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Mechanism of formation of multilayered 2D crystals of the Enzyme IIC-mannitol transporter

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Abstract

We have recently reported the crystallization by reconstitution into lipid bilayer structures of Enzyme IIC mtl, the transmembrane C-domain of the mannitol transporter from E. coli. The projected structure was determined to a resolution of 0.5 nm [J. Mol. Biol. 287 5 (1999) 845]. However, further investigation proved that these crystals were multilamellar stacks instead of 2D crystals, and therefore were unsuitable for three-dimensional structural analysis by electron crystallography. Understanding the crystallogenesis of these crystals could reveal the mechanism of formation of multilayers. In the present study, cryo-electron microscopy (cryo-EM) and turbidimetry are used to study the successive steps of reconstitution of Enzyme IIC mtl into phospholipid-containing structures and its crystallization under different conditions. Our experimental approach enabled us to distinguish the separate steps of reconstitution and crystallization. The salt concentration especially influenced the nature of the vesicles, either half open unilamellar or aggregated multilamellar, formed during reconstitution of Enzyme IIC mtl. The presence of DOPE and DOPC and the temperature influenced the type of lipid structures that were formed during the crystallization phase of Enzyme IIC mtl. Cryo-EM showed that protein crystallization is closely associated with the formation of isotropic lipid (cubic) phases. We believe that DOPE is responsible for the formation of these lipid cubic phases, and that crystallization is driven by exclusion of protein from these phases and its concentration into the lamellar phases. This mechanism is inextricably associated with the formation of multilayers.

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Keywords: 2D crystallization; Cryo-electron microscopy; CHAPS; Reconstitution

1. Introduction

Electron crystallography of protein 2D crystals is a powerful method of high-resolution structure determination of membrane proteins [27]. Its application depends on the crystallization into 2D protein crystals, which are one unit-cell-thick and preferably several square-micrometers in size. Several 2D crystals of membrane proteins have been obtained and their structure was determined at medium to high resolution. However, several studies [23] have also reported the formation of thin 3D crystals, which are several unit-cells in thickness, for which no method of 3D structural analysis is available yet. Understanding the process of formation of these thin 3D crystals may reveal their underlying mechanisms and provide indications for growing either 2D crystals or 3D crystals that are large enough for X-ray crystallography (as recently shown in the case of Ca2+ -ATPase [26]).

This study focuses on the transmembrane domain of the mannitol transporter Enzyme II (IIC mtl), for which we reported the formation of highly ordered crystals [10]. A 0.5 nm projection map was determined from these crystals by cryo-electron crystallography. Further analysis showed that these crystals are actually thin 3D crystals (Koning, Oostergetel, Stuart and Brisson, unpublished data). As for many membrane proteins, IIC mtl crystals were obtained by
reconstitution into a lipid bilayer (for a review, see Refs. [6,9,11]). In general, reconstitution is believed to take place upon detergent removal at the micelle to vesicle transition of the lipids [2] followed by self-organization of the protein into 2D crystals. In this study we combined turbidimetry and cryo-electron microscopy (cryo-EM) to characterize the crystallization process. Turbidimetry is widely used to follow the solubilization of liposomes by detergents [14,20] and is used here to study the crystallization process. The detergent concentration, which is recognized to be a critical parameter in the micelle-to-vesicle transition of phospholipids, was measured by quantitative thin-layer chromatography (TLC). The morphology of the lipid aggregates and of the protein-lipid ordered assemblies at different stages of the crystallization were studied by cryo-EM.

The influence of various parameters, like lipid composition, detergents, salt concentration, pH and temperature, on the crystallization process was investigated. First, to determine what parameters are crucial in a successful crystallization and second, to optimize the conditions to obtain large 2D crystals. It is shown that the formation of (multilayered) crystals of IIC\textsuperscript{mtl} depends on the presence of DOPE. We propose that these crystals are formed through a mechanism of protein concentration by exclusion from lipid cubic phases and that this mechanism might be relevant for other systems. The mechanism described here resembles the mechanism that is proposed for the 3D-crystallization of membrane proteins using lipid cubic phases from mono-olein [13].

2. Materials and methods

2.1. Materials

Decylmaltoside (DM) and -[(3-cholamidopropyl)-dimethylammonio]-propanesulfate (CHAPS) were obtained from Sigma. Dioleoyl-phosphatidylethanolamine (DOPE) and dioleoyl-phosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Ni-NTA-agarose was from Qiagen (Qiagen GmbH, Hilden, Germany). Bio-beads SM2 (Bio-Rad, Veenendaal, The Netherlands) were washed with methanol and rinsed with double-distilled water prior to use. All other chemicals were of the highest purity.

2.2. Protein purification

The membrane-bound C domain of enzyme II mannitol (IIC\textsuperscript{mtl}) from \textit{E. coli} was purified from membrane vesicles in one step [15]. In brief, membrane vesicles (0.5 ml, total protein \( \pm 25 \) mg/ml) derived from \textit{E. coli} homogenate were solubilized in 1% DM, 10 mM HEPES, 100 mM NaCl, 15 mM imidazole, 0.5 mM NaN\textsubscript{3}, pH 7.5 for 15 min on ice, in a total volume of 2 ml. The solution was centrifuged at 200,000 \( \times g \) for 20 min at 4 °C (Beckman TL 100 ultracentrifuge, TLA 100.3 rotor) and the supernatant was mixed with 0.5 ml pre-washed Ni-NTA agarose beads for 20 min at 4 °C in a column (Bio-Rad spin column). The column was washed with a buffer containing 0.3% DM, 10 mM HEPES, 100 mM NaCl, 15 mM imidazole, 0.5 mM NaN\textsubscript{3}, pH 7.5. IIC\textsuperscript{mtl} was eluted in 150–200 \( \mu l \) fractions with the same buffer containing 200 mM imidazole. The protein concentration was measured by UV absorption. The eluted fractions were pooled until a concentration of 1 mg/ml IIC\textsuperscript{mtl} was reached (the protein concentration of the fractions ranges from 2 to 0.2 mg/ml). Directly after purification, EDTA and mannitol were added (both at 1 mM final concentration) to improve the protein stability. Protein purity was checked by SDS-PAGE, no endogenous lipids were present after the purification as checked by TLC.

Fig. 1. (a) Thin layer chromatogram of detergents and phospholipids. Lanes 1 – 5 show calibration spots for CHAPS (1 – 8 \( \mu g/\)spot), lanes 6 – 9 show calibration spots for DM (1 – 4 \( \mu g/\)spot), lanes 10 – 17 show the detergent concentration determination from a crystallization mixture obtained 0 – 60 min. after bio-bead admission. The sample volume was increased in time to retain sufficient sensitivity for CHAPS and DM. Therefore, the intensity of the phospholipid spots increases (phospholipids are not extracted by bio-beads). (b) The intensity of the calibration spots was found to be linearly correlated with the amount of detergent that was deposited. The detergent concentration in the crystallization mixture was determined by linear regression.
2.3. Detergent concentration measurement

Since the detergent is a major parameter in the course of the crystallization process, special attention was given to accurately measure of the detergent concentration. This was done by quantitative TLC according to standard methods [7]. Samples containing 1–10 µg detergent were deposited on non-activated TLC plates (silica gel 60, aluminum sheets, Merck) and developed in chloroform/methanol/water (45:45:10, v/v/v). On each TLC plate, spots containing a known amount of detergent were deposited for calibration, since the spot intensities varied between plates, due to variations in staining. After development the plates were air-dried and stained by spraying with 2N H2SO4 followed by heating for 15 min at 100 °C. As shown in Fig. 1a, D M, CHAPS and the phospholipids used in the crystallization mixture were well separated. The plates were scanned on a flatbed scanner (Linotype, Saphir Ultra) and the spot intensities were measured using the Scion-image software package (Scion corporation, Frederick, MD, USA) [28]. The intensity of the detergent spots was found to vary linearly with the quantity deposited (Fig. 1b). The sensitivity of this assay is about 0.5–1 µg per spot of either detergent or phospholipid.

2.4. 2D crystallization

Phospholipid mixtures were prepared from chloroform stock solutions, dried under argon, and hydrated to a final concentration of 2 mg/ml in a buffer containing 10 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mM mannitol, 0.5 mM NaN₃. The phospholipids were completely solu-

Fig. 2. Characterization of IICmtl two-dimensional crystals by negative stain electron microscopy and cryo-electron diffraction. (a) Cigar-shaped crystal obtained at 100 mM NaCl and an LPR of 1 (w/w) in pure DOPE. (b) Crystals obtained at 100 mM NaCl with DOPE/DOPC (9:1) and an LPR of 1. Elongated crystals emerge from aggregates, with a high tendency to stack. (c) Large thin crystals of IICmtl obtained at 300 mM NaCl, DOPE/DOPC (9:1) and an LPR of 2. In each of the crystals, the striations can clearly be seen (inset). The difference in LPR with (b) did not cause the difference in appearance. (d) Electron diffraction of a frozen hydrated crystal as shown in panel (c) showed resolution spots extending to 8 Å in all directions (4.2 Å at – 7.15). Bar 200 nm in (a), and 1 μm in (b) and (c).
bilized by addition of CHAPS to a final concentration of 20 mg/ml.

In contrast to our former study [10], IIC\textsuperscript{mtl} crystals were grown in synthetic phospholipids in order to have a better control of the lipid composition and to minimize variations between batches. IIC\textsuperscript{mtl} was mixed with phospholipids at the desired lipid-to-protein ratio (LPR) (w/w). The NaCl concentration of the final crystallization mixture was adjusted to the desired value; 300 mg/ml wet bio-beads were used to remove the detergent [19]. Detergent removal was performed under continuous stirring at room temperature or at 4 °C. The evolution of the turbidity and the decrease of the detergent concentration were followed by measuring the optical density at 540 nm (Ultrospec 3000, Pharmacia Biotech) and performing TLC on aliquots. The morphology of the formed structures was studied by cryo-EM.

2.5. Cryo-EM

A few microliters of suspension were deposited on glow discharged holey carbon films. After blotting away the excess of liquid, the grids were plunged quickly in liquid ethane [3]. Frozen-hydrated specimens were mounted in a cryo-holder (Gatan, model 626) and observed in a Philips CM 10 or CM 120 electron microscope, operating at 100 or 120 KV, respectively. Micrographs were recorded under low-dose conditions on Agfa Scientia EM films or on a slow-scan CCD camera (Gatan, model 794).

3. Results

3.1. Formation of crystals of IIC\textsuperscript{mtl}

Using the procedure reported by Koning et al. [10], as a starting point, crucial parameters in the crystallization of IIC\textsuperscript{mtl} were determined in order to find optimal conditions (large two-dimensional crystals, no aggregates) for which the crystallization process could be studied in more detail. The formation of IIC\textsuperscript{mtl} crystals, and also the type of crystals that were formed, were highly dependent on the type of lipids and detergents that were used for reconstitution of

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Fig. 3. (a) The turbidity of crystallization mixtures upon detergent decrease. The crystallization mixture consists of IIC\textsuperscript{mtl} with DOPE/DOPC (9:1) at an LPR of 2 at 300 mM NaCl. The turbidity showed two transitions. Liposome formation takes place between 3 and 4 mg/ml detergent and liposome aggregation starts around 1 mg/ml detergent. At 4 °C liposome formation occurs but liposome aggregation and fusion at low detergent concentration is absent. At 100 mM NaCl there is a steep increase in turbidity (secondary Y-axis) during the reconstitution. (b) Cryo-electron microscopic image taken at 3.5–4 mg/ml total detergent of a crystallization mixture at 300 mM NaCl. Many open unilamellar vesicles are present with putative reconstitution of IIC\textsuperscript{mtl} in progress. (c) The unilamellar vesicles formed at high salt have a strong tendency to aggregate (∓ 2 mg/ml detergent). (d) Cryo-electron microscopic image taken around 3 mg/ml total detergent of a crystallization suspension at low salt. Many multilamellar vesicles are present with a high tendency to aggregate. The bar represents 100 nm.
IIC\textsuperscript{mnl}. The presence of a mixture of DM and CHAPS was required for the formation of large IIC\textsuperscript{mnl} crystals. When CHAPS was omitted and either pure decyl- or dodecylmal- toside were used, no crystals were formed and only aggregates were observed. In CHAPS alone, the protein was not stable and started to aggregate before crystallization could be achieved (data not shown).

Also the lipid DOPE was essential for crystal formation. When pure DOPE solubilized in CHAPS was used for crystallization of IIC\textsuperscript{mnl} purified in DM, stacked 2D crystals of IIC\textsuperscript{mnl} were obtained at an LPR around 1. These crystals were thick, cigar-shaped and often originated from large aggregates (Fig. 2a). When 10–25% (w/w) DOPC was added to the DOPE, crystals appeared thinner and larger, but still multilayered (Fig. 2b). When 30–50% (w/w) DOPC was added to DOPE, no crystals were formed but only large vesicles were observed (data not shown).

The NaCl concentration was varied from 50 mM up to 2 M. A large difference was found between crystallizations carried out at 100 and 300 mM NaCl, but not between 300 mM and 2 M. At 100 mM NaCl the crystals were relatively thick and a lot of aggregates were observed (Fig. 2b), whereas at 300 mM NaCl the crystals were larger (several \mu m\textsuperscript{2}) and thinner while no aggregates were observed (Fig. 2c). At 300 mM NaCl crystals could be obtained over an extended LPR range from 1.5 to 5. As characterized by electron diffraction of frozen hydrated specimens, many of these crystals diffracted up to 4 Å (Fig. 2d).

Furthermore, changing the pH between 4 and 10 had no marked influence on the crystal formation, nor had the addition of bivalent cations (CaCl \textsubscript{2} 10 and 100 mM). The addition of glycerol (1–10% w/v) was found to be destructive for the formation of crystals. The optimal conditions that were selected for the standard formation of large multilayered crystals of IIC\textsuperscript{mnl} were: IIC\textsuperscript{mnl} 0.5 mg/ml, DOPE/DOPC (9:1; w/w) 1 mg/ml, 300 mM NaCl, 1.5 mg/ml DM, 10 mg/ml CHAPS, 1 mM mannitol, 1 mM EDTA, pH 7.5 in a 10 mM HEPES buffer at room

Fig. 4. (a) Cryo-electron microscopy during the crystallization stage of IIC\textsuperscript{mnl} taken 2 h after bringing the sample from 4 °C to room temperature (300 mM NaCl). Complex structures are formed as a result of proteoliposome fusion. Note the many interlamellar attachments (arrows). (b) High magnification of panel (a) clearly showing the interlamellar attachments (arrows). (c) After 24 h at room temperature, large crystalline areas can be found adjacent to complex lipid areas as seen by negative stain. P = protein-rich area, L = lipid-rich area. (d) Cryo-electron microscopy of the complex structures that were formed in time. Lamellar structures (l) were found adjacent to cubic-like structures (c). Bar represents 500 nm.
temperature. During the systematic analysis of the crystallization conditions of IIC<sub>mtl</sub> we were not able to find conditions in which true 2D crystals were formed. Analysis of the crystals by cryo-electron crystallography resulted in uninterpretable projection maps for different crystals, which is probably due to the fact that the crystals are composed of a variable number of layers, shifted with respect to each other along the main lattice directions.

3.2. The crystallization process

To find the cause of the formation of multilayered IIC<sub>mtl</sub> crystals, which are unsuitable for 3D image analysis, the crystallization process was investigated by combining turbidity measurements and cryo-EM for different salt concentrations and temperatures. The turbidity was measured as a function of detergent concentration during gradual removal of the detergent. Samples for cryo-EM were taken at discrete times during the crystallization. At the standard crystallization conditions (300 mM NaCl and room temperature), the evolution of the turbidity upon detergent removal showed two transitions (Fig. 3a, closed diamonds). The first transition was observed by a small increase of the turbidity at a total detergent concentration of 3–4 mg/ml. Cryo-EM showed that at this point (proto)liposomes were formed via open vesicle intermediates (Fig. 3b). Between 3 and 1 mg/ml total detergent, the turbidity is stable and unilamellar proteoliposomes were present (Fig. 3c). The second transition marked the aggregation and fusion of unilamellar proteoliposomes resulting in a large increase in turbidity at a detergent concentration of 1 mg/ml and below. In these complex structures, many interlamellar attachments can be seen (Fig. 4a,b). The result of these fusion events is a phase separation between protein-rich (multiple layers of 2D-crystals) and lipid-rich domains (Fig. 4c). The lipid-rich domains consist of clusters of membrane connections looking like lipid cubic phase structures (Fig. 4d) [5,25] and were found adjacent to areas that show a first indication of protein crystallization. It seems that the protein is concentrated in layers by the transition of the phospholipids from a lamellar phase to cubic-like structures. Finally, after several hours large crystalline sheets (Fig. 2e) are obtained, almost without any protein aggregates. When the detergent removal at high salt was carried out at 0 °C the first stage of the crystallization develops similar to that at room temperature (although slower in time), but the second stage does not take place. In this sample, only proteoliposomes were observed and no protein aggregates or 2D-crystals were present. The second stage of the crystallization could however be induced by bringing the sample to room temperature (even after a week of storage at 4 °C). After the temperature increase, 2D-crystals started to appear within several hours, and evolves similar as crystallizations carried out at room temperature.

At low salt (100 mM NaCl) the turbidity increased abruptly around 4 mg/ml total detergent (Fig. 3a). In these conditions, aggregates of multilamellar liposomes were observed by cryo-EM (Fig. 3d). Open vesicles were not observed as intermediates. The multilamellar vesicles finally evolved to small thick crystalline areas with large aggregates of protein and lipid (Fig. 2b).

These results clearly show that the crystallization of IIC<sub>mtl</sub> at 300 mM NaCl (and above) comprises two distinct steps, reconstitution into proteoliposomes, followed by a temperature-dependent fusion and crystallization. At low salt the two stages could not be distinguished.

4. Discussion

In this study we attempted to gain insight into the mechanism of formation of (multilamellar) 2D crystals of IIC<sub>mtl</sub> by investigating the protein reconstitution and crystallization processes and the behavior of protein-containing liposomes. Reconstitution of membrane proteins into liposomal structures and their subsequent crystallization are two processes that are often difficult to observe separately. For the crystallization of IIC<sub>mtl</sub> at 300 mM NaCl however, we were able to distinguish between reconstitution and crystallization. At room temperature the two stages occur at clearly distinct detergent concentrations, 3–4 mg/ml and below 1 mg/ml, respectively. By carrying out the reconstitution at 4 °C the crystallization could be postponed until the sample was brought back to room temperature. The observed effects of the salt concentration, the type of lipids and the temperature with respect to the successive stages are discussed separately.

4.1. Reconstitution

The strong influence of the NaCl concentration on liposome formation and the appearance of the resulting crystals can be explained by the difference in the mixed-micelle to proteoliposome transition during the reconstitution phase. At low salt, aggregated multilamellar structures were observed by cryo-EM during the reconstitution. The large amount of aggregated protein indicates a poor reconstitution. At high salt, reconstitution occurred via unilamellar open-vesicle intermediates. No multilamellar liposomes were observed and the absence of aggregated protein indicates an efficient reconstitution.

Although the CMC of detergents is known to decrease with increasing ionic strength, as studied for CHAPS [18], there are no reports of the change in liposome reformation characteristics as we show here (unilamellar vesicles at 300 mM NaCl versus multilamellar vesicles at 100 mM NaCl). From experiments with DOPE/DOPC mixtures (without proteins) solubilized with CHAPS and vesicles reformation after detergent removal similar unilamellar and multilamellar vesicles were observed depending on the NaCl concentration (Stuart, in preparation). With CHAPS–phospholipid mixtures, threadlike micelles have been observed at low salt
concentrations [16], while no such thread-like micelles were found at high salt. Long thread-like micelles have also been observed in phospholipid–dodecylmaltoside mixtures [12]. Whether or not threadlike micelles can be formed, depends not only on the ionic strength, but is determined by the packing parameter [8] of the mixture (Stuart, in preparation) which depends on the nature of the phospholipids, the detergent, the presence of protein and also on the ionic strength. The presence or absence of thread-like micelles can explain the different structures that we found after detergent removal. Upon detergent depletion at low salt concentrations, it is likely that these threadlike micelles bend and coalesce, resulting in the formation of multilamellar bilayer structures as was found for dodecylmalto- side [12]. At low salt, when the threadlike micelles coalesce, a large fraction of the protein is possibly trapped between these micelles, and thus is not properly inserted into the bilayer. This would lead to the aggregation of protein and prevent optimal formation of proteoliposomes. This agrees with our observations that resulting crystals are surrounded by (protein) aggregates (Fig. 2b) and the turbidity increase at the time liposomes are formed (between 3 and 4 mg/ml detergent in Fig. 3a). The coalescence of threadlike micelles also explains the formation of multilamellar vesicles. The thick stacks of 2D crystals that were observed at low salt concentration are likely to be related to these multilamellar structures, observed during the reconstitution.

In the crystallization of EIIC mt we have used a mixture of two detergents, CHAPS and DM. The presence of CHAPS is required for an efficient reconstitution via open-vesicle intermediates at high salt, whereas DM is required to stabilize the protein until it is embedded and protected in the lipid bilayer. From the observations by Meyuhas et al. [16] on CHAPS–phospholipid mixtures at different salt concentrations, it is likely that CHAPS (and not DM) is responsible for the two different observed reconstitution mechanisms. As discussed by Mosser, the use of a mixture of two different detergents often proved the key to a successful 2D crystallization of a membrane protein [17].

4.2. Crystallization

After DOPC/DOPE-IIC mt proteoliposomes are formed, being either monolayered or multilayered, they aggregate and fuse into complex and inverted lipid structures. Liposome fusion is facilitated by the presence of DOPE in our crystallization mixtures. The time- and temperature-dependent fusion of liposomes has been reported earlier for DOPE/DOPC-mixtures [1,4]. These authors proposed the existence of an isotropic phase (H I) in mixtures of DOPE and DOPC of which the transition temperature (T I) starts roughly 20 °C below the transition temperature (T H) of the lamellar (Lα) to inverted hexagonal (HII) transition, which in our crystallization mixture is most likely above room temperature. By carrying out the reconstitution at 4 °C, protein crystallization could be postponed by preventing liposome fusion and the formation of isotropic lipid phases. After the reconstitution, the sample was brought to room temperature where both protein crystals and isotropic phases were observed by cryo-EM (Fig. 4) within a few hours. This indicates that the crystallization of IIC mt is related to the formation of isotropic phases.

The isotropic phase transition temperature (T I) is increased by the ratio between DOPC and DOPE. When the amount of DOPC was increased to 30–50%, no fusion and formation of cubic-like structures were observed and the crystallization process stopped after proteoliposome formation (data not shown). These observations explain the absence of crystal formation using DOPE/DOPC-liposomes (9:1; w/w) at 4°C and using DOPE liposomes with over 25% DOPC at room temperature.

The isotropic/cubic structures that were observed in the crystallization mixtures (Fig. 4c) are similar to those observed in various lipid systems bearing the same isotropic behavior [5,25]. Our explanation for crystal formation is that the protein does not fit into the high curvature of the isotropic/cubic phase and is pushed out. This will lead to
a separation into a lipid-rich phase (cubic, HII) and a protein-rich phase (multilamellar). Self-organization of the protein in this protein-rich phase will finally lead to crystallization in multilayers. The coexistence of the isotropic lipid phases and protein-rich lamellar phases, as seen in Fig. 4b–d is a strong indication for such a model. A schematic representation based on the HII precursors as presented by Siegel [24] is proposed in Fig. 5. This mechanism of crystal formation explains the formation of multiple crystalline layers, either randomly stacked or in true 3D-crystals. Therefore, it is intrinsically associated with the formation of multilayered crystals. Since DOPE is responsible for the formation of the isotropic phases, the formation of true 2D crystals from IIC\textsuperscript{plt} seems not possible when major amounts of DOPE are present.

The proposed mechanism could also explain the wide range of LPR (1–5; w/w) at which IIC\textsuperscript{plt} crystals were found, which is unlike other examples of 2D crystallization (for a review see Ref. [6]). No crystals were found in our system at an LPR below 0.5, both at low and high salt. In most cases, a protein crystallizes only at a narrowly defined LPR around or below 1 (w/w), and even changes of crystal types and packing are reported upon a small change of the LPR [22]. Here the higher LPR is accommodated by the formation of the isotropic/cubic phase, and the final LPR in the crystal is unknown, but probably well-defined by the crystal packing. The salt concentration is believed not to play an important role during the crystallization stage since it does not effect the HII transition temperature [1].

The mechanism described here presents an obvious analogy with the formation of 3D crystals of membrane proteins observed with lipids forming cubic phases [13,21].

5. Conclusions

The 2D-crystallization of IIC\textsuperscript{plt} falls apart in a reconstitution-phase and a crystallization-phase. The reconstitution is markedly dependent on the NaCl concentration. At 100 mM NaCl a poor reconstitution into multilamellar vesicles was found, whereas at 300 mM NaCl (and above) unilamellar proteoliposomes were formed. The crystallization is driven by the formation of lipid cubic phases and possibly inverted-hexagonal phases that are temperature and lipid-composition depended. This mechanism is inextricably associated with the formation of multilayers and therefore not suitable to produce thin 2D-crystals of IIC\textsuperscript{plt} for electron crystallography.

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