A Leucine Zipper Stabilizes the Pentameric Membrane Domain of Phospholamban and Forms a Coiled-coil Pore Structure*

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Phospholamban is a phosphoprotein regulator of cardiac sarcoplasmic reticulum which is phosphorylated in response to β-adrenergic stimulation. Previous results have shown that phospholamban forms Ca$^{2+}$-selective channels in lipid bilayers. The channel-forming domain has been localized to amino acid residues 26–52, which form a stable pentameric, helical structure. The specific residues responsible for stabilizing the pentameric membrane domain of phospholamban have been identified by mutational analysis. Residues 26–52 were individually mutated to Ala or Phe, and the ability of the resulting mutant to form a pentamer or other oligomer was assessed by SDS-polyacrylamide gel electrophoresis analysis. Replacement of Leu$^{27}$, Ile$^{40}$, Leu$^{44}$, Ile$^{47}$, or Leu$^{51}$ by Ala prevented pentamer formation, indicating their essential involvement in the oligomeric assembly. The heptad repeats, and 3-4-residue spacing of the essential amino acids suggest that residues 37–52 adopt a pentameric coiled-coil structure stabilized by a leucine zipper motif formed by the close packing of Leu$^{27}$, Ile$^{40}$, Leu$^{44}$, Ile$^{47}$, and Leu$^{51}$. The resulting symmetric structure contains a central pore defined by the hydrophobic surface of the five stabilizing leucine zippers, which are oriented to the interior and form the backbone of the pentamer.

PLB$^1$ is a small oligomeric phosphoprotein of cardiac SR that regulates Ca$^{2+}$ transport across this intracellular membrane organelle (Tada and Inui, 1983; Sham et al., 1991). Phosphorylation of PLB activates the Ca$^{2+}$ pump of SR and increases Ca$^{2+}$ uptake (Lindemann et al., 1983) by a mechanism that remains to be completely understood (J. Ames et al., 1989; Cantilina et al., 1993; Colyer, 1993; Toyofuku et al., 1994; Voss et al., 1994). Structural analysis of PLB has been instrumental in the discovery of its activity as a channel protein (Kvakcs et al., 1988), but the physiological relevance of this activity to Ca$^{2+}$ sequestration is not yet clear (Reddy et al., 1995).

Following the development of a method to purify PLB to homogeneity from SR vesicles, analysis by SDS-PAGE suggested that PLB was a pentamer of identical 5-6-kDa subunits (Wegener and J. ones, 1984; J. ones et al., 1985) and further indicated that each subunit was dually phosphorylated by cAMP- and Ca$^{2+}$/calmodulin-dependent protein kinases (Simmerman et al., 1986; Wegener et al., 1986). Peptide mapping studies showed that each subunit contained two domains: a cytosolic, hydrophilic domain incorporating the two phosphorylation sites and a hydrophobic domain responsible for the oligomeric subunit interactions (Wegener et al., 1986). Sequence analysis of PLB confirmed that it is a noncovalent oligomer of identical subunits (Simmerman et al., 1986), each containing 52 amino acids (Fujii et al., 1987). The amino-terminal hydrophilic domain contains the residues serine 16 and threonine 17 phosphorylated by cAMP- and Ca$^{2+}$/calmodulin-dependent kinases, respectively (Simmerman et al., 1986), whereas the hydrophobic domain is located within residues 26–52. Empirical analysis of the carboxyl-terminal residues 26–52 suggested that they could form an amphipathic helix sufficiently long to traverse the SR membrane and that five such helices could assemble to a pentameric pore-forming structure (Simmerman et al., 1986). Single channel recording experiments have demonstrated that PLB in planar bilayers does in fact exhibit voltage-regulated Ca$^{2+}$ channel activity (Kvakcs et al., 1988). Although the primary structure of PLB suggests that the membrane-embedded region of the protein is localized to residues 26–52, the role of specific residues in this domain to the oligomeric stability and channel activity is largely unknown. Solubilized preparations of residues 26–52 have been shown by circular dichroism spectroscopy to adopt a predominantly helical configuration (Simmerman et al., 1989). Residues 26–52 incorporated as pentamers into phospholipid bilayers are also mainly helical (Tatulian et al., 1995). Further questions remain regarding the arrangement, orientation, and topography of the native membrane-spanning domain of PLB in SR vesicles.

To probe the structure and stabilizing interactions of the membrane-spanning channel domain of PLB, mutational analysis has been implemented. Mutagenesis has been used successfully to determine the sequence specificity of interacting helices in glycoporphin A (Lemmon et al., 1992a, 1992b), heat shock factor (Rabindran et al., 1993), and the GCN4 DNA-binding domain (van Heeckeren et al., 1992; Harbury et al., 1993). It is assumed that replacement of a residue with Ala, which results in disruption of the PLB pentameric assembly, indicates the essential role of that residue in the stabilization of the quaternary structure. Such has been found the case for a de novo synthetic approach to examining the stability of two-stranded coiled-coils (Zhou et al., 1992). The results of PLB mutagenesis indicate that a leucine zipper stabilizes the pentameric membrane domain of PLB and forms a coiled-coil pore structure. A preliminary account of this work appeared earlier in abstract form (Simmerman et al., 1994).
In vitro translation of wild-type PLB and PLB mutated in alanine residues. cRNA encoding wild-type (WT) PLB and PLB with single alanine mutations (listed at top of figure) was in vitro translated in the presence of [35S]methionine. After 24 h at room temperature, SDS sample buffer was added and electrophoresis was conducted using a 10% polyacrylamide gel (Porzio and Pearson, 1977). The resulting autoradiograph is shown. Pentamer and Monomer denote the pentameric and monomeric mobility forms of PLB, respectively. The diffuse area of radioactivity visible above the PLB monomer band contained PLB dimers, which were poorly resolved in this gel, but were identified clearly with use of a 7–18% gradient gel (data not shown). *Boil indicates whether WT samples were boiled in SDS prior to PAGE. No RNA is a control showing that negligible [35S]methionine incorporation occurred when PLB cRNA was omitted from the translation mix. After autoradiography, the dried gel was placed in a GS-250 Molecular Imager (Bio-Rad), and the amount of recombinant PLB in each lane was quantified. Background radioactivity in the No RNA lane was deducted for each determination. Eight replicate experiments of this type were performed, and the averaged results for the percentages of PLB pentamer formation for each mutation are listed in Table I. Molecular weight standards (× 10^3) are shown on the left.

(5942) Residues Stabilizing the Phospholamban Pentamer

(Krieg and Melton, 1984). Base pairs -3 through +159 of canine cardiac PLB cDNA (Reddy et al., 1995) were amplified by polymerase chain reaction using primers with flanking BglII sites and four extra nucleotides at the 5' ends. The 176 base pair product was digested with BglII, electrophoresed from a 1% agarose gel, and ligated into the BglII site of pSP64T. For mutagenesis, the coding region was subcloned into the pSP64T. For mutagenesis, the coding region was subcloned into the pAlter-1™ vector using HincII and four extra nucleotides. Site-directed mutagenesis from pAlter-1™ was conducted with an Altered Sites II Mutagenesis System™ (Promega) according to the manufacturer’s instructions. Site-directed mutagenesis from pAlter-1™ was conducted with an Altered Sites II Mutagenesis System™ (Promega) according to the manufacturer’s instructions. After pentamer formation was approximately 50% of the total protein synthesized, and the characteristic dissociation of pentamers by boiling in SDS (Wegener and Jones, 1984) was retained. After pentamers, monomers were the next most stable species detected, followed by dimers.

RESULTS

In Vitro Translation of PLB—in agreement with the earlier work of Cook et al. (1989), we observed that PLB monomers synthesized in a rabbit reticulocyte lysate spontaneously associated to form pentamers, as assessed by SDS-PAGE analysis (Fig. 1, WT). The percentage pentamer formation was approximately 50% of the total protein synthesized, and the characteristic dissociation of pentamers by boiling in SDS (Wegener and Jones, 1984) was retained. After pentamers, monomers were the next most stable species detected, followed by dimers.

The same oligomeric forms of PLB were observed previously in cardiac SR vesicles (Jones et al., 1985), suggesting that recombinant PLB behaved in similar fashion to the natural protein. Interestingly, we observed that when translates were analyzed by SDS-PAGE immediately after reactions were terminated, only approximately 25% of the PLB monomers had associated into pentamers. Incubating the recombinant monomers for 24 h at room temperature prior to SDS-PAGE increased the proportion of pentamers to approximately 50%, the maximal value obtained. This observation suggested that pentamer formation occurred prior to addition of SDS for PAGE. Inclusion of pancreatic microsomes in the translation system did not affect the extent of pentamer formation (data not shown). However, as pointed out by Cook et al. (1989), rabbit reticulocyte lysates contain some membranes, so the data do not distinguish whether membranes are required for pentamer formation.

Alanine Mutations—Residues 26 through 52 of PLB were individually changed to Ala by in vitro translation of the mutated cRNAs and each mutated protein was then analyzed by SDS-PAGE (Fig. 1 and Table I). Most of the mutated proteins retained the ability to form pentamers, with the pentameric mobility form contributing 25% or greater of the total protein synthesized. The notable exceptions occurred at Leu residues 37, 44, and 51 and Ile residues 40 and 47, occupying positions a and d of a heptad repeat pattern of residues a-g beginning at Leu37. Changing any of these amino acids to Ala prevented pentamer formation (Table I). Replacement of Phe12 with Ala also prevented pentameric assembly, although this residue does not belong to the repeating heptad pattern of Leu and Ile.
The percentage pentamer formation by each mutant was quantified using a GS-250 Molecular Imager (Bio-Rad) as described in the legend to Fig. 1. Percentage values listed for alanine and phenylalanine mutants are the averages ± S.D. from eight and five separate in vitro translation experiments, respectively.

| Residue mutated | Heptad position | Percentage pentamer formation |
|-----------------|-----------------|------------------------------|
| None            |                 | 47.5 ± 5.6                   |
| Gln126          |                 | 47.5 ± 5.1                   |
| Asn27           |                 | 45.3 ± 7.1                   |
| Leu28           |                 | 48.8 ± 6.8                   |
| Gln29           |                 | 42.5 ± 6.1                   |
| Asn30           |                 | 49.7 ± 3.9                   |
| Leu31           |                 | 43.7 ± 4.1                   |
| Phe32           |                 | 11.2 ± 10.3                  |
| Ile33           |                 | 35.5 ± 6.1                   |
| Asn34           |                 | 32.4 ± 5.8                   |
| Phe35           |                 | 44.0 ± 5.6                   |
| Cys36           |                 | 40.3 ± 9.2                   |
| Leu37           | a               | 0.7 ± 1.1                    |
| Ile38           | b               | 438.4 ± 4.9                  |
| Leu39           | c               | 273.7 ± 5.9                  |
| Ile40           | d               | 1.6 ± 1.5                    |
| Cys41           | f               | 28.3 ± 9.6                   |
| Leu42           | g               | 44.6 ± 7.0                   |
| Leu43           | h               | 34.8 ± 6.0                   |
| Leu44           | i               | 25.2 ± 5.0                   |
| Ile45           | j               | 432.5 ± 5.1                  |
| Cys46           | k               | 42.1 ± 5.4                   |
| Ile47           | l               | 3.9 ± 2.2                    |
| Ile48           | m               | 381.8 ± 6.6                  |
| Val49           | n               | 443.3 ± 7.5                  |
| Met50           | o               | 356.0 ± 6.6                  |
| Leu51           | a               | 34.4 ± 10.9                  |
| Leu52           | b               | 49.1 ± 7.0                   |

Phenylalanine Mutations—In order to further assess site-specific contributions to pentamer stability and to map surface orientations, we changed each amino acid in the transmembrane region to Phe (Fig. 2 and Table I). Leu residues 37 and 47 and Ile residues 40 and 47 were again observed to play key structural roles. Changing any of these amino acids to Phe eliminated pentamer formation. Interestingly, substitution of Ala for several other Phe mutations was also sharply reduced. This included Phe substitutions at Gln29, Ile33, Asn34, Cys41, Met40, and Leu51. It is notable that all residues occupying the b, c, and f positions of the heptad pattern beginning at residue Leu17 tolerated replacement with Phe, viz. residues Ile38, Leu39, Leu42, Ile45, Cys46, Val49, and Leu52.

Changing Cys41 to Phe was previously reported to destabilize the pentamer (Fujii et al., 1989), as is confirmed in Fig. 2 and Table I. We also tested if substitution with another hydrophobic residue at this position would disrupt the pentamer. Unexpectedly, changing Cys41 to Leu led to a new oligomeric species running with a mobility expected for a tetramer (Fig. 2). Formation of tetratomers by the C41L mutation is characterized below.

Leucine/Isoleucine Mutations—To further analyze the roles of the critical Leu and Ile residues at positions 37, 40, 44, 47, and 51 in maintaining pentamer stability, we mutated each of these residues to its structural isomer (Fig. 3 and Table II). The requirement for Ile at position 40 appeared to be stringent, as replacement of this amino acid with Leu completely prevented pentamer formation. The stability of the pentamer was also substantially reduced, but not entirely eliminated, when Leu37 was changed to Ile. In contrast, changing Leu44 to Ile, Ile47 to Leu, or Leu51 to Ile did not have a major effect on pentamer formation.

Mutant Proteins Purified from Sf21 Cells—In order to confirm the reliability of the in vitro translation system for identifying the amino acid residues contributing to PLB pentamer stability, and to provide additional information on the different PLB mobility forms, we expressed and purified PLB containing two of the more revealing mutations from Sf21 insect cells (Reddy et al., 1995). The mutations chosen for further analysis, L37A and C41L, preferentially formed monomers and tetramers, respectively, when analyzed by the in vitro translation method. PLB mutated at these two positions, as well as recombinant wild-type PLB and natural PLB purified from SR vesicles, were subjected to gradient gel electrophoresis followed by Coomassie Blue staining, in order to permit direct visualization of all five mobility forms of the protein.

Fig. 4 demonstrates that wild-type PLB, purified from either SR vesicles or Sf21 cells, migrated predominately as a pentamer, with some monomers and dimers also present. Boiling the wild-type proteins in SDS prior to PAGE dissociated the pentamers and allowed the visualization of tetramers and trimers, as well as the other two mobility forms. Most importantly, the C41L mutation migrated principally as a tetramer, aligning exactly with tetramers formed from wild-type PLB boiled in SDS. A very weak pentamer band could be detected with the C41L mutation, which eliminated the trivial possibility that the tetrameric form of C41L was actually a pentamer but with an aberrant mobility. As observed by in vitro translation, the L37A mutation migrated principally as a monomer. Some dimers were also detected, which were also observed with in vitro translates when gradient gels were used. In other experiments we were able to detect a minor amount of pentamer formation by L37A, but only when electrophoresis through the stacking gel was conducted at a very slow rate with a very high concentration of L37A (≥ 20 μg of protein/gel lane) (data not shown). Thus, unique association constants for each of the mutant proteins analyzed may exist, the determination of which is beyond the scope of this study.

**DISCUSSION**

In the present site-directed mutagenesis study, residues responsible for stabilizing the pentameric structure of PLB were identified by mutation to Ala and were found to be Leu37, Ile40, Leu42, Ile47, and Leu51 (Fig. 1). The mechanism by which substitution of Ala for the critical stabilizing residues prevents the pentameric structure is not by disrupting the native alpha helical structure, since Ala is a strong helix-forming residue (Chou and Fasman, 1978). Furthermore, residues neighboring the stabilizing amino acids may be changed to Ala with no effect on quaternary structure and, therefore, presumably no effect on secondary structure either. We conclude that specific characteristics of the aliphatic side chains of Leu37, Ile40, Leu42, Ile47, and Leu51 stabilize the oligomeric structure of PLB.

The most striking observation from the results is the spacing between critically sensitive residues: an alternating series of three leucines and two isoleucines with each isomeric position separated from the next by seven residues. The heptad repeat pattern is a diagnostic feature of the leucine zipper structural motif (Landschulz et al., 1988), in which the region containing the leucine heptad repeats forms a helix, and the leucines line up along one face of the helix (at a pitch of 3.5 residues/turn) to promote oligomerization of the helices in a parallel orientation. In PLB the repeating isoleucines are offset at 3–4-residue intervals from the leucine heptad repeat (Fig. 5A), conforming to the model of a coiled-coil in which hydrophobic residues...
occupy positions a and d of a repeating heptad of a–g residues (O'Shea et al., 1989; Zhou et al., 1992; Zhu et al., 1993). Thus Leu<sup>37</sup>, Leu<sup>44</sup>, and Leu<sup>51</sup> occupy the a position, whereas Ile<sup>40</sup> and Ile<sup>47</sup> occupy the d position in the motif. The present mutagenesis results are thus consistent with a model of PLB in which residues 37–52 form a 3.5 residue/turn helix, creating a leucine zipper of three helical turns and a complimentary pair of aligned isoleucines with the appropriate axial spacing of two helical turns to interdigitate with the leucines, forming a symmetric, coiled-coil pentamer (Fig. 5B). Five identical zippers formed by interaction between the three leucines of one helix with the two isoleucines of the adjacent helix thus stabilize the PLB quaternary structure. Mixed Leu/Ile zippers have been observed previously (Cohen and Parry, 1990; Atkinson et al., 1991), and the coiled-coil model for the parallel PLB helices is consistent with the observation that parallel orientation of helices is very unusual except in a coiled-coil structure (Oas et al., 1990). The length of the region containing sites important for PLB pentamer formation further suggests extensive interhelical interactions that are more consistent with a coiled-coil model (Zhou et al., 1992) and are not consistent with a single closest approach crossover point between adjacent rigid helices.

The proposed model of PLB residues 37–52 (Fig. 5B) suggests that residues occupying the b, c, and f axial positions are oriented to the exterior surface of the structure and that the PLB pentamer would be insensitive to a bulky residue substitution at these sites. Our observation that all residues predicted to occupy the b, c, and f positions of the heptad repeat, viz. Ile<sup>38</sup>, Leu<sup>39</sup>, Leu<sup>42</sup>, Ile<sup>45</sup>, Cys<sup>46</sup>, Val<sup>49</sup>, and Leu<sup>52</sup>, accepted Phe without loss of pentamer formation supports our model of this domain as a coiled-coil bundle of leucine zipper helices with these residues oriented to the exterior.

### Table II

| Residue mutated | Percentage pentamer formation |
|-----------------|-------------------------------|
| None            | 44.1 ± 2.9                    |
| L37             | 11.7 ± 9.2                    |
| L44             | 27.1 ± 7.0                    |
| L51             | 38.8 ± 4.6                    |

### Table III

| Residue mutated | Percentage pentamer formation |
|-----------------|-------------------------------|
| None            | 44.1 ± 2.9                    |
| Leucine mutations | 0.9 ± 0.8 (d)             |
| Isoleucine mutations | 34.6 ± 2.4 (d)          |
In contrast to the absence of steric hindrance inherent to the b, c, and f axial positions of the leucine zipper helical domain model, residues occupying the e and g positions are in closer proximity in the cleft between adjacent helices (Fig. 5B). Although not specifying the primary stabilizing interactions between helices, these sites may contribute secondarily to oligomeric stability through interactions between their side chains (Hu et al., 1993), and these sites are more likely to exhibit a restricted range of acceptable substitutions based on the volume occluded by adjacent structure. Residues Cys41 and Ile40 in the e position and residues Leu43 and Met50 in the g position all accepted replacement with Ala without preventing pentamer formation. However, replacement with Phe at positions 41 and 50 prevented pentamer formation, but the bulky side chain did not prohibit pentamer formation at positions 43 and 48. The results suggest that the cleft positions within the PLB leucine zipper helical structure are not equivalent, with positions 41 and 50 more restricted than positions 43 and 48. The formation of a PLB tetramer upon substitution of Cys43 with Leu (Figs. 2 and 4) further indicates a unique role for this site in the higher order structure of PLB. A site-directed mutagenesis study of the role of PLB Cys residues in pentamer stability (Fuji et al., 1989) has shown previously that PLB quaternary structure is most intolerant of changes in Cys43. Pentamer formation and stability decreased as the size of the substituted side chain increased. Confirmed by the present work, these results are best interpreted as the effect of steric hindrance on pentamer formation and the disruption of close packing on stability, consistent with the model of PLB presented here in which Cys42 is confined to an interfacial cleft between adjacent helices.

It has been proposed that replacement of the Leu at positions a or d in a leucine zipper with the β-branched Ile may influence the stoichiometry of the oligomer formed through the specificity of packing interactions (DeGrado et al., 1989; Zhu et al., 1993). This has been demonstrated using mutants of the GCN4 leucine zipper (Harbury et al., 1993). However, that study did not address structural determinants for oligomers larger than a tetramer. Our results indicate that the PLB pentamer is primarily stabilized by interactions between leucines in the heptad position a (residues 37, 44, and 51) with isoleucines in position d (residues 40 and 47) (Fig. 4). According to the algorithm derived from GCN4 mutants, occupancy of the a position with Leu and the d position with Ile would be expected to specify a tetramer. As this is not observed with PLB, other residues in the PLB heptad pattern must therefore be involved in specifying the number of subunits in the oligomer. Our observation that substitution of Cys41 for Leu results in a PLB tetramer argues that this site, in the position of the heptad, is important in determining the aggregation number of the PLB oligomer. It thus appears that whereas occupancy of positions a and d with either Leu and Ile is necessary for the coiled-coil motif and primarily responsible for the stability of the resulting oligomer, it is not sufficient to specify the number of helices in the coiled-coil quaternary structure.

To probe the role of β-branched residues at positions a and d in determining the oligomeric state of PLB, we independently mutated Leu37, Leu44, and Leu51 to Ile and changed Ile40 and Ile60 to Leu. Mutations of Leu44, Ile47, and Leu51 had no effect on the formation of the native PLB pentamer, whereas mutation of Leu37 to Ile nearly completely eliminated pentameric assembly of PLB and mutation of Ile60 to Leu completely abolished the ability of the PLB monomers to associate (Fig. 3). Intermediate sized oligomers were not observed for any of these mutations. These results confirm the concept that in PLB the a and d heptad positions stabilize the pentameric assembly but do not specify the number of PLB helices in the oligomer. The tolerance for substitution of Leu/Ile isomers at the three C-terminal positions 44, 47, and 51 may indicate a greater structural flexibility in this region of the oligomer. This suggests that the contributions of the leucine zipper residues to the PLB pentamer are not equivalent, with residues Leu37 and Ile60 contributing more importantly than residues Leu44, Ile47, or Leu51. Taken together with the special sensitivity of Cys43 to mutation, the results suggest that specific contacts in the region of residues 37–41 are primarily responsible for stabilizing the pentameric PLB assembly, with contacts in other regions of the coiled-coil serving as secondary sources of stabilizing energy. The location of these critical residues at the N terminus of the leucine zipper motif suggests that correct initiation and propagation of the nascent leucine zipper helix during translation is important for native PLB quaternary structure.

The prevention of pentamer formation observed by mutation of Ile33 to Phe and also by mutations at Gin29, Phe32, Ile60, and Asn34 indicates that residues 26–36 must contribute in some way to the PLB pentameric structure, as suggested previously (Simmerman et al., 1986). Although Ile33 fits the PLB pattern of alternating Ile and Leu residues at 3–4 residue spacing, the zipper must not extend N-terminally from Leu37 to Ile43, as Ala is accepted at the latter site without loss of pentamer formation. Therefore, the side chain of Ile33 does not contribute to the essential hydrophobic interactions stabilizing the pentamer. The aromatic side chain of Phe32 appears obligatory at this position, as its replacement with Ala prevents formation of the PLB pentamer. The essentiality of a specific, outward facing orientation at this structural site to enable pentamer formation suggests that Phe32 may play a key function in correct folding of secondary structural elements during translation, prevent-
ing premature initiation of the zipper-type helix at Ile$^{25}$. Thus we propose that although residues 26–36 appear uninvolved in the close-packed hydrophobic interactions stabilizing the pentamer, they are critical for appropriate initiation and folding of PLB leucine zippers and their post-translational orientation and assembly into the pentamer. Interestingly, the belt of Phe$^{23}$ and Phe$^{26}$ aromatic side chains encircling the pentameric structure defines the interface between the polar and nonpolar regions of PLB. PLB shares this structural motif with membrane channel pores (Cowan et al., 1992; Weiss and Schulz, 1992), in which the band of aromatic residues may protect polar structural elements at the membrane interface from conformational fluctuations during vertical displacements of the protein within the membrane (Kreusch et al., 1994). It may be that PLB phenylalanines also serve to correctly position PLB within the bilayer and to protect polar interfacial residues.

The PLB oligomeric structure is stabilized only by contacts between hydrophobic residues without contribution from side chain electrostatic or hydrogen bonding interactions, commonly observed in other natural coiled-coils (Talbot and Hodges, 1982; Cohen and Parry, 1990). Synthetic two-stranded coiled-coils having only Leu at the a and d heptad positions are much more stable than naturally occurring two-stranded coiled-coils containing a significant proportion of Val and Ala at the critical a and d heptad positions (Hodges et al., 1990). It was thus suggested that natural coiled-coil proteins have not evolved for maximum stability. Considering that the PLB coiled-coil pentamer is stabilized only by Leu and Ile interactions, it appears that the PLB oligomeric structure has uniquely evolved very specifically for great stability.

The PLB membrane domain does not conform to the model of integral membrane proteins as “inside-out” with respect to the relative polarity of surface and buried residues (Engelman et al., 1986). Instead, it is a cylinder of close-packed hydrophobic helices (Fig. 5), which may fulfill predictions that the coiled-coil leucine zipper motif may serve as the defining structure for certain channels (Lear et al., 1988; Karle et al., 1988; DeGrado et al., 1988; Popot and Engelman, 1990; Harbury et al., 1993). Modeling suggests the pore formed by a pentameric coiled-coil would have a diameter of 5 Å (Lear et al., 1988), sufficient to accommodate a water molecule (2.8 Å diameter) or a hydrated small ion. The association of channel activity with the pentameric domain of PLB residues 26–52 emphasizes the utility of PLB as a natural model system for elucidating channel structure/function relationships and mechanisms of ion conductance, as suggested previously (Kovacs et al., 1988).

While this work was in progress (Simmerman et al., 1994), a study was published using chimeric protein analysis to examine PLB transmembrane domain structure (Arkine et al., 1994). The two approaches concur that amino acids stabilizing the pentamer line up on faces of a 3.5 residues/turn helix, and the resulting pore structure is defined by hydrophobic residues. However, the wild-type chimeric protein studied by Arkine et al. (1994) did not associate with the same oligomeric distribution as native PLB. The random substitutions created by saturation mutagenesis were confined to residues 35–52, and the enabling role of residues 26–36 in PLB pentameric assembly was not reported. The use of an indirect, qualitative detection method also limited identification of some structurally important residues. For example, the PLB fusion protein containing C41L was expressed as a monomer by Arkine et al. (1994), whereas we clearly show that when native PLB is substituted with C41L, the protein is a tetramer. Our results provide evidence that the leucine zipper helical motif can indeed stabilize an oligomeric membrane domain to form a coiled-coil structure with potential relevance to ion channels.

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