Structural organization of the pentameric transmembrane $\alpha$-helices of phospholamban, a cardiac ion channel

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Communicated by J. Schlessinger

Phospholamban is a 52 amino acid calcium regulatory protein found as pentamers in cardiac SR membranes. The pentamers form through interactions between its transmembrane domains, and are stable in SDS. We have employed a saturation mutagenesis approach to study the detailed interactions between the transmembrane segments, using a chimeric protein construct in which staphylococcal nuclease (a monomeric soluble protein) is fused to the N-terminus of phospholamban. The chimera forms pentamers observable in SDS–PAGE, allowing the effects of mutations upon the oligomeric association to be determined by electrophoresis. The disruptive effects of amino acid substitutions in the transmembrane domain were classified as sensitive, moderately sensitive or insensitive. Residues of the same class lined up on faces of a 3.5 amino acids/turn helical projection, allowing the construction of a model of the interacting surfaces in which the helices are associated in a left-handed pentameric coiled-coil configuration. Molecular modeling simulations (to be described elsewhere in detail) confirm that the helices readily form a left-handed coiled-coil helical bundle and have yielded molecular models for the interacting surfaces, the best of which is identical to that predicted by the mutagenesis. Residues lining the pore show considerable structural sensitivity to mutation, indicating that care must be taken in interpreting the results of mutagenesis studies of channels. The cylindrical ion pore (minimal diameter of 2 Å) appears to be defined largely by hydrophobic residues (140, 143 and 147) with only two mildly polar elements contributed by sulfur residues C36 and M50.

Key words: Ca$^{2+}$ ATPase/calcium regulation/ion channels/membrane protein/protein structure

Introduction

Understanding the detailed structures of mammalian ion channels remains one of the most challenging problems in the field of structural biology. The large size of the proteins in question (often thousands of amino acids), the difficulty of overexpressing and purifying the channels in large amounts, and the inherent difficulties in handling membrane proteins have hampered traditional methods of structure determination, such as crystallography and NMR. The large disparity between the paucity of structural information and the wealth of enzymological and physiological information further motivates structural investigation. Simpler model systems have, therefore, been studied in an effort to gain insights on more complex proteins. The chosen model systems are normally small peptides of fungal origin, such as alamethicin and gramicidin; however, these model peptides have several shortcomings that limit the applicability and relevance of structural information derived from their study. Gramicidin is composed of alternating D and L amino acids, resulting in a unique secondary structure, the $\beta$-helix (Wallace and Ravikumar, 1988). Furthermore, it is presumed that ions traverse the membrane through the middle of the helix and not in an aqueous pore defined by several helices, as is thought to be the case for mammalian ion channels (Roux and Karplus, 1991). Alamethicin contains (amongst other amino acids) $\alpha$-aminoisobutyric acid, a non-chiral amino acid not found in mammalian ion channels, with a high propensity for helix formation (Karle and Balaram, 1990; Karle et al., 1990). In addition, the oligomeric size and the degree of membrane association of alamethicin are still a subject of some debate (Fox and Richards, 1982; Cascio and Wallace, 1988).

Crystallographic structures of these model peptides strongly indicate that transmembrane $\alpha$-helices play a major role in defining the structure of such ion channels. In addition, the classical voltage-gated Na$^+$ and Ca$^{2+}$ channels are predicted to contain four bundles of $\alpha$-helices (Catterall, 1991), while the K$^+$ channel is a homotetramer of proteins, where each protomer is thought to be an $\alpha$-helical bundle (Wann, 1993). The acetylcholine receptor was predicted to contain five bundles of helices, based on algorithms aimed at finding transmembrane $\alpha$-helical sequences (Engelman et al., 1986). However, Unwin and co-workers (Unwin, 1993) have recently reported that they were able to identify only the five pore-lining $\alpha$-helices using electron microscope image analysis, and suggested that other transmembrane structures might be found in that protein.

We report here studies of interactions between transmembrane helices of phospholamban, a small channel-forming membrane protein native to human cardiac sarcoplasmic reticulum. Its small size (52 amino acids containing a single transmembrane $\alpha$-helix), defined oligomeric state [pentameric in SDS–PAGE (Kovacs et al., 1988)] and observed selective conductance (Kovacs et al., 1988; Arkin et al., 1993) make it an ideal system for investigation of the detailed interactions between the five single transmembrane $\alpha$-helices that form an ion pore. Phospholamban’s presumed function is the inhibition
(reversible upon phosphorylation) of the SR-resident Ca^{2+}-
ATPase (Kirchberger et al., 1975). Inhibitory association of phospholamban with the Ca^{2+} pump and disassociation upon phosphorylation is one mechanism of phospholamban function (James et al., 1989). However, it has also been shown that phospholamban itself is a Ca^{2+}-selective channel (Kovacs et al., 1988; Arkin et al., 1993), suggesting additional regulation through the collapse of the Ca^{2+} gradient.

Structurally, phospholamban is a type II non-covalent homopentameric membrane protein that reversibly dissociates upon boiling, as determined by SDS-PAGE (Simmerman et al., 1989). It consists of two regions: residues 1–28 comprise the cytosolic, phosphorylatable portion, while residues 29–52 are presumed to contain the bilayer-spanning α-helix (Simmerman et al., 1989). The putative transmembrane domain responsible for both the pentamerization and the channel properties of the molecule (Kovacs et al., 1988) is composed of bulky hydrophobic amino acids and three cysteines that are not disulfide bonded. Fujii et al. (1989) have shown that substitution of these cysteine residues with alanine or serine does not abolish pentamer formation, although these substitutions do result in a decrease in the temperature that is needed to disrupt the pentamers. CD spectroscopy of phospholamban in detergents has shown that the protein is mostly α-helical under the conditions studied (Simmerman et al., 1989).

In this study, we have used the chimeric protein approach developed by Lemmon et al. (1992a) for the study of transmembrane α-helix association. Fusion of the N-terminus of phospholamban to the C-terminus of staphylococcal nuclease (via a short linker region) resulted in a chimera that oligomerizes on SDS-PAGE, as does native phospholamban. We have extensively mutated residues 35–52, corresponding to the transmembrane domain, and have determined the oligomeric state of each mutant. Residues exhibit differential roles in pentamer stability and were divided into three categories: sensitive, moderately sensitive and insensitive. The results reveal a pattern of sensitivity that leads to a structural model of a five-membered coiled-coil helical bundle for the pore-forming segment of the molecule. In addition, search protocols employing molecular dynamics calculations were undertaken (details described elsewhere; Adams et al., 1994) in an effort to obtain a detailed structural model for the protein. A molecular model is presented that is consistent with the results of the mutagenesis.

Results

Chimeric protein construction

In order to study the oligomerization properties of the putative transmembrane region of phospholamban, a chimeric protein was constructed in which phospholamban is fused to the C-terminus of staphylococcal nuclease, which also contains a PhoA signal sequence (Lemmon et al., 1992a). The resulting protein was under the control of the PhoA promoter, inducible to low levels of expression upon phosphate starvation. A Western blot of solubilized whole cells expressing the chimeric proteins (Figure 2A) shows that the chimera oligomerizes in SDS-PAGE. The apparent molecular weights of the observed bands confirm that the chimeric protein pentamerizes in SDS gels like wild-type phospholamban. Furthermore, the pentamerization is not affected by reducing agents [concentrations of dithiothreitol (DTT) or β-mercaptoethanol (βME) as high as 0.5 M].

Mutagenesis

Each of 110 non-redundant mutants in the hydrophobic transmembrane domain was screened by Western blotting for SDS-PAGE oligomerization state and sequenced. A selected example of electrophoretic mutant phenotypes is shown in Figure 2B. The mutants obtained were classified into two groups: group 1, containing substitutions to strongly polar amino acids (D, E, H, K, N, Q and R) and P (data not shown), and group 2 (97 mutations), containing all other amino acids (A, C, F, G, I, L, M, S, T and V) (Table 1). Only three substitutions with W and Y were found, and thus these were not included in the interpretation. Diverse effects of mutations were seen throughout
the transmembrane helix. Disruptive changes are not limited to a short stretch of amino acids or to a specific type of amino acid substitution. There are locations in the helix that were able to accept any amino acid substitution in group 2 (e.g. I38) with no effect on pentamer formation, while other residues exhibited extreme sensitivity and were not able to accept any of the observed substitutions without disruption of pentamerization (e.g. I40). Some residues exhibited moderate sensitivity (e.g. L43) and were able to accept some substitutions (e.g. L43F), while other substitutions either abolished pentamerization (e.g. L43T) or reduced it (e.g. L43V). The specific pattern resulting from the mutagenesis will be addressed in the Discussion.

**Strongly polar amino acids disrupt pentamerization**

Every substitution to a highly polar amino acid (or P), irrespective of its position in the transmembrane domain, resulted in complete disruption of electrophoretically observed pentamer formation (with the exception of I38R, see Discussion). Since the nature of such disruptions does not yield any structural information (see Discussion), we based our interpretation on amino acids in group 2.

**Insensitive residues**

Residues F35, I38, L42, I45, I48, M50 and L52 are assigned as insensitive since they will accept any group 2 substitution and retain wild-type pentamer formation. This insensitivity is independent of the size of the substituted residue since, for example, I38 will accept both smaller residues (A and G) and larger ones (F). Furthermore, insertion of slightly polar amino acids (S) into the bilayer at these positions does not affect pentamer formation.

**Sensitive residues**

Residues L37, I40, L44 and I47 are termed sensitive, since every substitution of these residues resulted in pentamer dissociation. The degree of specificity is striking, e.g. I40 is not able to accept its isomer leucine and retain pentamer formation, and removal of a methylene in the case of I47V results in pentamer disruption.

**Moderately sensitive residues**

The remaining residues (C36, L39, C41, L43, C46, V49 and L51) exhibit differential effects as a function of the substituting residue. Some amino acid substitutions do not reduce pentamer formation, while other residues may partially or fully disrupt pentamer formation. Possible correlations between the type of substituting amino acid and the observed phenotype will be addressed in the Discussion.

**Modeling**

Detailed results of the modeling process will be described elsewhere. Briefly, the dimer searches yielded several defined clusters of structures when starting from left-handed coiled-coil tilt angles. Right-handed structures were not found nearly as frequently, clearly favoring a left-handed coiled-coil conformation. Several of the obtained structures were found to be pentameric or nearly pentameric when propagated upon superposition of the dimer monomers. Left-handed coiled-coil pentamer searches yielded five clusters of structures. The cluster of models arrived at most frequently also retained the best 5-fold symmetry. This model coincided with the mutagenesis results. In the center of the structure is a cylindrical pore extending the entire length of the helical bundle. The pore is mostly lined by aliphatic side chains with a minimal diameter of 2 Å.

**Discussion**

**The chimeric protein as a model system**

Our objective is to define structural relationships between the transmembrane domains of the pentameric phospholamban ion channel. In an earlier study, we studied helix–helix interaction by fusing the dimerizing transmembrane domain of human glycoporphin A (Lemmon et al., 1992a,b) to the C-terminus of staphyloccocal nuclease (a monomeric soluble protein). In this study, we have used the same approach to study the pentamerization of the putative transmembrane domain of human phospholamban. When the entire phospholamban protein is fused to the C-terminus of staphyloccocal nuclease, the resulting chimeric protein self-associates to form oligomers in SDS–PAGE, including strong pentamer and progressively weaker tetramer, trimer and dimer bands (Figure 2A), while wild-type phospholamban migrates mainly in pentamer and monomer forms. This difference may be attributed to somewhat reduced stability of the chimeric protein pentamer, due in part to the steric clashes between the large extra-membranous domains. This apparent decreased stability of the pentamer of chimeric protein aids in distinguishing finer nuances separating the stability of the different mutants.

The pentameric behavior of the chimeric protein is not surprising considering the fact that phospholamban pentamerization is thought to be largely driven by interactions between the transmembrane domains, which retain oligomeric behavior when cleaved from the full-length protein (Kovacs et al., 1988). Native phospholamban can then be considered as a two-part assembly of the pentamerizing domain containing the transmembrane α-helix, and a separate phosphorylatable cytosolic region. Thus, it is reasonable to suppose that the pentamerization of the transmembrane domain of the chimeric protein is similar to that of native phospholamban, and that the phenotypes seen in our mutational study would have corresponding effects in native phospholamban. Supporting this statement is the correlation between the effects of mutations constructed by Fuji et al. (1989) in native phospholamban and mutations in the chimeric protein. Reduced pentamer formation in our study correlated with an increased heat lability in native phospholamban (C41S), as did complete disruption of pentamerization of the chimeric construct (C36S). Mutations found to disrupt pentamer formation in native phospholamban also did so in the chimeric construct (C41F).

**Conservative versus strongly polar substitutions**

The two-stage model for the folding of α-helical integral membrane proteins posits that individually stable monomeric α-helices associate to form folded polytopic proteins or higher-order oligomers (Popot and Engelman, 1990).
The final assembly can be disrupted by mutations that affect either the stability of the monomeric transmembrane helices or the association of helices. The mutations that would affect association between the helices are those that generate information regarding the packing interactions of pore-forming transmembrane α-helices.

The first four amino acids to be mutated in the course of the study, L37, I38, L39 and I40, were mutated with polymerase chain reaction (PCR) primers containing totally random nucleotides at the respective site, resulting in substitutions of the whole spectrum of amino acids. Irrespective of the site of substitution, highly polar (or proline) substitutions abolished pentamer formation. Highly polar substitution may destabilize the helix due to interactions with the charged micelle surface, while proline substitutions reduce the overall helix stability. The exception to this finding was the mutant I38R. The ability to insert a positive charge at this highly insensitive position, while retaining pentamer formation, may be attributed to the proximity of this residue to the end of the transmembrane domain. This would allow ionic interactions between the guanidino side chain and the negatively charged head group region. The lack of disruption by I38R is not indicative of a general tolerance to charged residues at that site, as both I38H and I38D disrupted the pentamer.

That these substitutions occurred in four consecutive residues, encompassing more than a single α-helical turn, indicated a lack of specificity in affecting the helix–helix interactions. Such non-specific effects from the incorporation of strongly polar amino acids (and proline) in a transmembrane α-helix were previously observed in a study of the dimerization of glycoporphin A (Lemmon et al., 1992b).

**Conservative substitutions**

The ability of conservative substitutions to abolish pentamerization indicates specificity in the pentamer interaction. Conservative substitutions most likely do not affect the stability of the protein monomer (Lemmon et al., 1992a), but do affect the detailed interactions in complex formation via helix–helix interactions (stage II). It is likely that such residues are located in the helical interface that may be forming the specific packing interactions required for pentamerization (Lemmon et al., 1994).

As discussed previously, three groups of residues were defined with respect to their ability to retain pentamer formation based on the averaged effects of substitutions:

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**Fig. 3. Mutagenesis table.** A table summarizing the effects of conservative substitutions (group 2, see the text) on the oligomeric state of the chimeric protein. Oligomerization properties of point mutants of the phospholamban transmembrane domain by SDS–PAGE/Western blot analysis.

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**Fig. 4. Helical wheel projections.** Helical wheel projections at three different pitches: 3.6 (canonical helix); 3.9 (right-handed coiled-coil) and 3.5 (left-handed coiled-coil) amino acids/turn of the transmembrane domain of human phospholamban. ● indicates sensitive residues, ○ indicates moderately sensitive residues and ◦ indicates insensitive residues as defined by the mutagenesis. The presumed interaction surface is noted on the 3.5 amino acids/turn projection.
sensitive (abolish pentamer), insensitive (retain pentamer) and moderately sensitive (reduced pentamer) residues (Figure 3). Helical wheel diagrams at three different pitches, 3.6 (canonical helix), 3.5 (left-handed coiled-coil) and 3.9 (right-handed coiled-coil), indicated that, in the left-handed coiled-coil configuration, substitutions of each type aligned on faces of the helix. This result lends itself to defining an interaction surface between the helices involving four contact points, as shown in Figure 4.

Specific examination of the differential effects of substitutions upon pentamer formation in the moderately sensitive residues may yield structural information. For example, substituting V49 to smaller (A) or larger residues (F) results in reduced pentamer formation. However, residues closer to it in size (S and I) do not affect pentamerization.

**Modeling**

Molecular dynamics simulations similar to those previously used (Treutlein et al., 1992; Lemmon et al., 1994) were employed in an effort to construct a model for the transmembrane domain of phospholamban. Dimer searches and pentamer calculations resulted in left-handed coiled-coils much more frequently than right-handed coiled-coils. This is consistent with the correlations seen using a 3.5 amino acids/turn helical wheel diagram suggested from the mutagenesis. The two independent modeling strategies yielded several converging clusters of structures. It is encouraging that the pentamer model arrived at most frequently is concordant with the model predicted by the mutagenesis (Figure 5). Two inconsistencies between the modeling and the mutagenesis results were observed. Firstly, the modeling predicted a high energy of interaction for L43, a residue that was found to be only moderately sensitive by mutagenesis and, secondly, the last five amino acids of the protein (not found to be very sensitive towards substitutions) were assigned a high energy of interaction by the modeling process. This finding may be attributed to the nature of modeling in vacuo, not taking into account van der Waals or electrostatic interactions with the solvent or lipid/detergent yielding a compact model. An α-carbon molecular model of the pentamer predicted by both the mutagenesis and the modeling is presented in Figure 6.

**The ion pore**

One of the most striking results of this study is the finding that, in our model, the ion pore is lined almost entirely by hydrophobic residues. The only polar regions of the pore are the sulfhydryl side chains of C36 and the sulfurs of M50 and, in fact, the pore is most constricted by the three large side chains of I40, L43 and I47 (Figure 7). These residues, in fact, form inter-monomer contacts amongst adjacent helices. This finding contradicts the widely held notion that the ions must pass through the bilayer in a pore lined with hydrophilic groups (side or main chain). This preconception is based on the unfavorable energy required to place a charged ion in a hydrophobic environment. However, if the ion pore were lined by charges, the traversing ions might bind tightly to complementary charges and inhibit ion flow. Furthermore, the formation of hydrophobic channels appears possible on energetic grounds (Popot and Engelman, 1990). The phospholamban channel described here could not be occupied by the lipids and would not act as an ion binding site. It would, however, allow ions to traverse the bilayer according to their electrochemical gradient. Further evidence to support this is found in the crystal structure of...
the Trp synthase (Hyde et al., 1988). In this protein, a largely hydrophobic channel connects two parts of the complex to allow faster transit of intermediates from one catalytic site to another. The fact that no substrates were found inside this channel in the crystal structure may imply that no significant energy minimum is present.

Another intriguing finding of this study is that we were not able to distinguish between residues that define the pore and residues that contribute to the stability of the complex. Indeed, the residues which line the narrowest region of the pore in the model (L40, L43 and L47) show considerable sensitivity to substitution. While this sensitivity can be explained by the tight packing interactions amongst these residues, the fact that residues can be involved in both the stability of the pentamer and the definition of the pore points to the inherent difficulty in assessing the effects of mutations on the conductivity of channels. It is our expectation that the effect of the disruptive mutations identified here will be to abolish phospholamban conductivity by dissociating the pentamer. Non-disruptive mutations, on the other-hand, might alter conductivity by occluding or enlarging the pore, or by specific side chain/ion interactions, such as hydrogen bonding, thus yielding information about the specificity of the channel–ion interaction.

This result calls into question the interpretation of mutagenesis studies of channels where correlations between changes in ionic conductivity and residue type are made (reviewed in Catterall, 1991). In these studies, it is assumed that the change in the measured conductance is due only to the localized effect on the pore in the vicinity of the mutated residue. Clearly, in the phospholamban system, a number of disruptive mutations (which would almost certainly abolish conductivity) at sites that
do not line the pore could mislead in such a study. The effects of the two conservative, disruptive mutations C41L or L39V (Figure 2B) in isolation would argue that the respective sites lined the pore, but these residues are on opposite sides of a helix (Figure 5). One model that would allow both these residues to line the pore is a β-structure, a possibility that can be readily eliminated by considering more data points.

It would be difficult to correlate the minimal diameter of the phospholamban pore, as found in the model, with that calculated by the resulting conductance of the channel due to the fact that the simulations were done in vacuo. Further, the model may represent the closed state of the channel. However, it is interesting to note that the minimal diameter of the pore, 2 Å, is just slightly larger than the diameter of a dehydrated calcium ion (1.98 Å), as seen in Figure 8.

Conclusions

Extensive mutagenesis studies of the pentameric association of the phospholamban ion channel transmembrane α-helices have revealed differential sensitivity towards substitutions. Of 18 transmembrane residues, four show absolute sensitivity and seven show partial sensitivity to substitution. Residues of similar sensitivity lined up on the same side of a 3.5 amino acids/turn helical projection. The character of the circumferential distribution of effects suggests that the transmembrane helices form a five-
membered left-handed coiled-coil. This result is also obtained by modeling the helix interaction using molecular dynamics simulations. Residues lining the pore exhibited sensitivity towards substitution, alluding to their role in the structural stability of the complex. The model predicts that residues L37, I40, L44, I47 and L51, which comprise two sides of the helical projection, interact with the two sides of the projection containing residues C36, L39, L43, C46 and M50. The proposed ion pore appears to be composed of entirely hydrophobic residues, with the exception of the two sulfur of M50 and C36. The 2.0 Å minimal diameter of the pore is defined by three hydrophobic residues (I40, L43 and I47). The fact that these residues are highly sensitive to substitution indicates that the determinants of channel specificity and of structural stability may not be separable.

Materials and methods

Materials and general procedures
All restriction endonucleases were purchased from New England Bio Labs, T4-DNA ligase was purchased from New England Bio Labs or from Boehringer Mannheim. Taq DNA polymerase was purchased from Perkin Elmer Cetus or from Boehringer Mannheim. Alkaline phosphatase was purchased from Boehringer Mannheim. Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Research Organics. Double-stranded sequencing was performed using the Sequenase kit (US Biochemicals) and [γ-32P]ATP. PCR amplifications were performed in the following manner and employed Taq DNA polymerase: four cycles of annealing at 50°C, followed by 30 cycles of annealing at 60°C, strand denaturation at 94°C and chain elongation at 72°C. All other molecular biological manipulations were performed according to standard protocols (Sambrook et al., 1989).

Electrophoresis and Western blotting
SDS–PAGE was performed using homogeneous 12.5% Phast gels using the Phastsystem (Pharmacia). Gels were run at 16°C. Protein sample buffer contained at least 0.1 M DTT as reducing agent. SDS–PAGE of solubilized whole cells was followed by Western blotting using affinity-purified rabbit anti-nuclease antibodies (kindly provided by Dr B.J. Borman, Boehringer-Ingehelm, Inc.). Blots were developed either using Bio-Rad goat anti-rabbit alkaline phosphatase conjugate with the NBT/BCIP color reaction, or with the ECL chemiluminescence kit (Amersham), using a goat anti-rabbit–horseradish peroxidase (HRP) conjugate as secondary antibody.

Construction of phospholamban–staphylococcal nuclease chimera

The sequence of the first and last 15 nucleotides of the human phospholamban coding region (kindly provided by Dr T.Scott) was used to design PCR primers with additional restriction sites to facilitate cloning. The 5′ oligo (N-terminal) was TACAGCTTATGCCCACATTTGTGCTCCATGGAGAGATGAGTATATCAGAGGAGCAGT, which contains a BamHI and NcoI sites. The 3′ oligo (C-terminal) was ATCTGGAGGTTGGATCTTATATTCCAGAGGAGCAGT, which contains a BamHI site. PCR amplification. Using 0.1 μg of human placenta DNA (kindly provided by Dr G.Mitra) as template resulted in a single product of the anticipated size. This PCR product was ligated into the TA cloning vector (Invitrogen), and the resulting vector was digested with Apal and BamHI restriction endonucleases to release the inserted fragment with cohesive ends. The derived Apal–BamHI fragment was gel purified and ligated into the vector pSN1GpA (Lemmon et al., 1992a) appropriately restricted. In the resulting construct, the N-terminus of phospholamban is linked to the C-terminus of staphylococcal nuclease Q149P, a monomeric soluble protein containing a PhoA signal sequence. The linking sequence is shown in Figure 1. Correct construction of the vector was confirmed by sequencing, and the human phospholamban coding region was found to be identical to that published previously (Fujii et al., 1991). To facilitate cloning, an Ascl site was inserted in some vectors at residue 69 of the phospholamban coding region by the double-primer PCR method (McPherson et al., 1991). This change in the nucleotide sequence did not cause any alteration in the amino acid sequence.

Mutagenesis

Amino acids F35—L44 (numbered according to their position in native phospholamban) were mutated using two rounds of PCR. Mutagenic PCR primers (non-coding strand) were designed to extend from the codon corresponding to residue M50 to 15 bases 3′ of the desired mutation site. PCR amplification was performed with the mutagenic oligonucleotide (C-terminal) and a primer located in the N-terminal region of nuclease. The resulting product was used as a template for a second PCR amplification employing the same nuclease N-terminal primer with the phospholamban C-terminal primer (listed above) which overlaps the mutagenic oligonucleotide. The resulting PCR product was restricted with HindIII (located at residue 110 of nuclease) and BamHI to be subsequently ligated into the appropriately restricted and phosphatased pSN1GpA vector.

Amino acids I45—L52 were mutated in a single round of PCR amplification, C-terminal primers (non-coding strand) that extended from the BamHI site to 12 nucleotides 3′ of the mutated codon and the nuclease N-terminal primer described above yielded PCR products that were restricted and ligated as above.

Amino acids L37—I40 were mutated using completely random codons, while all other residues employed semi-random codons at the position of desired mutation (disallowing C and A in the first and second site of the mutated triplet, respectively). This procedure limited the possible resulting amino acid substitutions to F, L, S, C, W, I, M, T, R, V, A and G.

Screening of mutants

Screening was performed in a manner identical to that described by Lemmon et al. (1992b). Briefly, mutagenic plasmids were transformed into DH5α Escherichia coli (Gibco BRL). Single colonies were used as inocula for 4 ml Luria broth (200 μg/ml ampicillin) and were grown overnight. Then 5 μl of each growth were used as inoculum for 1 ml glucose–MOPS medium (Neidhardt et al., 1974) under limiting phosphate concentrations (0.1 mM), while the rest of the growth was used for plasmid extraction and DNA sequencing. Glucose–MOPS growths were allowed to reach saturation, causing induction of the PhoA promoter due to phosphate starvation, and were harvested after 10-14 h. The cultures were centrifuged and resuspended in 100 μl of protein sample buffer for subsequent electrophoresis. Detection of chimeric protein was achieved with Western blotting as described above. The mutated chimeras were classified into three groups: a pentamerizing mutation was defined as one that resulted in wild-type levels of pentamer, a disrupting mutation was defined as one that resulted in the detection of no pentamer band, and a reduced pentamer mutation was defined as one that resulted in an increased proportion of monomer (relative to wild type). For a subset of the mutants that had initially yielded poor quality Western blots, the sequenced plasmid was transformed into DH5α bacteria and single colonies were used as direct inocula for glucose–MOPS medium under limiting phosphate concentrations. Growth was harvested after 24–48 h and processed as described above.

Modeling

The modeling procedures employed will be described in detail elsewhere (Adams et al., 1994). Two conceptual approaches were used: one method attempts to identify the most favored structure by extensively exploring the interaction of two monomers, while the other examines the interactions in a set of symmetric pentamers.

In the first approach, an array of starting structures was computationally generated in which two helices were associated with one another. These differed only in the rotational orientation (rotational increments of 45°) of each of the helices about its long axis. Two different tilt angles between the helices were modeled (±50°), leading to a total of 128 different starting structures. Each of these starting structures was energy minimized and then subjected to molecular dynamics protocols using four different sets of initial velocities, followed by a final energy minimization. Favorable final structures were those which were observed to occur multiple times; the goal of this search protocol was to examine the width as well as the depth of the energy minima. The feasibility of pentamer formation based on a given dimer was tested by propagating the dimeric relationship into an oligomer, asking whether it produced a satisfactory pentamer. In the second approach, a symmetrical pentamer of canonical helices in a left-handed coiled-coil configuration was modeled, varying the degree of rotation of each pentamer simultaneously. The same methodology of molecular dynamics was then employed, resulting in several clusters of structures. The degree of pentamer symmetry was assessed for each final structure.

Transmembrane α-helices of phospholamban
Acknowledgements

The authors thank the Macnab laboratory for helpful advice and use of equipment. Helpful discussions with members of the Smith and Engelman laboratories are acknowledged. This work was supported by grants from the National Institutes of Health (5P01-GM39546), National Science Foundation (DMB8805587), funds from Boehringer Ingelheim Inc. and the National Foundation for Cancer Research to D.M.E., and by a grant from the National Science Foundation (ASC 93-181159) to A.T.B. M.A.L. was a recipient of a predoctoral fellowship from the Howard Hughes Medical Institute.

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Received on May 25, 1994; revised on July 18, 1994