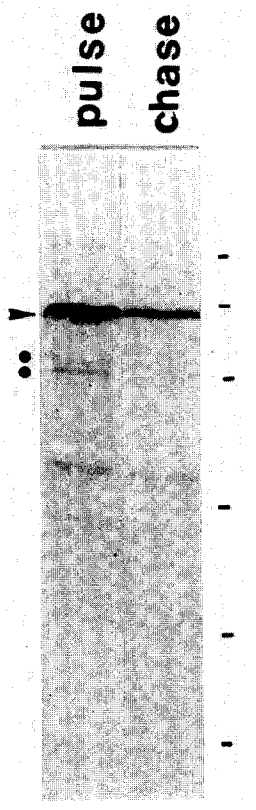

Fig.1. Nascent colicin A and large intermediates can bind membranes. The samples of membranes (density 1.16–1.21) from pulse-labelled cells (lane 1) and 2 min chased cells (lane 2) were solubilized in immunoprecipitation buffer as in [7] and nascent colicin A (arrowhead) as well as elongation intermediates (dots) were immunoprecipitated and analyzed on an SDS-polyacrylamide gradient gel (10–15%). M_r standards are indicated: 94 000; 67 000; 46 000; 30 000; 20 000; 14 400 (on the right).

munorecognized by colicin A and none was chased. They are also proteolytic products of membrane of contaminating proteases.

3.2. *Nascent colicin A can form stable dimers*

Ultracentrifugation studies have demonstrated that between pH 8 and 4, colicin A is a monomer (Sauve, P. et al., submitted). We have also previously demonstrated that by 1 h after induction by mitomycin C, there is a shut off of chromosomally encoded proteins in *Citrobacter* CA31 cells, and colicin A is the only protein detected in pulse-labelled cells because it is by far the major protein produced [4,7]. When colicin A labelled under such conditions was analyzed, 30% of the nascent protein from the culture migrated as a dimer (fig.2). Since we know that colicin A is released more than 60 min after synthesis [4,5], the nascent colicin A in the culture was cytoplasmic colicin A. The colicin A dimer migrated as a 116 kDa polypeptide. Indeed, this protein form was not observed in uninduced cells (fig.3) and was recognized by a monoclonal antibody directed against colicin A (fig.4). In the immunoblot analysis, we observed that in addition to the monomeric and dimeric forms of colicin A, significant amounts of prematurely arrested translation

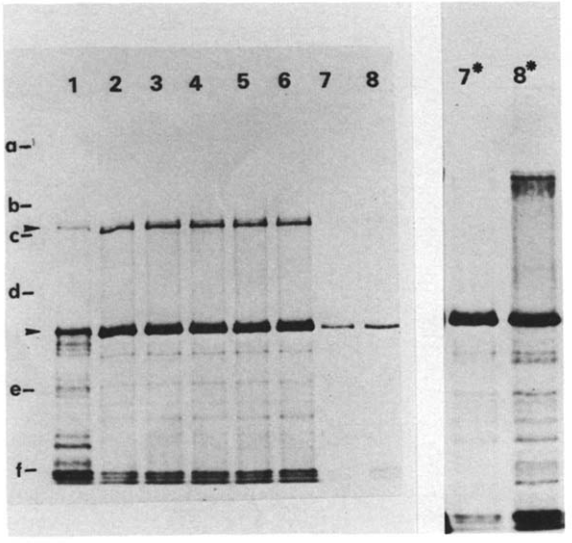

Fig.2. Cytoplasmic colicin A can form stable dimers. A culture of *C. freundii* CA31 ColA was pulse-labelled for 1 min with [^{35}S]methionine (150 μCi/ml) 1 h after addition of mitomycin C (0.1 μg/ml) (lane 1) and chased in the presence of unlabelled methionine (20 mM) for 3 min (lane 2), 7 min (lane 3), 11 min (lane 4), 15 min (lane 5), 1 h (lane 6). Samples were removed at the times indicated and total proteins from the cell culture (lanes 1–6) and from the extracellular medium (sample removed at 1 h after induction, lane 7), were analyzed by SDS gel electrophoresis and fluorography. A sample from the culture was also removed at 1 h after induction but the cells were disrupted by sonication; after low-speed centrifugation the proteins from the supernatant were analyzed (lane 8). Lanes 7*,8*, gel was exposed overnight instead of 1.5 h (lanes 1–8). M_r standards are indicated: (a) 205 000, (b) 116 000, (c) 97 400, (d) 67 000, (e) 46 000 and (f) 29 000. Colicin A monomer and dimer are indicated by arrowheads.

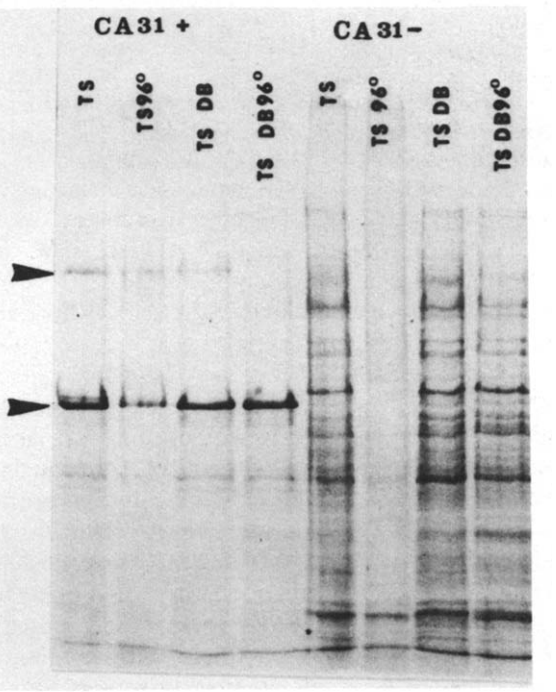

Fig.3. Colicin A dimer is only dissociated at high temperature. A culture of the strain *C. freundii* CA31 ColA was pulse-labelled for 1 min with [^{35}S]methionine (150 μCi/ml) either 1 h after addition of mitomycin C (CA31 +) or in the absence of mitomycin C (CA31 –) and total proteins in the culture samples were analyzed by SDS gel electrophoresis and fluorography. Samples were taken up and applied either in TS buffer or in TS-DB buffer as described in section 2. Some samples were heated at 96°C for 5 min just before application, as indicated.

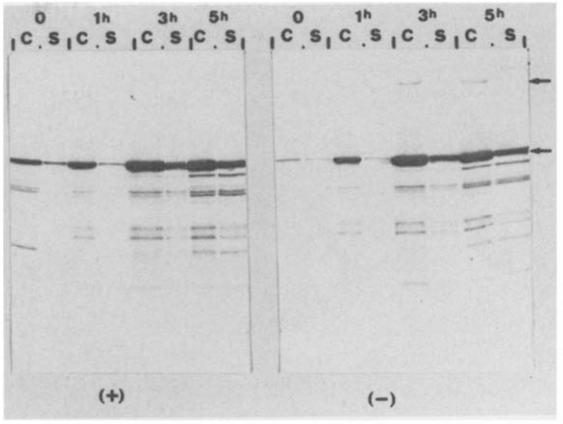

Fig.4. Colicin A dimer is immunorevealed by a monoclonal antibody directed against colicin A. Mitomycin C was added to a culture of W3110 ColA9. At intervals, as indicated, samples were removed, a subfraction was centrifuged and the proteins in the culture (c) and supernatant (s) were analyzed by SDS gel electrophoresis followed by immunoblotting. The samples were applied either after heating for 5 min at 96°C (+) or without heating (−). The arrows indicate the migration of colicin A monomer and dimer.

products were accumulated in the cells as noted in [7].

A major difference between cytoplasmic and extracellular colicin A could be deduced from the results presented in figs 2 and 3. At 1 h after labelling, a sample of extracellular colicin A (released to the medium) was analyzed and no dimer was detected although 17 μg pure carrier colicin A was added (fig.2, lane 7*). In contrast, when the cells from a similar subfraction were disrupted by sonication after low-speed centrifugation, proteins from the supernatant (which still contained unsedimented membrane vesicles) contained some colicin A dimer (fig.2, lane 8*).

By immunodetection after Western blotting, we also observed that even after 5 h of induction, when a large amount of colicin was released to the medium, no dimer was detected (fig.4). In contrast, even at 1 h for the cellular colicin A, a thin band of dimer can be detected by immunoblot analysis. Therefore, the lack of detection in the medium was not due to a lower colicin A concentration relative to that of cellular colicin A.

The colicin A dimer did not represent a

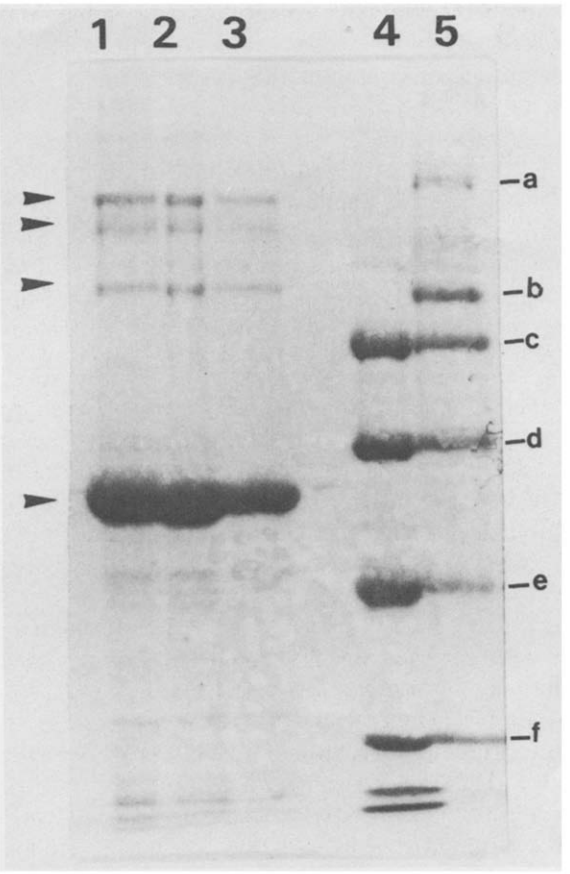

Fig.5. Extracellular colicin A can aggregate but does not spontaneously form a specific dimer. A solution of pure colicin A (17 μg) was lyophilized, resuspended in various buffers and analyzed by SDS gel electrophoresis. The samples were resuspended in TS buffer (lane 1); TS-DB buffer (lane 2); TS-DB buffer with heating at 96°C (lane 3); lanes 4,5, M_r standards as indicated in fig.2 (a–f) plus a marker of M_r 14400. Arrowheads indicate the migration of colicin A monomer and oligomers.

nonspecific aggregated form (fig.2). Indeed, it was stable during migration on SDS gels when applied in TS, even if heated for 3 min at 96°C. The dimeric form was also stable in the presence of denaturation buffer and heating in this buffer was required to disrupt it. Similar results were observed in the immunoblot analysis (fig.4). This behavior is quite different from that of nonspecific aggregation. In highly concentrated samples of pure colicin A we did observe aggregation which resulted in the formation of low amounts of dimer, trimer and

tetramer in samples which were not heated at 96°C (fig.5).

4. DISCUSSION

The present results strongly suggest a difference in conformation between extracellular and cytoplasmic colicin A. It is of interest to review first some of the properties of colicin A purified from the extracellular medium.

The insertion of colicin A into monomolecular films and liposomes has been previously studied [9]. At neutral and basic pH, colicin A is a weakly penetrating agent and the protein is unable to bind phospholipid vesicles and probably biological membranes. In contrast, at acidic pH (below pH 5) colicin A is able to insert into such vesicles and induces aggregation and fusion of liposomes [9,10]. Colicin A between pH 8 and 4 is a monomer with a sedimentation coefficient of 3.5.

The presence of a lipid-binding site in the colicin A monomer at these pH values was demonstrated both by hydrodynamic studies with micelles of non-ionic detergent and by differential sensitivity to a proteolytic enzyme (Sauve, P. et al., submitted). However, this lipid-binding site in extracellular colicin A is either different or oriented in a different way from that of cytoplasmic colicin A since it promotes neither dimerization nor membrane insertion [9].

In strong contrast with the results mentioned above, we have demonstrated here that cytoplasmic colicin A as well as nascent chains comprising more than 450 amino acid residues can bind to membranes at neutral pH. The hypothesis of aggregation in a particulate fraction can be ruled out since samples were applied in sucrose from the bottom of the sucrose gradient and colicin A was recovered from the membrane fraction equilibrated at the correct position.

Cytoplasmic colicin A can also form dimers, in contrast to extracellular colicin A. We do not know if the dimers preexist in the cells or if they are formed in the presence of SDS. The latter process may occur since, for example, it has been shown that the monomeric α-toxin can self-associate to form a hexamer in the presence of deoxycholate above the critical micellar concentration [16,17]. Under no circumstances, even in the presence of SDS, can the extracellular colicin A form any dimers. This result again points to a difference in behavior between cytoplasmic and extracellular colicin A.

How can these results be explained? Colicin A is produced in free polysomes in the cytoplasm [4] and not in precursor form [4,18]. Its polypeptide chain elongation is quite discontinuous and discrete intermediates have been demonstrated [7], thus allowing the early acquisition of secondary structure long before the occurrence of long-range interactions in the polypeptide chain. Here we have confirmed our previous result [8] demonstrating that as long as less than 450 amino acids of the 592 residues of colicin A have been assembled, the nascent chains have no affinity for membranes. This result can easily be interpreted since we have deduced the primary sequence of colicin A from the nucleotide sequence of the gene [18]. There is a 48 amino acid hydrophobic stretch in the C-terminal region which is involved in pore formation [3,11]. It is thus likely that when this region is synthesized, the nascent chains become competent for membrane insertion.

The secretion of the nascent chains occurs long after synthesis and they reach a high intracellular concentration [19]. Their secretion is promoted by a small protein which appears to cause a non-specific increase in the envelope permeability [6]. As soon as nascent chains are secreted into the extracellular medium, the high dilution from 0.7 μm^3 (intracellular volume) to the 'pacific ocean' (the extracellular medium) may promote a slow conformational change by which the polypeptide loses its competence for membrane insertion and the ability to form dimers. Indeed, when colicin A was released from the cells by sonication and immediately analyzed in SDS–PAGE some dimer was detected (fig.2, lane 8*). In contrast, colicin A slowly released through the normal pathway did not form any dimer (fig.2, lane 7*).

To explain our results we are led to hypothesize that the same polypeptide region which confers competence for membrane binding on the cytoplasmic colicin A also confers the ability to form dimers. These dimers are remarkably stable since they are not dissociated in the presence of high concentrations of SDS as long as the protein is not heated.

A simplified working model accounting for the results presented here is presented in fig.6. Because

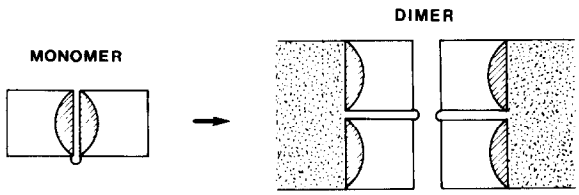

Fig.6. Model for the dimerization of cytoplasmic colicin A. The monomer consists of two domains linked by a hinge. On assembly the monomer opens up about the hinge, revealing an occluded hydrophobic surface (hatched area). The dotted region represents a lipid environment (membrane or detergent micelles).

we know from previous studies that very little change in secondary structure of colicin A occurs in the presence of detergent micelles [20], it seems reasonable to suggest that correlated with the hydrophilic to amphiphilic transition, a change in the tertiary structure in cytoplasmic colicin A takes place. In the structural rearrangement, the protein turns partly inside out before forming dimers in which the hydrophilic residues that were formerly on the surface of the monomer take part in subunit-subunit interactions. Hydrophobic residues that were occluded between the two domains in the monomer are revealed during assembly and form the outer surface of the membrane-inserted dimer or of the dimer interacting with SDS micelles.

The structure of extracellular colicin A would be such that the hydrophobic region would be more firmly occluded in the interior of the monomer so that the conformational change described above would no longer occur spontaneously. The interaction of extracellular colicin A with its specific receptor [21] in biological membranes might be required to induce the conformational change allowing membrane insertion.

What might be the possible origin for a difference in conformation between cytoplasmic and extracellular colicin A? There are many possible reasons but the most likely explanation is that the concentration and ionic environment of cytoplasmic nascent colicin A are the causes. Although we do not know whether the possible conformational change of colicin A occurring upon secretion has any physiological significance, it is tempting to suggest that it does. Conformational changes of the same sort have been demonstrated for the α-toxin of *Staphylococcus aureus* [16,17] which, like colicin A, can form channels in biological membranes [16,22].

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