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MUTAGENESIS OF BACTERIOPHAGE IKE MAJOR COAT PROTEIN TRANSMEMBRANE DOMAIN: ROLE OF AN INTERFACIAL PROLINE RESIDUE

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SUMMARY: The transmembrane (TM) domain of the 53-residue major coat protein of the M13-related bacteriophage IKe (residues 24-42: LISQTWPVVTTVVVAGVLI) has been subjected to randomized mutagenesis to probe the conformation and stability of the TM domain, as well as the effect of structurally-important residues such as proline. TM mutants were obtained by the Eckstein method of site-directed mutagenesis using the IKe genome as template so as to eliminate the need for subcloning. Over 40 single- and double-site viable mutants of bacteriophage IKe were isolated. Every residue in the TM segment, except the highly conserved Trp^{29}, could be mutated to at least one other residue; polar and charged mutations occurred in the TM segment adjacent to the N-terminal domain (residues 24-28), while non-polar substitutions predominated in the C-terminal portion (residues 30-42). The Pro^{30} locus tolerated four mutations - Ala, Gly, Cys, and Ser - which represent the four side chains of least volume. Mutant coat proteins obtained directly from the phage in milligram quantities were studied by circular dichroism spectroscopy and SDS-PAGE gels. Wild type IKe coat protein solubilized in sodium deoxycholate micelles was found to occur as an α-helical, monomeric species which is stable at 95°C, whereas the mutant Pro^{30}→Gly undergoes an irreversible conformational transition at *ca.* 90°C to an aggregated β-sheet structure. The result that Pro^{30} stabilizes the TM helix in the micellar membrane suggests a sterically-restricted location for the wild type Pro pyrrolidine side chain in the bulky Trp-Pro-Val triad, where it may be positioned to direct the initiation of the subsequent TM core domain helix.

The major coat proteins of filamentous bacteriophage have been widely studied as model membrane proteins (*e.g.*, M13 [1,2] and Pf1 [3]) by virtue of their small size and facile purification from phage in abundant quantities. The major coat protein spans the inner membrane of its bacterial host *Escherichia coli* prior to packaging phage DNA into the mature virion. The apparent transmembrane (TM) domain of these proteins - which consists of an uninterrupted stretch of *ca.* 20 hydrophobic residues - is representative of single-span membrane proteins predicted to cross the bilayer in an α-helical conformation [4,5]. Increasing evidence suggests that TM residues are not fulfilling solely a hydrophobic

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requirement for stable insertion into the bilayer, but in fact may mediate TM helix structure/dynamics [6], helix-helix oligomerization [7], and/or events such as transmembrane signalling [8] and subcellular retention [9]. As a result, the structural and functional properties of individual TM residues in the context of the hydrophobic lipid bilayer need to be clarified.

Similarly to the coat proteins of related filamentous bacteriophage such as M13, IKe coat protein is synthesized as a precursor (procoat) with an N-terminal leader peptide and subsequently cleaved by host leader peptidase after post-translational, *sec*-independent assembly into the membrane [10]. The IKe mature coat protein is 53 residues in length (50 in bacteriophage M13) and is made up of three domains: an acidic N-terminus of 23 residues (20 in M13), a 19-residue hydrophobic segment, and a 10-residue highly conserved basic C-terminus [11]. Bacteriophage IKe and M13 share identical phage symmetry and have 55% overall identity with 37% homology in the major coat protein [11]. M13 and IKe differ in their host specificity since IKe requires the presence of the N-conjugative plasmid which encodes N-pili for adsorbtion to bacteria while M13 requires F-pili [13]. The dual character of the major coat protein, as both membrane protein (in the bacterial inner membrane) and structural protein (in the virion), has led to their widespread use as a model membrane protein system since it is possible to exploit the character of the water soluble (DNA-bound) structural coat protein during the expression and purification of the phage, and then revert to the membrane protein form upon solubilization of the phage in detergent micelles or lipid vesicles.

The IKe coat protein is an attractive target for mutational analysis of the TM domain since this domain (residues 24-42: LISQTWPVVTTVVVAGLVI) is characteristic of TM domains in general [4,5], but in addition contains specific residues which may influence TM helical structure, such as Pro^{30} and Gly^{39}. This work complements analyses of the TM segments in glycophorin [7] and M13 coat protein [2,12,14], and may serve to clarify the structural and functional properties of the filamentous bacteriophage coat proteins. The application of recombinant DNA techniques to generate site-directed mutants directly in the phage genome expands the utility of this system since it allows for direct expression of mutant proteins in abundant, pure form to facilitate biophysical study [12]. We now report the preparation of an initial library of viable IKe TM domain mutants, and the first characterization of wild type and mutant IKe major coat protein structure in a micellar medium.

MATERIALS AND METHODS: *Growth of bacteriophage IKe and purification of the major coat protein.* Bacteriophage IKe was a gift of Dr. D.E. Bradley (Memorial University of Newfoundland, St. John's, Newfoundland) and the plasmid pCU109 containing the IncN transfer genes was a gift of Dr. V.N. Iyer (Carleton University, Ottawa, Ontario). *E. coli* JM101/pCU109 was used as bacterial host because of its high efficiency of transformation with phosphorothioate DNA. Wild type and mutant coat proteins were prepared [12,15] by growing JM101/pCU109 in 2 x TY media to OD_{550} of 0.3 and adding the phage at a multiplicity of 20. The phage was grown 4-5 hours and the bacteria removed by centrifugation. Phage was precipitated with PEG/NaCl, resuspended in TE buffer at pH 8.0 and concentration determined by absorbance at $OD_{265} = 3.5$ corresponding to 1 mg IKe phage [16]. Wild type phage was obtained in yields of 60-80 mg per liter of media. The coat protein was solubilized in sodium deoxycholate (DOC) pH 9.0 and separated from phage DNA by gel filtration [12,15]. The protein concentration was adjusted to 1 mg/ml (where $OD_{279} = 1$ corresponds to 1 mg/ml coat protein determined according to the Peterson-Lowry method [17]) in 30 mM DOC and lyophilized.

Site-directed mutagenesis. Mutagenesis of the TM domain of bacteriophage IKe major coat protein gene VIII was performed using an oligonucleotide-directed mutagenesis kit (Amersham Co., Chicago, IL) based on the Eckstein method of strand selection [18]. IKe single-stranded DNA was isolated from phage and employed directly as template to allow for direct expression of mutant coat proteins from the IKe genome thus eliminating the requirement for subcloning and shuttling the mutant DNA back to IKe genomic DNA for expression. Randomized oligos were employed to mutagenize the full TM region (3'-CTA**GAGTAA-AGAGTCTGAACCGGACAACATTGCTGA**-5'; 3'-**CATTGCTGACAACATCAA-CGACCAGAACATTAAGCG**-5'), which were prepared with 96.4% wild type base and 1.15% contamination of each of the other three bases for each of the nucleotides in bold [19], and 100% wild type at the three bases on either side. As well, an oligo designed to subject Pro^{30} to saturation mutagenesis (3'- AGAGTCTGAACCNNNCAACATTGCTGAC - 5'), was prepared with 25% of each base supplied at the three bases coding for the Pro residue. All oligonucleotides were synthesized and purified with oligonucleotide purification cartridge by the Biotechnology Centre, Banting Institute, University of Toronto. Modifications to the mutagenesis protocol included lengthening the exo III digestion to 1.5 hours and then increasing the level of nucleotides, DNA polymerase and DNA ligase to that employed in the first repolymerization step. Transformation was by the $CaCl_2$ method, with cells plated on 2 x TY plates (plus chloramphenicol at 30 μg/μl), held at 25°C overnight, and then transferred to 37°C for 8 hours to generate large, distinct plaques. Sequencing of individual plaques was performed using the T7 kit (Pharmacia, Uppsala, Sweden) based on the dideoxy method [20] using the sequencing oligo, 5'-CAATTACGCCTTAGCTGAAT-3'. Approximately 150 plaques were sequenced for each of the oligos; mutagenesis efficiency (including silent and multiple isolations of several mutants) was *ca.* 30%.

RESULTS AND DISCUSSION: Molecular biology techniques similar to those developed for M13 [2,12] were applied directly to the IKe genome to exploit the characteristics of single-stranded DNA filamentous bacteriophage. The presence of an NciI site in the IKe genome facilitated the use of the Eckstein method of improving mutational efficiency [18]. Modifications required to optimize this application included extension of the exonuclease digestion and supplying of additional reagents during repolymerization.

As seen from the *ca.* 40 single- and double-site viable IKe TM domain mutants isolated (Figure 1), the IKe transmembrane segment is highly tolerant to amino acid substitution.

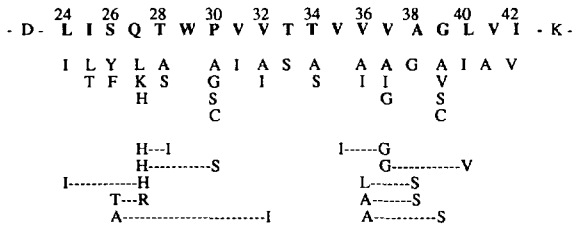

Figure 1. Viable mutant bacteriophage obtained from Eckstein mutagenesis of the transmembrane segment of bacteriophage IKe major coat protein. Each amino acid listed below the wild type sequence represents a single mutant at that position. Double mutants are connected by a dashed line. Mutants P30G, P30C, and P30S were obtained by saturation mutagenesis at the Pro^{30} codon (see Materials and Methods).

Every residue in the TM segment with the exception of Trp^{29} could be mutated to at least one other residue. Polar and charged mutations are enriched in the N-terminal segment (residues 24-30), while non-polar substitutions predominate in the C-terminal region (residues 31-42). These observations parallel findings with M13 coat protein [12] and suggest that an N-terminal sub-domain of the effective TM segment is exposed to the aqueous environment during the life cycles of both phage. Trp^{29} is conserved among the class I filamentous bacteriophage, and thus likely serves an important role either as an interfacial residue in the membrane, and/or in protein-protein or protein-DNA interactions in the phage. The C-terminal portion of the IKe hydrophobic domain was more easily mutated in IKe than that in M13 [12], and while this may be due to slight methodological differences, it more likely reflects the circumstance of fewer restrictions on the residues in this region of the IKe coat protein. A locus of small residues in the region of V^{37}-$AGLV^{41}$ in IKe may play a role in packing, as has been suggested for a corresponding region in M13 (G^{34}-$ATIG^{38}$) [14]; "small" residue substitutions, such as Ala and Gly, appeared as favored residues in this segment. Pro^{30} occurs at the interface between the two sub-domains of the effective TM segment. The Pro^{30} locus tolerated four substitutions - Ala, Gly, Cys, and Ser - which correspond to the four side chains of smallest volume, suggesting a sterically-restricted location for the Pro pyrrolidine side chain between the Trp indole ring and β-branched Val *iso*-propyl moiety.

Wild type IKe major coat protein is largely α-helical in conformation as shown by circular dichroism (CD) spectroscopy (Figure 2), exhibiting spectra similar both in shape and magnitude to that of M13 coat protein [*e.g.*, 14]. Yet, the IKe coat protein helix is remarkably

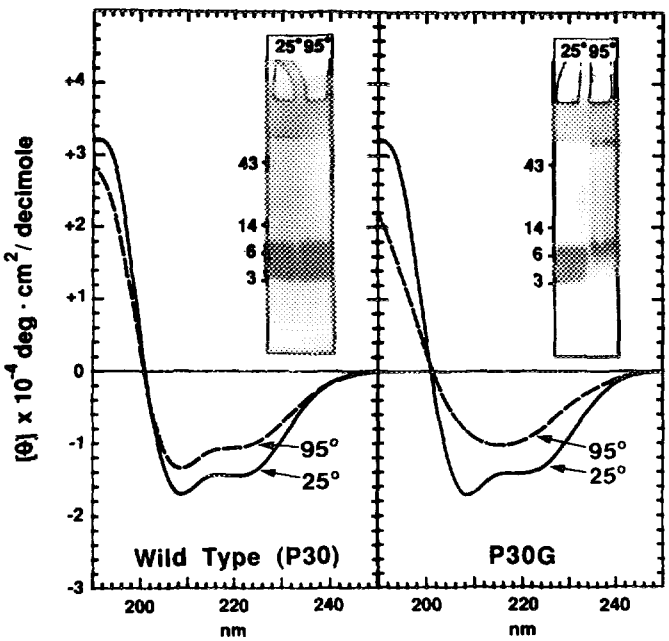

Figure 2. Circular dichroism spectra and corresponding SDS-PAGE gels (insets) at 25°C and 95°C of IKe wild type major coat protein (left panel) and IKe mutant P30G (right panel). CD spectra were recorded on a Jasco J-720 spectropolarimeter using 1 mg/ml coat protein in 30 mM deoxycholate (DOC) at pH 9. Each curve is the average of 4 scans, recorded between 250-190 nm in a cell with path length of 0.01 cm. Parallel SDS-PAGE experiments were performed on the CD samples in a 15% acrylamide separating gel prepared according to Laemmli [21] with 5 μg protein loaded per lane and stained with coomassie blue.

stable, as shown by only a slight decrease in helicity upon heating to 95°C [which reverses upon return to room temperature (data not shown)]; under similar conditions, M13 coat undergoes quantitative irreversible transition to a highly aggregated β-sheet structure [2, and references therein]. Parallel SDS-PAGE gels (Figure 2, left panel) show wild type protein as a monomeric species indistinguishable at 25°C and at 95°C. The P30G mutant produced a CD spectrum very similar to that of wild type at 25°C and was similarly monomeric on SDS gels (Figure 2, right panel). In contrast, however, P30G underwent an irreversible transition at *ca.* 90°C to a CD spectrum characteristic of a β-sheet, as confirmed by the observation of high molecular weight aggregates observed on the SDS gel at 95°C. Though both Pro and Gly residues might have been predicted to be helix-destabilizing in the context of a membrane, these latter results - albeit using elevated temperatures - reinforce the notion of a specific (helix initiation? kink-inducing?) role for the TM Pro residue at position 30 [6], which cannot be matched by the more flexible, non-imino, replacement Gly residue. Detailed analyses of wild

type and mutant IKe major coat proteins are presently underway to explore the properties of these and other transmembrane residues.

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