Minireview

Solid-state NMR investigations of interaction contributions that determine the alignment of helical polypeptides in biological membranes

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Received 11 July 2001; accepted 23 July 2001

First published online 6 August 2001

Edited by Andreas Engel and Giorgio Semenza

Abstract Helical peptides reconstituted into oriented phospholipid bilayers were studied by proton-decoupled ^{15}N solid-state NMR spectroscopy. Whereas hydrophobic channel peptides, such as the N-terminal region of Vpu of HIV-1, adopt transmembrane orientations, amphipathic peptide antibiotics are oriented parallel to the bilayer surface. The interaction contributions that determine the alignment of helical peptides in lipid membranes were analysed using model sequences, and peptides that change their topology in a pH-dependent manner have been designed. The energy contributions of histidines, lysines, leucines and alanines as well as the alignment of peptides and phospholipids under conditions of hydrophobic mismatch have been investigated in considerable detail.

Key words: Oriented bilayer; Transmembrane polypeptide; Amphipathic peptide; Membrane equilibrium; Hydrophobicity scale; Interface

Although membrane proteins constitute about one third of the expressed reading frames, only a few high-resolution structures of this important class of proteins have become available [1,2]. This lack of conformational information is due to the difficulties encountered during the expression and purification of hydrophobic proteins and their resistance to crystallization, which would be necessary for X-ray or electron diffraction. The organization of integral membrane proteins is characterized by the separation of hydrophobic membrane-spanning and hydrophilic membrane-flanking regions (reviewed in [1–3]) with two major motifs, i.e. helical domains and, for proteins of the bacterial outer membranes, β-barrel structures being observed [1].

At the same time DNA sequence analysis is fast and routine; therefore, structure prediction algorithms identifying membrane-spanning domains remain an important source of information. These protocols involve a first step in which the primary sequence is searched for hydrophobic, putatively transmembrane helical domains [4–6]. Clearly the accuracy of these predictions is strongly dependent on the quality of the underlying data base which assigns hydrophobicity values to individual amino acids.

An accurate knowledge of the interaction contributions that determine the alignment of membrane-associated helices is of great importance not only for the prediction of membrane protein structure, but also for our understanding of the functional mechanisms of smaller single helical domain polypeptides. Hydrophobic or amphipathic helical peptides exhibit a multitude of functional properties, including antibiotic, fusogenic and channel activities [7,8]. However, due to the multitude of conformations and the dynamic properties of these sequences in the presence of lipid bilayers, it is often difficult to directly correlate structural data with functional aspects [9,10]. For example, it has been suggested that the membrane-permeabilizing properties of magainin/PGS-type antibiotic peptides are caused by transmembrane peptide configurations (reviewed in [8]). This contrasts with structural studies, which indicate that these peptides are α-helical with an orientation parallel to the membrane surface [8]. It is possible that only a minor peptide population in a high-energy configuration is active. However, it is the abundant and inactive low-energy state that is observed by structural techniques [11]. The situation is even more complicated as the distribution between active and inactive configurations can be shifted in the presence of transmembrane electric fields, which constitutes an experimental condition very difficult to attain in high-resolution structural investigations. It is, therefore, of importance to understand the interaction contributions that determine the structures and topologies of α-helical polypeptides in membrane environments [5,12,13].

Theoretical estimates of the interaction contributions of in-plane and transmembrane peptides allow one to validate the suggested models of peptide–membrane interactions [11]. The membrane association of amphipathic and hydrophobic polypeptides has been described by a multitude of equilibria of the type (Fig. 1): water-dispersed aggregates ↔ soluble in aqueous buffer ↔ membrane surface associated ↔ IP ↔ TM_1 ↔ TM_2 ↔ ... ↔ TM_n, where IP represents in-plane and TM_n transmembrane peptide oligomers of n subunits [9]. Our ongoing studies focus on the investigation of the IP ↔ TM equilibrium using solid-state NMR spectroscopy in combination with other biophysical techniques.

The following energy contributions are of major importance

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Abbreviations: di-C10:0-PC, 1,2-dicapryl-*sn*-glycero-3-phosphocholine; HIV, human immunodeficiency virus; IP, in-plane (alignment); NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TM, transmembrane (alignment)

0014-5793/01/$20.00
PII: S0014-5793(01)02741-7

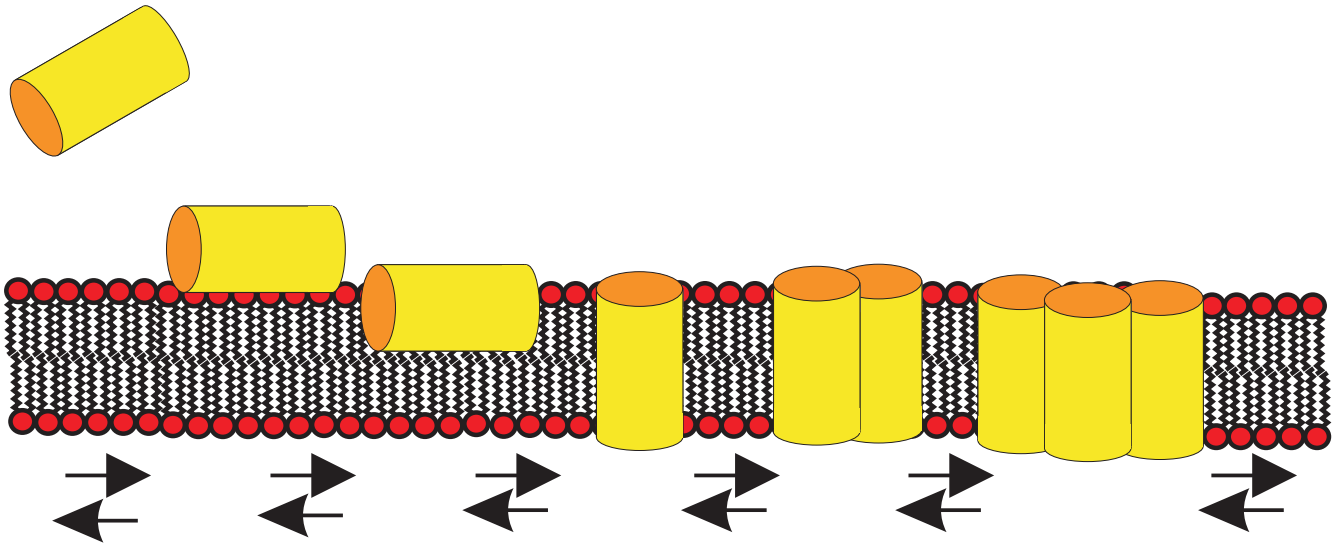

Fig. 1. Model for peptide bilayer association equilibria. The following sequence of events is schematically shown (from left to right): helical peptide soluble in aqueous buffer ↔ membrane surface associated ↔ IP ↔ TM_1 ↔ TM_2 ↔ TM_3 ↔ ....

during the IP → TM transition of helical peptides: $\Delta G = \Delta G^{h} + \Delta G^{d} + \Delta G^{p} + \Delta G^{m} + \Delta G^{\psi} + \Delta G^{\#}$, where ΔG^{d} is the pH-dependent energy of discharge of an amino acid side chain. Furthermore, changes in hydrophobic (ΔG^{h}), polar (ΔG^{p}), and mismatch interactions (ΔG^{m}) (e.g. [14–16]), energetic changes that arise from interactions with transmembrane electric fields (ΔG^{ψ}) as well as other contributions ($\Delta G^{\#}$) are considered. If not already part of ΔG^{m}, changes in lipid–lipid interactions, such as lipophobic [17], van der Waals and lipid head group interactions are considered separately ($\Delta G^{l} + \Delta G^{W} + \Delta G^{hh}$). The energy of discharge is derived from the corresponding chemical potentials and is calculated according to: $\Delta G^{d} = n_i \cdot RT \cdot \ln r + 2.3 RT \Sigma_i (pK_i - pH)$, r being the ratio of the charged over uncharged state of a functional group acceptable for membrane insertion (typically a value of 99 is assumed), n_i is the number of potential charge carriers i, and R and T have their usual meaning [18]. If $pK - pH = 3$, this term amounts to $\Delta G^{d} = 25$–30 kJ/mole, a value that is considerably smaller than the corresponding Born energy of transferring a charge from a high to a low dielectric environment (estimated as 50–200 kJ/mole [19]).

Solid-state NMR spectroscopy has been used to study structural and topological aspects of polypeptides reconstituted into lipid bilayers (reviewed e.g. in [8,20–25]). In particular, oriented samples of membrane-associated peptides, isotopically labelled with ^{15}N, have been investigated by proton-decoupled ^{15}N solid-state NMR spectroscopy. The resulting ^{15}N chemical shift is a sensitive indicator of the membrane alignment of α-helical peptides [18]. Whereas transmembrane alignments correlate with ^{15}N chemical shifts > 190 ppm, values < 100 ppm are observed for amphipathic peptides oriented along the membrane surface. This technique has been used to characterize in detail charged amphipathic α-helices that orient perpendicular to the membrane normal (e.g. [21,26]). In contrast, many hydrophobic sequences, including the channel-forming M2 domain of influenza A [21,27], the peptide antibiotic alamethicin [28,29] or the $\beta^{6.3}$-helix of gramicidin A [23], adopt transmembrane alignments. Investigations of the viral protein Vpu of HIV-1 involving a multitude of labelled sites indicate an approx. 20° tilt angle of the N-terminal hydrophobic helix, a close to 90° in-plane alignment of the amphipathic helix encountering residue 45, and the lack of efficient membrane interactions of residues 51–81 [26,30]. Interestingly, the alignment of helix 32–51 is independent of phosphorylation of serines 52 and 56 [26].

The spectral line shapes of proton-decoupled ^{31}P NMR spectra of phospholipid membranes have been used to analyse the orientational order of the phospholipid head group in an analogous manner [21]. Proton-decoupled ^{15}N and ^{31}P solid-state NMR techniques have, therefore, proved highly valuable during investigations of the topologies of amphipathic and hydrophobic model peptides, thereby increasing our understanding of the interactions that govern the transition between in-plane and transmembrane peptide helices.

Several α-helical model peptides have been designed and their orientation with respect to the lipid membrane analysed. The first peptide in the series, LAH_4 (Fig. 2A, KKALLALALHHLAHLALHLALALKKA), has been synthesized in such a manner that four histidines are interspersed in a sequence of leucines and alanines [18]. The hydrophobic core region is 21 amino acids long and, therefore, sufficient to span the lipid bilayer. Two lysines at the N- and C-terminus act as membrane anchors and help to solubilize the peptide. The peptide also exhibits pronounced amphipathic properties as the four histidines all lie on one face of the helix when viewed in a helical wheel representation (Fig. 2A). Proton-decoupled ^{15}N solid-state NMR measurements on LAH_4 reconstituted into oriented 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers indicate that at low pH the peptide is aligned parallel to the surface of the membrane as suggested by its pronounced amphipathic character [18]. At pH < 5 ΔG^{d} is high and the peptides reside at the membrane interface. At acidic pH hydrophilic interactions exceed the pH-independent hydrophobic contributions of two leucines, one alanine and 2–4 histidines. Increasing the pH results in a decrease of ΔG^{d}. As a result, at pH 7 interactions that favour membrane insertion dominate and transmembrane alignments are obtained. In dodecylphosphocholine detergent micelles the pK values of the histidines range between 5.4 and 6.0 [18]. Previous studies indicate that the energy contributions due to changes in the

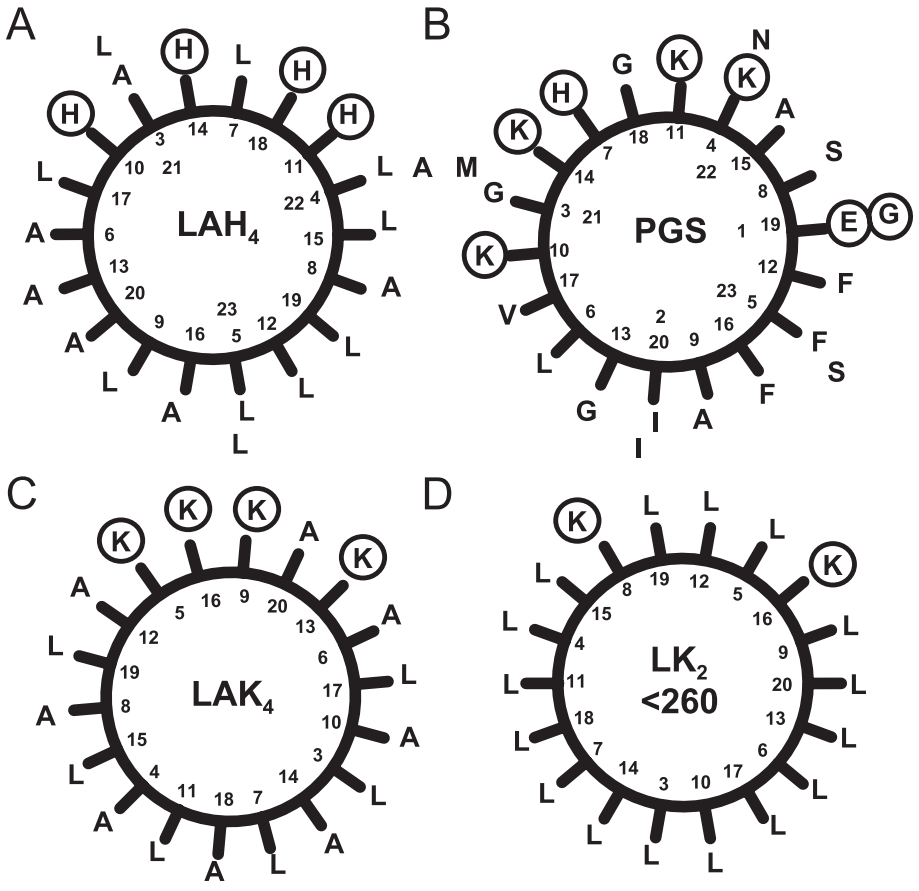

Fig. 2. Edmundson helical wheel representations of A: LAH_4, B: magainin 2/PGS, and of residues 3–20 of C: LAK_4 and D: $LK_2^{<260}$. The peptides are described in detail in [8,18,41].

lipid packing during peptide reorientation ($\Delta G^{\#}$) are small [17]; therefore, our results indicate that the gain in hydrophobic energy during the IP→TM transitions are sufficient to compensate for the polar contributions of uncharged histidine side chains. The IP→TM transition of LAH_4 is reversible and its midpoint has been determined at pH 6.1 by proton-decoupled ^{15}N solid-state NMR as well as ATR-FTIR spectroscopies [18,31].

Other histidine-containing peptides have been designed in such a manner that the contributions of ΔG^{h} vary. For example, replacing leucines by alanines shifts the transition pH to higher values [32]. Other more hydrophilic residues such as glycines or tyrosines abolish the IP to TM transition. The difference in transition pH has been used to calculate the relative hydrophobicity of leucine in the context of an α-helical peptide and in a lipid bilayer to be about 5–6 kJ/mole when compared to alanine. This difference is of the same order of magnitude when compared with previously obtained hydrophobicity values of these amino acids when, for example, the transition from water to a hydrophobic environment [4,13] or from water to the membrane interface [13] has been studied. It should be noted, however, that the changes in Gibb's free energy for the transfer of alanine or leucine from the membrane interface into octanol are reduced by approx. 30% when compared to water→octanol transitions [13].

When comparing the present results with those of previous studies it should be kept in mind that octanol represents the membrane interior only to first approximation. In addition, the dielectric properties quickly change within the interfacial region of the membrane [13,33]. Therefore the exact dielectric environment of individual amino acids and, therefore, the transfer energies strongly depend on the conformational details and the interfacial penetration depth of the peptide.

Whereas at all pH tested about 10 μM of the lysine-rich peptide magainin 2 (Fig. 2B), an antibiotic first isolated from frogs, is needed to lyse *Escherichia coli* cells in antibiotic assay, LAH_4 is about 1–2 orders more active at pH 5.5 [34]. In contrast, at pH 7.4 LAH_4 adopts transmembrane alignments and at the same time exhibits much reduced lytic activities [18,34]. This result indicates that in-plane oriented peptides are responsible for the antibiotic activities. Models that are based on the detergent-like properties of amphipathic peptides have, therefore, been suggested [7,11,35].

The insertion behaviour of $K_3A_{18}K_3$ has also been studied [47]. This peptide has been designed to test whether polyalanine inserts into the membrane in a transmembrane fashion as would be predicted by some of the currently used hydrophobicity tables [4]. On the other hand, more recent investigations suggest that alanine prefers to remain in the water phase due to considerable hydrophilic contributions of the peptide backbone [13,36]. Previously, both efficient membrane insertion as well as lack of such interactions have been observed experimentally with alanine-based peptide sequences such as $(SK)_2A_6WA_3(KS)_3$ and $A_{27}YK_6$, respectively [37,38]. When the interactions of $K_3A_{18}K_3$ with oriented membranes are tested by proton-decoupled ^{15}N solid-state NMR spectrosco-

py a mixture of in-plane and transmembrane helices is observed [47]. The highest transmembrane contribution of $K_3A_{18}K_3$ as observed in ^{15}N NMR spectra was about 70% [47]. This result indicates that the energy of transfer of one alanine from in-plane to transmembrane is close to ≥ -0.2 kJ/mole when averaged over 18 alanine residues within this α-helical peptide. Alanine, therefore, is a versatile amino acid found equally likely in aqueous and transmembrane helical domains. It also provides a point of reference for, to first approximation, inert behaviour during the in-plane to transmembrane transition. Replacing one or a few alanines by leucines results in a considerable increase in the stability of transmembrane alignments when this family of peptides is studied [38,47].

Whereas the ΔG^h value for alanine was taken from transfer experiments of $K_3A_{18}K_3$ (-0.2 kJ/mole; [47]), a value of -5.6 kJ/mole for leucine is correspondingly calculated (cf. above). Interestingly, comparison of these data with previously established values for water→oil transitions [5] suggests that the presence of hydrophilic contributions from the polypeptide backbone amounts to about 6–7 kJ/mole [13,36].

Lysines are abundant in many amphipathic helices exhibiting antibiotic and membrane-permeabilizing activities. Despite their high charge density lysine-containing helices have been suggested to form macroscopic structures including transmembrane helix orientations when active [35,39,40]. We have, therefore, investigated the topological equilibria of lysine-containing model peptides in some detail [41]. Interestingly, hydrophobic model peptides with one lysine adopt stable transmembrane alignments (Fig. 3A), indicating that hydrophobic interactions compensate efficiently for the large overall hydrophilic energy contributions associated with this basic amino acid ($pK \approx 10.5$). However, when three or more lysines are located on the hydrophilic face of an amphipathic helix (Fig. 2C) stable in-plane alignments are obtained (Fig. 3C). These latter peptides represent well the situation of the cecropin or magainin/PGS family of antibiotic peptides where four or more positively charged amino acids constitute the hydrophilic face of amphipathic α-helices (Fig. 2B; reviewed in [8]). When two lysines are placed in the central core of the otherwise hydrophobic sequence (Fig. 2D) an equilibrium between in-plane and transmembrane orientated peptides is observed (Fig. 3B), indicating that during the in-plane to transmembrane transition changes in hydrophobic, discharge, polar, and other interactions (cf. above and Fig. 3B) compensate each other [41]. These experimental solid-state NMR results are in good agreement with molecular modelling calculations where the alignment of monomeric peptides is tested in a slab of low dielectricity flanked by interfaces where the dielectric constant changes in a sigmoidal manner within 4.5 Å [41].

Additional investigations from this laboratory are aimed at a better understanding of the interactions between peptides and lipids when the length of the peptide does not match the thickness of the bilayer. We, therefore, synthesized a series of alanine-leucine or polyleucine peptides, which are flanked by lysine residues at both termini [16]. The hydrophobic core was chosen to be between 10 and 30 residues long. Our results indicate that within a wide range of hydrophobic mismatch conditions these peptides adopt transmembrane alignments. Only when the peptides are >14 Å too long or at least 3 Å too short are large deviations from transmembrane alignments observed.

At the same time, peptides that are too short do not disturb the phospholipid head group order. In contrast, a low degree of order is observed in proton-decoupled ^{31}P solid-state NMR spectra when the peptides exceed the tolerance for transmembrane alignments. This indicates that strong interactions between long hydrophobic sequences and the phospholipid bilayer cause pronounced macroscopic rearrangements.

A constant decrease in the ^{15}N chemical shift values is observed when the calculated hydrophobic mismatch is increased by up to 14 Å, indicating that adjustments to the bilayer thickness occur by increasing the helical tilt angle. Such a mechanism for adjustments is applicable for peptides that exceed the hydrophobic thickness of the bilayer, thereby explaining the pronounced asymmetry in hydrophobic mismatch tolerance. However, a quantitative analysis also indicates that the measured increase in tilt angle is insufficient to fully compensate for the calculated hydrophobic mismatch [16]. Therefore, other compensating mechanisms such as adjustments of the lipid order parameters or conformational changes of the peptides are equally active [15,42].

Peptides that are much shorter than the bilayer hydrophobic thickness predominantly adopt alignments parallel to the membrane surface [16]. Similarly, the uncharged 16 amino acid peptide antibiotic zervamicin II is too short to span POPC phospholipid membranes in a stable transmembrane fashion. This peptide, therefore, exhibits mostly in-plane alignments [29]. However, when the bilayer hydrophobic thickness is reduced to 1,2-dicapryl-*sn*-glycero-3-phosphocholine (di-C10:0-PC) transmembrane alignments of zervamicin

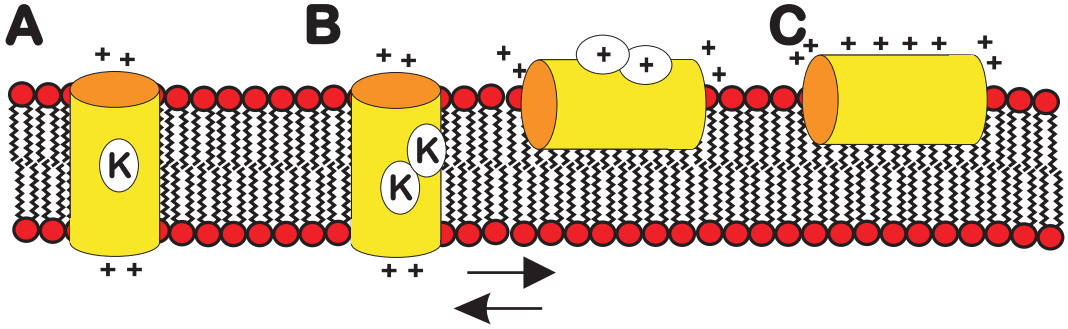

Fig. 3. Schematic representation of the alignments of A: LAK_1 (TM), B: LK_2 (TM↔IP), and C: LAK_4 (IP). The approximate positions of lysines are indicated by K when membrane inserted, and + when exposed to the aqueous environment. See text and [41] for details.

II are observed. In contrast, alamethicin, which is 20 amino acids long, adopts transmembrane alignments under all conditions tested [28,29]. The lengths of zervamicin II and alamethicin differ by four amino acids only. This corresponds to a calculated difference in length of ≤6 Å. At the same time the thickness of the lipid bilayers has to be decreased by more than 10 Å (from POPC to di-C10:0-PC) in order to accommodate zervamicin II in a stable transmembrane fashion [29]. Clearly other factors, such as conformational details in membrane environments or the length of the terminal anchoring sequences, contribute [43]. In addition, when compared to alamethicin the hydrophobic moment of zervamicin II is increased [44], thereby favouring in-plane orientations.

The studies presented in this review have helped to understand the interaction contributions which are important during the in-plane to transmembrane transitions. Not only is the knowledge of these interactions important for a quantitative evaluation of functional models of peptide activity [8], the study of model sequences [16] has also helped during the rational design of experimental conditions where the alignment of naturally occurring peptides shows the lowest mosaic spread [29]. Well-ordered samples are important for several structural techniques including oriented solid-state NMR spectroscopy [21,23]. Future work is required to improve our understanding of peptide–membrane interactions. Improved hydrophobicity scales taken from structured polypeptides and in true membrane environments will greatly improve membrane protein prediction algorithms. Furthermore, the effects on polypeptide structure and topology of transmembrane electric fields and peptide oligomerization need to be investigated further (recently reviewed in [45]). Ongoing studies are, therefore, aimed to also include these interactions in quantitative analyses [32,46].

Acknowledgements: The help of Josefine März, Martin Grimme, Susan Schinzel and Ingrid Neidhard during peptide synthesis and HPLC purification is gratefully acknowledged. I would also like to thank my co-workers and collaborators for their contributions and for interesting discussions. The Deutsche Forschungsgemeinschaft provided valuable financial contributions through Sonderforschungsbereich SFB266. I am grateful to Erich Sackmann for organizing this programme grant and for his willingness to accept my group as a partner.

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