Reexamination of the Role of the Leucine/Isoleucine Zipper Residues of Phospholamban in Inhibition of the Ca^{2+}-Pump of Cardiac Sarcoplasmic Reticulum*

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Phospholamban is a small phosphoprotein inhibitor of the Ca^{2+}-pump in cardiac sarcoplasmic reticulum, which shows a distinct oligomeric distribution between monomers and homopentamers that are stabilized through Leu/Ile zipper interactions. A two-faced model of phospholamban inhibition of the Ca^{2+}-pump was proposed, in which the Leu/Ile zipper residues located on one face of the transmembrane α-helix regulate the pentamer to monomer equilibrium, whereas residues on the other face of the helix bind to and inhibit the pump. Here we tested this two-faced model of phospholamban action by analyzing the functional effects of a new series of Leu/Ile zipper mutants. Pentameric stabilities of the mutants were quantified at different SDS concentrations. We show that several phospholamban mutants with hydrophobic amino acid substitutions at the Leu/Ile zipper region retain the ability to form pentamers but at the same time give the same or even stronger (i.e. L37I-PLB) inhibition of the Ca^{2+}-pump than do mutants that are more completely monomeric. Steric constraints prevent the Leu/Ile zipper residues sequestered in the interior of the phospholamban pentamer from binding to the Ca^{2+}-pump, leading to the conclusion that the zipper residues access the pump from the phospholamban monomer, which is the active inhibitory species. A modified model of phospholamban transmembrane domain action is proposed, in which the membrane span of the phospholamban monomer maintains contacts with the Ca^{2+}-pump around most of its circumference, including residues located in the Leu/Ile zipper region.

PLB1 is a 52-amino acid phosphoprotein integral to the cardiac sarcoplasmic reticulum membrane, which is an inhibitor of the Ca^{2+}-pump in its dephosphorylated state (1, 2). The small phosphoprotein is composed of a cytosolic N-terminal domain (residues 1–30) and a highly hydrophobic, C-terminal transmembrane domain (residues 31–52). In cardiac sarcoplasmic reticulum, PLB mediates the hormonally controlled regulation of the Ca^{2+}-pump by modulating the apparent affinity of the ATPase for calcium (3, 4). Dephospho-PLB inhibits the Ca^{2+}-pump by lowering its apparent affinity for ionized calcium. Phosphorylation of PLB at Ser-16 and/or at Thr-17 in the cytosolic domain restores the high apparent Ca^{2+} affinity, causing an activation of ATP hydrolysis and Ca^{2+}-transport at submicromolar ionized calcium while leaving the V_{max} of the enzyme measured at saturating ionized calcium concentration largely unchanged (2).

On SDS-PAGE, PLB exhibits a remarkable pattern of oligomerization, being distributed mainly between populations of monomers (20%) and homopentamers (80%) (5). Alaine- and phenylalanine-scanning mutagenesis has been used to identify key amino acid residues in the transmembrane domain of PLB that are critical for pentamer formation. The pentamer-stabilizing amino acids are Leu residues 37, 44, and 51 and Ile residues 40 and 47, all of which are located in the transmembrane α-helix (6). When plotted on a heptad repeat, these residues appear at a 3–4-residue spacing, with the critical Leu and Ile residues required for pentamer stabilization found clustered on one face of the PLB transmembrane α-helix, at positions a and d, respectively (Fig. 1A). On SDS-PAGE, single Ala (or Phe) substitutions at any one of these Leu and Ile residues is sufficient to substantially disrupt pentamer formation (6). In contrast, mutation to Ala (or Phe) at sites b, c, e, and f (i.e. on the other face of the PLB transmembrane helix (Fig. 1A)) does not affect pentamer formation substantially. A Leu/Ile zipper model of PLB was proposed to explain its unique oligomerization, in which the aliphatic side chains of the Leu and Ile residues at positions a and d of adjacent PLB protomers interdigitate and, by hydrophobic forces, promote the assembly of protomers into pentamers (Fig. 1B). Further supporting the Leu/Ile zipper model was the observation that isomeric mutations at positions 37, 44, and 51 (Leu to Ile), and at position 47 (Ile to Leu) facilitated a partial restoration of PLB oligomerization (6).

In an independent study employing alanine-scanning mutagenesis of the transmembrane domain of PLB, Kimura et al. (7) confirmed the critical amino acid residues in the Leu/Ile zipper region that are essential for pentamer formation. Moreover, these investigators extended the earlier work by co-expressing the mutated PLB molecules with the Ca^{2+}-ATPase and measuring functional effects. A relatively strong correlation was observed in which Ala point mutations in the Leu/Ile zipper region of PLB that disabled PLB pentamerization on SDS-PAGE actually enhanced PLB inhibitory function on Ca^{2+} transport activity (7). These strong monomeric inhibitors of the Ca^{2+}-pump were called “supershifters” because they lowered the apparent Ca^{2+} affinity of the enzyme substantially more
than wild type PLB (WT-PLB) (7). Based on these results, the hypothesis was proposed that the monomeric form of PLB is, in fact, the inhibitory species that is responsible for binding to the Ca\(^{2+}\)-pump and inhibiting it. In parallel studies, Autry and Jones (8) observed that the monomeric mutant of PLB, L37A-PLB, was a much stronger inhibitor of the Ca\(^{2+}\)-ATPase than was pentameric WT-PLB and also proposed that the PLB monomer is the species inhibiting the Ca\(^{2+}\)-pump. This active monomer hypothesis (7, 8), based on correlations between SDS-PAGE analyses of oligomeric states of different PLB mutants and the abilities of these mutants to inhibit the SERCA2a Ca\(^{2+}\)-pump in expression systems, was supported by measurements of the intramembrane oligomeric states of purified WT- and L37A-PLB in lipid bilayers. When reconstituted in phospholipids, the oligomeric states of WT- and L37A-PLB were found to be mainly pentameric and mainly monomeric, respectively, as predicted from SDS-PAGE results. Moreover, the distribution of PLB oligomers in lipid bilayers appeared to be in a dynamic equilibrium regulated by PLB phosphorylation (9). Thus, dissociation of PLB monomers from pentamers was proposed to occur in native sarco/plasmatic reticulum membranes, where Ca\(^{2+}\)-pump molecules are present and may interface with inhibitory domains of the PLB monomers. In support of this, using spectroscopic techniques capable of detecting intramembrane protein interactions, Reddy et al. (10) recently observed that co-reconstitution of the Ca\(^{2+}\)-pump in lipid membranes along with native PLB specifically promoted the depolymerization of the PLB homopentamers into populations of PLB monomers and smaller oligomers.

In the alanine-scanning mutagenesis study of the transmembrane region of PLB in which PLB was co-expressed with the Ca\(^{2+}\)-pump, Kimura et al. (7) noted that several point mutations of PLB disrupted its inhibitory interaction with the Ca\(^{2+}\)-pump. Remarkably, all of these were located on the face of the PLB transmembrane helix opposite to the Leu/Ile zipper residues (i.e., at positions e, b, and f in Fig. 1A), suggesting that this region of the PLB membrane-spanning helix is the one responsible for binding to and inhibiting the Ca\(^{2+}\)-pump. Thus, for the first time, specific residues that might be necessary for Ca\(^{2+}\)-pump inhibition were identified in the transmembrane domain of PLB (7).

In summary, mutagenesis results addressing PLB topology and structure/function suggest that the transmembrane region of PLB contains two major protein interface domains; at one face of the transmembrane helix are located residues whose function is to promote PLB pentamer stabilization-destabilization (6–9), and at the other face of the transmembrane helix are located residues whose function is to bind to and inhibit the Ca\(^{2+}\)-pump (7, 11). In this “two-faced” model of PLB transmembrane domain interaction with the Ca\(^{2+}\)-pump (2, 7), amino acid residues located at positions a and d of the transmembrane helix (Fig. 1) control the pentamer to monomer equilibrium (6–9), whereas residues at positions b, c, and f of the PLB transmembrane helix are responsible for Ca\(^{2+}\)-pump inhibition (7, 11). However, not all mutations in the membrane-spanning region of PLB that have been investigated produce functional effects that are consistent with this two-faced mode of action. For example, the C41F mutation of PLB produces all monomers on SDS-PAGE but gives a somewhat weaker inhibition of the Ca\(^{2+}\)-pump than does WT-PLB (12, 13); the I40L mutant of PLB, in contrast to I40A-PLB, produces a substantial fraction of pentamers on SDS-PAGE but nonetheless remains a much stronger inhibitor of the Ca\(^{2+}\)-pump than WT-PLB, being comparable in inhibitory potency with I40A-PLB (13). N27A-PLB, which harbors the Ala substitution in the cytoplasmic domain of PLB, is the strongest SERCA2a supershifting inhibitor of all of the PLB point mutants tested to date, including the monomeric transmembrane mutants, yet retains pentameric stability comparable with WT-PLB (14, 15).

Here, we completed our previous mutational study on the Leu/Ile zipper residues of PLB (6) by correlating hydrophobic residue substitutions in this region with functional effects on Ca\(^{2+}\)-pump activity. Previously, only Ala substitutions in this region have been analyzed in any detail for functional effects on Ca\(^{2+}\)-pump activity, and it is from results of these Ala mutagenesis studies that the two-faced model of PLB action was largely developed (2, 7). Since several of the Leu/Ile isomeric substitutions in this region retain pentamer stability, whereas all of the Phe substitutions are pentamer-disruptive (6), we reasoned that if the two-faced model of PLB transmembrane action is correct, then pentamer-retaining mutations in the Leu/Ile zipper region of PLB should all be relatively weak inhibitors of the Ca\(^{2+}\)-pump compared with the monomeric mutants, whereas pentamer-disruptive mutations in this region should all be strong inhibitors. Surprisingly, this supposition proved to be wrong. Some point mutations in the Leu/Ile zipper region of PLB produced very strong superinhibition of the Ca\(^{2+}\)-pump but nevertheless were quite effective in maintaining a considerable fraction of stable pentamers, whereas other mutations in this region were completely pentamer-disruptive but were substantially weaker inhibitors of the Ca\(^{2+}\)-pump. The combined results of the mutational analysis call into question the two-faced model of monomer action and suggest that a more complicated mechanism must be invoked to rationally explain how PLB inhibits the Ca\(^{2+}\)-pump.
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EXPERIMENTAL PROCEDURES

Materials—DNA synthesizing, modifying, and restriction enzymes were obtained from Promega. All oligonucleotides were purchased from Life Technologies, Inc. The Sequenase® sequencing kit was purchased from Amersham Pharmacia Biotech, and α-[35S]dATP and [3H]-labeled protein A were obtained from PerkinElmer Life Sciences. SF21 insect cells were purchased from Invitrogen, and the BaculoGold™ transfection kit was obtained from Pharmingen. Other chemicals were purchased from Sigma.

Site-directed Mutagenesis of PLB and Construction of PLB Recombinant Baculovirus—Point mutations were introduced in the canine isoform of PLB, as described previously, using the Altered Sites II Mutagenesis System™ (6). All DNA mutations were confirmed by dyeodeoxy sequencing. Mutated inserts were subcloned into the BglII site of the pVL1393 transfection vector and co-transfected into SF21 with Baculogold™-linearized baculovirus DNA using the BaculoGold™ transfection kit (Pharmingen). Recombinant baculovirus clones were isolated using a plaque assay as described previously (8, 13). Plaques were amplified individually, and PLB expression was confirmed by immunoblotting.

Co-Expression of SERCA2a and PLB in SF21 Cells and Isolation of Microsomes—Canine SERCA2a and PLB were co-expressed in SF21 insect cells as described previously (8, 13). Briefly, the SF21 cells were maintained at 27 °C in suspension cultures that were split every 72 h with Grace’s insect cell medium supplemented with 10% fetal bovine serum (Atlanta Biologicals), 0.1% Phorbiq® F-68 (Life Technologies, Inc.), 50 μg/ml gentamicin, and 2.5 μg/ml amphotericin B. The cells were maintained at densities between 3.0 and 4.0 × 10^7 cells/ml. For expression of infectivity, 15–50 μl of baculovirus were added to each 25 ml of medium containing 10 mM NaHCO₃ and 0.2 mM leupeptin (0.2 mg/ml), pepstatin A (1 mg/ml), and Pefabloc SC (0.1 mg/ml). The cells were transferred to JA-20 centrifuge tubes and homogenized with a Polytron three times for 30 s at 14,000 rpm with 10-s breaks. The homogenates were centrifuged at 12,000 × g for 45 min in a Beckman JA-20 rotor, and the supernatants were collected and spun in a Beckman type 70 Ti rotor at 50,000 × g for 35 min. The pellets were resuspended in 1.5–2 ml of medium containing 10 m NaHCO₃ and 0.2 mM CaCl₂, supplemented with the protease inhibitors aprotinin (1 μg/ml), leupeptin (0.2 μg/ml), pepstatin A (1 mg/ml), and Pefabloc SC (0.1 mg/ml). The pellets were resuspended in 25 ml of medium containing 10 mM NaHCO₃, and 0.2 mM CaCl₂, supplemented with the protease inhibitors aprotinin (1 μg/ml), leupeptin (0.2 μg/ml), pepstatin A (1 mg/ml), and Pefabloc SC (0.1 mg/ml). The cells were transferred to JA-20 centrifuge tubes and homogenized with a Polytron three times for 30 s at 14,000 rpm with 10-s breaks. The homogenates were centrifuged at 12,000 × g for 45 min in a Beckman JA-20 rotor, and the supernatants were collected and spun in a Beckman type 70 Ti rotor at 50,000 × g for 35 min. The pellets were resuspended in 1.5–2 ml of medium containing 10 mM MOPS, 0.3 mM sucrose, and 5 mM NaN₃ (pH 7.0) supplemented with the protease inhibitors above and subjected to a final homogenization in a 2-ml Potter-Elvehjem tissue grinder. The protein concentration of the microsomal suspension was determined by the method of Lowry et al. (16) and was between 4 and 6 mg/ml. The yield of microsomal protein was typically 10 mg from 120 ml of cell culture suspension. Microsomes were stored frozen in small aliquots at −80 °C.

SDS-PAGE and Immunoblotting—Microsomal samples were solubilized with an equal volume of SDS-dissociation medium containing 62.5 mM Tris (pH 6.8), 5% glycerol, 5 mM diethiothreitol, 0.0025% bromophen blue, and different SDS concentrations as described under “Results.” SDS-PAGE was performed in 8% polyacrylamide and 0.1% SDS using blue, and different SDS concentrations as described under “Results.” SDS-PAGE was performed in 8% polyacrylamide and 0.1% SDS using blue, and different SDS concentrations as described under “Results.”

RESULTS

SDS Concentration Effects on PLB Pentameric Stability after Mutation at Leucine Residue 37—Leu-37 is a key residue localized in the Leu/Ile zipper region of PLB (2, 6–9). In initial experiments, we correlated effects of single amino acid substitutions here on the pentameric stability of PLB with the functional effects on SERCA2a activity. Fig. 2 shows the effect of SDS concentration on the oligomeric stability of WT-PLB and PLB mutated at Leu 37 with amino acids of varying hydrophobicity. Consistent with previous results (8), when PLB was co-expressed with the Ca²⁺-pump in SF21 cell microsomes and solubilized in 3.5% SDS, WT-PLB migrated predominantly as a pentamer on SDS-PAGE, whereas L37A-PLB was almost totally monomeric (Fig. 2A and B). L37F-PLB solubilized in 3.5% SDS was also virtually completely monomeric, whereas L37I-PLB formed a substantial fraction of pentamers (Fig. 2C and D). Similar analyses on the oligomeric structure of PLB have been conducted previously, in which a single high concentration of SDS was used for PLB solubilization (6–9, 19). By using a range of SDS concentrations in the sample solubilization buffer, however, some interesting new features on the ability of PLB to oligomerize became apparent (Fig. 2). First, the two mutants thought previously to be incapable of forming significant amounts of pentamers, L37A- and L37F-PLB (6–8), in fact formed appreciable amounts of stable pentamers when the concentration of SDS in the solubilization buffer was lowered. For example, at SDS concentrations between 0.5 and 1.0%, L37A- and L37F-PLB formed 20–45% stable pentamers; only at 3.5% or higher SDS concentrations were these mutants completely monomeric, as reported previously (6). Second, L37I-PLB was a much more stable pentamer than either L37A- or L37F-PLB, rivaling WT-PLB in its ability to oligomerize (Fig. 2C). Between 0.5 and 1.5% SDS, this mutant formed 50% stable pentamers (Fig. 2E). Third, WT-PLB formed the same amount of pentamers regardless of the SDS concentration. Between 0.5 and 7.5% SDS, the fraction of WT-PLB pentamers remained virtually constant at 70–80% (Fig. 2, A and B). By plotting the fraction of pentamers formed at each SDS concentration for each mutant (Fig. 2E), a much better estimate of pentameric stability was allowed than by using a single high concentration of SDS as in previous reports (6–8, 19). Using this analysis, the relative pentameric stabilities of WT-PLB and of the other mutants were WT-PLB > L37I-PLB > L37A-PLB > L37F-PLB (Fig. 2E). Assessment of SERCA2a co-expression in the same membrane samples indicated that similar levels of Ca²⁺-pump were co-expressed in all of the samples analyzed (see Fig. 4).
Residue 37 Mutation Effects on SERCA2a Inhibition—If the two-faced model of PLB inhibition of the Ca\(^{2+}\)-pump is correct, then the ability of PLB to monomerize when mutated at position 37 should correlate strongly with its ability to inhibit the Ca\(^{2+}\)-pump when the two proteins are co-expressed. WT-PLB or PLB molecules mutated at Leu\(^{37}\) were co-expressed with SERCA2a in Sf21 cells, and microsomes were isolated and analyzed for Ca\(^{2+}\)-ATPase activity as described previously (8). The strength of inhibition of the Ca\(^{2+}\)-pump was quantified by the shift in \(K_{Ca}\) produced by each PLB mutant, where \(K_{Ca}\) is the concentration of ionized Ca\(^{2+}\) required for half-maximal activation of enzyme turnover. Potential artifacts caused by the effect of PLB mutation on microsomal permeability to Ca\(^{2+}\) (20, 21) were circumvented by measuring Ca\(^{2+}\)-dependent ATPase activity in the presence of the Ca\(^{2+}\)-ionophore A23187 (8), rather than by assaying for Ca\(^{2+}\)-transport activity. Consistent with previous reports (7, 8), co-expression of WT-PLB with SERCA2a in Sf21 microsomes increased the \(K_{Ca}\) by more than 2-fold, from 0.13 ± 0.01 to 0.33 ± 0.04 \(\mu M\), whereas co-expression of the mostly monomeric mutant L37A-PLB increased the \(K_{Ca}\) by 5-fold, to 0.64 ± 0.10 \(\mu M\) (Fig. 3, A and C). Unexpectedly, however, we observed that co-expression of L37F-PLB, the most monomeric of all the PLB mutants at this position tested, with SERCA2a gave little more inhibition (\(K_{Ca} = 0.44 ± 0.02 \mu M\)) than did co-expression of WT-PLB (Fig. 3, A and C). In contrast, L37I-PLB, which formed the highest percentage of stable pentamers of all of the mutants tested, was also the strongest supershifter, giving a \(K_{Ca}\) of 1.18 ± 0.15 \(\mu M\) (Fig. 3, A and C). This \(K_{Ca}\) value was nearly double the value achieved by co-expression of L37A-PLB with SERCA2a. Thus, when several single amino acid substitutions at Leu\(^{37}\) of PLB were made (rather than substituting only alanine (7, 8)), the degree of pentamer destabilization did not correlate strongly with the strength of SERCA2a inhibition.

To ensure the specificity of the PLB-SERCA2a interactions for each of the PLB mutants analyzed, we preincubated Sf21 microsomes with the PLB monoclonal antibody prior to the ATPase assay in order to reverse inhibition of the Ca\(^{2+}\)-pump (8, 13, 15) (Fig. 3, B). Substantial, but incomplete, reversal of the inhibitory effects of all of the different mutated PLB molecules on Ca\(^{2+}\)-ATPase activity was achieved by use of the antibody (Fig. 3, B). The inability of the monoclonal antibody (8, 15) or of PLB phosphorylation (22) to completely reverse the inhibitory effect of supershifting PLB molecules was noted previously. The magnitude of antibody-reversible shift in \(K_{Ca}\) (Fig. 3, C, ordinate) correlated strongly with the strength of SERCA2a inhibition by each mutant (Fig. 3, C, abscissa), however, further demonstrating the specificity of the protein-protein interac-

![Image: Immunoblot showing SDS concentration effects on PLB pentameric stability after mutation at residue 37.](https://example.com/immunoblot.png)
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Effects of Phenylalanine Mutations at Leu/Ile Zipper Residues 40, 44, 47, and 51—The substitution at Ile40, Leu44, or Ile47 of PLB virtually completely abolished pentamer formation at low or high SDS concentration, giving the same effect on oligomeric stability as the L37F mutation (Figs. 4 and 5). However, in contrast to L37F-PLB monomers, I40F- or L44F-PLB monomers remained very strong SERCA2a inhibitors, supershifting $K_Ca$ values by similar amounts as the corresponding alanine point mutants at the same sites (Fig. 6). For I47F-PLB, however, the $K_Ca$ value obtained was actually 25% lower than the value obtained with use of I47A-PLB but still substantially greater than that obtained after co-expression of WT-PLB or of L37F-PLB (Fig. 6).

**DISCUSSION**

In order to further test the two-faced model of PLB transmembrane domain action on SERCA2a, we used the SF21 insect cell baculovirus expression system to co-express SERCA2a with a series of mutated PLB molecules, all of which carried either alanine or bulky hydrophobic residue substitutions targeted to the Leu/Ile zipper domain. The correlation between PLB pentamer-monomer equilibrium and SERCA2a inhibition was then analyzed. The critical assumption made in this and previous studies was that the relative oligomerization propensities revealed by SDS-PAGE should also apply in the membrane environment and in the presence of Ca$^{2+}$-pumps. This assumption has recently been supported by spectroscopic studies assessing the intramembrane oligomerization of PLB, measured in the absence (9) and presence (10) of the Ca$^{2+}$-pump. Furthermore, it was observed that the Ca$^{2+}$-pump actually facilitated depolymerization of the PLB pentamers into smaller oligomers and/or monomers (10). Here, by varying the SDS concentration, we were able to provide more refined information on the relative tendencies of the different PLB mutants to oligomerize (Figs. 4 and 5) and then correlate these results with the degree of inhibition of Ca$^{2+}$-pump activity (Fig. 6).

In agreement with previous reports, single Ala mutations at...
all five Leu/Ile zipper positions in the transmembrane domain of PLB disrupted pentamer formation (6) and enhanced inhibitory action on SERCA2a by increasing the $K_{Ca}$ value (7). Previously, such data have been interpreted to suggest that PLB monomers are the active agents inhibiting the Ca\(^{2+}\)-pump (7, 8) and that the Leu/Ile zipper domain does not interact directly with the Ca\(^{2+}\)-pump but instead is solely responsible for controlling the pentamer-to-monomer equilibrium (2). Here, we noted several inconsistencies with this idea. First, considering the Phe substitutions in the Leu/Ile zipper domain, introducing Phe at position 37 completely disrupted PLB pentamer formation but resulted only in a small gain of inhibitory function on the Ca\(^{2+}\)-pump compared with that achieved with co-expression of WT-PLB. Since the expression levels of the
Ca\(^{2+}\)-pump and PLB were comparable in all samples, it is reasonable to assume that there was a higher concentration of PLB monomers in the membrane with co-expression of L37F-PLB than with co-expression of WT-PLB. This leads to the conclusion that WT-PLB monomers are more effective inhibitors of the Ca\(^{2+}\)-pump than are L37F-PLB monomers. The difference between the inhibitory potencies of the PLB monomers is more evident when comparing the effects of SERCA2a co-expression with L37A-PLB and L37F-PLB. L37A-PLB has been shown to be predominantly monomeric in lipid membranes as well as in SDS (9), and the SDS concentration effects reported here reveal that L37F-PLB is even less even capable of pentamer formation than L37A-PLB (Fig. 6). However, Phe substitution at position 37 almost completely reversed the gain in inhibitory function relative to the Ala substitution at position 37, down to almost the level of inhibition elicited by WT-PLB (Fig. 6). These contrasting functional effects exerted by the L37A- and L37F-PLB monomers by themselves raise doubts about the involvement of residue 37 only in pentamer formation. The interference of Phe at position 37 with the inhibitory function of PLB suggests that this position may be part of or adjacent to a region of PLB that interacts with the Ca\(^{2+}\)-pump. Steric hindrance caused by the bulky aromatic ring of Phe could prevent both PLB pentamer formation and the optimal assembly of PLB-Ca\(^{2+}\)-pump inhibitory complexes. At residue 47 of PLB, we noted a similar but less pronounced effect. Substitution with Ala or Phe here was equally effective in disrupting PLB pentamers, yet I47F-PLB was 25% less potent in inhibiting the Ca\(^{2+}\)-pump than was I47A-PLB. Therefore, the conclusion can be drawn that at position 47 of PLB (arm d in Fig. 1A), as well as at position 37 (arm a in Fig. 1A), steric factors are importantly involved in maintaining proper contact with the Ca\(^{2+}\)-pump.

A second line of evidence that argues against the zipper domain of PLB being inert with respect to Ca\(^{2+}\)-pump interactions comes from the isomeric amino acid replacements analyzed. For several of the Leu to Ile and Ile to Leu PLB mutants, we found an effect directly opposite to that caused by the Phe substitutions discussed above, specifically, enhanced Ca\(^{2+}\)-pump inhibition associated with enhanced PLB oligomerization. The most striking case was noted with L37I-PLB, which was very capable of forming pentamers, especially in comparison with L37A-PLB, leading us to expect that the inhibitory potency of L37I-PLB on the Ca\(^{2+}\)-pump would be substantially reduced compared with that of L37A-PLB. We were quite surprised, however, to find that L37I-PLB was the strongest supershifter of all of the PLB mutants analyzed, decreasing the apparent affinity of SERCA2a for Ca\(^{2+}\) nearly 2 times more effectively than the decrease achieved with use of L37A-PLB. In fact, direct comparison of the inhibitory potency of L37I-PLB on the Ca\(^{2+}\)-pump with that of N27A-PLB, the strongest Ca\(^{2+}\)-pump superinhibitor previously identified (14, 15, 22), revealed that L37I-PLB was a significantly stronger inhibitor even than this PLB mutant. L37I-PLB increased the \(K_{ca}\) of SERCA2a to 1.18 \(\pm\) 0.15 \(\mu\)M, whereas N27A-PLB increased the \(K_{ca}\) to 0.83 \(\pm\) 0.16 \(\mu\)M.\(^2\) Similar isomeric mutations at three other Leu/Ile positions (Ile to Leu at position 40 and Leu to Ile at positions 44 and 51) affected pentamer formation and SERCA2a-inhibitory function in a fashion resembling that of L37I-PLB. For all of these mutants, strong pentamer-forming capability was preserved; however, all inhibited the Ca\(^{2+}\)-pump about as strongly as the Ala mutants, which were consistently more monomeric. Assuming that the PLB monomer is the active inhibitory species, these results suggest that the monomers bearing the branched-chain amino acid substitutions are stronger inhibitors of the Ca\(^{2+}\)-pump than are monomers bearing the Ala substitutions. Thus, it is apparent that zipper residues 37, 40, and 51 of PLB affect the inhibitory potency of PLB in a fashion that is consistent with a direct physical contact of these residues with the Ca\(^{2+}\)-pump.

Do our results support or refute the concept that the PLB monomer is the active agent inhibiting the Ca\(^{2+}\)-pump? On the one hand, we noted a consistent positive correlation between monomeric propensities of Ala mutants in the Leu/Ile zipper domain of PLB and Ca\(^{2+}\)-pump inhibition (7) and, on the other hand, a frequent negative correlation between monomeric propensities of other PLB mutants and Ca\(^{2+}\)-pump inhibition when hydrophobic-isomeric and Phe substitutions are made in this region. How does one reconcile such seemingly disparate results?

We believe the work reported here, in combination with previous studies (7–9), makes it exceedingly unlikely that the PLB pentamer is the active species inhibiting the Ca\(^{2+}\)-pump, as suggested in two recent reports (22, 23). For example, considering just the L37I-PLB mutation, we noted a strikingly potent inhibition of Ca\(^{2+}\)-pump activity. If the pentameric form of PLB were responsible for ATPase inhibition, then residue 37 would be buried in the interior of the PLB pentamer in the zipper region (Fig. 1B) (6, 24) and sterically incapable of binding to and inhibiting the Ca\(^{2+}\)-pump. Clearly, the functional data reported here suggested a strong interaction of residue 37

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\(^2\) R. L. Cornea and L. R. Jones, unpublished data.
of PLB with the Ca\(^{2+}\)-pump. Also supporting the active monomer model, the L37F-, I40F-, L44F-, and I47F-PLB mutations all produced PLB molecules that were virtually totally monomeric yet remained stronger inhibitors of the Ca\(^{2+}\)-pump than was WT-PLB. It is difficult to conceive how all of these mutated PLB molecules could assemble into trace levels of pentamers in the membrane, which would then be solely responsible for the substantial inhibition of the Ca\(^{2+}\)-pump that was achieved.

The results reported here, however, cannot be accounted for easily by a two-faced model of PLB action at the transmembrane domain, in which residues participating in Ca\(^{2+}\)-pump inhibition are located only on the side of the \(\alpha\)-helix (opposite from the zipper domain; Ref. 7). The two-faced model does not explain how the more stable pentamer L37I-PLB is a 2-fold stronger inhibitor of the Ca\(^{2+}\)-pump than is L37A-PLB or how the less stable pentamer L37F-PLB is a 2-fold weaker inhibitor of the Ca\(^{2+}\)-pump than is L37A-PLB. Such effects can be reconciled, however, in the context of the two-state association model of PLB with the Ca\(^{2+}\)-pump, as originally proposed by Kimura et al. (7). In this model, \(K_{d1}\) and \(K_{d2}\) are the dissociation constants describing the PLB pentamer to monomer binding equilibrium (9), and the PLB monomer to Ca\(^{2+}\)-pump binding equilibrium, respectively (Fig. 7). Considering just the mutations at position 37, for example, L37I-PLB can be a much stronger inhibitor of the Ca\(^{2+}\)-pump than is L37A-PLB, yet at the same time less “monomeric,” if the affinity of the L37I-PLB monomer for the Ca\(^{2+}\)-pump is much greater than is the affinity of the L37A-PLB monomer (i.e. L37I-PLB \(K_{d1}\) is much less than L37A-PLB \(K_{d1}\)), offsetting the increased tendency of L37I-PLB to form pentamers (resulting from its relative decrease in \(K_{d1}\)). Although L37F-PLB is more monomeric than L37A-PLB (Fig. 2), it can be a weaker inhibitor of the Ca\(^{2+}\)-pump than L37A-PLB, if the \(K_{d2}\) value for L37F-PLB is substantially increased relative to the \(K_{d2}\) value for L37A-PLB. Implicit in this interpretation of the functional effects caused by the different PLB mutations reported here is that the Leu/Ile zipper residues of PLB interact directly with the Ca\(^{2+}\)-pump. According to this scheme, residues of PLB at positions \(a\) and \(d\) of the 3.5-residue/turn transmembrane \(\alpha\)-helix, along with residues at positions \(b\), \(e\), and \(f\) (7), all interact directly with the Ca\(^{2+}\)-pump (Fig. 7). In the context of this model, it would be impossible for a PLB protomer that binds to and inhibits the Ca\(^{2+}\)-pump to simultaneously be a part of the homopentameric assembly of PLB (Figs. 1 and 7).

In conclusion, these data are consistent with and further support the model in which the inhibitory action of PLB on the Ca\(^{2+}\)-pump is exerted by PLB monomers (7, 8), not by PLB pentamers (22, 23). Furthermore, the results suggest that the PLB transmembrane domain is virtually embedded in the transmembrane structure of the Ca\(^{2+}\)-pump when it exerts its inhibitory action. Recently, it has been suggested that the transmembrane helix of PLB interacts with transmembrane helix M6 of the Ca\(^{2+}\)-pump (11). In the recently published high resolution crystal structure of SERCA (25), transmembrane helix M6 appears in a mostly buried location, where the putative binding sites for the PLB transmembrane domain would be flanked by transmembrane helices M9 and M2 or M2 and M1. Therefore, the conclusion that PLB forms contacts with the Ca\(^{2+}\)-pump around nearly its entire transmembrane circumference is supported by recent biochemical (11) as well as crystallographic (25) data.

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