Microphase-Separated Structure in Triblock Copolypeptide Membranes Composed of L-Glutamic Acid and L-Leucine

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ABSTRACT: Microphase-separated structure in cast triblock copolypeptide membranes was examined by fluorescence spectroscopy and electron microscopy. One of these membranes was composed of γ-benzyl-L-glutamate and L-leucine ($PBLG_x-PLL_y-PBLG_x$), ($x = 0.18$, $y = 0.64$, mole fraction), and the other was composed of L-glutamic acid and L-leucine ($PLGA_x-PLL_y-PLGA_x$), which was prepared via saponification of the benzylglutamate groups of the PBLG−PLL−PBLG membrane. Electron micrograph of an ultrathin section of PBLG−PLL−PBLG membrane (cut perpendicular to the surface) showed that domains composed of the poly(γ-benzyl-L-glutamate) chains were embedded in a continuous matrix of the poly(L-leucine) phase. The shape of the domains was nearly cylindrical, and the domain size (diameter) and the distance between two adjacent domains were estimated to be 100 and 140 nm, respectively. This domain structure was retained after the saponification reaction following which the domains become hydrophilic. Fluorescence emission spectra of 8-anilino-1-naphthalenesulfonic acid ammonium salt incorporated into wet PLGA−PLL−PLGA membrane showed three peaks. This suggests that the membrane has three regions with different degrees of hydrophobicity, which are presumed to represent the PLGA, interfacial, and PLL regions, respectively.

Introduction

Microdomain formation in vinyl block copolymer systems has received the most attention to date.[1,2] However, polypeptide systems, which are biocompatible materials, are of considerable interest as well. These latter systems are not so well-studied, although there are a few reports which provide evidence for microphase-separated morphology in these systems based on dynamic mechanical, spectroscopic, and wide angle X-ray diffraction techniques.[3−6]

We synthesized, following the experimental procedure of Hayashi et al.,[7] a triblock copolypeptide of the form (γ-benzyl-L-glutamate)$_x$−(L-leucine)$_y$−(γ-benzyl-L-glutamate)$_x$ and obtained thin membranes of the polymer by a solvent-casting procedure using benzene as the solvent. By properly tailoring the chain lengths of the component blocks, we were able to control the microphase-separated morphology of the membranes so that poly(γ-benzyl-L-glutamate) blocks formed cylindrical domains in a continuous matrix of poly(L-leucine). Interestingly, these cylindrical domains formed transmembrane passages that connect the two faces of the membrane. Further, we were able to render these passages hydrophilic by saponifying the benzylglutamate groups to glutamic acid groups. In this state, the membrane functions as a "mesogel"[8,9] in which the poly(L-glutamic acid) domains are selectively solvated by aqueous solutions while the poly(L-leucine) matrix remains hydrophobic and dry.

We recently reported that the saponified triblock copolypeptide membrane described above generated spontaneous electrical pulses under a concentration gradient of KCl.[10] This shows that the membrane is capable of serving as an analog system potentially useful in studying the mechanisms underlying biomembrane excitability.[11] Interestingly, we did *not* observe spontaneous oscillations in the membrane potential with a cross-linked homopolymer membrane cast from poly(L-glutamic acid), under conditions identical with those employed in the experiment with the triblock copolypeptide membrane.

The purpose of this study is to elucidate and substantiate, through experimental evidence, the above-described microphase-separated structure of the triblock copolypeptide membrane. We explore the structure using fluorescence spectroscopy and electron microscopy and determine the average size and the spacing between the cylindrical domains.

Experimental Section

Polypeptides were synthesized by ring-opening polymerization of the *N*-carboxy amino acid anhydride (NCA) monomers. NCAs of γ-benzyl-L-glutamate and L-leucine were first synthesized by reacting γ-benzyl-L-glutamate or L-leucine, respectively, with phosgene in a dioxane solution.[12]

Synthesis of Block Copolypeptide. The polymerization conditions for (γ-benzyl-L-glutamate)$_x$−(L-leucine)$_y$−(γ-benzyl-L-glutamate)$_x$ triblock copolypeptide (PBLG−PLL−PBLG) were as follows: The L-leucine block was synthesized using the L-leucine-NCA in a benzene−dioxane (19:1 vol ratio) solution with 1,6-hexamethylenediamine as the initiator. The ratio of the L-leucine-NCA to the initiator, [B]/[I], was 1000. The polymerization reaction was monitored by measuring the carbon dioxide that evolved. After 3 days the reaction reached more than 90% conversion. On completion of the polymerization of the L-leucine blocks, γ-benzyl-L-glutamate-NCA dissolved in benzene−dioxane solution was added to the polymerization solution. In this solution, the ratio of the γ-benzyl-L-glutamate-NCA to the initiator, [A]/[I], was 600. A total polymerization time of 14 days was allowed. All polymerizations were carried out at room temperature at a concentration of 20 g/L relative to the benzene−dioxane solution. The copolymer was precipitated by pouring the polymerization solution into methanol and then washed several times with methanol. To remove coexisting homopolymers, poly(L-leucine) and poly(γ-benzyl-L-glutamate), the mixture was fractionated using chloroform and trifluoroacetic acid. The product was then dried under vacuum for 5 days.

Monomer composition of the block copolypeptide was estimated by elemental analysis and high-resolution NMR spectroscopy (measured in trifluoroacetic acid). Mole fractions were $x = 0.18$ and $y = 0.64$. Thus, the measured monomer composition was almost the same as the loading ratio of

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L-leucine-NCA and γ-benzyl-L-glutamate-NCA. Intrinsic viscosity of the block copolypeptide determined in a Ubbelohde type viscometer in trifluoroacetic acid (TFA) at 25 °C was 0.689.

Synthesis of Homopolypeptides and a Random Copolypeptide. Homopolymers, poly(L-leucine) (PLL) and poly(γ-benzyl-L-glutamate) (PBLG), were synthesized by polymerization of the corresponding NCA in benzene or dichloromethane, respectively, as a solvent for polymerization. The reactions were carried out at room temperature for 7 days. Triethylamine was used as an initiator in the synthesis of both the homopolymers. Intrinsic viscosities of PBLG in dichloroacetic acid (DCA) and TFA were 1.48 and 0.775, respectively. The molecular weight of the polymer was 270 000, which was calculated by Doty's relationship.[13] The ratio of weight-average molecular mass to number-average molecular mass (M_w/M_n) of PBLG, analyzed with gel permeation chromatographic analysis, was 1.05.

A random copolypeptide of L-leucine and γ-benzyl-L-glutamate, P(LL−BLG), which has similar monomer composition to that of the block copolypeptide (7:3 mol ratio), was synthesized using a mixture of L-leucine-NCA and γ-benzyl-L-glutamate-NCA (7:3 mol ratio) in a benzene−dioxane (7:3 vol ratio) solution with triethylamine as the initiator. The ratio of the sum of both the NCAs to the initiator was 500. The reaction was carried out at room temperature for 7 days. Monomer composition of the random copolypeptide was checked using the same methods as those used with the block copolypeptide. In the case of the random copolypeptide, the measured monomer composition was almost the same as the loading ratio of L-leucine-NCA and γ-benzyl-L-glutamate-NCA. Intrinsic viscosity of the random copolypeptide in TFA was 0.677.

Preparation of the Membranes. Thin membranes (about 0.02 mm thick) of PBLG−PLL−PBLG, P(LL−BLG), and homo-PLL were cast on a glass plate from a benzene solution (0.5%) of the corresponding polymers, and thin membranes of homo-PBLG were cast from the dichloromethane solution. These membranes were dried by slow evaporation of the solvent at 20 °C with subsequent drying under vacuum at 80 °C for 5 days.

Hydrolysis of the Membranes. The PBLG−PLL−PBLG membrane was hydrolyzed in a solvent mixture consisting of methanol−2-propanol−5M NaOH aqueous solution (2:2:1, by vol) for 20 days at 18 °C to eliminate the carboxyl-protecting groups. The hydrolyzed membrane was thoroughly washed with methanol and subsequently with 100 mM HCl and then dried in air. Thus, a triblock copolypeptide membrane (PLGA−PLL−PLGA) composed of L-glutamic acid and L-leucine was obtained. In the case of the random copolypeptide, the hydrolysis reaction was done in the same solvent mixture for 10 days, and a random copolypeptide membrane (P(LL−LGA)) composed of L-leucine and L-glutamic acid was obtained.

Electron Microscopy. The microphase-separated structure of the block copolypeptide membrane was observed using a Nippon Denshi transmission electron microscope JEM-1000CE. Ultrathin sections of about 350 Å thickness were cut perpendicular to the membrane surface with an ultramicrotome, stained by osmium tetraoxide, and viewed under the microscope. The PBLG and PLGA block components were preferentially stained by osmium tetraoxide because these components are more polar than the PLL block component.

Fluorescence Spectroscopy. A fluorescence probe, 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS), was purchased from Wako Chemical Co., Ltd. ANS-incorporated membranes were obtained by contacting the membranes with a 10^{-5} M aqueous ANS solution. Fluorescence spectra of the ANS-incorporated membranes were measured in water with a fluorescence spectrophotometer (Hitachi Co., Ltd., F-3000). The excitation wavelength of ANS was 350 nm.

Hydration Measurement. The degree of hydration of the membrane, i.e., the volume fraction of water in the water-swollen membrane, was determined as follows: The membrane was swollen, blotted, and weighed until a constant weight of the swollen membrane was obtained within an experimental error at 20 °C. The membrane was then dried to a constant weight under vacuum at 80 °C. From these two weights the degree of hydration was computed.

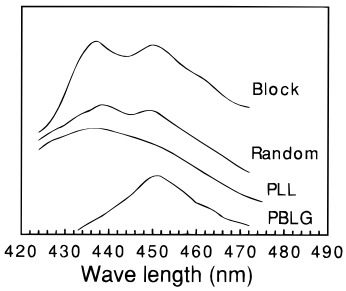

Figure 1. Fluorescence emission spectra of ANS in wet membranes of PBLG−PLL−PBLG membrane (block), P(LL−BLG) membrane (random), poly(L-leucine) membrane (PLL), and poly(γ-benzyl-L-glutamate) membrane (PBLG).

Density Measurement. The densities of the PLL and PBLG membranes were examined using a flotation method with an aqueous calcium chloride solution at 20 °C, and were 1.07 and 1.28, respectively.

Results and Discussion

In order to produce a membrane with a well-defined microphase-separated structure, we synthesized ABA-type block copolypeptide with end blocks of equal chain length. Since preformed polypeptide blocks containing free amino groups can act as initiators for polymerization of the peptide chain with other α-amino acid NCAs,[10] the center block was initially prepared using a diamine initiator followed by simultaneous polymerization of the end blocks.

Fluorescence Spectra of ANS in Membrane. Solvent polarity is known to have an effect on the emission spectrum of dissolved ANS. As the polarity of the solvent becomes lower, the peak in the emission spectrum shifts toward shorter wavelengths: for example, for the solvents water, ethanol, acetone, and hexane, the peaks are observed respectively at 528, 470, 460, and 443 nm. Therefore, the presence of regions of different polarities in the membrane structure can be recognized from the fluorescence spectra of ANS in membranes. This, in turn, could provide clues on the morphological features of the membrane.

Figure 1 shows the fluorescence emission spectra of ANS in PBLG−PLL−PBLG membrane. Two peaks are observed, suggesting that two phase-separated regions which have different polarities exist in the membrane. By comparing this spectrum with the spectra of the PLL and PBLG membranes, the peak at the shorter wavelength in the spectrum of the PBLG−PLL−PBLG membrane is attributed to a phase composed of a poly(L-leucine) block, while the peak at the longer wavelength is attributed to a phase composed of poly(γ-benzyl-L-glutamate) block. However, the random copolypeptide P(LL−BLG) with the corresponding monomer composition also has two peaks similar to PBLG−PLL−PBLG. It should be noted that monomer reactivity ratios of γ-benzyl-L-glutamate-NCA and L-leucine-NCA are 1.57 and 0.61, respectively.[14] This suggests that γ-benzyl-L-glutamate-NCA is more reactive than L-leucine-NCA when both are simultaneously polymerized. The two peaks seen in the spectrum of the random copolypeptide may result from the fact that the two types of monomers are not perfectly arranged at random along the polymer chain. Therefore, a clear conclusion regarding the existence of a *phase-separated* structure cannot be drawn from Figure 1.

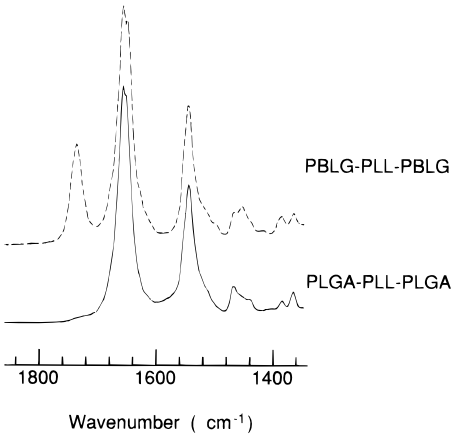

Figure 2. Infrared absorption spectra: (a) PBLG−PLL−PBLG membrane and (b) PLGA−PLL−PLGA membrane after immersion in an aqueous solution of HCl.

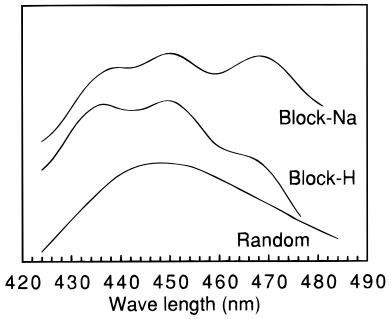

Figure 3. Fluorescence emission spectra of ANS in wet membranes of hydrolyzed PLGA−PLL−PLGA membrane after immersion in the NaOH solution of pH = 10.0 (block-Na), PLGA−PLL−PLGA membrane after immersion in the HCl solution of pH = 2.0 (block-H), and hydrolyzed P(LL−LGA) membrane (random).

Figure 2 shows the infrared absorption spectra of PBLG−PLL−PBLG and its hydrolyzed membrane (PLGA−PLL−PLGA). The absorption peak at 1735 cm^{-1}, which is assigned to the ester-stretching vibration of the γ-benzyl-L-glutamate in the spectra of PBLG−PLL−PBLG membrane, disappeared in the spectrum of the PLGA−PLL−PLGA membrane. This means that the benzyl groups in poly(γ-benzyl-L-glutamate) block chains are converted almost completely by the hydrolysis reaction.

Figure 3 shows the emission spectra of ANS in a PLGA−PLL−PLGA membrane. When the glutamic acid in the PLGA−PLL−PLGA membrane is in the form of sodium salt, three peaks are observed, suggesting that three regions with different polarities exist in the membrane. The peak at the longest wavelength in the spectrum is attributed to the domain composed of the poly(L-glutamic acid) block. When the glutamic acid sodium salt was changed to the glutamic acid form, the peak at the longer wavelength became small. This behavior results from a decrease of hydrophilicity of the poly(L-glutamic acid) domain. The peak at the middle in the spectrum is attributed to the interface region between poly(L-leucine) domains and poly(L-glutamic acid) domains because the position of this peak tallies with the position of the peak of the spectrum of the random copolypeptide P(LL−LGA) with a corresponding monomer composition. These results support, in a more conclusive manner, the notion that a phase-separated structure exists in the PLGA−PLL−PLGA membrane.

Morphology of Block Copolypeptide Membrane. Figure 4 shows an electron micrograph of an ultrathin section cut perpendicular to the surface of the PBLG−PLL−PBLG membrane. The dark portions correspond to the domains composed of the poly(γ-benzyl-L-glutamate) block chains embedded in a continuous matrix of the poly(L-leucine) phase. The structure of the domains is supposed to be nearly cylindrical, extending from one surface to the other. Figure 5 schematically shows the hexagonally packed structure of the cylindrical domains in the block copolypeptide membrane. In order to estimate the diameter of the domains and the distance between two adjacent domains, we measured the width of the dark portions (black stripes) and the distance between the centers of the width of adjacent black stripes on a large number of transmission electron micrographs. Figures 6 and 7 show the histograms of the width and the distance. The data of these histograms are widely spread because the cut surface seen in the electron micrographs is not always an ideal cross section passing through the center of the domains, and also the microstructural elements are actually not well-ordered. But still, these results

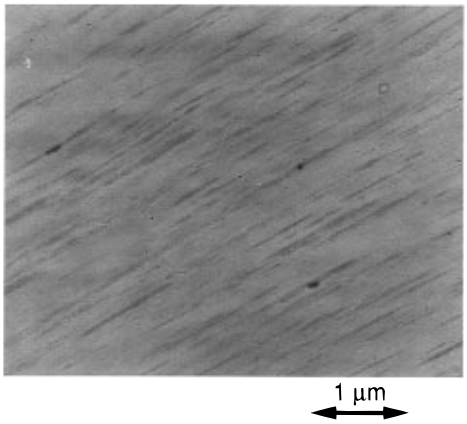

Figure 4. Electron micrograph of an ultrathin section of PBLG−PLL−PBLG membrane (cut perpendicular to the surface).

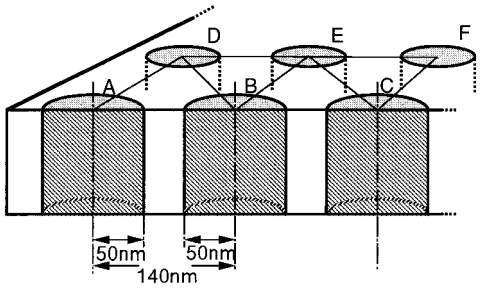

Figure 5. Schematic view of a block copolypeptide membrane with hexagonally packed cylinders. Shaded portions correspond to poly(γ-benzyl-L-glutamate) domains which are surrounded by a matrix of poly(L-leucine).

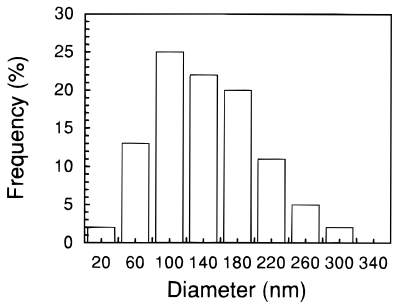

Figure 6. Histogram of the width of black stripes on the electron micrographs.

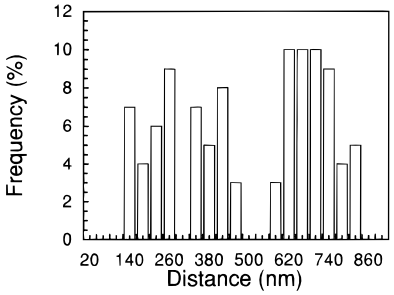

Figure 7. Histogram of the distance between the centers of the width of the adjacent black stripes on the electron micrographs.

suggest that the diameter of the domains is about 100 nm and the distance between two adjacent domains is about 140 nm. When, as in Figure 5, the distance between the central axes of domains A and B is 140 nm, the distance between the central axes of domains A and E is calculated to be 242 nm, and the distance between the central axes of domains A and F is calculated to be 370 nm. Therefore, the wide distribution of the distances seen in Figure 7 seems to be reasonable.

Assuming the domains to be perfect cylinders, the volume fraction of the domain V_d was calculated to be 46%. This value is in good agreement with the theoretical expectation of 48%, calculated from the chemical composition of the copolypeptides and the densities of PLL and PBLG (1.07 or 1.28, respectively), assuming that the volume of the copolymer simply is the sum of the component homopolymer volumes:

$$V_d = \frac{C_B \frac{G_B}{\rho_B}}{C_B \frac{G_B}{\rho_B} + C_L \frac{G_L}{\rho_L}} \times 100$$

where C_B and C_L are the chemical compositions and G_B and G_L are the molecular weights of the γ-benzyl-L-glutamate and L-leucine residues of PBLG−PLL−PBLG, respectively, and ρ_B and ρ_L are the densities of PBLG and PLL homopolymers, respectively. These results provide further justification for the morphological structure suggested above.

Information about the packing of the polypeptide chains in the domains is provided by comparing the diameter of the domains with the length of the polypeptide helices calculated by the formula:

$$D = hP_n$$

where D is the average length of the helix, h (=1.5 Å) is the projection of the distance between two residues on the helix axis, and P_n is the number-average degree of polymerization of the polypeptide block. The calculated lengths of the poly(γ-benzyl-L-glutamate) helix and the poly(L-leucine) helix are 45 and 150 nm, respectively. Since the length of the poly(γ-benzyl-L-glutamate) helix is about one-half the diameter of the domains, these values seem to be reasonable. Poly(L-leucine) chains are obliged to fold in the matrix among the domains.

Influence of Chemical Modification on the Morphology and Degree of Swelling. Figure 8 shows an electron micrograph of an ultrathin section cut perpendicular to the surface of the hydrolyzed membrane (PLGA−PLL−PLGA). The dark portions correspond to the domains composed of the poly(L-glutamic acid) block chains embedded in a continuous matrix of the poly(L-leucine) phase. In comparison with Figure 4, the domain size and the distance between two adjacent domains in PLGA−PLL−PLGA are *the same* as those in PBLG−PLL−PBLG, indicating that the membrane retained its morphology following hydrolysis of the benzylglutamate groups to glutamic acid groups.

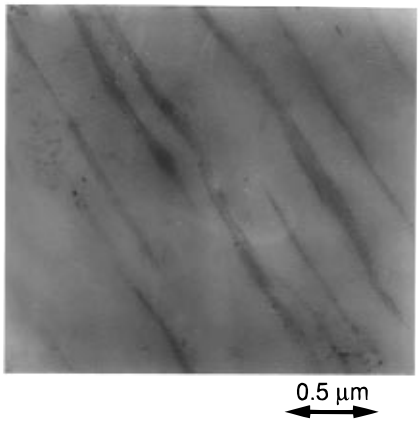

Figure 8. Electron micrograph of an ultrathin section of hydrolyzed PLGA−PLL−PLGA membrane (cut perpendicular to the surface).

Further, since the saponification reaction converts the hydrophobic PBLG domains to hydrophilic PLGA domains, one would expect an increase in the degree of hydration of the membrane. Indeed, our measurements indicate that the degree of hydration had changed from 0.02 to 0.14 following the saponification reaction. However, the hydrolyzed membrane, somewhat surprisingly, did *not* show a macroscopic swelling (i.e., did not change its size). This was the case whether the membrane was immersed in the HCl solution (pH = 2.0) or in the NaOH solution (pH = 10.0). The domain size and spacing also did not change following these wide pH variations, although a conformational change of poly(L-glutamic acid) chains had occurred from an α-helix to a random-coil structure (absorption bands at 1645, 1545, and 615 cm^{-1} shifted to absorption bands at 1653, 1580, and 650 cm^{-1})[15,16] within the cylindrical domains.[17]

This behavior of the hydrolyzed triblock copolypeptide membrane was in stark contrast to that of a glycerin-cross-linked poly(L-glutamic acid) membrane, which was prepared using Noguchi's procedure.[18] In the latter case, the membrane was already swollen tremendously (degree of hydration = 0.96) when the pH of its surroundings reached values between 6 and 7. One could not even hope to study its swelling and hydration behavior in the alkaline pH range because the electrostatic repulsive forces of the ionized glutamic acid groups would disintegrate the gel in this pH range. On the basis of these observations, we concluded that in the case of the triblock copolypeptide membrane, the rigid hydrophobic poly(L-leucine) matrix, surrounding the cylindrical poly(L-glutamic acid) domains, resists the tendency of the domains to swell, even when the glutamic acid chains are totally ionized.

Conclusions

Membranes solvent-cast from the triblock copolypeptide poly(γ-benzyl-L-glutamate)$_x$−poly(L-leucine)$_y$−poly(γ-benzyl-L-glutamate)$_x$, in which the mole ratio of the constituent blocks was properly tailored, displayed a mesophase structure comprised of cylindrical domains

of poly(γ-benzyl-L-glutamate) in a continuous matrix of poly(L-leucine). Further, the poly(γ-benzyl-L-glutamate) domains formed transmembrane channels that connected the two faces of the membrane. We examined the morphology of these membranes by fluorescence spectroscopy and electron microscopy and determined the size and spacing of the cylindrical domains in the membrane. Next, these channels were rendered hydrophilic by saponifying the benzylglutamate groups to glutamic acid groups. In this state, water acts as a solvent that selectively solvates the poly(L-glutamic acid) domains while leaving the poly(L-leucine) matrix dry. While the hydrophilization process increased the water content of the poly(L-glutamic acid) domains, these domains retained their size and shape even when the pH and ionic strength of the aqueous medium was sufficient to ionize all the glutamic acid groups in the domain. We presume that the resistance offered by the rigid poly(L-leucine) matrix prevents expansion of these domains.

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