Engineered nanostructured β-sheet peptides protect membrane proteins

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We designed β-strand peptides that stabilize integral membrane proteins (IMPs). β-strand peptides self-assemble in solution as filaments and become restructured upon association with IMPs; resulting IMP–β-strand peptide complexes resisted aggregation when diluted in detergent-free buffer and were visible as stable, single particles with low detergent background in electron micrographs. β-strand peptides enabled clear visualization of flexible conformations in the highly dynamic ATP-binding cassette (ABC) transporter MsBA.

IMPs, which comprise a third of proteins encoded in genomes, perform essential functions as receptors, transporters and channels. The hydrophobic nature of IMPs requires their solubilization as isolated stable and functional particles but also presents a challenge for many biochemical and biophysical studies. The development of novel amphiphilic reagents, including protein-based nanodiscs1, amphiphilic polymers2, peptide-based detergents3–5 and maltose neopentyl glycol detergents6, among others7, has greatly facilitated functional and structural studies of IMPs. However, each type of amphiphile has its own limitations, and no universal reagent has been developed to facilitate general application in biophysical studies (for example, X-ray crystallography, NMR spectroscopy, electron microscopy and others).

β-sheet peptide assemblies, such as β-barrel proteins in the outer membrane of Gram-negative bacteria8, are characterized by high thermodynamic stability resulting from extensive hydrogen-bond interactions between neighboring strands. This exceptional structural stability has led to interest in de novo design of β-sheet peptides or proteins to address specific biotechnological applications. Here we demonstrate the design of short amphiphilic β-strand peptides that improve the stability of IMPs in solution. The designed β-strand peptides self-assemble into filamentous structures that disperse when mixed with IMPs and detergents, resulting in highly stabilized IMPs that are readily visualized as individual particles by electron microscopy. This approach allowed us to directly visualize multiple conformations of the ABC exporter MsBA in a single electron microscopy preparation, thus providing new opportunities to investigate the structural dynamics of this family of proteins.

We designed three β-strand peptides, with the general sequence acetyl-(octyl)Gly–Ser–Leu–Ser–Leu–Asp–(octyl)Gly–Asp–NH2 (Fig. 1a), to include the following features: (i) alternating polar and apolar residues to present opposite hydrophilic and hydrophobic faces in an extended, β-strand conformation9; (ii) a short, 8-amino-acid sequence of appropriate length to span the central nonpolar region of membrane bilayers (~3 nm)8; (iii) elongated alkyl side chains at each end of the peptide to increase hydrophobicity, a modification important for IMP stabilization as implicated by lipopeptide detergent design9; and (iv) incorporation of N-methyl amino acids that diminish hydrogen-bond donors to regulate interstrand association and avoid the formation of insoluble macroscopic fibril structures10,11. β-strand peptides 1–3 (BP-1, BP-2 and BP-3 with one, two and three N-methyl substituents, respectively) exhibited moderate water solubility (~3 mM for BP-1 and BP-2, and ~1 mM for BP-3), whereas the same sequence lacking N-methyl amino acids was barely soluble in aqueous solution and most organic solvents, making it difficult to purify as a result of its tendency to aggregate due to strong hydrogen-bond interactions. In contrast to the majority of amphiphiles, we designed β-strand peptides to sequester IMP hydrophobic surfaces by forming an ordered, stabilizing β-barrel–like structure, and to decrease dynamic dissociation from IMP surfaces by introducing intermolecular hydrogen-bond interactions (Fig. 1b).

Circular dichroism spectroscopic analysis of the β-strand peptides indicated the formation of β-sheet secondary structures, with ellipticity minima at ~222 nm for BP-1, and ~225 nm for BP-2 and BP-3 (Fig. 1c). These apparent redshifts from the typical β-sheet ellipticity minimum at ~218 nm can be attributed to a conformational twist or distortion due to the N-methylated peptide backbone11,12. Fourier transform infrared spectroscopic analysis of BP-1 also indicated mainly β-sheet secondary structures (Supplementary Fig. 1). Electron micrographs of negatively stained β-strand peptides revealed elongated, flexible filaments ~3 nm across (Fig. 1d and Supplementary Fig. 2), consistent with the dimensions of the designed peptides. Electron micrographs of serial dilutions of BP-1 showed that the number of filaments correlated with the dilution, consistent with the BP-1 filaments being present in solution (Supplementary Fig. 3), and the filamentous structures also remained in supernatant after...
ultracentrifugation (300,000g for 1 h). β-strand peptides formed filaments immediately upon dissolution, even at very low concentration (<1 μM), and we observed no evident growth or aggregation of these filaments even after incubation at room temperature (20 °C) for more than 2 months (data not shown). Thus, filaments of β-strand peptides have features distinct from protein amyloids, which are typically bundles of β-sheets and subject to aggregation through nucleation-dependent polymerization.

We solubilized the ABC exporter MsbA in a solution of β-strand peptides by a complete detergent exchange through dialysis (Online Methods). MsbA is a bacterial homolog of the human multidrug resistance P-glycoprotein and functions natively to transport lipid A using energy generated by ATP hydrolysis. As was the case when MsbA had been reconstituted in liposomes or solubilized in stabilizing detergents, the ATPase activity of MsbA in a solution of BP-1 remained high (~4 μmol min⁻¹ mg⁻¹), comparable to the measured activity of MsbA reconstituted in lipid nanodiscs (Supplementary Fig. 4). The activity of MsbA in a solution of BP-1 exhibited little change throughout 40 d of incubation at room temperature, demonstrating both protein stability and retention of functionality (Fig. 2a). In contrast, measured ATPase activity of MsbA in a solution of β-α-decyl maltoside (UDM), a detergent commonly used for purification and crystallization of MsbA (which performed better than several other commercial detergents; data not shown), was lower (2.2 μmol min⁻¹ mg⁻¹). MsbA solubilized in UDM lost ~80% of its ATPase activity within the first day of incubation at room temperature (Fig. 2a), and this loss was accompanied by protein precipitation.

BP-1 also substantially improved the stability of three other IMPs: bacteriorhodopsin, the tetrameric voltage-gated potassium channel KcsA and full-length glucagon receptor, a class-B G protein-coupled receptor (Supplementary Figs. 5–7). The enhanced stability of IMPs in a solution of BP-1, relative to the case with BP-2 and BP-3, as well as compared to conventional detergents that form micelles driven mainly by hydrophobic effects, may be attributed to the less dynamic association of BP-1 with the hydrophobic surfaces of IMPs, which is likely strengthened by hydrogen bonding among surface-bound BP-1 molecules.

An intriguing question is how IMPs are stabilized by β-strand peptides, which preassemble into filamentous structures. Negative-stain electron microscopy imaging confirmed the disappearance of BP-1 filaments upon addition of UDM-purified MsbA (Supplementary Fig. 8). Subsequent removal of UDM by dialysis lowered the background resulting from the presence of detergent and decreased the particle clustering observed in the electron micrographs, allowing us to visualize single MsbA particles (Supplementary Fig. 8). Circular dichroism spectroscopic analysis of the MsbA–BP-1 sample indicated that the β-sheet secondary structures of BP-1 were preserved (Supplementary Fig. 9). We conclude that the BP-1 filaments undergo a restructuring process directed by the IMP template; in a recent study a
cylindrical β-barrel crystal structure for an 11-residue amyloid-forming protein fragment had been reported19, supporting the feasibility of transformation between the two types of structures. However, a detailed understanding of the process and binding modes involved, whether by formation of a β-barrel or through other types of structures, will require higher-resolution structural studies.

BP-1 enabled us to analyze single particles of MsbA by electron microscopy. When we diluted BP-1–solubilized MsbA samples as much as 100-fold using detergent-free buffer, electron microscopy analysis revealed apparently stable and well-preserved particles (Fig. 2b). We clearly identified multiple conformations of MsbA even in individual particles in the unprocessed electron micrographs. Two-dimensional class-average images of thousands of particles from a single MsbA preparation clearly showed the two nucleotide-binding domains separated at varied distances in the absence of nucleotides (Fig. 2c and Supplementary Fig. 10). Random conical tilt analysis provided unbiased 3D reconstructions (~26 Å resolution for three selected conformations), confirming the conformational heterogeneity of MsbA (Fig. 2d and Supplementary Figs. 10 and 11). In contrast, it was difficult to discern structures of MsbA from UDM preparations because of the pronounced particle clustering and high background resulting from the detergent (Fig. 2e and Supplementary Fig. 8), and dilution of MsbA–UDM complexes with detergent-free buffer resulted in immediate protein aggregation (Fig. 2f). An additional advantage of using BP-1 for electron microscopy analysis, particularly for small IMPs (<150 kDa), is that it contributes very little background clutter as compared, for example, to detergent micelles (Fig. 2e and Supplementary Fig. 12) or to the relatively large nanodiscs (10–15 nm) commonly used in IMP preparations20.

In conclusion, nanostructured β-strand peptides offer a new strategy for stabilizing IMPs. We engineered the short monomers of β-strand peptides (~8 residues) to span the thickness of the membrane lengthwise and to form an ordered secondary structure. Controlled hydrogen bonding between β-strands decreased dynamic dissociation from IMP surfaces as compared to more loosely bound and less ordered detergent molecules. Our electron microscopy studies of the ABC transporter MsbA illustrated the utility of BP-1 as a stabilization reagent to visualize dynamic conformations. The unique features of β-strand peptides confer enhanced IMP stability without constraining protein functionality, demonstrating their potential as broadly useful reagents for IMP research. The enhanced IMP stability and potential to form well-ordered IMP–β-strand peptide complexes suggests the utility of β-strand peptides for high-resolution structural studies such as by X-ray crystallography. We anticipate that β-strand peptides could be engineered to introduce additional features and to enable challenging IMP studies thus far limited by the currently available reagents.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

Peptides were synthesized by H.T. and R.S.R.; peptide assembly was characterized by H.T., S.C.L., A.M. and J.Z.; protein stabilizations were performed by H.T. and S.C.L.; GLR was prepared by F.Y.S. with oversight by R.C.S.; MsbA samples for electron microscopy were prepared by S.C.L.; the electron microscopy study was performed by A.M. with oversight by B.C. and C.S.P.; and data were interpreted by all authors. The manuscript was written by H.T., S.C.L., A.M., B.C. and Q.Z.; and the study was conceived and overseen by Q.Z.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Peptide synthesis. The peptides were synthesized by Fmoc strategy using Rink Amide AM resin (EMD). HCTU (2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) was used as the amino-acid coupling reagent, except that coupling of N-methyl amino acids used HATU (2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate). Fmoc-L-octylglycine ((octyl)Gly) was custom-synthesized or purchased directly from Acros. Peptides were acetylated at the N terminus, cleaved from resin in the presence of 95% trifluoroacetic acid (TFA) and 2.5% triisopropylsilane, then purified by reverse-phase HPLC (CH_3CN-H_2O, 0.1% TFA). Molecular weight of each peptide was verified by electrospray mass spectrometry.

Circular dichroism spectroscopy. Far-UV circular dichroism (CD) spectra were recorded on an AVIV 202-SF CD spectrometer (AVIV Instruments) at 25 °C using a quartz sample cell with 1 mm path length (1 nm step size, 3 s integration per step, average of three scans). Samples of β-strand peptides (BPs) were prepared at concentrations of ~0.1–1 mM in buffer containing 100 mM Tris (pH 7.35) and 40 mM NaCl. Molar ellipticity [θ] was calculated from the equation [θ] = (100 × θ(ϵ × l), where θ = observed ellipticity (in degrees), c = concentration (in moles per liter), l = cell path length (in centimeters).

Fourier transfer infrared spectroscopy. Fourier transfer infrared (FTIR) spectra were collected on a Bruker Equinox 55 FTIR spectrometer equipped with liquid N_2 cooled HgCdTe infrared detector, which was continuously purged with dry N_2 gas. BP-1 (2.5 mM) was dissolved in D_2O solution containing 100 mM Tris (pH 7.5) and 40 mM NaCl. Approximately 8 µl of sample volume was loaded into a liquid cell with CaF_2 windows and a 75 µm Teflon spacer. Four thousand scans with 2 cm⁻¹ resolution were averaged and a Blackman-Harris 3-term apodization function was used in the Fourier transform. Absorbance of BP-1 was corrected by subtracting measured absorbance from that for buffer alone. Absorbance in the amide I band (1,700–1,600 cm⁻¹) was used for the assignment of peptide secondary structures.

Preparation of MsbA, bacteriorhodopsin and KcsA. E. coli MsbA and Halobacterium bacteriorhodopsin (br) were prepared as described and purified in UDM and β-d-octylglucoside (OG) (Affymetrix), respectively. Full-length KcsA was prepared as described, except that the step for cleavage of C-terminal fragment was eliminated and β-d-decylmaltoside (DM) was used throughout membrane solubilization and protein purification. BPs were mixed with the purified protein typically in a 50:1–100:1 molar ratio. However, a ~25-fold molar excess of BP-1 was found to sufficiently stabilize MsbA. The primary detergents were removed by dialysis against detergent-free buffer (6 kDa cutoff membrane, 4 °C, 2 d), monitored by thin-layer chromatography. Complete detergent exchange of UDM to BP-1 in MsbA samples was achieved in ~8 h, and E. coli lipids in the protein sample were stained by iodine vapor and molybdenum blue, which gave the lipid:MsbA ratio ~0.03:1 (wt/wt). IMPs solubilized in BPs remained in supernatant after centrifugation at 300,000g for 2 h at 4 °C, and were analyzed by SDS-PAGE. Extraction and purification of MsbA in lauryl maltose neopentyl glycol (LMNG, Affymetrix) was conducted similarly as in UDM. MsbA was also solubilized in Amphipol A8-35 (Affymetrix) at a weight ratio of 5:1 (A8-35: MsbA) according to a published procedure. Reconstitution of MsbA and KcsA into nanodiscs comprised of MSP1D1 and dimyristoylphosphatidylcholine (DMPC) was carried out following published procedures.

Preparation of glucagon receptor. Human wild-type glucagon receptor (GLR), a class-B G protein–coupled receptor (GPCR), containing a PreScission protease site, 10× His and Flag tag at the C terminus was expressed in Spodoptera frugiperda (Sf9) insect cells. Sf9 membranes were prepared with 1 wash cycle of hypotonic buffer (25 mM HEPES (pH 7.5), 10 mM MgCl₂ and 20 mM KCl) in the presence of EDTA-free protease inhibitor cocktail tablets (Roche) and four wash cycles of high-salt buffer (25 mM HEPES (pH 7.5), 1 M NaCl, 10 mM MgCl₂ and 20 mM KCl). Two grams of washed membranes containing the human wild-type GLR were incubated with 270 µM of antagonist NNC359 (provided by Novo Nordisk) and 2 mg/ml of iodoacetamide (Sigma) for 30 min at room temperature. The receptor was solubilized in a solution containing 1.00% and 0.20% (w/v) of n-dodecyl-β-d-maltopyranoside and cholesterol hemisuccinate (DDM-CHS), respectively, or UDM-CHS for 2 h at 4 °C.

The NaCl and DDM-CHS concentrations of the solubilized protein were adjusted to 800 mM and 0.50%-0.10%, respectively. The solubilized protein was bound to talon resin (Clontech) overnight in the presence of 15 mM imidazole (pH 7.5) and 100 µM of NNC359. The talon resin was washed with 10× bed volume of wash buffer (25 mM HEPES (pH 7.0), 800 mM NaCl, 10% glycerol, 0.05%-0.01% DDM-CHS or 0.10%-0.02% UDM-CHS, 50 µM NNC359, 40 mM imidazole (pH 7.5)). Wild-type GLR was eluted with elution buffer (25 mM HEPES (pH 7.0), 150 mM NaCl, 10% glycerol, 0.05%-0.01% DDM-CHS or 0.10%-0.02% UDM-CHS, 50 µM NNC359, 300 mM imidazole (pH 7.5)). The eluted GLR was concentrated using a Vivaspin centrifuge concentrator (GE Healthcare) with a 100,000 kDa molecular weight cut-off, and protein concentration was determined by analytical size-exclusion chromatography. The protein was then diluted to 1.5 mg/ml with elution buffer containing no imidazole or ligand.

For stability test, GLR–BP-1 samples were prepared in two different approaches, either by 20× dilution of purified GLR (DDM-CHS preparation) into the same buffer containing BP-1 (0.50 mM) or by dialysis as described above for other IMPs. Each set of samples contained the same concentration of NNC359. To facilitate detergent exchange, GLR purified in UDM-CHS was used in the dialysis experiment in which no GLR ligand was present in the dialysis buffer, and NNC359 was added back to keep the ligand concentration as about the same as in the control sample before dialysis.

Stability assays. Stability of MsbA, bR and KcsA in BPs and control detergents was assayed at sample concentrations ranging from 0.2 mg/ml to 0.5 mg/ml according to literature procedures. BPs samples in buffer (100 mM Tris and 40 mM NaCl, pH 7.4) were incubated at 37 °C for 2 months, and stability of bR was monitored by recording its characteristic UV–vis absorption spectrum. MsbA samples prepared in 20 mM Tris (pH 7.5) plus 20 mM NaCl buffer were incubated at room temperature for 40 d, and stability was monitored by measuring MsbA ATPase activity.
using a standard linked enzyme ATPase assay\textsuperscript{25}. KcsA samples in buffer (20 mM Tris and 20 mM NaCl, pH 7.5) were incubated at 37 °C for 5 d, and then analyzed using SDS-PAGE\textsuperscript{26}.

The stability of GLR was monitored using a fluorescence thermal denaturation assay\textsuperscript{27}, which has been used for several GPCRs and other classes of IMPs. Briefly, 4 µg of GLR was incubated with 0.4 µg of thiol-reactive fluorophore, N-(4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl)maleimide (CPM), for 20 min at 4 °C in a total volume of 60 µl. Fluorescence emission was then measured on a Cary Eclipse fluorescence spectrophotometer (\(\lambda_{ex} = 387 \text{ nm}; \lambda_{em} = 463 \text{ nm} \)) from 20 °C to 90 °C with 1 °C intervals and a ramp rate of 2 °C/min, and the background fluorescence of buffer in the absence of protein was subtracted. Midpoints of the thermal transitions (\(T_m\)) were obtained using a least-squares nonlinear regression analysis (GraphPad Prism) of fluorescence signal versus \(T\) plots as described previously\textsuperscript{28}.

**Electron microscopy.** All samples were applied onto freshly glow-discharged carbon-coated copper grids. The sample was diluted to distribute particles homogenously over the grid surface; in most cases a 1:20 dilution with detergent-free buffer was sufficient. Sample (3 µl) was applied to the grid and allowed to adhere for 15 s before being blotted from the side. Immediately after blotting, 3 µl of 2% uranyl formate solution was applied onto the grid and blotted off directly from the same side. This was repeated three times. Ten milliliters of 2% uranyl formate was freshly prepared, aliquotted into 40-µl portions, flash-frozen in liquid nitrogen and stored at \(-80^\circ \text{C}\). For every grid a fresh aliquot was used. Data were acquired using a Tecnai F20 Twin transmission electron microscope operating at 200 kV, using a dose of \(\sim 45 \text{ e}^-/\text{Å}^2\) and nominal underfocus ranging from 1.5 µm to 2.5 µm. Images were collected automatically at a nominal magnification of 80,000\times and pixel size 0.136 nm onto a Tietz F415 4,000 × 4,000 pixel charge-coupled device (CCD) camera (15 µm pixel) using the Leginon data collection software\textsuperscript{29}. Experimental data were processed by the Appion software package\textsuperscript{30} interfaced with the Leginon database infrastructure. Particles were automatically selected using a difference of Gaussian (DoG) algorithm\textsuperscript{31} and extracted with a box size of 128 pixels. XMIPP (X-windows based microscopy image processing package) reference-free maximum likelihood alignment\textsuperscript{32} was applied to identify the best particles from the selection using 20 averages. All resulting class averages of the maximum likelihood alignment were used as reference for Spider 2D alignment\textsuperscript{33} with consecutive Coran classification. Random conical tilt reconstructions were automatically carried out on selected 2D class averages\textsuperscript{31}. Each volume contained 100–200 single particles.

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