Mg\textsuperscript{2+}-binding triggers rearrangement of the IM30 ring structure, resulting in augmented exposure of hydrophobic surfaces competent for membrane binding.

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ABSTRACT

The inner membrane-associated protein of 30 kDa (IM30), also known as vesicle-inducing protein in plastids 1 (Vipp1), is found in the majority of photosynthetic organisms that use oxygen as an energy source, and its occurrence appears to be coupled to the existence of thylakoid membranes in cyanobacteria and chloroplasts. IM30 is most likely involved in thylakoid membrane biogenesis and/or maintenance, and has recently been shown to function as a membrane fusion protein in presence of Mg\textsuperscript{2+}. However, the precise role of Mg\textsuperscript{2+} in this process and its impact on the structure and function of IM30 remains unknown. Here, we show that Mg\textsuperscript{2+} binds directly to IM30 with a binding affinity of approximately 1 mM. Mg\textsuperscript{2+}-binding compacts the IM30 structure coupled with an increase in the thermodynamic stability of the proteins’ secondary, tertiary and quaternary structure. Furthermore, the structural alterations trigger IM30 double ring formation in vitro due to increased exposure of hydrophobic surface regions. However, in vivo Mg\textsuperscript{2+}-triggered exposure of hydrophobic surface regions most likely modulates membrane binding and induces membrane fusion.

An important part of photosynthesis takes place in a specialized structure within chloroplasts and cyanobacteria, the thylakoid membrane (TM) system. Although several molecular details of photosynthesis are already understood, it essentially still remains a mystery how TMs are synthesized and maintained in chloroplasts and cyanobacteria. Clearly, TMs develop in the light (1-3), and the TM network is reorganized in response to changing environmental (light) conditions, involving TM assembly and disassembly (1-4). Thus, a mechanism controlling dynamic organization of TMs must exist. In fact, within the last fifteen years, several proteins were discussed to be involved in TM biogenesis, rearrangement and maintenance, including the inner membrane-associated protein of 30 kDa (IM30). IM30 was initially discovered in chloroplasts of \textit{Pisum sativum} where it was found to localize at both, the inner envelope membrane and TMs (5).
Most likely, IM30 has evolved from the phage shock protein A (PspA) in a cyanobacterial ancestor as a result of a gene duplication event (6). PspA is a 25 kDa protein involved in bacterial stress response (7,8) and solely encoded in bacteria. While in chloroplasts exclusively IM30 is present, cyanobacterial genomes code for both proteins (8). Due to their common origin, IM30 and PspA share several structural and functional similarities: both proteins are peripheral membrane-binding proteins (5,8-11) that have a conserved secondary structure, which is mainly α-helical, interrupted by small loop regions (11-14). The most obvious structural difference between PspA and IM30 monomers is the existence of an extra α-helix at the IM30 C-terminus (6,14). Both, PspA and IM30, form large ring assemblies with molecular masses >1 MDa (11-13,15). However, while PspA appears to form one type of ring with a well-defined size and a 9-fold internal symmetry (13), IM30 forms diverse oligomeric rings with varying rotational symmetries of at least 7-22 (11,12). We were recently able to generate a 3D surface representation of several IM30 rings, and based on these structures and mutant analyses we could show that the ring top and bottom clearly differ, e.g. the IM30 specific C-terminus is located exclusively at the ring top side (16). However, since a detailed structure of full-length IM30 or PspA is not available yet, it is still unclear how the monomers precisely pack inside these rings.

While the exact physiological function of IM30 still is under debate, due to its dual localization at the chloroplast inner envelope membrane plus at the TM, it has early been assumed that the protein is involved in TM biogenesis in chloroplasts (5). Depletion of IM30 in Arabidopsis thaliana (9,17) and the cyanobacterium Synechocystis sp. PCC 6803 (6,18) resulted in a reduced and disordered thylakoid structure and in an overall disturbed photosynthesis efficiency, which supports the assumption of an involvement in TM biogenesis and maintenance. In the last two decades, various IM30 functions have been proposed, including lipid and/or protein transport, protein complex formation, organization of thylakoid centers, the formation of cytoskeleton-like structures and membrane protection (for a recent review see (19)). Since under defined experimental conditions structures reminiscent of budding vesicles were observed at the inner chloroplast envelope membrane in IM30-depleted A. thaliana chloroplasts (9), renaming the protein to “vesicle inducing protein in plastids 1, Vippl” has been suggested. However, subsequent indications for an involvement in vesicle formation are lacking. Rather, we recently established that IM30 can trigger membrane fusion (10), an activity that explains several of the observations made before with IM30-depleted cyanobacteria and chloroplasts. Importantly, for IM30-induced membrane fusion, the presence of Mg$^{2+}$ is absolutely required (10). This was an important observation, as Mg$^{2+}$ is globally involved in regulating light-dependent processes in chloroplasts and appears to regulate the activity of several chloroplast proteins (20-23). But, does IM30 simply act as “glue” between the membrane and IM30, or does Mg$^{2+}$ bind to IM30 and induces structural rearrangements necessary for membrane binding and/or fusion? Thus, does Mg$^{2+}$ directly activate IM30?

Here we show that Mg$^{2+}$ binds to IM30, and Mg$^{2+}$-binding stabilizes the IM30 secondary as well as the tertiary and quaternary structure. The Mg$^{2+}$ binding affinity is about 1 mM, a value that would allow “activation” of IM30 and controlling its activity by the changes in the Mg$^{2+}$ concentration observed in vivo. Mg$^{2+}$-binding induces reorganization of the protein structure resulting in increased exposure of hydrophobic surfaces. In vitro, this results in ring clustering and double ring formation. However, in vivo exposure of increased hydrophobic surface regions most likely modulates membrane binding and induces membrane fusion.

**Results**

Mg$^{2+}$-binding changes the hydrophobicity of the IM30 ring surface

Recently, we have demonstrated that IM30 mediates membrane fusion in presence of Mg$^{2+}$ (10). However, the exact role of Mg$^{2+}$ in the fusion process is still enigmatic, and (i) either Mg$^{2+}$-binding to IM30 results in a rearrangement of the IM30 structure that, in the end, triggers membrane fusion or (ii), alternatively, Mg$^{2+}$ serves as a bridging ion that, glue-like, connects IM30 with the membrane.

It is well established that Mg$^{2+}$ binds to negatively charged lipid bilayers, e.g. formed of 1,2-dioleoyl-sn-glycerol-3-phospho-(1′-rac-glycerol) (DOPG) (24). We could verify this via addition of Mg$^{2+}$ to DOPG liposomes containing the fluorescence
probe Laurdan, which senses changes in the polarity of the lipid head-group region (25) (Fig. 1A). Thus, Mg$^{2+}$-binding to negatively charged membrane surfaces could indeed mediate membrane attachment of IM30. However, this does not exclude that Mg$^{2+}$ also directly binds to IM30 and that this is (additionally) needed for membrane fusion.

In order to follow binding of Mg$^{2+}$ to IM30, we at first employed isothermal titration calorimetry (ITC). We had to use a reverse ITC protocol, since injection of highly concentrated Mg$^{2+}$-solutions to an IM30 protein solution, as would be necessary for “normal” ITC experiments, caused a very high heat of dilution (data not shown), masking the signal for the IM30/Mg$^{2+}$ interaction. While even with the reverse ITC setup the heat of dilution background for Mg$^{2+}$ was very high (Fig. S1A), a direct interaction between Mg$^{2+}$ and IM30 was observed (Fig. 1B). In order to estimate the binding affinity, a simple hyperbolic binding model was fitted to the data, revealing a $K_d$ of 0.6 ± 0.3 mM (Fig. 1B).

Noteworthy, data analysis was limited to the concentration range up to 15 mM Mg$^{2+}$ due to the large heat of dilution at higher Mg$^{2+}$ concentrations. While binding of divalent cations, such as Mg$^{2+}$, typically is a result of electrostatic interactions, which are characterized by negative enthalpy changes, binding of Mg$^{2+}$ to IM30 resulted in a positive $\Delta H$ of up to 20 kcal/mol, and thus appears to be entropically driven. This could e.g. be explained by a Mg$^{2+}$-induced rearrangement of the IM30 structure that involves an altered protein surface chemistry, resulting in the release of water molecules, or by an involvement of proton transfer between protein and buffer, which can severely affect observed binding enthalpies (26).

To tackle these possibilities, we analyzed whether the surface properties of IM30 change after addition of increasing Mg$^{2+}$ concentrations. The fluorescence dye 8-anilinonaphthalene-1-sulfonic acid (ANS) binds to protein surfaces via hydrophobic interactions to exposed hydrophobic patches as well as via salt bridge formation between the dyes’ sulfonate group and positively charged amino acid side chains (27). Binding of the dye to a protein is observed as an increase in the ANS fluorescence intensity coupled with a blue shift of the fluorescence emission maximum (28). As can be seen in Figure 1C, addition of Mg$^{2+}$ led to increased fluorescence intensities and a blue shift of the fluorescence emission maximum from 525 nm to 490 nm, demonstrating increased binding of ANS to the protein surface in presence of increasing Mg$^{2+}$ concentrations. Importantly, significant differences in the relative ANS fluorescence were not observed in the absence of protein, clearly indicating that Mg$^{2+}$ does not directly influence the ANS fluorescence (data not shown). Furthermore, the observed effect is Mg$^{2+}$-specific, since increasing the ionic strength by addition of NaCl did not significantly affect ANS binding (Fig. S2).

Most likely, the observed changes in the fluorescence were caused by increased ANS-binding to hydrophobic patches on the protein surface, since ANS-binding mediated by ionic interactions is dramatically weaker than binding via hydrophobic interactions (27). Thus, the above-described observations strongly suggested that Mg$^{2+}$-binding to IM30 induces structural rearrangements, which result in increased exposure of hydrophobic surface regions. The determined increase in the fluorescence intensity with increasing Mg$^{2+}$ concentrations (Fig. 1D) could be well described by a hyperbolic function, yielding a dissociation constant $K_d$ of 0.6±1.9 mM, a value in good agreement with the $K_d$ value determined before in the ITC experiments (Fig. 1B).

To further support the assumption that Mg$^{2+}$-binding alters the IM30 structure, we next measured ANS fluorescence at increasing Mg$^{2+}$ concentrations, using chemically cross-linked IM30 rings (IM30-X). We anticipated that cross-linking the protein in absence of Mg$^{2+}$ would stabilize the Mg$^{2+}$-free ring conformation and would abolish (or at least hinder) Mg$^{2+}$-binding and/or structural rearrangements occurring upon Mg$^{2+}$-addition. Indeed, the ANS fluorescence spectra did not change upon addition of Mg$^{2+}$ to IM30-X (Fig. 1C+D), and thus, cross-linking clearly inhibits ANS binding. Noteworthy, IM30-X and wt IM30 have identical secondary and quaternary structures in absence of Mg$^{2+}$ (Fig. S3A), and neither is altered significantly by addition of Mg$^{2+}$ to IM30-X (Fig. S3B). Noteworthy, we cannot completely rule out that Mg$^{2+}$ still binds to the cross-linked protein. While in ITC measurements the titration of Mg$^{2+}$ into an IM30-X solution led to reaction heats undistinguishable from the control (data not shown), this does not completely rule out Mg$^{2+}$-binding because of the large heats of dilution (control).

Together, the observations described strongly suggest that Mg$^{2+}$ directly binds to IM30 with a $K_d$ of ...
of about 1 mM. Mg$^{2+}$-binding involves structural rearrangements of flexible and solvent accessible domains, resulting in increased exposure of hydrophobic regions.

**Mg$^{2+}$ binding results in deprotonation of carboxyl groups**

Exposure of hydrophobic surface regions is typically accompanied by increased ordering of surrounding water molecules and with a decrease of entropy, which, however, seems to contradict the results of the ITC measurement (Fig. 1B). However, the increase in entropy deduced from the ITC measurements might be due to deprotonation events coupled with Mg$^{2+}$-binding and/or subsequent structural rearrangement of IM30. Depending on the pH and buffer conditions, the influence of the protonation enthalpy on observed binding enthalpies can be significant. Since the entropy is calculated from the difference between binding enthalpy and free energy obtained from the binding constant, a shift in the experimentally determined binding enthalpy due to protonation of the buffer would lead to an incorrect value for the binding entropy. Thus, in case of proton transfer the here calculated binding enthalpy $\Delta H_{binding}$ would not represent the intrinsic binding enthalpy.

In fact, difference absorption FTIR spectroscopy of IM30 in absence and presence of Mg$^{2+}$ indicated that Mg$^{2+}$-binding to IM30 involves deprotonation events. The absorption in the 1720 cm$^{-1}$ region (Fig. 2), which monitors COOH stretching vibrations, is decreased in the presence of Mg$^{2+}$, which strongly indicates deprotonation of carboxyl groups, most likely from Glu and/or Asp side chains (29). Furthermore, the absorption of the IM30 amide I band (~1650 cm$^{-1}$) is increased in presence of Mg$^{2+}$ (Fig. 2). This difference, which is even more pronounced at higher Mg$^{2+}$ concentrations (Fig. S4A), clearly suggests a Mg$^{2+}$-induced rearrangement of the peptide backbone (amide I band) and thus, the secondary structure (30).

**Mg$^{2+}$-binding stabilizes IM30**

As IM30 is a predominantly $\alpha$-helical protein (11,12), we next used CD spectroscopy to further examine the Mg$^{2+}$-induced structural alterations indicated in our FTIR measurements. Since exposure of hydrophobic patches should destabilize the protein, the stability of IM30 against urea-induced denaturation was investigated. The CD spectrum of IM30 in absence of urea suggests that the protein has a largely $\alpha$-helical structure, regardless of the Mg$^{2+}$ concentration (Fig. 3A), which is in line with previous results (11). However, in presence of Mg$^{2+}$, the denaturation curve transition point ($D_{1/2}$) is clearly shifted to higher urea concentrations (Fig. 3C+D), and the slope of the curve is increased compared to the protein in absence of Mg$^{2+}$ (Fig. 3C). While the original level of secondary structure could be regained after removal of urea, the denaturation and renaturation curves do not overlap. Thus, the system shows hysteresis (Fig. S5A), and therefore we refrained from calculating $\Delta G$ values.

While the above described experiments suggest that Mg$^{2+}$-binding stabilizes the IM30 secondary structure, the molecular details of this stabilization were unclear. However, we speculated that a more tightly packed IM30 ring structure eventually stabilizes the secondary structure by reducing the accessibility of the protein core for the denaturant, thereby increasing the apparent thermodynamic stability. This assumption was supported by the notion that an increased slope of the denaturation curve as observed in presence of Mg$^{2+}$ could indicate a reduced solvent accessible surface area (31). In fact, a closer inspection of the CD-spectra in absence and presence of Mg$^{2+}$ further supports the assumption of a more compact structure. The ratio of the CD signal at 222 nm and 208 nm is a measure for coil-coiled-type interactions of $\alpha$-helices (32), and with a 222/208 ratio of >1.0, coiled-coil-type interactions are clearly involved in formation and stabilization of the IM30 structure (Fig. 3B), in line with previous reports (33). However, the 222/208 ratio increases in presence of Mg$^{2+}$, indicating increased formation of intra- and/or inter-molecular helix-helix interactions (Fig. 3B).

To better understand the impact Mg$^{2+}$-binding has on the IM30 tertiary and quaternary structure, we next determined the accessibility of Trp71, which is located in Helix 2 (14), for the fluorescence quencher acrylamide. The Trp71 fluorescence was quenched successively with increasing acrylamide concentration, with a higher slope, denoted as Stern-Volmer constant, in absence of Mg$^{2+}$ (Fig. 3E). The Stern-Volmer constant changed from 3.24 M$^{-1}$ in absence of Mg$^{2+}$ to 2.46 M$^{-1}$ in presence of Mg$^{2+}$ (Fig. 3E), and is in the same range as observed in previous studies analyzing proteins with buried Trps (34). Thus, the Trp fluorescence quenching measurements indicate that Trp71 is already buried.
within the IM30 structure in absence of Mg\textsuperscript{2+}. However, in presence of Mg\textsuperscript{2+}, the accessibility of Trp71 for acrylamide is decreased (Fig. 3E), and thus the Mg\textsuperscript{2+}-induced changes in the IM30 tertiary and/or quaternary structure further shield Trp71 within the oligomeric IM30 structure.

**Mg\textsuperscript{2+}-binding protects the IM30 C-terminal domain against proteolytic cleavage**

To further elucidate the impact Mg\textsuperscript{2+}-binding has on the IM30 structure, IM30 was digested by trypsin in presence and absence of Mg\textsuperscript{2+}, as an altered IM30 structure could result in altered proteolytic stability of the protein. When analyzed via SDS-PAGE, the full-length protein started to disappear already 2 minutes after addition of trypsin and a weak band with an apparent molecular mass of ~40 kDa appeared instead, which remained stable for about 40 min (Fig. 4A). In presence of Mg\textsuperscript{2+}, the original band remained visible significantly longer and the 40 kDa band was hardly detectable (Fig. 4A). The small fragment removed in absence of Mg\textsuperscript{2+} contains the IM30 C-terminus, as in the corresponding Western-blot, the 40 kDa band was not detected by an antibody specifically recognizing the IM30 C-terminal domain (Fig. 4B). Noteworthy, a direct impact of Mg\textsuperscript{2+} on the trypsin activity was excluded (Fig. S6). Thus, in presence of Mg\textsuperscript{2+} the IM30 structure is altered and the C-terminus has a decreased trypsin accessibility, i.e. is differently structured and/or located.

All results obtained thus far clearly indicate that Mg\textsuperscript{2+}-binding induces structural rearrangements, and the proteolysis data suggest a different structure and/or position of the IM30 C-terminus in presence of Mg\textsuperscript{2+}. Consequently, we next investigated the spatial proximity of IM30 C-termini in presence and absence of Mg\textsuperscript{2+}. FRET measurements were performed using IM30 rings composed of IM30 monomers that are labeled at their respective C-terminus with CFP or Venus, a YFP variant. Importantly, proper ring formation of both mutants was demonstrated via SEC and TEM (Fig. S8), and we have just recently demonstrated that the IM30 in vivo activity is not compromised by the fusion tag (35).

When differently labeled IM30 rings were mixed in a 1:1 ratio, the ratiometric FRET efficiency \(E_{\text{rat}}\) increased continuously from initially ~0.15 up to ~0.4, reached at Mg\textsuperscript{2+} concentrations >20 mM (Fig. 5A). Additionally, the time required to achieve a stable \(E_{\text{rat}}\) value was altered when Mg\textsuperscript{2+} was present: while in absence of Mg\textsuperscript{2+} it took about 7 h to reach the final value, in presence of 7.5 mM Mg\textsuperscript{2+} a constant value was reached already after ~2 h (Fig. 5B).

The observed increasing FRET efficiencies could originate from (i) either Mg\textsuperscript{2+}-binding enforcing IM30 monomers or lower ordered oligomeric structures to form high oligomeric ring structures, (ii) individual monomers exchanging more easily between two rings upon Mg\textsuperscript{2+}-binding or (iii) a dye-dye crosstalk between different IM30 rings, e.g. due to ring-ring interactions. Based on previous analyses we ruled out the possibility that a significant fraction of IM30 is monomeric or present as low-oligomeric forms (36). To be able to tackle option (ii), we next determined the maximal \(E_{\text{rat}}\) value by measuring the fluorescence emission of IM30 rings carrying both IM30-CPF and IM30-Venus monomers in an about 1:1 ratio. Using these dual-labelled IM30 rings, the maximum \(E_{\text{rat}}\) value was calculated to be 0.54 ± 0.01 (data not shown). This value was by far not reached after mixing single-labeled IM30 rings, even after incubation for 15 h, and thus we excluded a substantial monomer exchange between rings. Thus, a ring-ring interaction (iii), resulting in increased FRET, seemed to be most likely.

In fact, TEM of negatively stained IM30 indeed indicated an increased formation of IM30 double rings and a small number of rod structures in presence of Mg\textsuperscript{2+} (Fig. 5C). Such aggregates can also be observed in absence of Mg\textsuperscript{2+}, but to a much lower extent (11). When analyzed more quantitatively via SLS, the average IM30 particle size increased with increasing Mg\textsuperscript{2+} concentrations (Fig. 5D), supporting the assumption that Mg\textsuperscript{2+}-induced ring stacking is the major cause of the increasing \(E_{\text{rat}}\) value in presence of Mg\textsuperscript{2+}. Importantly, we can rule out the possibility that IM30 ring stacking is caused by an unspecific effect of increased ionic strength, since we did not observe an increased particle size when we increased the cation concentration by equivalent levels of Na\textsuperscript{+} (supplemental Fig. 7).

Thus, the structural rearrangements observed upon addition of Mg\textsuperscript{2+} result in ring stacking, which leads to an overall shielding of the C-terminus.

**Discussion**

Recently, we have identified Mg\textsuperscript{2+} as an important cofactor involved in IM30-mediated membrane fusion (10). However, its exact mode of action was
unclear and we hypothesized that Mg\textsuperscript{2+} either acts as a bridging-ion, mediating interaction of the protein with negatively charged membrane surfaces, or that Mg\textsuperscript{2+} directly binds to IM30, resulting in structural rearrangement and finally in formation of fusion-competent IM30 rings. While Mg\textsuperscript{2+} clearly binds to negatively charged membrane surfaces (24)(Fig. 1A), and could thus act as a bridging ion, such a “glue” is not required, as IM30 rings already bind to negatively charged membrane surfaces in absence of Mg\textsuperscript{2+} (10,36). However, the results presented here suggest that Mg\textsuperscript{2+} directly binds to IM30 rings resulting in structural rearrangements, coupled with an increased exposure of hydrophobic surface regions, finally resulting in ring stacking, at least in vitro. Albeit we are not yet able to pinpoint the exact Mg\textsuperscript{2+} binding site(s) at IM30, the FTIR data suggest that Asp and/or Glu side chains are involved in Mg\textsuperscript{2+}-binding (Fig. 2B). In fact, Mg\textsuperscript{2+} favors an octahedral coordination sphere with strong oxygen containing Lewis bases, such as carboxyl groups (37).

In the past, the activity of several pro- and eukaryotic proteins has been demonstrated to depend on Mg\textsuperscript{2+} (38-41). Most importantly, regulation of enzymatic activity via Mg\textsuperscript{2+} is well described in chloroplasts, and e.g. the activities of the CO\textsubscript{2}-fixing enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBISCO) (42), fructose-1,6-bisphosphatase (43) and sedoheptulose-1,7-bisphosphatase (43) are regulated by Mg\textsuperscript{2+}. Mg\textsuperscript{2+} is the most abundant metal ion in the TM lumen and in the chloroplast cytosol (20,23), and the concentration of free Mg\textsuperscript{2+} in chloroplasts is estimated to be in the range of 0.4 to 120 mM for different plant tissues and compartments (reviewed in (44)). However, the spatio-temporal Mg\textsuperscript{2+}-concentration in the chloroplast stroma depends on the environmental light conditions, as light exposure induces an increase in the stromal Mg\textsuperscript{2+} concentration (21,45-47) due to a Mg\textsuperscript{2+} release from the TM lumen (22). This change in the stromal Mg\textsuperscript{2+} concentration is key for regulation of above described enzymatic activities. Thus, it is not far-fetched to assume that Mg\textsuperscript{2+} plays a role also in regulation of TM biogenesis, maintenance and dynamics, since TMs develop and remodel in the light (1-3).

Binding of Mg\textsuperscript{2+} to IM30 could be key for TM maintenance and dynamics since the here determined $K_d$ of ~ 1 mM lies in the transition range of the stromal Mg\textsuperscript{2+} concentration. Likely, a light-induced increase in the Mg\textsuperscript{2+} concentration within the chloroplast stroma and the cyanobacterial cytoplasm activates the IM30 inherent fusion activity. Mg\textsuperscript{2+}-binding to IM30 induces a rearrangement of the IM30 structure involving formation of new or constriction of existing helix-helix interactions (Fig. 3B), leading to a more tightly packed structure with increased thermodynamic stability plus an exposure of extra hydrophobic surface regions. As we observed increased double ring formation in presence of Mg\textsuperscript{2+} (Fig. 5), it is reasonable to assume that, in absence of a membrane, the newly exposed hydrophobic surface triggers homo-dimerization of IM30 rings. In principle, such double rings could form via head-to-head, tail-to-tail or head-to-tail interaction of two rings. However, a head-to-tail interaction would ultimately result in formation of longer IM30 rods, which was observed only in a few cases here. Furthermore, as the Förster distance of the CFP/YFP pair is in the range of 50 Å (48) and as a single ring has a height of about 140 Å (i.e. a double ring has a height of ~280 Å) (16), solely formation of double rings via the ring top surfaces containing the C-termi (that carry CFP/YFP) satisfactorily explains the observed increase in the FRET signals upon Mg\textsuperscript{2+}-induced double-ring formation (Fig. 5A). Thus, IM30 double rings likely form via the upper ring surface that contains the C-terminal tail, and this C-terminal IM30 tail (Helix 7) is then buried within the ring-ring interface, as indicated by the decreased protease-sensitivity. However, in vivo, in presence of a cellular membrane, the hydrophobic patches of IM30 rings likely interact with the membrane, bringing the C-terminus in contact with the membrane surface. Indeed, the isolated IM30 C-terminus forms an amphipathic α-helix that can interact with membranes (49). Actually, the $K_d$ for the IM30-membrane interaction was recently determined to be in the μM range already in the absence of Mg\textsuperscript{2+} (36), indicating that a significant amount of IM30 rings likely is already membrane-bound in absence of Mg\textsuperscript{2+}. Thus, increasing the Mg\textsuperscript{2+}-availability in the stroma/cytosol on one hand might result in an increased membrane binding affinity of soluble IM30 rings due to the increased hydrophobicity of the protein surface, but could also alter the structure of membrane-attached IM30 rings. Membrane fusion involves the ability to connect two membrane layers, and thus, Mg\textsuperscript{2+}-activation of IM30 rings has to involve formation of IM30 rings...
exposing hydrophobic regions at both, the ring top and bottom (16). Although we show an increased hydrophobic surface on the top side of IM30 rings, likely promoting membrane interactions, our observations do not exclude that the ring bottom can also interact with membranes after IM30 activation, as suggested recently (16).

**Experimental Procedures**

**Cloning, expression and purification of IM30**

Construction of the pRSET-His-IM30 plasmid for expression of His-tagged IM30 is described in detail in (18). The plasmid for expression of the His-IM30-CFP mutant was constructed using the pRSET-His-IM30 expression plasmid (11), which contains the His-tagged IM30 coding sequence of *Synechocystis* sp. PCR 6803, pRSET-His-IM30 as well as pRSET-CFP, containing the sequence for the cyan fluorescent protein as described in (50) with the two modifications R27K and H232L, were restriction digested by *BamHI* and *XbaI*. The respective fragments were isolated from a 1% agarose gel and ligated by T4 ligase, resulting in the plasmid pRSET-His-IM30-CFP. The plasmid for expression of the His-tagged IM30-Venus mutant was generated using the pRSET-His-IM30-CFP expression plasmid as a template. From a pVenus plasmid (51), the *venus* gene was amplified using the primers 5'-TAA GCA GGA TCC GTG AGC AAG GGC GAG GAC-3' and 5'-TGC TTA GAA TTC TTA CTT GTA CAG CTC GTC CAT GCC-3'. Via the primers, a *BamHI* restriction site was introduced at the 5'end and an *EcoRI* restriction site at the 3'end (underlined). Insert and template were restriction digested with *BamHI* and *EcoRI* and ligated using T4 ligase. All enzymes were from New England BioLabs (Frankfurt am Main, Germany).

All proteins were heterologously expressed in *E. coli* BL21 (DE3) cells and purified with a nickel-nitritoltriacetic acid-agarose column (Qiagen, Hilden, Germany), as described in (10,36). Purified proteins were extensively dialyzed against 20 mM HEPES buffer, pH 7.6, and the purity of the proteins was analyzed on 14% SDS-polyacrylamide gels. Protein concentration was determined using a Bradford assay. For the labeled mutants, SDS-polyacrylamide gels calibrated with BSA standards were densitometrically evaluated to estimate the protein concentration.

**Protein cross-linking**

IM30 was cross-linked with glutaraldehyde (Sigma-Aldrich, Munich, Germany). This bifunctional cross-linker reacts with functional groups such as amines, thiols, phenols and imidazoles (52), thereby decreasing the structural flexibility and dissociation/oligomerization of proteins (53).

IM30 with concentrations between 3 and 10 µM (in 20 mM HEPES pH 7.6) were incubated with 5 mM glutaraldehyde for 5 min at 37 °C. The reaction was stopped by adding TRIS/HCl pH 8.5 to a final concentration of 50 mM. The reaction solution was extensively dialyzed against 20 mM HEPES buffer, pH 7.6. A nearly quantitative cross-linking of IM30 was confirmed on 14% SDS-polyacrylamide gels.

**Liposome preparation**

DOPG (dioleoylphosphatidylglycerol) (Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA)) dissolved in chloroform was mixed with Laurdan (6-Dodecanoyl-N,N-dimethyl-2-naphthylamine, Fluka Analytical, Seele, Germany) dissolved in chloroform in a molar ratio of 1:500 (Laurdan:Lipid). The mixture was dried under a stream of nitrogen. To remove traces of chloroform, the mixture was dessicated under vacuum over night. Lipids were hydrated in HEPES buffer with vigorous stirring to yield multi-lamellar liposomes. Unilamellar liposomes were formed by five freeze-thaw cycles.

**Fluorescence measurements**

If not mentioned otherwise, all fluorescence measurements were performed on a FluoroMax-4 fluorimeter (HORIBA Scientific, Kyoto, Japan) with an integration time of 0.1 s at 25 °C.

For the ANS fluorescence measurements, 1 µM IM30 or cross-linked IM30 (IM30-X) were mixed with 7.5 µM 8-anilinonaphthalene-1-sulfonic acid (ANS) (Sigma-Aldrich, Munich, Germany) in 20 mM HEPES buffer (pH 7.6), respectively, and incubated in the dark for 15 min at room temperature. Then, increasing amounts of MgCl₂ in a fixed buffer volume were added to reach a final sample volume of 200 µL. As a control, samples containing NaCl instead of MgCl₂ with the same equivalent concentration of cations, were prepared. The samples were incubated at room temperature in the dark for another 30 min. The total concentration of Mg²⁺ ranged from 0 to 128 mM. The excitation wavelength was 370 nm (slit widths 4 nm), the...
emission wavelength ranged from 400 to 650 nm (slit widths 4 nm). Fluorescence intensities of at least 3 independent measurements were averaged and plotted against increasing ionic strength of the monitored cation species. The dissociation constant $K_d$ of Mg$^{2+}$-binding to IM30 was obtained by fitting the following hyperbolic binding function to the data:

$$F = F_{min} + \frac{\Delta F}{1 + \frac{K_d}{x}}$$  (1)

Here, $F$ is the measured fluorescence intensity, $F_{min}$ is the fluorescence intensity in absence of Mg$^{2+}$, $\Delta F$ is the change in fluorescence intensity due to Mg$^{2+}$ binding and $x$ is the Mg$^{2+}$ concentration. The fit was performed with the non-linear regression tool implemented in OriginPro 8.6.0.

**Laudan fluorescence measurements**

DOPG liposomes (100 µM lipid) in 20 mM HEPES buffer, pH 7.6 were incubated with increasing concentrations of Mg$^{2+}$ (0-15 mM) in separate samples at room temperature for 30 min. Fluorescence intensities were measured with an Omega Fluostar plate reader (BMG Labtech GmbH, Ortenberg, Germany). The excitation filter was set to 360 nm and the emission filters were set to 420 and 520 nm. Fluorescence intensities at 420 nm ($I_{420}$) and 530 nm ($I_{530}$) of 3 samples were averaged and used to calculate the Generalized Polarization ($GP$) after substraction of buffer background (25):

$$GP = \frac{I_{420} - I_{520}}{I_{420} + I_{520}}$$  (2)

**Acrylamide Quenching**

1.6 µM IM30 in absence or presence of 15 mM MgCl$_2$, respectively, were incubated with 0 to 400 mM of freshly prepared acrylamide (Sigma-Aldrich, Munich, Germany) for 30 min at room temperature (20 mM HEPES pH 7.6). Trp fluorescence was measured at 25 °C from 310 to 370 nm (slit widths 3 nm) upon excitation at 300 nm (slit width 3 nm), to minimize the influence of Tyr absorbance. The fluorescence intensities at 333 nm of three individual measurements were averaged and corrected for inner filter effects by correlating the spectra to the intensities of the water Raman-peak under the same conditions without addition of protein. The Stern-Volmer equation

$$\frac{F_{eq}}{F_0} - 1 = K_{SV} \times [Q]$$  (3)

was used to determine the Stern-Volmer-constant ($K_{SV}$) from linear regression of the data. Here, $F_0$ is the measured fluorescence intensity at 333 nm, $F_{eq}$ is the intensity in absence of quencher and $[Q]$ is the acrylamide concentration (34).

**Förster Resonance Energy Transfer measurements**

For Förster Resonance Energy Transfer (FRET) measurements, IM30-CFP and IM30-Venus (1 µM each) were mixed and incubated for ~2 h at RT with the indicated Mg$^{2+}$ concentrations. The donor (CFP) was excited at 420 nm (slit width 2 nm) and fluorescence emission was recorded from 440 to 700 nm (slit width 2 nm). As reference for the actual concentration of acceptor for each sample, a spectrum between 517 to 700 nm was recorded (slit 1 nm) after acceptor excitation (510 nm, slit 1 nm). As a measure of FRET efficiency, the ratiometric FRET efficiency $E_{rat}$ was calculated using the equation

$$E_{rat} = \frac{1}{1 + \frac{I_D}{I_A}}$$  (4)

where $I_D$ is the fluorescence intensity of the donor emission at 475 nm and $I_A$ is the fluorescence intensity of the acceptor emission at 528 nm, both after donor excitation. $E_{rat}$ is suitable for comparing the relative ratio between donor and acceptor peak in a qualitative manner. Kinetics of changes in FRET were measured in presence or absence of 7.5 mM MgCl$_2$. Samples were prepared as described above and spectra were recorded at different time points. To estimate the $E_{rat}$-level of rings composed of a 1:1 ratio of monomers tagged with CFP and Venus, respectively, cells expressing either CFP- or Venus-labelled IM30 were broken and cell suspensions were mixed prior to purification following the standard procedure described above. As observed recently (36), IM30 tetramers are purified with this procedure, and the tetramers reassembly to IM30 rings directly after purification, resulting in mixed CFP/Venus-labelled rings. The ratio of the two constructs was determined via SDS-PAGE. $E_{rat}$ was determined as described above.
Static light scattering (SLS)
Static light scattering was used to follow particle size changes of IM30 in presence of increasing Mg\(^{2+}\) concentrations. 1.6 µM IM30 was incubated for 15 min at room temperature in presence of increasing Mg\(^{2+}\) concentrations in 20 mM HEPES buffer (pH 7.6). The samples were excited at 600 nm (slit width 5 nm) and emission spectra were recorded from 200 to 400 nm (slit widths 5 nm). The intensities of the Rayleigh scattering peaks of at least three samples were measured at the second order wavelength (300 nm) and were plotted against the Mg\(^{2+}\) concentrations.

Isothermal titration calorimetry (ITC)
ITC experiments were performed using a MicroCal VP-ITC microcalorimetry system (Malvern Instruments, Worcestshire, UK). The sample cell contained 0-50 mM MgCl\(_2\) solutions in 20 mM HEPES buffer (pH 7.6). The syringe was loaded with 20 µM IM30 in 20 mM HEPES buffer (pH 7.6). All solutions were degassed thoroughly under vacuum prior to the measurements. Three injections of 20 µL per Mg\(^{2+}\) concentration were carried out at 20 °C and changes in the heating rate were measured (reference heat rate 20 µcal/s). The origin based Microcal ITC software was used to automatically calculate the enthalpy change by integrating the heat rate peaks. The enthalpy change of three injections was averaged, assuming a negligible change in the molar ratio between ligand and protein due to the high dilution of the protein. The data were corrected for heats of dilution of Mg\(^{2+}\) by subtracting enthalpy changes of control experiments that were done with buffer instead of protein solutions and by subtracting the value for adding 0 mM Mg\(^{2+}\) from all data points. A hyperbolic binding function was fitted to the data in the concentration range of 0 to 15 mM Mg\(^{2+}\) to determine the dissociation constant (\(K_d\)) by the non-linear least square algorithm implemented in OriginPro 8.6.0. (54,55). Due to the low affinity of the binding process, the concentration of Mg\(^{2+}\) was significantly higher than the protein concentration, allowing to use the simple hyperbolic binding function.

Transmission electron microscopy (TEM)
6.3 µM IM30 were incubated for 30 min in presence of 15 mM MgCl\(_2\) in 20 mM HEPES buffer (pH 7.6). The control sample contained the same amount of protein from the same stock solution and was prepared under the same conditions without MgCl\(_2\). The samples were processed and imaged as described previously (49).

Fourier Transform Infrared (FTIR) Spectroscopy
First, a gold layer was deposited on the surface of a Si ATR crystal by etching the Si with HF and by reduction of AuCl\(_3\) as described previously (56). Prior to the deposition of the gold film, the ATR crystal was polished with 0.3 µm alumina, rinsed with copious amounts of Millipore water, acetone and water again. The crystal was then dried under an argon stream and immersed in 40% NH\(_4\)F (w/v) for 1 min, rinsed and dried again. It was then heated at 65 °C for 10 min together with the plating solution. This solution was a 1:1:1 mix (v/v/v) of A) 15 mM NaAuCl\(_4\), B) 150 mM Na\(_2\)SO\(_4\), 50 mM Na\(_2\)S\(_2\)O\(_3\) and 50 mM NH\(_4\)Cl and C) HF 2% (w/v; total volume: 1 ml). Once the plating temperature was reached, the prism was covered with the solution for 40 s, and the reaction was stopped by washing the plating solution off with water, followed by drying with a stream of argon. The resulting gold film was then tested for electrical conductance with a multimeter (the typical electric resistance of the layer as measured from one corner to another of the crystal should be around 15 Ω for a thickness of 50 nm).

The experimental procedure for the Ni-NTA SAM was adapted from (57,58). First, the gold modified silicon ATR crystal was covered with 1 mg/ml of 3,3′-dithiodipropionic acid di(N-hydroxy succinimide ester) (DTSP) (Sigma-Aldrich, Munich, Germany) in dry dimethylsulfoxide and the monolayer was allowed to self-assemble for 1 h. The excess DTSP was then washed away with dry DMSO and the crystal was dried under an Argon stream. Afterwards, it was covered with 100 mM Na\(^+\),Na\(^+\)-bis(carboxymethyl)-L-lysine (Sigma-Aldrich, Munich, Germany) in 0.5 M K\(_2\)CO\(_3\) at pH 9.8 for 3 h and then rinsed with water. Finally, the surface was incubated in 50 mM Ni(ClO\(_4\))\(_2\) for 1 h before being washed one last time with water. For immobilization of the protein, 5 µL of 21.2 µM IM30 in 20 mM HEPES (pH 7.6) were deposited on the modified gold surface for 1h.

A configuration allowing the simultaneous acquisition of Fourier Transform Infrared (FTIR) spectra in the ATR mode with perfusion of solutions with given composition was used. As a
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multi-reflection ATR-unit, we used silicon crystal with 3 mm surface diameter. All experiments were carried out with a Bruker Vertex 70 FTIR spectrometer (Globar source, KBr Beamsplitter, LN-MCT detector) at 8 mm aperture and 40 kHz scanner velocity. The measurements were carried out at ~7 °C. Solutions were kept on ice prior to use. The pump speed was kept constant at a flow rate of 0.2 mL/min. Before each perfusion step, the input tube was carefully washed with water and buffer. For recording IM30 differences spectra, MgCl$_2$ was dissolved in 20 mM HEPES (pH 7.6) to a final concentration of 10 and 90 mM. A second perfusion solution of the same composition but without MgCl$_2$ at a given pH value was also used. Before measuring perfusion-induced difference spectra, the system was perfused first with HEPES buffer without MgSO$_4$ for 30 min to equilibrate the system. A background spectrum was then recorded and the perfusion was changed to HEPES buffer containing MgCl$_2$. After a delay of 20 min for equilibration, the Mg$^{2+}$ (pH 7.6) minus Mg$^{2+}$-free (pH 7.6) difference spectra were recorded. Subsequently, a new background was recorded, and the solution was changed back to the solution without Mg$^{2+}$. The equilibration time was again 20 min and Mg$^{2+}$-free (pH 7.6) minus Mg$^{2+}$ (pH 7.6) spectra were obtained. The procedure described was repeated 5 times, and the difference spectra were averaged. Baseline correction and smoothing were done, where necessary. Full reversibility is indicated by a symmetrical shape of the two spectra obtained.

The secondary structure was determined on the basis of deconvolution of the amide I signal of the dried films in the presence and absence of Mg$^{2+}$. First, the wavenumber of each component was determined from the second derivative of the individual spectrum and Gaussian curves were fitted to each absorption spectrum (Origin 8.5 software) while the identified position was kept constant during the first fitting. Then, a consecutive optimization of amplitudes, band positions and the half-width of the individual bands were performed. The individual amide I band components were obtained at 1621, 1654, 1687 and 1699 cm$^{-1}$ and have been assigned to the secondary structure elements as follows: α-helices and unordered structures (1660-1640 cm$^{-1}$), anti-parallel β-sheet (two bands in 1621 and 1699 cm$^{-1}$) and β-turns (1687 cm$^{-1}$), on the basis of a large body of experimental data (59). Due to the fitting procedure, the error is +1%.

**Circular dichroism (CD) Spectroscopy**

Circular dichroism was measured using a JASCO-815 CD spectrometer (JASCO Corporation, Tokio, Japan). Spectra ranging from 200 to 250 nm were recorded at 20 °C with a scan rate of 100 nm/min, 1 nm steps and 1 s data integration time. For each sample, three spectra were averaged and smoothed by the JASCO software package (Savitzky-Golay filter), if necessary. All spectra were converted to molar ellipticity (60).

The helix-helix interaction ratio (coiled-coil ratio) of IM30 in presence and absence of Mg$^{2+}$ was calculated as the ratio of molar ellipticity at 222 nm and 208 nm (32). For this calculation, the ratio determined from at least six independent samples was averaged. To test the statistical significance of the differences, we performed One-way-ANOVA (OriginPro 8.6.0), testing for the population means of the 222/208 nm ratios with p-levels of 0.05, 0.01 and 0.001.

The stability of IM30 was determined by incubating 3.2 µM IM30 with increasing concentrations of urea (0–6.5 M) in 10 mM Na-Phosphate buffer (pH 7.6) for 30 min at room temperature. Protein denaturation was followed in presence of 0–15 mM Mg$^{2+}$. For protein renaturation, the urea concentration was lowered stepwise by dialysis for 90 minutes and spectra were recorded at each step. Three independent measurements were averaged.

The transition point of the denaturation curve ($D_{1/2}$) was calculated by a least-square non-linear fit of the following Boltzmann equation:

$$ f_D = \frac{y_N - y_D}{1 + e^{(x-D_{1/2})/dx}} + y_D \tag{5} $$

Here, $y_N$ is the molar ellipticity at 222 nm in the native state and $y_D$ the molar ellipticity at 222 nm in the denatured state. $f_D$ is the fraction of denatured protein and x the concentration of urea.

**Trypsin digestion of IM30**

2.5 µM IM30 in presence and absence of 10 mM Mg$^{2+}$ was incubated with 0.01 mg/ml trypsin (bovine pancreas, 5000 USP/mg, Sigma-Aldrich, Munich, Germany) at 37 °C for 0 to 60 min. The reaction was stopped immediately by adding 5xSDS loading buffer (containing 250 mM TRIS, 10% SDS (w/v), 0.2% bromphenol blue (w/v), 50% glycerol (w/v), 500 mM DTT) and by heating to 95...
°C for 3 min. The samples were analyzed on a SDS-PAGE gel and via immunoblotting using an antibody directed against the IM30 C-terminal domain (rabbit) (Griamsch Laboratories, Schwabhausen, Germany) and a peroxidase coupled anti-rabbit secondary antibody (Sigma-Aldrich, Munich, Germany) with subsequent visualization by the “ECL™ Prime Western Blotting Detection Reagent” kit (GE Healthcare, Munich, Germany).

As a control for a potential interaction of trypsin with Mg$^{2+}$, the trypsin activity was determined using a N$_a$-Benzoyl-L-arginine ethyl ester (BAEE) assay (Acros Organics, Geel, Belgium) in presence and absence of Mg$^{2+}$ (61). The trypsin activity of three samples was averaged. The activity of trypsin in absence of Mg$^{2+}$ was set as 100%. Activities of trypsin in presence of salts were calculated relative to this.

**Size exclusion chromatography**

In order to confirm formation of high-molecular weight particles of IM30-CFP and IM30-Venus, 350 or 250 µg protein, respectively, was loaded onto a Superdex-200-16/600 column at 4 °C using an Äkta Explorer system (GE Healthcare, Munich, Germany) at a flow rate of 0.5 ml/min. For calibration, the following proteins of the molecular weight maker kit (Sigma Aldrich, Munich, Germany) were used: ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), β-amylase (200 kDa) and apoferritin (443 kDa).

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**Conflict of interest**

The authors disclose any financial conflict of interest.

**Author contributions**

D.S., J.H., B.J., N.H., P.H., N.G., J.M., K.R. and W.G. designed the experiments and analyzed data. J.H., B.J., N.G., K.R. and W.G. performed the experiments. D.S., J.H., B.J., N.H., P.H., and N.G. wrote the paper.
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Figures

Figure 1: Mg$^{2+}$-binding to IM30 alters the protein surface hydrophobicity.

(A) Generalized Polarization (GP) of Laurdan fluorescence measured with DOPG liposomes in presence of increasing Mg$^{2+}$ concentrations. Mg$^{2+}$-addition increases the GP value in a concentration-dependent manner. Error bars represent standard deviation (N=3). (B) Binding of Mg$^{2+}$ to IM30 (■) is coupled to a positive enthalpy change. The ITC data were fitted with a standard one-site-binding model (——). Error bars represent standard deviation (N=3). (C) Fluorescence spectra of ANS in presence of IM30 +/− Mg$^{2+}$ (▲ IM30 wt, 0 mM Mg$^{2+}$; ▼ IM30 wt, 16 mM Mg$^{2+}$; ■ IM30-X, 0 mM Mg$^{2+}$; ● IM30-X, 16 mM Mg$^{2+}$). Binding of Mg$^{2+}$ to IM30 causes an intensity increase and a blue shift of the ANS fluorescence peak. Spectra were averaged from nine samples. (D) Fluorescence intensities at 490 nm in presence of increasing Mg$^{2+}$ concentrations (■ IM30-X, ◊ IM30 wt). Error bars: standard deviation (N=9 (IM30 wt); N=3 (IM30-X)).
Figure 2: FTIR difference absorbance spectra of IM30 +/- Mg$^{2+}$.

FTIR difference absorbance spectra of IM30 in absence of 10 mM Mg$^{2+}$ (20 mM HEPES - (20 mM HEPES +10 mM Mg$^{2+}$)) and in presence of 10 mM Mg$^{2+}$ (20 mM HEPES +10 mM Mg$^{2+}$)-20 mM HEPES). Significant changes can be observed in the range of 1400 – 1800 cm$^{-1}$. The data show increased absorbance of the amide I band and decreased absorbance in the 1720 cm$^{-1}$ region in presence of 10 mM Mg$^{2+}$.
Mg$^{2+}$-binding triggers rearrangement of the IM30 ring

Figure 3: Mg$^{2+}$ binding affects the secondary/tertiary/quaternary structure and stability of IM30.

(A) CD spectra of IM30 in absence (black line) and in presence of 15 mM Mg$^{2+}$ (gray line). The 208 nm peak amplitude is decreased in presence of Mg$^{2+}$, indicating a change in protein structure. Spectra were averaged from three samples. (B) Boxplots of the 222/208 ratio at increasing Mg$^{2+}$ concentrations. The boxes represent the lower and upper quartile relative to the mean value, whiskers represent the standard deviation. The arithmetic mean is marked by an asterisk (N=6-7). The 222/208 ratio increases with increasing Mg$^{2+}$ concentration, indicating increased formation of coiled-coil-type structures. Significance levels: $p\leq 0.05=*$; $p\leq 0.01=**$; $p\leq 0.001=***$. (C) Urea denaturation of IM30 in absence (■) and presence of 15 mM Mg$^{2+}$ (▼). The denaturation curves show a stability increase as a shift of the inflection point to a higher urea concentration in presence of 15 mM Mg$^{2+}$. The curves show averaged data of three independent experiments. Lines: fit, based on a Boltzmann function. Error bars represent Gauss errors of three samples (N=3). (D) $D_{1/2}$ values of IM30 calculated from urea denaturarion curves gained in presence of increasing Mg$^{2+}$ concentrations (■). The stability of the secondary structure increases with increasing Mg$^{2+}$ concentration for both values. Error bars represent error for $D_{1/2}$ of the least square non-linear fit of three averaged denaturation curves. (E) Stern-Volmer plot of IM30 Trp fluorescence quenching by acrylamide in presence (▲) and absence (■) of 15 mM Mg$^{2+}$. The slope of the linear regression decreases in presence of 15 mM Mg$^{2+}$. The $K_{SV}$ for the control is 3.24±0.12 M$^{-1}$, and 2.46±0.14 M$^{-1}$ in presence of Mg$^{2+}$. Thus, Trp is more buried within the IM30 ring structure in presence of Mg$^{2+}$. Error bars: standard deviation of three samples (N=3).
Figure 4: Mg$^{2+}$-binding affects tryptic digestion of IM30.

(A) SDS-PAGE following trypsin digestion of IM30 in absence (top) and presence of 10 mM Mg$^{2+}$ (bottom). F marks the full-length protein, C the protein core fragment after cleavage of an easily accessible fragment, and T trypsin. In absence of Mg$^{2+}$, a small fragment is cleaved rapidly, while a stable core fragment is detectable for at least 20 min. The full-length protein is only detectable for 2 min. In presence of Mg$^{2+}$, the full length protein can be detected for at least 10 min. (B) Immunoblot of IM30 after trypsin digestion in absence (top) and presence of 10 mM Mg$^{2+}$ (bottom) using an antibody directed against the IM30 C-terminus. In absence of Mg$^{2+}$, the C-terminal domain is detectable for 2 min, while it is detectable for at least 20 min in presence of Mg$^{2+}$. Therefore, Mg$^{2+}$-binding protects the C-terminal domain against tryptic digestion.
Figure 5: Mg$^{2+}$ triggers stacking of IM30 rings.

(A) FRET between IM30 CFP and IM30 Venus is caused by stacking of IM30 rings in presence of Mg$^{2+}$ (see text for details). FRET between IM30 CFP and IM30 Venus increases with increasing Mg$^{2+}$ concentrations. Error bars represent standard deviation of three samples (N=3). (B) FRET between IM30 CFP and IM30 Venus in presence (●) and absence (□) of 7.5 mM Mg$^{2+}$ followed over time. The plateau is higher and is reached faster in presence of Mg$^{2+}$. Error bars represent standard deviation of 3 samples (N=3). (C) Negative stain TEM of IM30 (6.3 µM) in absence (top) and presence (bottom) of 15 mM Mg$^{2+}$. Both samples were from the identical protein stock solution. The number of double rings and rod structures increases in presence of Mg$^{2+}$. Apparently, the amount of protein found on the grid is also increased in presence of Mg$^{2+}$, which might be caused by better binding to the grid as a result of the altered protein surface in presence of Mg$^{2+}$. Scale bars = 500 nm. (D) Light scattering of IM30 at increasing Mg$^{2+}$ concentrations. Scattering increases with increasing Mg$^{2+}$ concentrations, caused by an increased particle size. Error bars represent standard deviation of three samples (N=3).
Mg$^{2+}$-binding triggers rearrangement of the IM30 ring structure, resulting in augmented exposure of hydrophobic surfaces competent for membrane binding

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