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Identification of the membrane penetrating domain of *Vibrio cholerae* cytolysin as a β-barrel structure

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Summary

Vibrio cholerae **cytolysin (VCC) is an oligomerizing pore-forming toxin that is related to cytolysins of many other Gram-negative organisms. VCC contains six cysteine residues, of which two were found to be present in free sulphydryl form. The positions of two intramolecular disulphide bonds were mapped, and one was shown to be essential for correct folding of protoxin. Mutations were created in which the two free cysteines were deleted, so that single cysteine substitution mutants could be generated for site-specific labelling. Employment of polarity-sensitive fluorophores identified amino acid side-chains that formed part of the pore-forming domain of VCC. The sequence commenced at residue 311, and was deduced to form a β-barrel in the assembled oligomer with the subsequent odd-numbered residues facing the lipid bilayer and even-numbered residues facing the lumen. Pro328/Lys329 were tentatively identified as the position at which the sequence turns back into the membrane and where the antiparallel β-strand commences. This was deduced from fluorimetric analyses combined with experiments in which the pore was reversibly occluded by derivatization of sulphydryl groups with a bulky moiety. Our data support computer-based predictions that the membrane-permeabilizing amino acid sequence of VCC is homologous to the β-barrel-forming sequence of staphylococcal cytolysins and identify the β-barrel as a membrane-perforating structure that is highly conserved in evolution.**

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Introduction

The structural gene *hlyA* in *Vibrio cholerae* encodes for the *V. cholerae* procytolysin (pro-VCC) of approximately 80 kDa, which is secreted as a monomer and is proteolytically activated in solution or after binding to cells through removal of the 15 kDa protoxin domain at the N-terminus (Alm *et al*., 1988; Rader and Murphy, 1988; Yamamoto *et al*., 1990). Mature VCC subsequently oligomerizes to form transmembrane pores (Zitzer *et al*., 1995; 1999). Recent work from our laboratory has shown that VCC remains in solution as monomer, and only oligomerizes after binding to membranes (Valeva *et al*., 2004).

Three major structural domains of pro-VCC have been identified. First, the N-terminal domain has been shown to function as an intramolecular chaperone. In its presence, pro-VCC, stored in urea or guanidine hydrochloride, will spontaneously renaturate following removal of the denaturing agent (Nagamune *et al*., 1997). Second, a cytolytic domain was identified based on PSI-BLAST similarity with aerolysin and with members of staphylococcal haemolysin/leukocidins (Bateman *et al*., 2002) and by CLUSTALW alignment with members of this family (Olson and Gouaux, 2003). This predicted cytolytic domain of 250 amino acids starts approximately 80 amino acids from the proteolytic activation site. Structure similarity to leukocidin F and alpha-toxin suggests that the cytolytic domain of VCC forms an antiparallel β-sheet (Olson and Gouaux, 2003). Third, a ricin-like domain with lectin activity has been predicted that flanks the cytolytic domain, in line with reported carbohydrate binding capacity of the toxin (Saha and Banerjee, 1997; Zhang *et al*., 1999).

Single cysteine substitution mutants have been employed to identify the membrane insertion regions of pore-forming toxins. These proteins can be site-specifically labelled with polarity-sensitive probes, allowing changes in the environment of the respective amino acid side-chain to be detected (Palmer *et al*., 1996; Valeva *et al*., 1996; Shepard *et al*., 1998; Schindel *et al*., 2001; Promdonkoy and Ellar, 2003; Melton *et al*., 2004). Employment of this strategy to analyse VCC posed a novel challenge because, in contrast to the toxins studied before, this molecule harbours six cysteine residues. In order to apply the fluorophor-labelling strategy, we first identified the free and disulphide-bonded cysteine residues in VCC. Mutants could then be constructed containing single free