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Tuning membrane protein mobility by confinement into nanodomains

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Abstract

High-speed atomic force microscopy (HS-AFM) can be used to visualize function-related conformational changes of single soluble proteins. Similar studies of single membrane proteins are, however, hampered by a lack of suitable flat, noninteracting membrane supports and by high protein mobility. Here we show that streptavidin crystals grown on mica-supported lipid bilayers can be used as porous supports for membranes containing biotinylated lipids. Using SecYEG (protein translocation channel) and GlpF (aquaglyceroporin), we demonstrate that the platform can be used to tune the lateral mobility of transmembrane proteins to any value within the dynamic range accessible to HS-AFM imaging through glutaraldehyde-cross-linking of the streptavidin. This allows HS-AFM to study the conformation or docking of spatially confined proteins, which we illustrate by imaging GlpF at sub-molecular resolution and by observing the motor protein SecA binding to SecYEG.

Planar supported lipid bilayers (SLB) are formed on hydrophilic solid supports such as mica, silicon-oxide, or glass. They are well accessible to optical microscopy and other surface analytical techniques such as quartz crystal microbalance¹ and surface plasmon resonance². Due to their high mechanical stability and flatness, they are particularly suited for high speed atomic force microscopy³ to study model lipid rafts⁴, pore forming toxins⁵, antibody-antigen⁶, and membrane protein interactions^{7, 8}. Depending on their lipid composition,

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Author contributions

A.K. and J.P. performed HS-AFM experiments and performed data analysis. B.N., B.P., and E.K. performed fluorescence experiments and did data analysis. A.K., A.H., D.K., R.K., K.W., L.W., C.S., N.O., and J.P. developed sample preparation techniques. J.P. and A.K. designed the experiments. A.K., J.P. and P.P. prepared the final manuscript.

Competing financial interests

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supported membranes are separated by an up to 5 Å thin water layer from the support^{9–11}. Nevertheless, lipid mobility is lower than in free-standing bilayers¹². Depending on the size of transmembrane protein domains that protrude out of the membrane, the lateral mobility of transmembrane proteins can vary to a much larger extent: from completely immobile^{13, 14}, to intermediately mobile^{7, 8}, or even highly mobile^{8, 15}. The mobility can even exceed the detectable range of HS-AFM so that they are no longer detected as laterally extended objects, but as spike-like topographical noise observed in HS-AFM images¹⁶. Interactions with the support are also expected to prevent conformational changes related to function of transmembrane proteins^{17, 18}, or substrate translocation by transmembrane transporters as there is insufficient space on the support side of the membrane into which a translocating substrate¹⁹ could be released.

In contrast, free standing bilayers²⁰ or polymer supported bilayers²¹ provide the necessary motional freedom and space on both membrane sides. However, single membrane proteins embedded in such bilayers exhibit high lateral mobility of $\sim 1 \mu\text{m}^2/\text{s}$ ²⁰ that is far beyond the mobility accessible for HS-AFM^{6, 8}. The lack of mechanical stability of the free standing membrane itself or the roughness of its surface do not allow for HS-AFM high resolution imaging of single membrane proteins (unlike membrane proteins embedded in a 2D crystal²²). Our approach combines the advantages of free-standing and mica-supported membranes, that is, there is sufficient space on the support side to minimize uncontrolled interactions, as well as flatness and mechanical stability. It further allows for local tuning of the membrane protein's lateral mobility and its confinement into nanometer sized raft-like domains that could serve as model systems for compartmentalized biochemical processes in the cell membrane. We demonstrate the capability of our platform by providing the bacterial translocation channel SecYEG^{19, 23} (*E. coli*; heterotrimer, ~ 74 kDa) and the bacterial glycerol uptake facilitator GlpF^{24, 25} (from *E. coli*; homotetramer, ~ 134 kDa) with a defined lateral mobility. We further show that the mechanical stability is sufficient to perform HS-AFM high-resolution imaging by revealing the tetrameric structure of GlpF and we demonstrate binding of the protein translocase motor SecA to SecYEG confirming that proteins are structurally and functionally intact.

Platform design

A sketch of our platform is depicted in Figure 1a: We use a bottom-up approach based on the self-assembling properties of streptavidin (SA) molecules to form regular two dimensional arrays on mica-supported fluid lipid bilayers containing biotinylated lipids^{26–28}. These arrays then serve as porous support for the upper reconstituted lipid bilayer. It is generated by spreading proteoliposomes that contain a small fraction of biotinylated lipids (5 %). First, the homo-tetrameric SA self-assembles on a lower supported fluid biotinylated planar lipid bilayer into highly ordered P2 symmetric crystalline arrangements (Fig. 1b) comprising a unit cell characterized by $a=b=5.90 \pm 0.07$ nm and $\gamma=90 \pm 1^\circ$ (Fig. 1c). Two of its monomeric biotin-binding subunits are thereby oriented towards the bilayer and occupied by biotin that is tagged to the dioleoyl phosphatidyl ethanolamine (DOPE) lipids, while the other two subunits are unoccupied facing away from the support. These subunits are accessible to high resolution HS-AFM imaging (Fig. 1c), and can be further used as specific sites for binding of biotinylated entities, as demonstrated for various soluble proteins²⁷.

The SA-crystal also provides small buffer-filled compartments that occupy ~ 37 % (volume ~ $4.5 \times 2.6 \times 4 \text{ nm}^3$, estimated from Fig. 1c and the height of the SA-crystal) of the unit cell area. Proteoliposomes containing either SecYEG or GlpF (for purification and reconstitution details cf. methods section and Supplementary Fig. 1) spread on the SA-crystal and fused to large periodically suspended membranes (PSM). Spreading was due to the specific interactions between SA and 5% of biotinylated DOPE lipids that were also included into the proteoliposomes. The size of the PSM patches is sensitive to the concentration of the proteoliposome suspension and the timespan it is applied to the SA-crystal (cf. Supplementary Fig. 2). The resulting membranes appear flat and stable, with a thickness of ~ 4 nm, indicating that they closely adhere to the underlying SA-crystal. The presence of SecYEG and GlpF could be recognized in HS-AFM movies (Supplementary Movie S1) as characteristic, spike-like topographical noise indicative for fast diffusing objects¹⁶. These fast lateral dynamics suggest that interactions with the support are vastly reduced. To fully resolve membrane proteins by HS-AFM, it is necessary to reduce their mobility to match the instrument's imaging capabilities that allows us to capture diffusional processes of up to a few hundreds of nm^2s^{-1} 6–8. Therefore, we introduced additional membrane obstacles in the form of glutaraldehyde (GA) crosslinks of neighboring SA molecules subsequent to SA-crystal formation (cf. methods). After spreading proteoliposomes on the SA-crystal, we finally observed membrane proteins of different mobilities (in this case SecYEG) in this uppermost bilayer (note that the incubation time interval used for Fig. 1d was chosen to be small in order to demonstrate the layer-by-layer composition of the platform; Full coverage: Supplementary Movie S2; Supplementary Fig. 2).

Apparent protein abundancies on SA-crystals and mica SLBs

The extent of chemical fixation of SA arrays prior to the final membrane deposition process can be used to fine-tune the lateral mobility of embedded proteins, as examined for SecYEG^{19, 23} and GlpF^{24, 25}. PSMs were generated on SA-crystals that were treated for a defined time (5 min) with different GA concentrations. Apparent protein abundance per scan area was then determined by HS-AFM imaging (Supplementary Movie S2, cf. methods) and compared to the corresponding values from conventional mica SLBs (Fig. 2a). The number of SecYEG detected via HS-AFM depended on the GA exposure during SA-crystal preparation. At the highest GA concentration, it twofold exceeded the number observed in SLBs on mica (black dashed line). In contrast, the abundance of GlpF detected by HS-AFM did not depend on glutaraldehyde treatment of SA-crystals, but resembled the value obtained from mica-SLBs (orange dashed line). For both SecYEG and GlpF, only proteoliposomes from the same reconstitutions were compared and therefore all membranes should have contained the same amount of proteins. The discrepancy in the apparent SecYEG abundancies can be rationalized by assuming that (i) ~50% of the SecYEG molecules on mica SLBs diffuse too fast to be captured by HS-AFM, and (ii) the SecYEG molecules on SA-crystals slow down thus enabling their identification and counting by HS-AFM imaging (which was not possible for PSMs on SA-crystals without GA treatment, compare Supplementary Movie S1). As the SecYEG and GlpF molecules we have used throughout this study carried a fluorescence dye (ATTO 488, cf. methods) we could test this hypothesis, by characterizing the mobility of SecYEG and GlpF that were reconstituted into mica SLBs

or SA-crystal PSMs (10 mM GA) by means of fluorescence single particle tracking. Single particle trajectories were recorded and examined (cf. methods). On mica SLBs the data was best fit by a model²⁹ including two independent mobility components. Following the suggestion of Occam's razor, we abstained from using models with a larger number of components, although they would also be compatible with the data. The analysis of trajectories (Supplementary Fig. 3) revealed that ~44% of SecYEG exhibited a high mobility of $D=9.8 \pm 3.3 \times 10^4 \text{ nm}^2\text{s}^{-1}$ that is indeed too fast to be captured by HS-AFM (limited up to a few hundreds of nm^2s^{-1} 6–8), whereas the remaining fraction (~56%) had a lower mobility of only $D=0.2 \pm 0.1 \times 10^4 \text{ nm}^2\text{s}^{-1}$. Fluorescence measurements of SecYEG in SA-crystal PSMs, GlpF in mica SLBs, and GlpF in SA-crystal PSMs only detected a single slow fraction. The excessive GA concentration for SA fixation was responsible for protein slow-down in SA-crystal PSMs (compare next paragraph).

Tailoring membrane protein mobility

HS-AFM imaging of SecYEG and GlpF revealed two fractions: An intermediate and a slow mobility fraction. Increase of GA concentration during the SA-crystal fixation step decreased the intermediate mobility fraction and increased the slow mobility fraction, respectively. To characterize both lateral mobility and the respective fractions, we performed a quantitative analysis of the corresponding HS-AFM movies by generating SecYEG and GlpF trajectories and subjected the ensemble to a diffusional analysis again using a model²⁹ including two independent mobility components (cf. methods; Supplementary Fig. 4): the first population of molecules possessed an intermediate mobility (i.e. they were less mobile than the fast molecules that were observed only by fluorescence), while the mobility of the second population was so low that the calculated diffusion constant was smaller than the positional accuracy. Nevertheless, we do not call them immobile because we observed molecules switching from intermediate to low mobility and back (Supplementary Movies S3 and S4). Depending on GA concentration during SA-crystal preparation, the average fraction of intermediately mobile proteins was tuned from ~25 - 30% (0.4 mM GA) to ~0 - 8% (10 mM GA), Figure 2b. Similarly, the mobility of these fractions was reduced from $80 \text{ nm}^2\text{s}^{-1}$ to ~ $1 \text{ nm}^2\text{s}^{-1}$ (SecYEG) and $40 \text{ nm}^2\text{s}^{-1}$ to $10 \text{ nm}^2\text{s}^{-1}$ (GlpF) when SA-crystals were treated with increasing GA concentrations (Fig. 2c). Fluorescence recovery after photo bleaching (FRAP) and single particle tracking experiments (cf., methods; Supplementary Fig. 5) served to assess membrane quality and fluidity of SA-crystal PSMs. The fluorescent lipids recovered homogenously with a diffusion coefficient of ~ $1.5 \mu\text{m}^2\text{s}^{-1}$ (determined from FRAP and single particle trajectories, Supplementary Fig. 5) indicating the presence of continuous SA-crystal PSMs.

Crowding is responsible for confinement into nanodomains

Without GA treatment of the SA-crystal, membrane proteins moved too fast to be fully resolved by HS-AFM (Supplementary Movie S1). A minimal treatment of 0.4 mM GA was necessary to effectively slow down their diffusive motion. Furthermore, a close inspection of trajectories (Fig. 3a and b, Supplementary Movie S3 and S4) revealed that the proteins did not randomly diffuse but that they were confined into nanodomains: The proteins repeatedly moved bi-directionally along the same tracks. The lateral dimension of these domains

depended on the GA concentrations, as indicated by the loss of the intermediately mobile protein fractions at the highest GA concentrations (Fig. 2b and c). A high resolution image of a SA-crystal (Fig. 3c; white and yellow axes) reveals the mechanism for the observed dynamics: The contact region between two adjacent SA molecules is not planar but features a groove that connects the holes in the SA-crystal, thereby allowing flexible membrane-protruding moieties¹³ to pass. As this groove propagates throughout the SA-crystal (white and yellow axes, Fig. 3c), we suggest that GA treatment effectively introduces obstacles within these grooves by covalently interlinking amine groups. We have generated a model of a SA-crystal (Fig. 3d; based on the SA structure pdb 3RY230, and lattice constants, Fig. 1c) and identified lysine and arginine residues within flexible loops on the SA surface as potential cross-linking sites within the groove at the interface between neighboring SA molecules (Fig. 3e and f). Without crosslinking, the parts of SecYEG and GlpF protruding the membrane are likely to slip between adjacent SA molecules, thereby retaining high lateral mobility. The passage of membrane protruding parts of SecYEG and GlpF became increasingly restricted as the number of crosslinks increased. Starting from a certain GA threshold concentration the tracks are no longer connected to other ones. That is, proteins are trapped within these GA-induced nanodomains and within these, their motion is restricted by the discrete template provided by the meshwork of holes and obstacle-free grooves between adjacent SA molecules. This results in trajectories that have parallel and perpendicular components (in accordance with the underlying grooves) as shown in Figure 3a and b, as well as in Supplementary Movie S3 and S4. As GA concentration is further increased the lateral dimension of nanodomains further shrinks so that finally all membrane proteins belong to the slow mobility fraction (Fig. 2).

Topographical and functional assessment of membrane proteins

The GlpF and SecYEG proteins were examined for their functionality in standard *in vitro* assays. GlpF was proven to be active by measuring its water permeability from osmotically challenged vesicles in a stopped flow assay¹⁴, and SecYEG was subject to a standard translocation assay³¹. We further analyzed the size (heights) of the membrane-protruding proteinaceous parts with respect to the surrounding lipids to determine their orientation (periplasmic/cytoplasmic surface up/down) in mica SLBs and SA-crystal PSMs. A probability density function (pdf) of height values was generated from height measurements of membrane protruding GlpF moieties on mica SLBs (dashed line) and SA-crystal PSMs (solid line, Fig. 4a, left panel). A detailed analysis (Supplementary Fig. 6a and b) revealed that the pdfs were best fit by a sum of three Gaussians (I-III). Comparison to the crystal structure (Fig. 4a, right panel) shows that for the SA-crystal PSMs, the mean values of these Gaussians match the heights of membrane protruding ends of transmembrane helices at the cytoplasmic side (I; fraction 0.40 ± 0.02), as well as the alpha-helical protrusions at the periplasmic side (II; fraction 0.44 ± 0.02). Occasionally (fraction 0.16 ± 0.03), some molecules appeared higher (III), very likely reflecting the unstructured, very flexible 40aa linker masking the lower protrusions (I) at the cytoplasmic side. In total, 56 % of molecules were oriented with the cytoplasmic side (I+III) and 44 % with the periplasmic side (II) facing upwards. For mica SLBs (Fig. 4a, dashed line), the shape of the pdf was comparable, however, the relative orientation of molecules was slightly different, with 43% of molecules

oriented with the cytoplasmic side (I+III) and 57 % of molecules with the periplasmic (II) side facing upwards. The quality of high resolution images obtained from GlpF tetramers embedded in SA-crystal PSMs (immobile, Fig. 4b, and mobile, Supplementary Movie S5) was comparable to that of images of GlpF embedded in mica SLBs taken previously¹³. A similar height-analysis was done for SecYEG (Fig. 4c, left panel; Supplementary Fig. 6c and d). For SA-crystal PSMs, the pdf (solid line) was best fit by a sum of three Gaussians. Comparison to the crystal structure (right panel) revealed that the mean values of Gaussians match the heights of secondary structural elements protruding from the membrane at the periplasmic side (I; fraction 0.47 ± 0.06), as well as the lower protrusion (II, fraction 0.43 ± 0.05) and the less frequently detected (since more flexible), higher protrusion (III, fraction 0.10 ± 0.04) both at the cytoplasmic side. In total, 47 % of proteins detected in SA-crystal PSMs were oriented with the periplasmic side upwards (I) and 53 % of molecules (II+III) were oriented with the cytoplasmic side facing upwards. For the mica SLBs, the pdf (dashed line; Fig. 4c, left panel) was best fitted by a sum of two Gaussians (Supplementary Fig. 6d). The mean value of the first Gaussian (I; fraction 0.73 ± 0.03) again matches the height of the periplasmic protrusion and the mean value of the second Gaussian (II) was found to be in between the values identified for the cytoplasmic protrusions of SecYEG in the SA-crystal PSMs and occurred less frequent (0.27 ± 0.03) than these. In total 73 % of the proteins detected in mica SLBs were oriented with the periplasmic side upwards and 27 % of molecules were oriented with the cytoplasmic side facing upwards. As the overall number of proteins detected in mica SLBs was ~ half the number detected in SA-crystal PSMs (cf. Fig. 2a) this 27% represent only 13.5% of the overall protein content suggesting that the majority of proteins with the cytoplasmic side facing upwards (37.5% of total protein content) are included in the fast-diffusing fraction as determined by single particle tracking (Supplementary Fig. 3), and are thus too fast for HS-AFM observation. As these protrusions are representing the binding site for the translocase motor protein SecA_{32, 33} (post-translational translocation) and the ribosome¹⁹ (co-translational translocation), SecA binding to this fraction cannot be observed by HS-AFM either.

Catching the act of ligand binding to membrane receptors

However, on SA-crystal PSMs, this fraction was accessible to HS-AFM imaging, so that SecA being bound to SecYEG was observed (Fig. 4d). SecYEG-containing SA-crystal PSMs were first incubated with 2 μ M SecA for 2 min, then rinsed to remove unbound SecA and subsequently subjected to HS-AFM imaging (Fig. 4d). The SecA particles protruded up to 4 nm from the SA-crystal PSMs (Fig. 4c, red curve) and were stably bound to SecYEG (Fig. 4d) which is in agreement with surface plasmon resonance measurements of SecA binding to SecYEG in inner membrane vesicles³⁴. This confirms that the involved epitopes on SecYEG₃₃ are structurally intact and functional, and that the involved rearrangements of SecYEG transmembrane helices necessary to facilitate this association³³ can occur without hindrance. On rare occasions SecA was released, thereby exposing the SecYEG molecule that was underneath (Fig. 4d, circle). Sometimes we observed its subsequent re-association (Supplementary Movie S6). In addition, we found relatively high topographical ‘spike’ noise¹⁶ under these conditions that we attributed to lipid bound SecA₃₅. These SecA molecules migrated along the membrane surface too fast (apparent lateral diffusion

coefficient $D=1.86 \pm 0.16 \times 10^6 \text{ nm}^2\text{s}^{-1}$; assessed by FRAP) to be fully resolved by HS-AFM imaging.

Conclusions

Protein reconstitution into SA-crystal PSMs allowed us to mimic the crowded environment of biological membranes. In this way, we have tuned the mobility of membrane proteins with protrusion sizes ranging between $\sim 0.7 - 3 \text{ nm}$ (13 to 51 residues) which covers the vast majority of extra-membraneous domains of integral membrane proteins found throughout eubacterial, archaean, and eukaryotic organisms³⁶. While retaining their natural lateral and rotational mobility, reconstituted membrane proteins can now be imaged at sub-molecular resolution by virtue of their transient confinement into nanodomains that are similar in size to rafts or other cytoskeleton assisted nanodomains.

Thus SA-crystal PSMs offer the possibility to use high speed atomic force microscopy³ to capture (i) dynamic conformational changes of single membrane and soluble proteins^{37, 38} alike and (ii) interactions of soluble proteins and membrane proteins with nm-spatial and millisecond temporal resolution.

The technique may also be of interest to conventional AFM as the proteins may be rendered practically immobile without being tethered or being in contact to the support. The platform is well suited for studying reconstituted membrane proteins not only by AFM but also by other techniques - like by optical microscopy (e.g. single dye tracing and fluorescence resonance energy transfer), surface plasmon resonance, or quartz crystal micro balance. It thus eases the investigation of interactions between membrane proteins and peripheral proteins, or compartmentalized biochemical processes.

Confinement of mobile membrane proteins into small patches is also possible by suspending lipid bilayers on highly ordered functionalized pore arrays in silicon nitride. The main limitation here is not the pore size that can be reduced below the commonly used $\sim 1 \mu\text{m}$ ³⁹, but is rather represented by the strict border of these domains that no membrane protein can pass. In contrast, the nanodomains in SA-crystal PSMs can be tuned so that the confinement is transient, i.e. that a certain probability of protein escape remains. In this sense, they are a much better model of membrane rafts, which allow for protein and lipid exchange with their environment. It is also imaginable that the incorporation of photo-reactive amino acids into SA molecules at locations that form the SA-SA interfaces within the SA crystals might offer the possibility to use a patterned photo-crosslinking approach to generate domains with specifically tailored sizes, orientations, and shapes.

The combination of SA-crystal PSMs with HS-AFM will further deepen our knowledge about the dynamic structure of membrane proteins and their interplay with peripheral proteins. It will enable us investigation of receptor activation by peripheral ligands that migrate along the membrane surface to find their target. In the particular case of bacterial translocation machinery, it puts the direct visualization of protein translocation within tangible reach.

Methods

Streptavidin crystal suspended membranes (SA-crystal PSMs)

Streptavidin-crystals were prepared as described²⁷. Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) were mixed at a ratio of DOPC:DOPS:DOPE-cap-biotin = 6:2:2 (w/w) and dissolved in a 2:1 mixture of chloroform and methanol. The solvents were evaporated for 30 min and the lipids were dissolved in chloroform, which was again evaporated for 30 min. Drying was completed on a high-vacuum pump for 2h. The mixture was solved in 500µl Milli-Q H₂O in a test tube and flooded with argon gas to get rid of air. Then the mixture was sonicated for 5-10min to obtain small unilamellar vesicles and finally diluted with crystallization buffer (CB; 10 mM HEPES, 150 mM NaCl, 2 mM Ca₂Cl₂, pH 7.4) to a concentration of 1mg/ml. These vesicles were applied to freshly cleaved mica for 15 min followed by rinsing with CB. Then streptavidin (0.1 mg/ml in CB) was incubated on top of the resulting bilayer for 2.5 h. After another rinsing step with CB, the formed streptavidin crystals were chemically fixed with glutaraldehyde (0.4 - 10 mM in CB) for 5 min and then quenched with Tris-HCl (2 - 20 mM in CB). SecYEG and GlpF containing proteoliposomes were diluted in measuring buffer (MB; 50 mM Tris, 50 mM KCl, 50 mM NaCl, 5 mM MgCl₂, pH 7.9) to a concentration of 1 mg/ml and applied to the streptavidin crystals for 15 min. The sample was rinsed with MB and subsequently imaged in MB. For comparison, the same proteoliposome samples were applied to freshly cleaved mica for 15 min, rinsed and imaged in MB.

SecA binding to SecYEG

2 µM SecA was applied to SecYEG containing SA-crystal PSMs for 2 min. followed by rinsing with MB to remove unbound SecA from the sample volume.

Protein purification and reconstitution into proteoliposomes

SecYEG and GlpF were modified by site-directed mutagenesis to contain only one (SecY, A204C; periplasmic loop) or two cysteine residues (Glpf, C261 and C264; both located at the periplasmic C-terminus), respectively, thereby enabling covalent labeling with the fluorescent dye Atto 488 maleimide. His-tagged GlpF and SecYEG were expressed, purified on a Ni²⁺-column followed (in case of SecYEG) by a size-exclusion chromatography step (Äkta Pure, column SuperDex 200 Increase100/30), and reconstituted into E.coli total lipid extract and additional 5 % DOPE-cap-biotin (Avanti Polar Lipids, Alabaster, AL, U.S.A.) as previously described^{25, 31, 40}. All steps including the reconstitution step were controlled by SDS-PAGE (Supplementary Fig. 1). Proteoliposomes were extruded through two stacked 100 nm polycarbonate filters (Avestin, Ottawa, Canada). Reconstitution efficiency of SecYEG and GlpF into proteoliposomes was checked by fluorescence correlation spectroscopy¹⁴ (LSM 510 META/ConfoCor 3, Carl Zeiss, Jena, Germany). The reconstituted GlpF was further subjected to a functional test in a stopped flow apparatus. Its single channel water permeability was derived as previously described¹⁴.

SecA purification

SecA was overexpressed in *Escherichia coli* NiCo21 (New England Biolabs) from the pET30b SecA expression vector and induced by 1 mM isopropyl-1-thio- β -D-galactopyranoside at 37 °C⁴⁰. After 4 h, the cells were pelleted and lysed by a homogenizer in 0.5 M NaCl, 20 mM HEPES (pH 7.5) using three cycles of 20,000 p.s.i. After centrifugation for 60 min at 40,000 rpm and 4 °C, the supernatant was incubated with equilibrated Chitin Resin for 35 min to remove impurities. The flow-through was incubated with Ni²⁺-chelating beads for 1 h at 4 °C. The beads were loaded on a column and washed in the presence of 20mM imidazole. Subsequently SecA was washed in the presence of 400 μ M TCEP and incubated with 250 μ M ATTO532 dye for 45min at 4°C. SecA was washed again and eluted with 200 mM imidazole and then subjected to size exclusion chromatography using 100mM NaCl, 20mM HEPES(pH 7.5). Protein-containing fractions were pooled and protein concentration was determined by the Bradford protein assay.

In vitro translocation assay

The functionality of SecYEG and SecA was analyzed by a slightly modified standard translocation and protease protection assay. In brief, the reaction consisted of 10% SecYEG proteoliposomes, 20 μ g/ml SecA with 50 μ g/ml proOmpA-69-Dhfr-Hisx6-Cys, consisting of the first 69 Amino acids of OmpA, a Gly-Ser-Gly-Ser-linker, dihydrofolate reductase (Dhfr), a His tag and an atto 488 (Atto dyes) labeled cysteine, as translocation substrate. The reaction was done in 50mM Tris (pH 7.9), 5 mM MgCl₂, 50mM KCl, 50mM NaCl, 1mM DTT, 1mg/ml BSA, 5mM ATP, substituted with 10mM Creatine Phosphate and 50 μ g/ml Creatine Kinase (Roche) as an ATP regeneration system. Analysis was performed by Western Blot.

High speed atomic force microscopy

HS-AFM³ was operated in tapping mode at room temperature (25 °C) with free amplitudes of 1.5-2.5 nm and an amplitude set point of larger than 90 %. Silicon nitride cantilevers, (BL-AC10DS-A2, Olympus, Tokyo, Japan; USC-F1.2-k0.15, Nanoworld AG, Neuchâtel, Switzerland) with nominal spring constants of 0.1-0.15 N/m, resonance frequencies of ~ 500 kHz, and a quality factor of ~2 in liquids were used.

Image and trajectory analysis

HS-AFM images were analyzed in ImageJ (NIH) with a plug-in for multiple particle detection and tracking (ParticleTracker v. 1.5)⁴¹. Images were corrected for instrumental drift using a slice alignment plugin⁴². Trajectories were further processed using in-house algorithms implemented in MATLAB (MathWorks). For the analysis of the mobility, square displacements (SD) were calculated from trajectories for time-lags of 1 to 4 frames. The cumulative distribution function (CDF) of SDs was fitted with a double exponential function (Supplementary Fig. 4a-e) including two different mobility components with mean square displacements MSD₁ and MSD₂, and fractions α and (1- α), respectively: $CDF(SD) = 1 - (1 - \alpha) \exp(SD/MSD_2) - \alpha \exp(SD/MSD_1)$ ²⁹. The corresponding diffusion coefficients ($D_{1/2}$) were obtained from MSD_{1/2} vs. time-lag plots (Supplementary Fig. 4e). The number of analyzed trajectories and average trajectory length for samples with 0.4, 0.5, 1, 10 mM glutaraldehyde concentrations used for fixation of the SA-crystals were n=614 (9.7 frames),

n=2114 (12.4 frames), n=692, (12.1 frames), n=591, (11.5 frames) for SecYEG, and n=574 (12.1 frames), n=372 (15.8 frames), n=1653 (14.1 frames), n=184 (7 frames) for GlpF, respectively. For the analysis of protein heights, the maximum height with respect to the lipid bilayer surface at each point of a trajectory within a radius of 6 pixels was detected. Probability density functions of particle heights were generated from the mean height determined from each trajectory using MATLAB built-in kernel density estimation method⁴³. To assess the significance of peaks / shoulder peaks in such pdfs, a sum of Gaussian functions⁴⁴ was fitted to the pdfs yielding corresponding mean and standard deviation as well as relative fractions for each protrusion (shoulders). We used the degree-of-freedom adjusted R-square to judge the goodness of the fits and thus the optimal (minimal) number of Gaussians to reasonably reproduce the pdfs, cf. Supplementary Fig. 6.

The total numbers of different scan areas used for determination of mean protein abundance per scan area ($400 \times 400 \text{ nm}^2$) for 0.4, 0.5, 1, 10 mM glutaraldehyde concentrations used for fixation of the underlying SA-crystals and mica were n=4, 13, 25, 19 (SA-crystal), 23 (mica) for SecYEG, and n=17, 16, 14, 20 (SA-crystal), 18 (mica) for GlpF, respectively.

Fluorescence microscopy

For fluorescence microscopy, mica was freshly cleaved ($\sim 1 - 10 \mu\text{m}$ thick) and glued onto glass coverslips (No 1, Stoelzle, Austria) using an optically transparent UV-glue (optical adhesive NOA61, Norland Products Inc., Cranbury, NY). The mica surface was re-cleaved before the preparation of SLBs and SA-crystals. To confirm membrane integrity and determine lipid diffusion constants, bilayers were labelled with $1 \mu\text{g/ml}$ (for FRAP measurements), and $0.1 \mu\text{g/ml}$ fluorescent lipid DiD (single particle tracking) for 1 min (DiD was purchased from Life Technologies, Vienna, Austria). Following thorough washing, membrane integrity was confirmed by fluorescence imaging. As an additional marker for membrane integrity, membrane fluidity was determined by FRAP.

Fluorescence microscopy instrumentation

For single particle tracking and FRAP experiments, sample chambers were placed on an x-y-stage (CMR-STG-MHIX2-motorized table, Märzhäuser, Germany) and images were taken on a modified Olympus IX81 (Olympus Tokyo, Japan) inverted epifluorescence microscope equipped with an Olympus UApo N $100\times / 1.49 \text{ NA}$ oil objective lens (Olympus, Tokyo, Japan).

Samples were illuminated in objective-type widefield configuration via the epiport using 491 nm light from a diode laser (Cobolt Calypso 100TM, Solna, Sweden), 642 nm from a solid state laser (Omicron Laserage Laserprodukte GmbH—Phoxx[®]), and 532 nm from a solid state laser (Rodgau-Dudenhofen, Germany), with intensities of $3-10 \text{ kW/cm}^2$. After appropriate filtering, emitted signals were imaged on a back-illuminated, TE-cooled CCD-camera (Andor iXon Du-897 BV, Belfast, UK). For the precise control of the illumination timings, we used acousto-optical modulators (1205C, Isomet, Springfield, VA, USA) for the solid state laser or directly modulated via TTL signals for the diode lasers. Timing protocols were generated by an in-house program package implemented in LABVIEW (National Instruments, Austin, TX, USA). Illumination times were adjusted to values between 1 and 5

ms. Movies were recorded with a delay in the range of 50 ms to 200 ms between two consecutive images.

For Fluorescence Recovery After Photobleaching (FRAP) experiments we used the single molecule microscopy system as described above. A spherical spot was photobleached ($r = 4 \mu\text{m}$), and the recovery of the signal was recorded after 0.2 s up to 15 s; samples were illuminated in wide field epi-configuration. Each result represents the average of 15 membrane areas. Experiments were performed at room temperature. In general, after recording a pre-bleach image with an illumination time of 5 ms, samples were bleached with a laser pulse applied for 500 ms. First we took a series of images (same settings as for the recovery images) before bleach, which were used to correct for photobleaching in the data analysis. The recovery image was followed by a sequence of up to 10 images with an illumination time of 5 ms and a varying delay between 200 - 5000 ms between subsequent images. For FRAP analysis, images were analyzed using in-house algorithms implemented in MATLAB (MathWorks, Natick, MA). The image after 15 s recovery was used for brightness analysis. All experimental data were corrected for photobleaching during the measurement and for background. The recovery was calculated from the ratio of recovery image and pre-bleach image. Diffusion analysis was performed as described⁴⁵.

Single particle tracking

The fluorescence data was analyzed using custom written Matlab code (MathWorks) as described previously⁴⁶. In brief, nonmaximum suppression and thresholding were applied to determine possible locations of single fluorophores smoothing. The regions of interest were then fitted by a pixelated Gaussian function including a homogeneous photonic background using a maximum likelihood estimator (MLE) for Poisson distributed data by a freely available GPU fitting routine⁴⁷ on a GeForce GT 550 Ti (Nvidia). Single molecule localizations between successive frames were linked by minimizing the total displacement⁴⁸. The diffusion coefficient was determined by fitting the first two points of the mean square displacement vs. time lag plot⁴⁹. The CDF of the MSD was further fitted with a bi-exponential function to separate different mobility fractions as detailed above.

Modeling of the unstructured 40 aa tail of GlpF

The height of the unstructured region on the cytoplasmic side of GlpF (40aa; 3.9 kDa, including the his-tag used for purification) was estimated by summing up the volumes that the individual amino acids (according to the sequence) occupy in solvent and subtracting $10.4 \text{ \AA}^3$ for each peptide bond⁵⁰. This calculation yielded an estimate for the protrusion size of $\sim 2.7 \text{ nm}$ (when treated as a sphere) which very well agrees with the height of the minor fraction III as found in our analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Platform for transmembrane protein observation.

a, Sketch of the Streptavidin-crystal periodically suspended membranes (SA-crystal PSMs) containing transmembrane proteins (e.g. SecYEG). **b**, A SA-crystal was formed on a lipid bilayer containing biotinylated lipids (DOPC/DOPS/biotin cap DOPE 6:2:2 w/w) as described¹⁶. **c**, High resolution imaging of the SA-crystal reveals two-fold symmetry and a unit cell $a=b=5.90 \pm 0.07$ nm; $\gamma=90 \pm 1^\circ$. **d**, SecYEG containing proteoliposomes were incubated for ~ 3 min. forming lipid patches on top of the SA-crystal. Moderately diffusing SecYEGs could be observed.

Figure 2. Membrane protein mobility depends on extent of SA-crystal chemical fixation.

a, Apparent membrane protein abundance per scan-area ($A_s=400 \times 400\text{nm}^2$) in SA-crystal PSMs (left panel) vs. mica SLBs (right panel). **b**, Fraction of detected proteins exhibiting an intermediate mobility. **c**, Lateral diffusion constants of intermediate and slow fractions as a function of glutaraldehyde exposure during SA-crystal preparation. All values are mean $\pm$ s.e.m.

Figure 3. Confined lateral mobility of membrane proteins.

a, Overlay of trajectories of SecYEG embedded in a SA-crystal PSM, Supplementary Movie S3. The proteins repeatedly diffuse along the same pathways. **b**, Overlay of trajectories of GlpF embedded in a SA-crystal PSM, Supplementary Movie S4. Similar to SecYEG, the proteins repeatedly diffuse along the same pathways. **c**, 3D representation of the high resolution image of a SA-crystal (Fig. 1c). The meshwork of grooves between adjacent SA molecules is indicated by the yellow and white axis. **d**, Molecular model of the SA-crystal. **e**, Magnification of the SA-SA interface (top view). Potential sites (amine groups) for

Glutaraldehyde crosslinking of neighboring SA molecules are displayed (red circles). **f**, Side view on the SA-SA interface. Amino acids relevant for potential glutaraldehyde cross-links are indicated. Colorcode indicates their location on SA1 (blue), SA2 (yellow), and SA3 (green).

Figure 4. Characterization of membrane proteins embedded in SA-crystal supported lipid bilayers.

a, Probability density function (pdf) of heights of membrane protruding GlpF moieties measured on mica ($n=206$) and SA-crystal ($n=389$; 10 mM glutaraldehyde;) (left panel). The pdfs were best fit by a sum of three Gaussians (cf. Supplementary Fig. 6a and b). The mean values of these Gaussians (I-III) match the heights of structural features relative to the membrane as known from crystal structure (right panel, pdb: 1fx8²⁴ + unstructured 40 aa tag on the cytoplasmic side^{13, 14}, cf. methods). **b,** HS-AFM time series of a single GlpF

tetramer (periplasmic side)¹³ embedded in a SA-crystal PSM. **c**, Probability density function (pdf) of heights of membrane protruding SecYEG moieties measured on mica (n=697) and SA-crystal (n=1232; 10 mM glutaraldehyde;) (left panel). The pdf for mica SLBs was normalized so that the area under the curve reflects the fraction of proteins observed on mica relative to the number of proteins observed on SA-crystals per scan area (Fig.2a). The pdfs were best fit by a sum of three (SA-crystal) resp. two (mica) Gaussians (cf. Supplementary Fig. 6c and d). The *E. coli* SecYEG structure (right panel, pdb: 3J4519) suggests three different height populations (I at the periplasmic side, II and III at the cytoplasmic side) that can be collectively found in the height pdf collected from SecYEG embedded in the SA-crystal PSM (cf. Supplementary Fig. 6c). For the mica SLBs, the pdf (dashed line; Fig.4c, left panel) was best fitted by a sum of two Gaussians (Supplementary Fig. 6d) whereas the cytoplasmic fractions (II+III) were far less pronounced (arrow, cf. Supplementary Fig. 6d). A height pdf of SecA (n=134) bound to SecYEG reveals a pronounced height increase of up to 2 nm compared to the bare SecYEG (cytoplasmic side). Topographical noise introduced by the lipid associated, fast diffusing SecA prevented the detection of the lower height populations. **d**, HS-AFM time series taken from Supplementary Movie S6 of SecA (white circles) bound to SecYEG. Only very rarely could the dissociation of SecA from SecYEG be observed uncovering the underlying SecYEG channel.