Evidence for β-sheet conformation in vesicle-bound peptides derived from the transmembrane bacterial flagellar motor protein MotB from *Rhodobacter sphaeroides*

Clare E. Bostock-Smith,[a]† Gary J. Sharman,[a]‡ R. Elizabeth Sockett[b] and Mark S. Searle[a]*

[a] *Department of Chemistry, University Park, University of Nottingham, Nottingham, UK NG7 2RD. E-mail: mark.searle@nottingham.ac.uk*
[b] *Division of Genetics, School of Clinical Laboratory Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, UK*

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Circular dichroism experiments show that a 28 residue transmembrane peptide derived from the *R. sphaeroides* bacterial flagellar motor protein MotB adopts predominantly β-sheet conformation when bound within phosphatidylcholine vesicles. A peptide with a mutation at Asp32, which has been shown to destroy proton conductance, is also shown to insert into the model membrane in predominantly β-sheet conformation, suggesting that it is not the gross structural features of the transmembrane region that are disrupted by this mutation but perhaps only the electrostatic properties of the pore. A tentative structure for a MotB pore is proposed which consists of an eight stranded β-barrel.

Introduction

The bacterial flagellum is a rotating propeller which enables bacteria to swim in response to chemotactic stimuli. It consists of a thin, semi-rigid, helical filament attached to a rotary motor in the cell membrane and is driven by a transmembrane proton or sodium ion gradient.[1] *Rhodobacter sphaeroides* has just one proton-driven flagellum which can rotate in one direction only and re-orientation is a passive process occurring while the bacterium is motionless.[1] The flagella of most bacteria are similar in composition: their gross structure is well documented,[1–3] and the component proteins have been identified by electron microscopy and mutation studies. The flagellum is composed of three main elements: the filament, the hook and the basal body (Fig. 1), which passes through a series of rings in the inner and outer membranes and the periplasm. A functional flagellum, however, has other more loosely associated membrane-bound proteins, essential for torque generation and control, and these have been isolated genetically and deductions concerning their structure, location and function have been made, as discussed below. Their precise structures have not however been accurately determined and together with function are still a matter of some debate. Two of the major problems in solving the structures of these proteins are the size of the complexes and the fact that several are membrane bound and therefore not amenable to X-ray analysis or NMR spectroscopy.

Mutations in these proteins cause paralysis of the flagellum with no obvious defect in structure and have been divided into two functional categories. The flagellar switch proteins, FliG, FliM and FliN are thought to control the direction or stopping and starting of rotation and are located at the base of the basal body.[3] The motor proteins, MotA and MotB are thought to drive the motor by generating the transmembrane proton gradient.[4,5] They are membrane bound and together give rise to between ten and sixteen studs in the inner membrane located around the periphery of the basal body and visible by electron microscopy.[6]

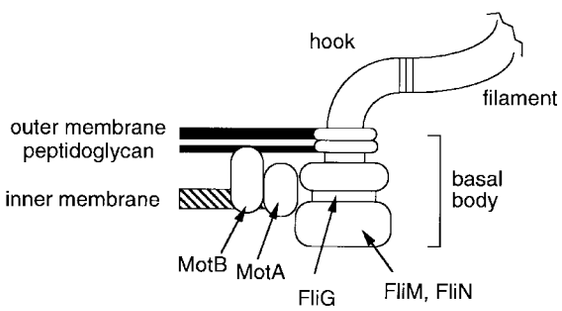

Fig. 1 Schematic representation of the structure of the bacterial flagellum.

Proton flux through the membrane is known to be tightly coupled to rotation, and of the order of 1000 protons per revolution. The flagellum of *R. sphaeroides* rotates at a speed of 100 Hz but the mechanism of energy conversion for flagella in general is not known. Conclusions concerning the structures and interactions of the Mot and Fli proteins have been drawn from mutagenesis experiments and analysis of the amino acid sequences. The MotA sequences of *E. coli* and *R. sphaeroides* have four hydrophobic regions, most likely transmembrane helices, with highly charged segments located between helices 1 and 2 and helices 3 and 4, indicating that these are in the cytoplasm, and the N and C termini therefore lie in the periplasm. This has been confirmed by membrane impermeant sulfydryl experiments.[7] Tryptophan scanning mutagenesis of *E. coli* MotA suggests that the protein takes the form of a four helix bundle.[8]

In contrast, MotB has just one hydrophobic region together with an N-terminal region that is highly charged, and a C-terminal region that lies in the periplasm,[9] suggesting that the hydrophobic domain must cross the membrane, but only once. Mutation studies have revealed the importance of one particular residue, Asp 32 of MotB, which is conserved across all known MotB sequences. It has long been thought that proton or sodium ion binding sites in the motor proteins must be important for function, and that these are likely to be charged amino acid side chains. In a study in which conserved acidic residues of all five motor proteins were mutated, only Asp 32 of

† Current address: Royal Society of Chemistry, Science Park, Cambridge, UK CB4 0FW.
‡ Current address: AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, UK SK10 2NA.

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MotB proved essential for torque generation. Of fifteen substitutions at this site the only one to retain any function was the conservative replacement of aspartate with glutamate.[10] The mutations appeared not to disrupt the structure of the protein, as is demonstrated in this work, but were shown to abolish proton conduction, indicating that MotB is part of the proton channel. Recent experiments on the conductance of over-expressed MotA in *E. coli* derived vesicles[11] and isolated membrane-spanning MotB peptides in artificial membranes (I. Mellor and R. E. Sockett, unpublished results) have demonstrated that MotB peptides are able to conduct protons alone, and at a far greater rate than MotA (10^6 protons per second compared with 250 protons per second). Mutation of the previously mentioned Asp32 of MotB to valine destroys conductance. We describe a conformational analysis by circular dichroism spectroscopy of the secondary structure of vesicle-bound peptides derived from the transmembrane spanning region of *R. sphaeroides* MotB and discuss possible models for a MotB proton pore.

Experimental

Materials and methods

Wild type and mutant *Rhodobacter sphaeroides* MotB peptides, sequences AWLATFADIATNLMAFFVLILGFAKFDE and AWLATFAVIATNLMAFFVLILGFAKFDE, and reference peptides VTIKANLITPSGTQTAEFKG and VTIKANLIFANGSTQTAEFKG were synthesised by standard solid-phase Fmoc chemistry and purified by reversed-phase HPLC. Purity was checked by analytical HPLC and plasma-desorption mass spectrometry. Phosphatidylcholine (L-α-PC from soybean) was purchased from Sigma Chemical Co. and used without further purification. Both the MotB peptides and phosphatidylcholine were the same as used in MotB conductance experiments. The MotB peptides incorporate the putative 22 residue transmembrane region with a 6 residue (FAKFDE) periplasmic tail.

Circular dichroism in TFE

Circular dichroism spectra were acquired using an Aviv 62DS spectrometer using supplied software. A 1 mm cell was used, with a bandwidth of 4 nm and a step size of 1 nm. 100 μM solutions of the wild type and mutant peptides in 50% (v/v) TFE–H_2O were prepared by dissolving the appropriate mass of the peptides in TFE and diluting with phosphate buffer (1 mM phosphate, pH 4.0). Aliquots of each of the solutions were then diluted with the appropriate proportions of TFE/buffer to yield 50% TFE, 100 μM peptide; 25% TFE, 50 μM peptide and 12.5% TFE, 25 μM peptide solutions. Solutions of the Asp32Val peptide were prepared similarly to give the following solutions: 50% TFE, 20 μM peptide; 25% TFE, 10 μM peptide and 12.5% TFE, 5 μM peptide. CD spectra were recorded at 293 K. 9 scans were acquired between 190 and 250 nm and the average used. A solvent baseline was acquired and subtracted from the average. Mean residue ellipticities (θ in degrees cm^2 $dmol^{-1}$) were calculated using standard methods,[12] however, due to uncertainties in peptide concentrations in vesicle solutions, ellipticities in Fig. 3 are left in experimentally measured units of millidegrees.

Circular dichroism in vesicles

Phosphatidylcholine (12 mg) was dissolved in distilled chloroform (2 ml). The solvent was evaporated under reduced pressure to give a thin film on the wall of the flask. This was then lyophilised for two hours to remove any residual solvent. H_2O (2 ml) was added and the mixture was shaken for 30 minutes. The opaque solution of multilamellar vesicles was sonicated for a total of 10 minutes using an ultrasonic generator with micro-tip probe (Ultrasonics Ltd, Dawe Instruments Ltd, Soniprobe type 7532B) by repeats of 15 seconds sonication followed by 30 seconds in an ice bath to yield a transparent solution of small unilamellar vesicles. After centrifugation for 5 min at 14,000 rpm in a bench top micro-centrifuge to remove any residual large vesicles, the vesicle suspension was diluted with H_2O to give a suspension containing 0.5 mM phosphatidylcholine. Aliquots of a stock solution of wild type or mutant peptide were evaporated under reduced pressure leaving the required mass of peptide to give a 0.05 mM peptide solution on addition of 0.5 ml vesicle suspension. The mixtures were sonicated as previously described to allow the vesicles to take up the peptide and microcentrifuged to remove residual peptide. An initial molar ratio of lipid : peptide of 10 : 1 was used, however, not all peptide was taken-up by the vesicles. The CD spectra of vesicle-bound peptides showed no apparent time-dependence over the course of the experiment. Because of the limited solubility of peptides in the presence of vesicles, and subsequent uncertainties in peptide concentrations, a detailed study of the concentration-dependence of CD spectra was not pursued. CD spectra in the presence of vesicles were recorded as described above averaging 25 scans and baseline corrected by subtracting the spectrum of pure vesicles. CD spectra of aqueous buffer solution with and without vesicles showed little difference in absorption, from which we conclude that light scattering by vesicles is relatively small.

Spectra of 0.05 mM solutions of L20, a 20 residue random coil reference peptide (sequence VTIKANLITPSGTQTAEFKG), were recorded both in the absence of vesicles and with 0.5 mM vesicles as the averages of 16 and 25 scans respectively and the appropriate baselines subtracted. These are plotted down to 198 nm only, owing to poor signal-to-noise below that wavelength. A spectrum of 0.15 mM L21, a water-insoluble β-sheet peptide (sequence VTIKANLIFANGSTQTAEFKG), in 0.5 mM vesicle solution was recorded as the average of 9 scans and the pure vesicle baseline subtracted.

Results

CD analysis of peptides in aqueous organic solvents

The CD spectra of both the wild type and mutant peptides in 50% TFE show clear evidence of α-helical conformation, with strong minima at 208 and 222 nm and positive ellipticity below 205 nm (Fig. 2). On lowering the concentration of TFE the two minima reflecting helical secondary structure give way to a broader minimum around 216 nm. The spectral changes observed suggest a shift towards a greater proportion of β-sheet conformation at low TFE concentration. The minimum at 216 nm is broader than would be expected for pure β-sheet, particularly for the wild type peptide (a), and is still negative at 208 nm, suggesting a mixture of helix and sheet in equilibrium. The spectral features for a helical conformation have a greater intensity than for sheet and so even a small proportion of helix is likely to distort the absorption envelope. The effect of peptide concentration was investigated in 12.5% TFE where the proportion of β-conformation appears to be greatest. A four-fold dilution from 25 μM to 6 μM does not alter the spectrum, neither increasing nor decreasing the proportion of β-sheet (data not shown). CD spectra of aqueous buffer solution were recorded after sonication with both wild type and mutant peptides but no signal could be detected showing the peptides to be water-insoluble.

TFE is known to stabilise helix formation, however, the results in TFE show that for both peptides helical and sheet conformations are stabilised by different concentrations of TFE. This is by no means a unique observation. In previous studies of β-hairpin peptides of 20 residues derived from the N-terminus of Ferrodoxin we showed that the folded conformation was highly sensitive to solvent composition. Despite the propensity of this peptide to adopt β-sheet in the native protein,

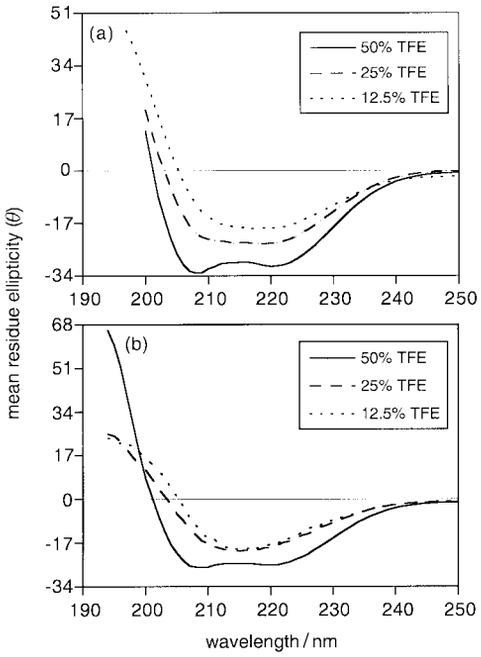

Fig. 2 CD spectra (mean residue ellipticity, θ in degrees cm^2 dmol^{-1} × 10^3) of wild-type (a) and Asp32Val mutant (b) MotB peptides in 50%, 25% and 12.5% aqueous trifluoroethanol solution at 20 °C.

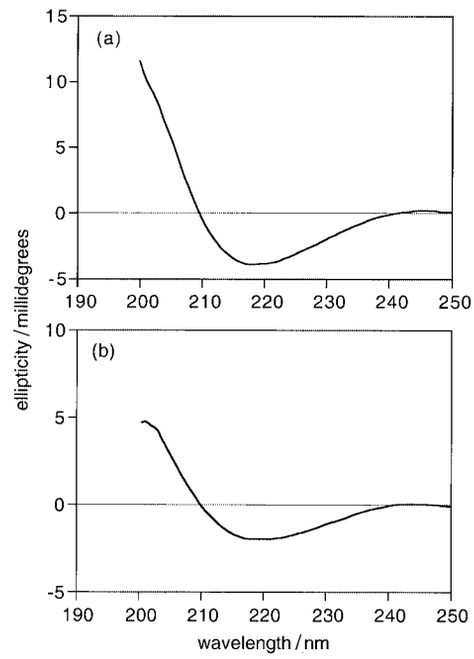

Fig. 3 CD spectra (ellipticity in millidegrees) of wild-type (a) and Asp32Val mutant (b) MotB peptides bound to phosphatidylcholine vesicles at 20 °C.

TFE is able to force an increasing proportion of α-helix above 50% (v/v) concentration.[13] At low TFE concentration both helical and β-sheet conformations appear to be in equilibrium. Low concentrations of methanol (<40%), in contrast, were shown to stabilise predominantly the β-hairpin conformation.

CD analysis of peptides bound within model membranes

Solvation-effects from added cosolvents, in particular the helix-inducing properties of TFE, make it difficult to draw conclusions regarding the preferred conformation of the MotB peptides in their natural environment, the cell membrane. A better mimic of this environment is to insert the peptide into vesicle membranes. These have previously been used as biological membrane mimics in CD spectroscopy[14–16] as well as in many other applications such as solution NMR[17] and IR[18] and are a more accurate simulation of the membrane environment than solubilising the peptide in mild detergent micelles. The major disadvantage of CD of peptides in lipid bilayers is distortion of the spectra due to light scattering from large particles, and differential absorption flattening.[19] The use of an homogeneous suspension of small unilamellar vesicles reduces this problem to a minimum, thus avoiding the need to use a scattered light collecting device or complex mathematical approaches to reduce optical artefacts.

A transparent solution of vesicles was prepared in which both peptides were soluble upon sonication. As neither peptide is soluble in water it is assumed that the hydrophobic peptides are inserting in the membrane and not simply associated with the surface of the vesicles. The spectra obtained gave good signal to noise down to 200 nm. CD of both peptides in small unilamellar vesicles are shown in Fig. 3. The results are similar to those in 12.5% TFE, but with a more pronounced minimum around 216–218 nm and positive ellipticity at 208 nm, strongly suggesting a β-sheet conformation in the membrane, and in good agreement with published CD spectra of β-sheet peptides inserted in phospholipid vesicles.[20–25] Both the wild type peptide and Asp32 Val mutant give similar CD spectra suggesting that the mutation does not perturb the gross structural features of the membrane-bound conformation.

To check for possible non-specific effects of vesicles on peptide conformation, control spectra were recorded on two

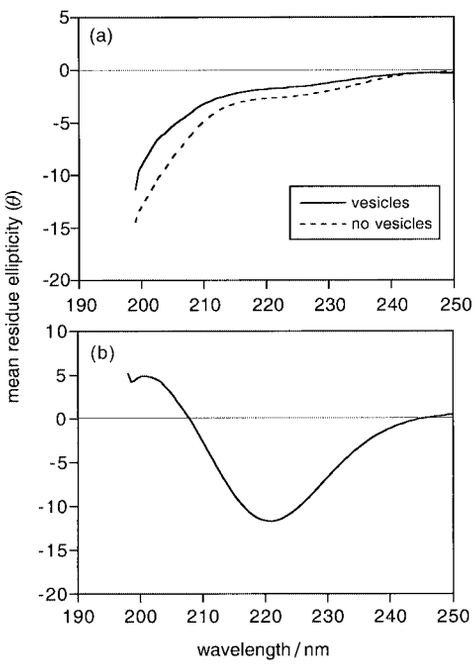

Fig. 4 CD spectra (mean residue ellipticity, θ in degrees cm^2 dmol^{-1} × 10^3) of peptide L20 (a) in aqueous solution and in the presence of vesicles; (b) peptide L21 bound within phosphatidylcholine vesicles at 20 °C.

unrelated peptides. The first, L21, is a water insoluble β-hairpin peptide derived from residues 1–21 of the immunoglobulin light chain binding domain of protein L.[26] The second, L20, is of similar sequence but with a different β-turn sequence (FANGS→TPSG) that disrupts the folded structure. In contrast, the peptide is water soluble and adopts a random coil conformation as judged by CD and NMR in aqueous solution. The spectrum of L20 was recorded both in the presence and absence of vesicles and gave essentially the same spectra typical of a largely unstructured conformation with slightly poorer signal to noise and lower intensity in the presence of vesicles (Fig. 4). The peptide showed no evidence for interaction with the vesicle. A spectrum of L21 in aqueous solution alone could

not be obtained due to its very low solubility. However, the peptide was readily taken up by the vesicle solution upon sonication, and gave a CD spectrum typical of β-sheet with a minimum at ~220 nm (Fig. 4), reflecting the conformational propensity of this sequence in the native protein structure. The CD spectrum in vesicles was similar to that of the MotB peptides in the same environment (Fig. 3).

These control experiments, together with the spectra of the wild type and mutant peptides in both TFE and vesicle solutions, suggest that both peptides adopt a high proportion of β-sheet conformation in biological membranes. Any non-β-contribution to the spectra is likely to come from the short periplasmic tail of the peptide. In addition, the experiments indicate that the lack of function imparted by mutation of Asp32 is not due to a gross structural change but to a loss of the necessary functionality for proton conductance, as previously deduced from double mutant experiments.[10]

Discussion

It has been demonstrated that a peptide taken from the MotB protein is able to conduct protons at a rate compatible with flagellar rotation (I. Mellor and R. E. Sockett, unpublished data) and that the membrane spanning region of this peptide inserts in a phosphatidylcholine membrane in predominantly β-conformation. The questions remaining concern the relevance of these experiments to the *in vivo* situation, and the detailed structure of a MotB pore. While the first question can only be answered by further investigation, it is possible on the basis of these experiments and of known protein structures to make some proposals concerning pore structure.

Until recently it was assumed that all transmembrane channels were lined by α-helices, but since the early 1990s many β-barrel channels have been discovered, the first of which were the porins: trimers of sixteen or eighteen stranded anti-parallel β-barrels in the outer membranes of gram negative bacteria, but eight stranded β-barrels have subsequently been identified and structures determined.[27–31] These transport small nutrient molecules but are not voltage-dependent. While to date none of the known β-barrel channels transport protons, instead conducting sodium, potassium or calcium, a β-barrel proton channel would not seem unlikely. The bacterial flagellar motor proteins have not been conserved in eukaryotes and so proteins homologous in either sequence or function are rare.

The MotB conductance experiments were performed under conditions under which all peptides should insert into the membrane in the same orientation. They were introduced from one side of the membrane and only a positive and not a negative applied voltage resulted in an active channel, implying an asymmetric pore with all peptides in the same orientation. Taken together with the evidence that MotB crosses the membrane once, and assuming that under the non-electrified conditions of the CD experiments the peptide is inserting into the membrane in the same manner as in the conductance experiments, all strands of the proposed β-barrel must be parallel. This is an unprecedented structure for a transmembrane β-barrel, but one that cannot be ruled out simply on the basis that it has not previously been observed. Investigations into a potassium channel peptide with a 22 residue transmembrane domain have suggested a structure consistent with either an antiparallel β-barrel in which the strands cross the membrane twice, or a parallel β-barrel in which the strands cross once and are tilted at 60° to the bilayer normal, allowing the complete 77 Å hydrophobic region to lie in the 37 Å wide bilayer.[32] A tilt such as this is not unusual, being found in porin β-barrels among others.[27–31] Molecular modelling studies have demonstated that some degree of tilting serves to stabilise the structure of both parallel and anti-parallel β-sheets.[32] The extent of β-sheet tilting is illustrated in Fig. 5 in the crystal

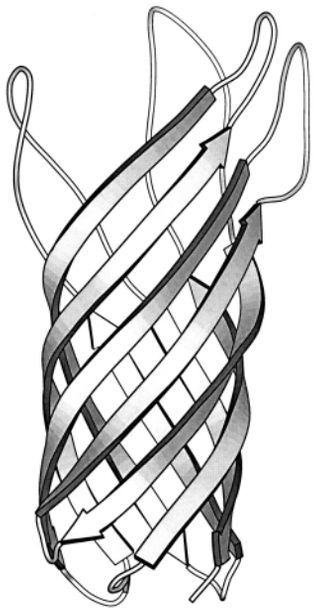

Fig. 5 Crystal structure of the outer membrane protein A of *Escherichia coli* illustrating the highly tilted nature of the eight-stranded β-barrel. Figure drawn with the program Molscript.[34]

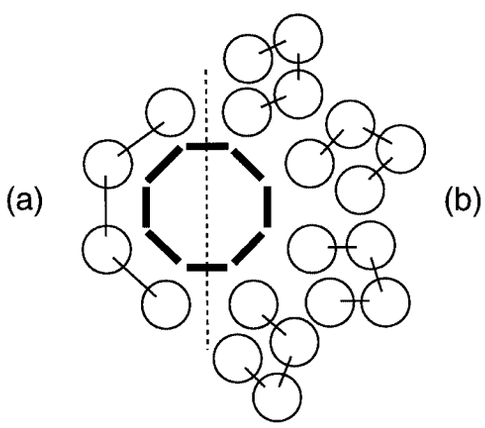

Fig. 6 Schematic representation of an eight-stranded MotB pore (cross-section in the plane of the membrane) (a) surrounded by two extended MotA proteins, and (b) surrounded by eight MotA proteins.

structure of the outer membrane protein A (protein data bank accession code 1BXW).[30]

The same studies have demonstrated that a four stranded β-barrel cannot form a pore large enough for ion permeation whereas an eight stranded barrel can form a pore of radius 2.9–4.8 Å, depending on the tilt angle.[32] The size of a pore may be calculated from its rate of ion conductance. The radius for the wild-type MotB pore has been calculated as 2.5–3.8 Å, although this is likely to be an underestimate as any interaction between the ions and residues in the pore, *i.e.* Asp32, will distort the results. These results could therefore be consistent with a barrel structure in which the β-strands are highly tilted. A further possiblity is that the channel is not purely β-structure in nature. The main precedent for this is the antibiotic zervamicin IIA which takes the form of a peptide pore in which the 16 residue parallel peptides have three distinct regions: 3,10 helix, α-helix and β-bend.[33] While the MotB peptides are predominantly β-structure, in contrast to the mixed structure of zervamicin IIA, and it is assumed that the non-β contribution to the MotB results from the small extra-membrane region, a structure in which the channel is not purely β-sheet remains a possibility.

In summary, it has been demonstrated that the transmembrane peptide of the *R. sphaeroides* MotB protein adopts a

predominantly β-sheet conformation when bound within a phosphatidylcholine vesicle. On the basis of this, and literature data, a tentative structure for a MotB pore has been proposed, consisting of an eight stranded parallel β-barrel (Fig. 6). However, while it may be surmised that a β-barrel pore is present *in vitro*, it remains to be seen whether or not this structure bears any relation to the *in vivo* structure of bacterial flagellar motors. A peptide with a mutation at Asp32, which has been shown to destroy proton conductance, is also shown to insert into the model membrane in predominantly β-like conformation, suggesting that the gross structural features of the transmembrane region are not disrupted by this mutation but perhaps only the electrostatic properties of the pore.

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