Organization into higher-ordered ring structures counteracts membrane binding of IM30, a protein associated with inner membranes in chloroplasts and cyanobacteria

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ABSTRACT

The inner membrane-associated protein of 30 kDa (IM30), also known as the vesicle inducing protein in plastids 1 (Vipp1), has a crucial role in thylakoid membrane biogenesis and maintenance. Recent results suggest that the protein binds peripherally to membranes containing negatively charged lipids. However, while IM30 monomers interact and assemble into large oligomeric ring complexes with different numbers of monomers, it is still an open question whether ring formation is crucial for membrane interaction. Here we show that binding of IM30 rings to negatively charged phosphatidylglycerol (PG) membrane surfaces results in a higher ordered membrane state, both in the head-group as well as in the inner core region of the lipid bilayer. Furthermore, by using gold nanorods covered with PG layers and single particle spectroscopy we show that not only IM30 rings, but also lower oligomeric IM30 structures interact with membranes, yet with higher affinity. Thus, ring formation is not crucial for, but even counteracts membrane interaction of IM30.

ABSTRACT

Notably, chloroplasts and cyanobacteria both contain an extra internal membrane system, the thylakoid membrane (TM) network. Furthermore, both perform oxygenic photosynthesis, and the TMs harbor all protein complexes and pigments involved in the photosynthetic light reaction. However, while assembly and maintenance of TMs is vital for photosynthesis, the details of TM biogenesis and maintenance are still largely unsolved mysteries (1).

In 1994 the inner membrane-associated protein of 30 kDa (IM30) was discovered in chloroplasts of Pisum sativum (2), and a homologous protein appears to be expressed in almost every organism that is able to conduct oxygenic photosynthesis (2-5). Several \textit{in vivo} studies have shown that the protein plays an essential role in TM formation and maintenance (reviewed in (6)), as \textit{e.g.} the depletion of the protein in Arabidopsis thaliana or the cyanobacterium Synechocystis sp. PCC 6803 has resulted in a decreased amount of TMs (3,7-9). Under cold-stress conditions, depletion of IM30 resulted in the formation of vesicular structures at the chloroplast inner envelope membrane in Arabidopsis, which led to the name vesicle inducing protein in plastids 1 (Vipp1) (3). Nevertheless, while diverse physiological functions were proposed for IM30 in the past two decades, the exact protein function remained

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Engulfment of an ancient cyanobacterium by a primordial cell has resulted in the development of modern day chloroplasts, the organelles where photosynthesis takes place. Consequently, the fine-structures of cyanobacterial cells and chloroplasts share many similarities.
elusive for a long time. Only recently, IM30 has been identified to be able to reorganize lipid bilayer structures and to mediate membrane fusion in chloroplasts and cyanobacteria (10). Similar to its bacterial homolog, the phage shock protein A (PspA), which is involved in a bacterial stress response in situations provoking membrane stress (11), IM30 specifically interacts with negatively charged membrane surfaces, finally triggering membrane fusion (10,12). IM30 does not exhibit membrane spanning helices, as typical for a canonical transmembrane protein (2,3), and thus it appears to peripherally bind to the cytoplasmic surface of the inner envelope/cytoplasmic membrane and TMs (5,13). In fact, IM30 appears to exist in equilibrium between a soluble and a membrane-attached form (5).

Based on computational analyses, IM30 contains seven α-helices, which are interrupted by small random coil domains, and these α-helices are predicted to be involved in the formation of coiled-coil structures (5,6,14,15). While the tertiary structure of IM30 is not yet resolved, it is clear that IM30 monomers interact and assemble into large oligomeric ring complexes with molecular masses exceeding 2 MDa (5,14). However, from a common building block not a single ring species is formed, rather IM30 forms various ring structures with different rotational symmetries (5,14-16). It was unclear for a long time whether these IM30 rings have any physiological meaning or just form to ‘store’ IM30. As ring formation as well as membrane binding was disturbed when the first 21 N-terminal amino acids of the Arabidopsis thaliana IM30 were deleted, it was concluded that formation of a ring structure is essential for lipid interaction (12), and it was indeed recently shown by electron microscopy that IM30 rings bind to PG-containing membranes (10). However, as IM30 forms diverse ring structures with different amounts of IM30 protomers (5), it appears to be unlikely that formation of a defined ring structure is prerequisite for membrane binding and membrane remodeling. Furthermore, due to their small size, IM30 monomers have potentially escaped analyses and were not yet observed to directly interact with membranes. Thus, it is currently unclear whether IM30 monomers might also bind to membrane surfaces.

Here we show that IM30-binding to negatively charged membrane surfaces increases the lipid order in the head-group as well as in the hydrophobic membrane core region. Additionally, we show that also low molecular weight IM30 species bind to membrane surfaces, using Nano-SPR (17) and an IM30 mutant that does not form stable ring structures anymore. In fact, the membrane-binding affinity of these lower ordered IM30 species is increased when compared to IM30 proteins organized in ring structures. Thus, ring formation is not a prerequisite but offsets membrane binding of IM30.

RESULTS

IM30 binding changes the order of PG membranes-Recently, we have shown that IM30 binds with high specificity to membranes containing negatively charged lipids, such as DOPG or sulfoquinovosyldiaclyglycerol (SQDG) (10), which together account for ~20% of all membrane forming lipids in chloroplasts and cyanobacteria (18,19). To examine the interaction of IM30 with negatively charged lipid membrane surfaces in greater detail, we first explored the effect of IM30 on the structure of pure PG membranes. As DOPG has a phase transition temperature $T_m$ of -18 °C, DMPG was chosen in these experiments, which has a main phase transition temperature $T_m$ at ~23 °C (20). DMPC, which has the same fatty acid composition as DMPG, was used as a control, as IM30 is not supposed to interact with the zwitterionic lipid head groups (10).

Melting curves of DMPG liposomes were recorded from 10 to 60 °C in presence of increasing IM30 concentrations, and the fluorescent dye Laurdan, which senses changes in the polarity of the membrane head-group region (21), was used as a probe. As can be seen in Fig. 1, addition of increasing IM30 concentrations did not affect the melting behavior and the phase transition of DMPC liposomes, confirming that IM30 does not bind to the zwitterionic PC head-groups (Fig. 1A), in perfect agreement with previous findings (10). In contrast, a shift in the melting curves became obvious when increasing IM30 amounts were added to DMPG liposomes (Fig. 1B), and the $T_m$ constantly shifted to higher temperatures. Furthermore, while the GP values were about identical at low temperatures, i.e. when
the DMPG membrane was in the gel state, the GP values of the fluid state (e.g. at 60°C, Fig. 1C) steadily increased with increasing IM30 contents. Together, these results indicate a stable interaction of IM30 with PG head-groups, and IM30-binding to PG surfaces has an ordering effect, at least in the lipid head-group region.

In contrast to Laurdan, the highly hydrophobic fluorescence probe TMA-DPH integrates deeply into the non-polar core region of a lipid bilayer membrane (22), and this membrane probe thus senses the acyl-chain order in the hydrophobic membrane core. Therefore, DMPG melting curves were also recorded upon addition of increasing amounts of IM30 using TMA-DPH as a membrane probe. The experiments were performed as before, except that the anisotropy $A$ was monitored. The results shown in Fig. 2 again demonstrate that IM30 stabilizes the liquid ordered membrane phase and increases the phase transition temperature of DMPG liposomes (Fig. 2B), but has no effect on DMPC membranes (Fig. 2A), in agreement with the results obtained before using Laurdan as a membrane probe. For a more quantitative analysis, the phase transition temperatures from both Laurdan and TMA-DPH experiments, were plotted against the IM30 concentrations. As can be seen in Fig. 2C the ordering effect of IM30 on PG membranes levels off at a protein concentration of around 2 µM, i.e. at a protein-lipid ratio of 1:50.

Together, the results clearly show that IM30 binds to negatively charged lipid surfaces and thereby affects the membrane structure in the interfacial lipid head-group region as well as in the hydrophobic membrane core. Binding of IM30 to PG membranes result in a more ordered lipid layer.

Assembly of IM30 monomers into oligomeric structures reduces the membrane binding affinity—When purified IM30 is analyzed via SEC (Fig. 3A,B), the protein is predominately found in the column’s void volume fraction and thus IM30 has a molecular mass $>660$ kDa. Therefore, IM30 almost exclusively exists as higher-ordered oligomeric ring structures (Fig. 3C), in line with previous analyses (5), and only a very minor fraction of the protein is monomeric. Thus, the IM30 solution can be taken as a solution of IM30 rings.

To quantify the interaction of IM30 rings with PG membranes, increasing IM30 concentrations (0.4 to 7 µM) were added to DOPG liposomes, which contained Laurdan as a membrane probe. Laurdan fluorescence emission spectra were recorded and the GP values were calculated (Fig. 4A). Fitting the resulting binding curve with Eq. 2 gave a $K_D$ of $7.20 \pm 0.95$ µM, when the IM30 monomer is part of the IM30 ring structure. It should be mentioned that, since the curve does not flatten out convincingly, the determined $K_D$ is a lower estimate compared to the IM30_FERM mutant (see below).

Recently, we have shown that IM30 binds as a ring, in a well-defined geometry to membrane surfaces (10). However, this observation raised the question whether IM30 binds exclusively as a ring or whether IM30 monomers and/or lower oligomeric structures can also bind to membrane surfaces. To address this question, we next determined the $K_D$ values of an IM30 mutant, which exhibits a drastic destabilization of the high molecular mass ring structures. Four amino acids conserved in PspA/IM30 proteins (16) are mutated in the IM30_FERM protein (see above), and the protein forms not well defined, irregular high molecular mass aggregates (Fig. 3D). Thus, these residues are likely important for IM30 ring formation. In fact, a significant amount of the mutated protein is present in a lower-ordered oligomeric form, potentially representing a tetramer (Fig. 3B,D). Changes in the lipid order upon addition of IM30_FERM to DMPG membranes were determined using Laurdan as a probe, and the resulting GP values were plotted against the IM30 FERM concentrations (Fig. 4B). Fitting these data with eq. (2) resulted in a $K_D$ of $1.39 \pm 0.22$ µM for the IM30 mutant, i.e. the determined $K_D$ value was about five-fold lower than determined before for the wild-type (wt) protein, which forms stable ring structures.

Thus, these data not only indicate that also lower ordered IM30 species bind to PG surfaces, the $K_D$ value is even lower in case of destabilized IM30 ring. Moreover, in case of the IM30_FERM mutant the increase of the Laurdan GP values is more pronounced compared to the IM30 wt, which reinforces the conclusion that the mutant has an increased membrane binding affinity.

Lower ordered IM30 oligomers can bind to DOPG covered gold nanorods—The
observations described above strongly indicated that IM30 monomers and/or lower oligomeric structures bind to membrane surfaces. To follow up on this observation, we studied the IM30 wt adsorption to lipid bilayers using the NanoSPR technique we recently introduced (17). NanoSPR works similar to conventional surface plasmon resonance (SPR) sensors with the important difference that the sensor elements, the gold nanoparticles, have dimensions approaching the molecular size of proteins. Due to the small size of its sensing elements, NanoSPR is able to detect a discrete protein binding event especially for large protein structures such as IM30 rings (23). As our sensor lies on a flow-cell surface, which shields around one third of the bottom part of the sensor surface we assumed 60% of the sensor surface area to be available for adsorption. Furthermore, proteins on the surface are not densely packed, but absorb up to an available surface coverage limit of around 55% (24). If we calculate the number of IM30 rings, whose diameter vary between 25-33 nm (5), that can, on the basis of the assumption made above, absorb on top of a single AuNR, we end up with a number of 4 to 7 rings, dependent on the number of protomers per IM30 ring. The values are calculated from the footprint of the IM30-ring on the particle surface area. The geometry of the NR is described to be a cylinder with spherical shaped caps. The dimensions are given by the dimensions of the NR itself with a 5 nm lipid bilayer on top. The binding of a single IM30 ring would cause a plasmon resonance shift of about $\Delta \lambda_{\text{ring}} = 0.47$ to 0.82 nm, dependent on the ring size (see above). If IM30 was adsorbing primarily as rings, the adsorption curve would therefore consist of not more than 7 discrete steps of at least 0.47 nm height (we calculated this shift for the smallest IM30 ring with 25 nm in diameter). However, the adsorption curves we obtained for single particles, where bigger steps do not average out, look relatively smooth and did not show such pronounced steps (compare Fig. 5A red data points for examples at two different IM30 concentrations $c_{\text{IM30}}$).

To get a more quantitative understanding, we calculate the percentage of $\Delta \lambda_i$ between two measuring points that exceed the shift we estimated for the ring binding ($\Delta \lambda_{\text{ring}}$) for each measured IM30 concentration. The percentages were calculated for binding of exclusively small rings of 25 nm in diameter and exclusively big rings of 33 nm in diameter. The average in the sample lies between those borders. This percentage gives an upper bound to the number of ring binding events within the time-step between those two measuring points as successive adsorption of monomers or lower order oligomers could add up, especially at higher protein concentrations. The results of this analysis are shown in Fig. 5B. The number of shifts that exceed the theoretically calculated threshold for adsorption of a single ring, even when only the smallest rings would bind, was usually below 25% (with an outlier for $c_{\text{IM30}} = 1.58 \mu M$). Even if we take into account that binding of smaller oligomers might leave little area for ring binding (sketch in Fig. 5B), the results clearly show that IM30 predominantly binds to PG membranes as monomers or small oligomers. Ring binding is either absent or occurs only with low probability (below 25%).

The lipid coated AuNRs (NanoSPR) can be used for quantifying membrane interaction of the lower ordered IM30 oligomers (including IM30 monomers) similarly as conventional SPR, by observing the equilibrium coverage as a function of IM30 concentration. Since we have shown above that the majority of binding events on the lipid coated gold nanoparticles do not represent IM30 ring binding, the binding constants determined from this IM30 titration experiment describes binding of monomers and/or small oligomers.

In this experiment, the bilayer covered gold nanosensors were incubated with increasing IM30 concentrations (0.01-12.67 µM) and their resonance wavelength shift over time was followed until equilibrium ($\Delta \lambda_{\text{Eq}}$) was reached (Fig. 6). The determined $\Delta \lambda_{\text{Eq}}$ values were fitted with a Langmuir equation, allowing calculation of the dissociation equilibrium constant $K_D = 0.42 \pm 0.22 \mu M$ (Fig. 6). As mentioned above, this $K_D$ value describes IM30 monomer/small oligomer binding, and not binding of IM30 rings. To further support this assumption, we also analyzed selected concentrations of the IM30 FERM mutant (Fig. 6, grey dots). As expected, the mutant protein, which does not form defined oligomeric ring structures anymore (Fig. 3D), binds as well to the membrane covered gold nanosensor as the wt protein. Thus, impaired ring
IM30/Vipp1 membrane binding

formation of the mutant protein does not affect membrane binding of lower ordered IM30 oligomers or monomers.

Together, these observations strongly support the assumption that lower ordered IM30 protein oligomers/monomers bind to negatively charged membrane surfaces. The membrane binding affinity of these IM30 oligomers/monomers ($K_D = 0.42 \pm 0.02 \mu M$) is higher compared to IM30 rings ($K_D = 7.20 \pm 0.95 \mu M$).

DISCUSSION

Membrane binding and lipid ordering are conserved features of IM30/PspA proteins-In the present study we analyzed the molecular impact of IM30-binding to pure PG membrane surfaces, using two different membrane probes. Due to its polar, chromophoric head-group, Laurdan integrates into the membranes polar head group region (21), whereas TMA-DPH stays parallel or perpendicular to the surface in the inner, unipolar membrane core region (22). The two dyes, which probe the lipid order in complementary membrane regions, show that IM30 binding to pure PG membrane surfaces in fact increases lipid packing both, in the head-group as well as in the acyl-chain region. This induced increase in lipid packing might be an ancient skill, inherited with the homologous protein PspA (8,25). Reducing the proton permeability of the membrane by affecting the membrane fluidity is discussed to be one physiological function of PspA in *E. coli* (11). As expression of the *Synechocystis* IM30 protein in an *E. coli ΔpspA* deletion strain can substitute the membrane protective function of PspA (26), membrane binding and lipid ordering most likely is an ancient feature of members of the PspA/IM30 protein family. When the cyanobacterial TM or the *E. coli* cytoplasmic membrane is destabilized, the membrane permeability eventually decreases and binding of PspA/IM30 could protect the structure and integrity of a lipid bilayer by increasing the lipid acyl chain order, thereby guarding the membrane and securing transbilayer proton and/or ion gradients. This assumption is further supported by the observation that IM30 is down-regulated in *Synechocystis* under cold stress conditions (27), where further decreasing the membrane fluidity would be counterproductive. However, it is worth mentioning that generally increasing the order of TM lipids can lead to damaged photosynthesis in *vivo* (28,29), and indeed IM30 appears to have acquired extra functions in cyanobacteria and chloroplasts (10). In fact, triggered by Mg$^{2+}$, IM30-binding to membrane surfaces appears to mediate membrane fusion, and it is currently unclear whether the lipid ordering effect is physiologically really relevant in chloroplasts and cyanobacteria.

**IM30 ring formation counteracts membrane binding**-Recently, we have shown that IM30 rings bind to negatively charged membrane surfaces (10). The observations of this study clearly demonstrate that IM30 rings as well as low molecular weight structures bind to the negatively charged PG membrane surfaces, and thus, ring formation is not crucial for membrane binding. However, while IM30 appears to essentially exclusively exists in an oligomeric form in solution (Fig. 3B), disassembly of the ring and binding of low molecular weight IM30 species increases the membrane binding affinity of the IM30 monomer almost 20fold (Fig. 4 and 6). Thus, binding of the low molecular weight species is thermodynamically more favored than binding of the oligomeric rings. But why is the membrane binding affinity reduced when IM30 forms rings? While the structure of a full-length PspA/IM30 protein has not been resolved yet, both proteins are predicted to mainly consist of $\alpha$-helices, which have a high propensity to form coiled-coil structures (5,16,30). In fact, the recently solved crystal structure of a PspA N-terminal protein fragment clearly shows that this region forms a stable coiled-coil (31). Formation of coiled-coil structures involves leucine-zipper-like packing of two $\alpha$-helices, where two amphipathic helices interact via their hydrophobic surface. Thus, amphipathic helices are involved in formation of quaternary contacts in the IM30 oligomers and are not available for membrane binding. As monomeric IM30 has a dramatically increased membrane binding affinity, it appears to be likely that monomerization of the protein results in exposure of amphipathic helices, which are then free for membrane binding, resulting in an increased membrane binding affinity. Such an assumption is further supported by our analysis of the IM30_FERM mutant, which forms a destabilized ring structure and low molecular mass
IM30 oligomers. In agreement with the assumption that lower molecular mass species (down to monomers) bind with higher affinity to membrane surfaces, also the IM30_FERM mutant binds with higher affinity to PG surfaces, and the determined \( K_D \) is about five-fold lower than the one determined for the wt protein (Fig. 4B).

However, proper evaluation of IM30 binding to membranes is complicated due to the fact that multiple equilibria have to be considered, as summarized in Fig. 7.

Oligomerization of monomers in solution and formation of higher-ordered oligomers and finally ring structures is represented by the dissociation constant \( K_{D,1} \). In fact, formation of IM30 rings from monomers might involve multiples steps and successive oligomerization of monomers or lower-ordered oligomers (6). While we did not succeed yet to experimentally determine \( K_{D,1} \), it is clear that the equilibrium favors protein oligomerization, and only a very minor fraction of IM30 is monomeric in solution (compare Fig. 3B). Binding of the lower-ordered structures (down to monomers) to membrane surfaces is described by the dissociation constant \( K_{D,2} \), which is in the range of 0.4 \( \mu M \) - 1.4 \( \mu M \), as determined for the wt protein (by using NanoSPR) and for the IM30_FERM mutant (Fig. 4 and 6).

However, determination of this constant in the NanoSPR experiment is complicated, as the feeder system affects the availability of the monomers. Thus, the \( K_D \) determined in this study for interaction of low molecular weight IM30 species with membranes (~0.4 \( \mu M \)) might in fact summarize at least the two equilibria \( K_{D,1} \) and \( K_{D,2} \). The \( K_{D,3} \) value, describing the membrane-IM30(ring) interaction, was determined to be about 7.2 \( \mu M \), when IM30 monomers are assembled and form a ring. Thus an IM30 monomer, organized in a ring, has a drastically decreased affinity to bind to PG membrane surfaces compared to the low molecular weight IM30 species.

Together, the results of this study show that binding of IM30 to negatively charged membrane surfaces affects the lipid order in the lipid head-group as well as in the core region of the membrane. Organization of IM30 into ring structures reduces the membrane binding affinity, most likely due to shielding of amphipathic helices involved in formation of the IM30 quaternary structure. In vivo, IM30 appears to cooperate with Mg\(^{2+}\), and Mg\(^{2+}\)-binding increases the IM30-membrane binding affinity as well as triggers IM30-mediated membrane fusion (10). Thus, it is possible that Mg\(^{2+}\) alters the IM30 ring structure, resulting in enhanced membrane binding.

**EXPERIMENTAL PROCEDURES**

**Expression and purification of IM30 and the IM30 mutant IM30_FERM**-The pRSET-His-IM30 expression plasmid (9), which contains the IM30 coding sequence of *Synechocystis sp. PCC 6803*, was used as a template for site directed mutagenesis, leading to the vector for expression of the IM30_FERM mutant, named after four mutations at the positions Phe168, Glu169, Arg170 and Met171 to alanine. The two primers 5’CCACTAGCAGTGCTACTAGTGCTGCTGCAAGCGCGGAGAACAAGGTACTGG-3’, 5’CCAGTACCTTGTTCTCCGCCGCTGCAGCACTAGTAGCACTGCTAGTGG-3’ and the QuikChange II Site-Directed Mutagenesis Kit, (Agilent Technologies, Santa Clara, CA, USA) were used.

IM30 from *Synechocystis* and the mutant IM30_FERM were heterologously expressed in *E. coli* BL21 (DE3) cells and purified on a Ni-NTA agarose column, as described in detail recently (5,9). To remove excess imidazole, the protein was finally dialyzed against 20 mM HEPES buffer, pH 7.6. Based on analyses on 14% SDS polyacrylamide gels, the purified IM30 protein was >95% pure.

**Size exclusion chromatography**-Size exclusion chromatography (SEC) was performed at 4°C using an Äkta Explorer system together with a Superose\textsuperscript{TM} 12, 10/300GL (GE Healthcare, Little Chalfont, UK) column at a flow rate of 0.5 mL/min. Protein samples were dialyzed after purification against 50 mM sodium phosphate buffer pH 7.6 containing 100 mM sodium chloride and 10 mM sodium isothiocyanate. Subsequently, 100 µg protein was loaded onto the column. For calibration of the column, the molecular weight marker kit (Sigma Aldrich, thyroglobulin (660 kDa; void volume), apoferritin (440 kDa), β-amylase (200 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (14 kDa)) was used.

**Electron microscopy**-Negatively stained protein samples were prepared on glow discharged
continuous carbon grids by a combination of the single droplet method (32) and the OpNS method (33), using a solution of 2% uranyl formate at pH 2. Images were taken on a FEI Tecnai 12 electron microscope (nominal magnification: 71,540x) equipped with a TVIPS TemCam-F416 4K CCD camera.

Liposome preparation—DMPC (dimyristoylphosphatidylcholine), DMPG (dimyristoylphosphatidylglycerol) and DOPG (dioleoylphosphatidylglycerol) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). Lipids were dissolved in chloroform/methanol 2:1 (v/v). The organic solvent was removed under a gentle nitrogen stream followed by vacuum dehydration. The dried lipid films were hydrated with HEPES buffer, and unilamellar liposomes were prepared by subjecting the samples to at least five cycles of freeze-thawing.

Determination of the membrane organization using fluorescent probes—For analyzing the lipid order in the lipid/water border region of the membrane, the fluorescent probe Laurdan (6-dodecanoyl-N,N-dimethyl-2-naphthylamine, from Sigma, Taukirchen, Germany), a fluorophore that is highly sensitive to changes in the fluidity of the environment, was added to each lipid sample at a 1:500 molar ratio. Steady-state Laurdan fluorescence emission was monitored on a Horiba Scientific FluoroMax-4 spectrometer. Spectra were recorded from 400-550 nm after excitation at 350 nm. For further evaluations, the Generalized Polarization (GP) (21) was calculated using Eq. 1:

\[
GP = GP_{\text{min}} + \frac{\Delta GP}{1 + \frac{K_D}{c_{\text{IM30}}}}
\]

Equation 2

To determine the lipid acyl-chain order within the hydrophobic membrane core region, steady state fluorescence measurements were performed using TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate, Life Technologies, Darmstadt, Germany) as a probe. TMA-DPH anisotropy was measured at 430 nm on a Horiba Scientific FluoroMax-4 spectrometer upon excitation at 360 nm with excitation and emission slits set at 4 nm. For measuring lipid melting curves, the samples were adjusted to different temperatures from 10 to 55 °C for 1 min before the measurement. The determined melting curves were fitted with a sigmoidal logistic fit from Origin7G (OriginLab, Northampton, MA, USA), from which the point of inflexion was calculated, which corresponds to the phase transition temperature \(T_M\).

Gold nanorod preparation, characterization and coating with lipids—Cetyltrimethylammonium bromide (CTAB) stabilized gold nanorods (CTAB-AuNRs) were synthesized...
and characterized as described previously (34). The particles used here showed a refractive index sensitivity \( S = \Delta \omega / \Delta n \) of \( S = (143.1 \pm 39.5) \text{ nm/RIU} \) (if immobilized on a surface), and their dimensions determined by electron microscopy were: diameter \( d = (31.1 \pm 5.6) \text{ nm} \); length \( l = (67.8 \pm 9.2) \text{ nm} \). If not stated otherwise, we report values as mean value ± standard deviation.

To replace the CTAB layer around the particles by a lipid bilayer, the CTAB-AuNRs were centrifuged at 4020 g, and the pellet containing the CTAB-AuNRs was resuspended in ultrapure water. The particle suspension was then mixed with liposomes with a high excess of lipids compared to the estimated surface CTAB concentration on the nanorods (~1:2,000). Subsequently, CTAB-AuNRs and liposomes were incubated for 18 h at room temperature. To remove unfunctionalized particles and remaining lipids from the solution, PG-AuNRs were separated on a 7K zeba spin desalting column (Thermo Scientific). Functionalization of the nanorods was verified via UV-vis spectroscopy and gel electrophoresis. By changing the particle surrounding, the plasmon resonance wavelength of the nanorods changes slightly and aggregation of particles caused by instability can be seen in increasing extinction at wavelength between 800-900 nm. UV-vis extinction spectra show a red shift of the plasmon resonance wavelength for PG-AuNRs of 2 nm and do not exhibit particle aggregation (Fig. S1). Comparing the mobility of functionalized (PG-AuNR) and unfunctionalized (CTAB-AuNRs) particles in gel-electrophoresis, PG-AuNRs show a higher gel mobility (Fig. S2; a1), while CTAB-AuNRs aggregate in the well of the gel in presence of the working buffer (Fig. S2; a2). Filtration of the particles lead to a reduction of unfunctionalized particles in the sample (Fig. S2; b1) and particle stability of the PG-AuNRs was proven, as they keep their stability after three days in Heps buffer (Fig. S2; b2). Vitamin B was run as a control (Fig. S2; b3).

**Optical dark-field spectroscopy:** To attach the lipid-coated particles on the negatively charged glass surface of the microscope flow-cell, the flow-cell was incubated first with 0.1 mg/mL poly-L-lysine hydrobromide (Sigma) for 5 min and thereafter thoroughly washed with 2 mL ultrapure water. Then, PG-AuNRs were injected and incubated with the glass surface until an adequate surface density of attached particles was reached. Unbound particles were washed out. After rinsing with working buffer (20 mM HEPES, pH 7.6), the position of each deposited nanoparticle within the field of view was recorded.

The optical dark-field spectroscopy setup used to record single particle spectra was described in detail previously (34). AuNRs, immobilized on the glass surface of a flow-cell, were illuminated by dark-field illumination so that only the scattered light of a single nanoparticle entered the spectrometer at a given time. In order to obtain statistics over many particles, a piezo scanning stage allowed for the sequential investigation of many particles within the field of view. The nanoparticles displayed a plasmon resonance with its central wavelength (\( \lambda_{\text{res}} \)) shifting when the refractive index near the nanoparticle changes, e.g., after adsorption of a protein (35,36).

To carry out an adsorption experiment, 200 µL of a protein solution with a given IM30 concentration \( c_{\text{IM30}} \) was flushed into the flow-cell at a flow rate of 75 µL/min, and the plasmon resonance wavelength shift (\( \Delta \lambda_{\text{res}} \)) of every particle (20-80 particles for each protein concentration) was recorded every 60-90 s at this IM30 concentration until an equilibrium value (\( \lambda_{\text{eq}} \)) was reached. The dissociation constant \( K_D \) was obtained by fitting the data with the Langmuir equation (Eq. 3).

\[
\Delta \lambda_{\text{eq}}(c_{\text{IM30}}) = \Delta \lambda_{\text{max}} \cdot \frac{c_{\text{IM30}}}{K_D + c_{\text{IM30}}} \quad \text{Equation 3}
\]

Calculating the plasmon shift caused by binding of a single IM30 ring (\( \Delta \lambda_{\text{ring}} \))—The plasmon resonance wavelength \( \lambda_{\text{res}} \) of AuNRs shifts due to changes in the polarizability or refractive index (\( \Delta n \)) in the immediate environment. Polarizability can change due to the formation of a dense layer around the particle or even due to binding of a single protein (17,23). We calculate the shift of the plasmon resonance wavelength induced by the binding of a single IM30 ring \( \Delta \lambda_{\text{ring}} \) in order to compare this shift with those shifts measured in experiments to estimate the presence or absence of ring-binding events.

The plasmon resonance shift \( \Delta \lambda_{\text{ring}} \) is proportional to the amount of refractive index change \( \Delta n \) with a proportionality constant or
sensitivity $S$. Furthermore, the magnitude of the plasmon resonance shift $\Delta \lambda_{res}$ decreases exponentially with increasing distance from the AuNR surface with a characteristic distance or ‘sensing distance’ $d_s$. Both $S$ and $d_s$ are functions only of the AuNRs geometry (length and width) and either measured or estimated theoretically by numerically solving Maxwell’s equations (using tabulated dielectric constants). In our case, we use $S = (143.1 \pm 39.5)$ nm/RIU (measured) and $d_s = (14.3 \pm 2.6)$ nm (calculated). Knowing $S$ and $d_s$, the resonance wavelength shift for a protein layer $\Delta \lambda_{layer}$ with thickness $t_{protein}$ is given by:

$$\Delta \lambda_{layer} = S \Delta n \left( 1 - e^{-\frac{t_{protein}}{d_s}} \right)$$

Equation 4

In our case, the proteins are not adsorbing directly on the particle surface but on a lipid bilayer (with thickness $t_{bilayer} = 5$ nm) surrounding it. The increased distance to the nanoparticle surface has to be taken into account by subtracting the shift produced by the lipid bilayer from the total shift of the bilayer and the protein. The resulting plasmon resonance wavelength shift $\Delta \lambda_{layer}$ of a complete and dense layer of IM30 is then:

$$\Delta \lambda = S \Delta n \left[ \left( 1 - e^{-\frac{t_{protein}+t_{bilayer}}{d_s}} \right) - \left( 1 - e^{-\frac{t_{bilayer}}{d_s}} \right) \right]$$

Equation 5

The plasmon shift induced by a single IM30 ring ($\Delta \lambda_{ring}$) follows by dividing by the number $N_{layer}$ of IM30 rings that would fit into the volume of a complete layer around the particle, which we estimated by the relative volumes $N_{layer} = V_{layer}/V_{ring}$. Using the known particle dimensions, the bilayer thickness and the IM30 ring dimensions reported in (5) (25-33 nm diameter, 22 nm height), we estimate a maximum of 23 to 40 rings per nanoparticle, dependent on the ring diameter. Using the standard assumption for the refractive index for biomolecules of $n = 1.5$ (37), i.e. $\Delta n = 0.17$, we arrive at a resonance wavelength shift for the binding of a single IM30 ring of $\Delta \lambda_{ring} = 0.47$ to 0.82 nm, dependent on the diameter of the ring.

To determine the amount of potential IM30(ring) binding events, we evaluated the shifts in the resonance wavelength position between two consecutive measuring points $\Delta \lambda_i$ for each individual particle until 90% of $\Delta \lambda_{eq}$ was reached. Only when the determined $\Delta \lambda_i$ between two measuring points exceeded the calculated level of $\Delta \lambda_{ring}$, the determined $\Delta \lambda_i$ could possibly be caused by binding of an IM30 ring to the membrane-covered AuNR. However, it is worth mentioning that a measured $\Delta \lambda_i \geq \Delta \lambda_{ring}$ could also be caused by consecutive binding of multiple lower-ordered IM30 oligomers or even monomers that occurred faster than the time resolution of the measurement.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: D.S., J.H., C.S., V.W., J.M. and M.S. designed the experiments and analyzed data. J.H., V.W., R.H. and M.S. performed the experiments. All authors wrote the paper.

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FOOTNOTES
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The abbreviations used are: IM30, inner membrane-associated protein of 30 kDa; Vipp1, vesicle inducing protein in plastids 1; PG, phosphatidylglycerol; TM, thylakoid membrane; PspA, phage shock protein A; SEC, Size exclusion chromatography; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; Laurdan, 6-decanoyl-\(N, N\)-dimethyl-2-naphthylamine; GP, Generalized Polarization; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate; CTAB, cetyl trimethylammonium bromide; AuNR, gold nanorods; SQDG, sulfoquinovosyldiaclylglycerol; SPR, surface plasmon resonance, wt, wild-type.

FIGURE LEGENDS

FIGURE 1. Phase transition of DMPC and DMPG membranes in presence of IM30. Melting curves of (A) DMPC and (B) DMPG membranes were recorded in presence of increasing IM30 concentrations, using the fluorescence probe Laurdan, which senses changes in the membrane environments polarity (expressed as Laurdan GP). Curves are shown at different IM30 concentrations (in µM) (C). The Laurdan GP value determined at 60 °C plotted against the IM30 concentration. The increasing GP values demonstrate that binding of IM30 increases the lipid acyl-chain order. The experiment was repeated two times.

FIGURE 2. Phase transition temperature of DMPC and DMPG measured with TMA-DPH. The fluorescence dye TMA-DPH, which integrates into the non-polar core region of a lipid bilayer membrane, was used as a probe to determine membrane phase transition temperatures of (A) DMPC and (B) DMPG membranes. Changes in the anisotropy \(A\) were recorded at different IM30 concentrations (in µM). (C) The phase transition temperature \(T_m\) was calculated from the curves shown in (A, B) and plotted against the IM30 concentration. Error bars were calculated from the fit via Gaussian distribution. Additionally, the phase transition temperatures from the Laurdan phase transition curve from Fig. 1A,B were inserted. Error bars were calculated as described in the experimental procedures. The experiments were repeated two times.

FIGURE 3. Oligomeric structure of IM30 and IM30_FERM. (A) Equal amounts of purified IM30 (a) and IM30_FERM (b) were analyzed by SDS-PAGE and subsequent Coomassie Blue staining. (B) SEC chromatograms of IM30 (straight line) and the IM30 mutant IM30_FERM (dotted line) exhibit destabilization of the oligomeric IM30 structure and formation of a lower-ordered IM30_FERM oligomer. The same experimental conditions were used to record the flow distribution of reference proteins (only peak maxima indicated): 660 kDa (thyroglobulin/ void volume), 440 kDa (apoferritin), 200 kDa (β-amylase), 66 kDa (albumin), 29 kDa (carbonic anhydrase), 14 kDa (cytochrome C). The experiments were repeated three times. (C, D) Electron micrographs of negatively stained wt IM30 (C) and IM30_FERM (D). In case of the wt protein the typical IM30 ring structures are visible, whereas the mutant protein forms irregular aggregates of variable size. Space bar for both frames, 100 nm.

FIGURE 4. IM30 binding to PG membrane surfaces. For calculating the \(K_D\) of the IM30-PG interaction, samples containing a constant amount of PG (75 µM) were incubated with an increasing amount of (A) IM30 or (B) IM30_FERM, respectively. The GP values were calculated and plotted against the IM30 concentration. Error bars represent the standard derivation (\(N=3\)). Note that the final lipid concentration in this experiment differs from the one used in the experiments shown in Fig. 1 and Fig. 2.
FIGURE 5. IM30 adsorption on lipid coated single gold nanosensors suggests binding of small IM30 entities. (A) Adsorption of IM30 was measured over time at different protein concentrations, as shown here for $c_{IM30} = 3.14$ µM (squares) and $c_{IM30} = 0.79$ µM (triangles). Black curves show the mean and standard error for all particles ($N=20$), red curves show single particle data which is used for the evaluation of the step sizes. (B) The percentage of step sizes that exceed the calculated threshold of small rings $\Delta\lambda_{IM30} = 0.47$ nm (grey) as well as for big rings $\Delta\lambda_{IM30} = 0.82$ nm (black) is shown as a percentage of all step sizes until 90% of the equilibrium resonance wavelength shift is reached ($N=20-80$ particles) for each measured concentration respectively.

FIGURE 6. Dissociation constant for IM30 binding on lipid coated gold nanosensors. Black data points show the mean equilibrium plasmon shift values for all particles after incubation with a defined IM30 concentration and their standard error ($N=20-80$ particles). Data was fitted with a Langmuir equation resulting in $K_D = 0.42 \pm 0.02$ µM. Grey dots show exemplary data points for the IM30_FERM mutant indicating identical plasmon shifts compared to the wild type protein.

FIGURE 7. Multiple equilibria are involved in the IM30-membrane interaction. The equilibrium between higher-ordered oligomers and ring structures in solution is represented by the dissociation constant $K_{D,1}$. Binding of IM30 higher order oligomers and IM30 rings to the membrane is described by $K_{D,2}$ and $K_{D,3}$, respectively.
FIGURE 1

A

B

C

FIGURE 2

A

B

C
IM30/Vipp1 membrane binding

FIGURE 5

A

B

FIGURE 6

FIGURE 7

$K_{d1} = 0.42 \mu M$

$K_{d2} = 1.39 \mu M$

$K_{d3} = 7.20 \mu M$

$K_{d3}$

$K_{d2}$
Organization into higher-ordered ring structures counteracts membrane binding of IM30, a protein associated with inner membranes in chloroplasts and cyanobacteria

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