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Effect of organic anions on the crystallization of the Ca^{2+}-ATPase of muscle sarcoplasmic reticulum

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The effect of varying the solute species on the crystallization of the Ca^{2+}-ATPase from rabbit muscle reticulum (SR) is reported. We have found that substitution of KCl with salts of organic acids in the crystallization protocol reported by Pikula et al. [1] has a profound effect on the size of two-dimensional crystalline arrays. Crystalline arrays of up to 3 μm diameter have been obtained by incubating purified calcium ATPase in standard crystallization medium but with 0.8 M sodium propionate substituted for KCl. These two-dimensionl (2-D) arrays display a reduced tendency to stack in addition to having larger planar dimensions. Increasing the KCl concentration does not have the same effect on stacking or crystal growth that sodium propionate has. The production of 2-D sheets has some dependence on the hydrocarbon chain length of the salt because crystals formed in propionate were larger and less stacked than those formed in acetate or formate. There seems to be no dependence on cation. These observations suggest that in addition to reducing the forces that lead to stacking of the sheets, propionate may facilitate incorporation of the detergent-solubilized protein into the 2-D sheet.

Introduction

The crystallization of membrane proteins into two-dimensional (2-D) arrays suitable for electron crystallography has enjoyed increased emphasis because of the potential to reconstruct the structure in three dimensions (3-D). Over the years, significant advances in specimen preservation methods [2–4], image processing techniques [5] and instrumentation [6–9] have developed electron crystallography to the stage where these membrane proteins can be viewed at near atomic resolution. The recent 3-D model of purple membrane obtained by electron crystallography [10] attests to the current development of the technique. However, a critical requirement for protein electron crystallography is the production of large, single-layered, two-dimensional crystalline arrays. Although the useful size of a crystalline sheet is dependent upon the system, the 2-D sheets must typically be several microns in diameter. Often, the 2-D crystalline arrays tend to stack in the third dimension to form small 3-D crystals. Such crystals have been dubbed Type I [11]. In such cases, the stacking in the third dimension can occur with varying degrees of registration between the individual layers. In the case where stacking is completely in register it is theoretically possible to determine the structure, provided the overall thickness is not so extensive that multiple scattering effects become significant. However, many practical difficulties in the processing of image data from 3-D crystals remain. In the case where stacking is without rotational registration, it is possible to computationally separate the individual layers. Finally, in the case where stacking is in rotational registration, but without translational registration, the image of a single layer can be obtained by exploiting a method to detect and compensate for the displacement between the separate layers [12]. Nevertheless, the ideal situation for protein electron crystallography is to have single-layered crystalline sheets.

Two-dimensional crystalline arrays of the SR Ca^{2+}-ATPase from detergent solubilized enzyme were first reported by Dux et al. [13]. These crystals consisted of extensive stacks of 2-D crystals which were characterized by a somewhat irregular stacking in the third

Abbreviations: $C_{12}E_8$, octaethylene glycol dodecyl ether; DTT, dithiothreitol; SR, sarcoplasmic reticulum.

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dimension. Within the 2-D sheets, the molecules extended out of both sides of a lipid-detergent phase to form a centered lattice in the 2-sided plane group c12 [14,15]. The small size and extensive stacking of these 2-D sheets presented a serious obstacle for electron crystallographic analysis.

Most studies on the crystallization of the Ca^{2+}-ATPase have focused on the effects of phospholipid and detergent on the formation of 2-D arrays. The conditions for crystallization of the Ca^{2+}-ATPase were extensively investigated by Pikula et al. [1] who found that crystals were formed only in the presence of nonionic detergents in a narrowly defined pH range and with high calcium concentration. A key aspect of their work was the elucidation of a set of conditions that preserve activity of the solubilized enzyme for the extended periods of time required for crystallization. Stokes and Green [15] observed that in-plane crystal growth was enhanced at particular detergent : lipid : protein ratios. The crystals formed were nevertheless multilayered.

As part of our studies on Ca^{2+}-ATPase structure utilizing the crystals prepared according to Pikula [1], we investigated treatments that would disrupt the stacking of preformed Ca^{2+}-ATPase crystals. These treatments indicated that high concentrations of organic anions disrupted the stacking and suggested that the crystallization conditions could be modified to reduce stacking. Subsequently, we found that the size and stacking of 2-D crystalline arrays was dramatically modified by using salts of organic acids such as acetate and propionate. Under certain conditions, we also succeeded in getting single layer sheets of these 2-D crystals. This report summarizes our observations on the effects of organic salts on the growth and stacking of crystalline arrays of Ca^{2+}-ATPase.

Materials and Methods

Isolation and purification of Ca^{2+}-ATPase

SR membranes were the kind gift of Dr. Anthony Martonosi. These membranes were isolated from predominantly white muscle of rabbits following Nakamura et al. [16]. The preparations were suspended at a protein concentration of 42 mg/ml in 0.3 M sucrose, 10 mM Tris-maleate buffer (pH 7.0), rapidly frozen in liquid nitrogen and stored at $-70°C$ until needed. Purified Ca^{2+}-ATPase was obtained following Meissner et al. [17].

Crystallization conditions

For crystallizaiton experiments, the frozen SR microsomes were thawed, diluted 15-fold in wash buffer consisting of 0.1 M KCl, 10 mM imidazole (pH 7.4) and centrifuged for 90 min in a Beckman SW 28 rotor at 25000 rpm. The pellet was then taken up either in the appropriate crystallization buffer or in the wash buffer at the initial concentration. Protein concentration was estimated according to Lowry et al. [18].

Our crystallization protocol followed closely that reported by Pikula et al. [1] with the exception of the substitution of KCl in the medium. Standard crystallization medium of Pikula et al. consisted of 0.1 M KCl, 10 mM Mops (pH 6.0), 3 mM $MgCl_2$, 20 mM $CaCl_2$, 20% glycerol, 3 mM NaN_3, 5 mM dithiothreitol, 25 IU/ml trasylol and 2 μg/ml 1,6-di-*tert*-butyl-*p*-cresol. Crystals were more readily obtained with the detergent Brij 36T than with $C_{12}E_8$ (both from Sigma). Best crystals were obtained with Brij 36T at a protein to detergent ratio of 1 : 4 and so for all the crystals reported here, Brij 36T was used as the detergent. Although pH was varied in some experiments, crystals were obtained most readily at pH 6.0. In these experiments we substituted KCl by varying strengths of organic anions with increasing hydrocarbon chain lengths (such as ammonium formate, ammonium acetate and sodium propionate). The concentration of these organic salts was varied between 0.1 M to 1.0 M. Usually for crystallization, an appropriate amount of protein was diluted in corresponding buffer to which was added the required amount of Brij 36T. The suspension was vortexed and stored under nitrogen at 2°C.

Electron microscopy

Specimens for electron microscopy were prepared by depositing 3 μl of crystalline suspension on carbon coated electron microscope grids, which were previously glow-discharged in air to render them hydrophilic. After about 30 s, the grid was washed with 8–10 drops of aqueous uranyl acetate (2%), side blotted and air dried. The grids were prepared in the cold room.

Electron micrographs were obtained with the Philips EM301 or EM420 electron microscopes. The samples which contained single layered crystals were imaged in the low-dose mode using the Philips EM420 and micrographs developed in full strength D-19 for 12 min. Otherwise, the micrographs were developed for 4 min in diluted D-19 (1 : 2) developer.

Thin sections of crystals were obtained by embedding and pelleting the crystalline suspension using a modification of the method of Wray and Sealock [19]. Briefly, 20 μl ($\approx$ 10 μg) of crystalline suspension in 100 μl of buffer (crystallization) was spun onto the bottom of pretreated polyvinyl chloride (PVC) microtitration wells, at 20000 $\times$ *g* for 20 min in the cold. The supernatant was replaced with fixative consisting of 2.5% glutaraldehyde, 2% paraformaldehyde, 1% tannic acid (Mallinkrodt #1764) in buffer (pH 5.6) and left overnight at 2°C. The samples were rinsed in buffer, postfixed in buffer (0.5% osmium tetroxide, rinsed in water, stained *en bloc* in 2% uranyl acetate in 50% ethanol and dehydrated, before embedding in Epon. The wells were

removed and the blocks re-embedded in fresh Epon to replace the PVC supporting the thin sample layer. Silver-gray sections were cut on a Reichert Ultracut and contrasted in Sato's lead stain [20], prior to electron microscopy using Philips EM301 at 80 kV. Micrographs were recorded at ×24700.

Image processing

Electron micrographs of negatively stained crystals were surveyed initially by optical diffraction to assess the image quality and resolution. The seven best crystalline areas were digitized using a Perkin-Elmer PDS 1010M microdensitometer at a spacing of 7 Å with respect to the original object. The digitized images were processed as described previously [21] except that correction for lattice distortion [5] was carried out prior to calculating structure factors. Structure factor data from the different images were aligned and averaged in the two sided plane group p1. For the final average, the symmetry of the actual two-sided plane group of the crystals, i.e., c12, was imposed after a suitable origin search.

Results

Effect of high ionic strength on the stacking of 2-D crystals of Ca^{2+}-ATPase

Crystals of Ca^{2+}-ATPase grown in standard medium from detergent solubilized SR consisted invariably of extensive stacks of small crystalline arrays (Fig. 1a). To facilitate their structural analysis, these stacks need to be disaggregated into single layers. Disruption of stacking of Ca^{2+}-ATPase crystals was investigated by sedimenting preformed crystals grown in standard medium and resuspending the pellet in buffers of varying solute composition buffered to pH 6.0 with 10 mM Mops. The most useful effects were observed when pellets were resuspended in high ionic strength buffers composed of 0.8 M KCl or 0.8 M ammonium acetate. Electron microscopy of these specimens preserved in negative stain revealed large quantities of small crystalline patches whose lateral size approximated those of the 2-D sheets that make up the stacks (Fig. 1b and c). Although both KCl and ammonium acetate at a concentration of 0.8 M disrupted stacked crystals, ammonium acetate was more efficient at producing single layer crystals (Fig. 1c). This observation suggested that replacing KCl with organic salts in the crystallization medium might produce crystals with less stacking.

Effect of hydrocarbon chain length of organic salt on the crystallization of detergent solubilized SR

Detergent solubilized SR was crystallized under otherwise standard conditions at protein concentrations of 2 mg/ml at two different salt concentrations (0.4 and 0.8 M) of three different organic salts having increasing hydrocarbon chain length (i.e., formate, acetate and propionate). Crystalline sheets were formed under all crystallization conditions tried. However, the crystal quality and stacking depended not only on the concentration of organic salt, but also on its type. Crystallization in 0.4 and 0.8 M ammonium formate produced crystals which were small in size and showed poor extent of crystallization (Fig. 2a, b). In 0.4 M ammonium acetate or sodium propionate, extensive stacking of crystalline sheets was seen (Fig. 2c and e), reminiscent of crystallization under standard conditions. More dramatic effects on the crystallization pattern

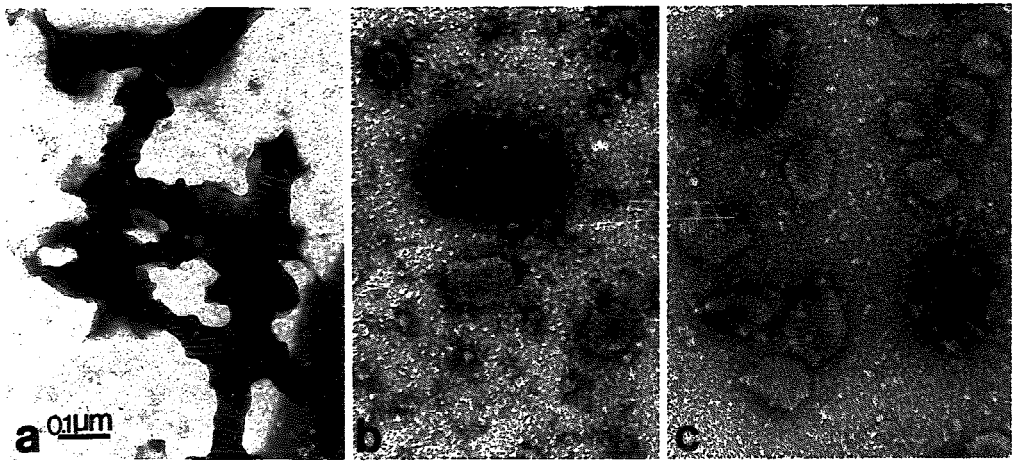


Fig. 1. Disaggregation of preformed stacks with high ionic strength. (a) Crystals grown under standard conditions [1]. (b) After resuspension in 0.8 M KCl. (c) After resuspension in 0.8 M ammonium acetate. Some residual aggregation is evident after KCl treatment; less residual aggregation is evident after ammonium acetate treatment.

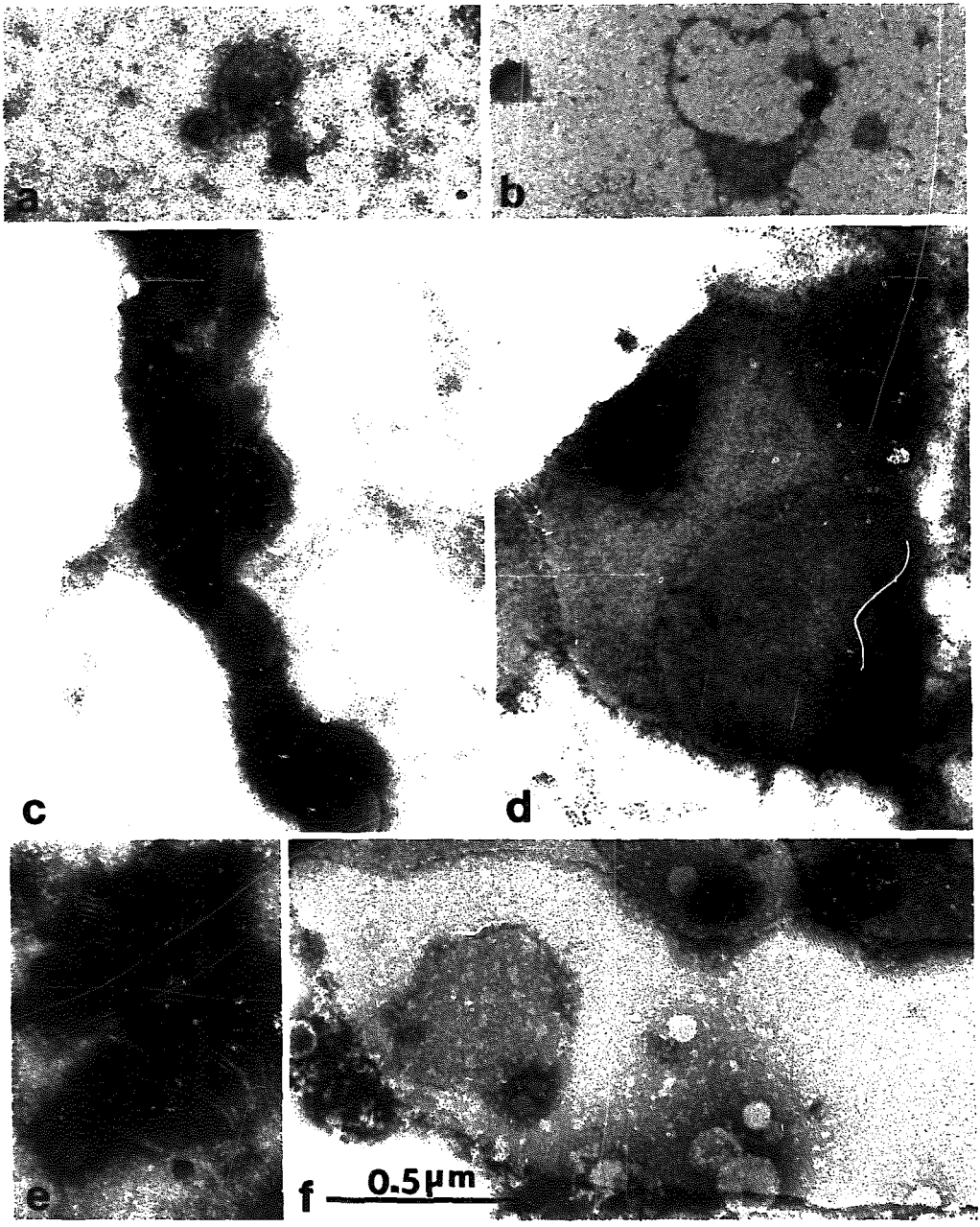


Fig. 2. Effect of hydrocarbon chain length and anion concentration on crystallization of detergent solubilized SR. Crystals shown in (a), (c) and (e) were grown in 0.4 M and the ones shown in (b), (d) and (f) were grown in the presence of 0.8 M of ammonium formate, ammonium acetate and sodium propionate, respectively. Protein concentration was 2 mg/ml and protein to detergent ratio was 1:4.

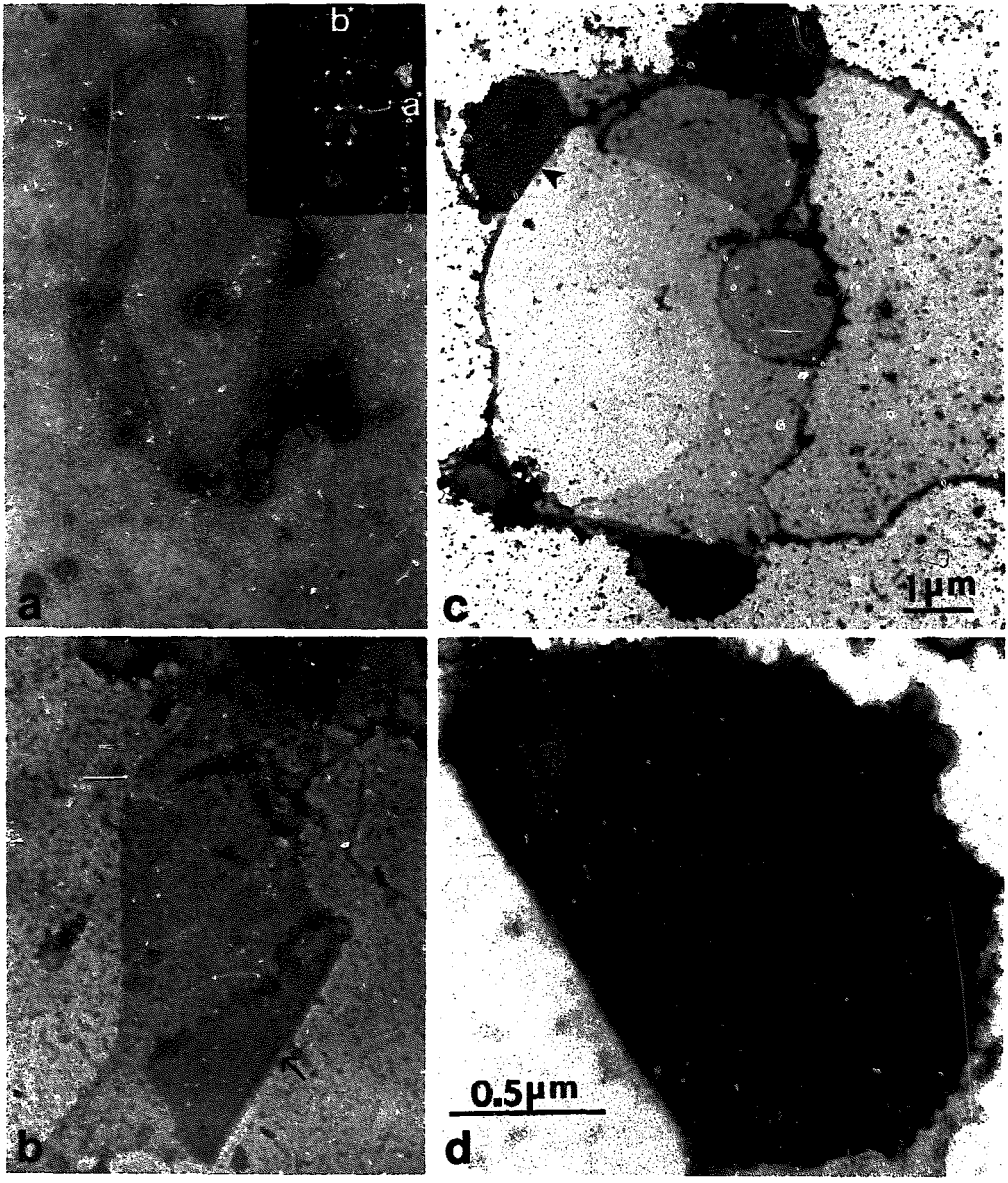
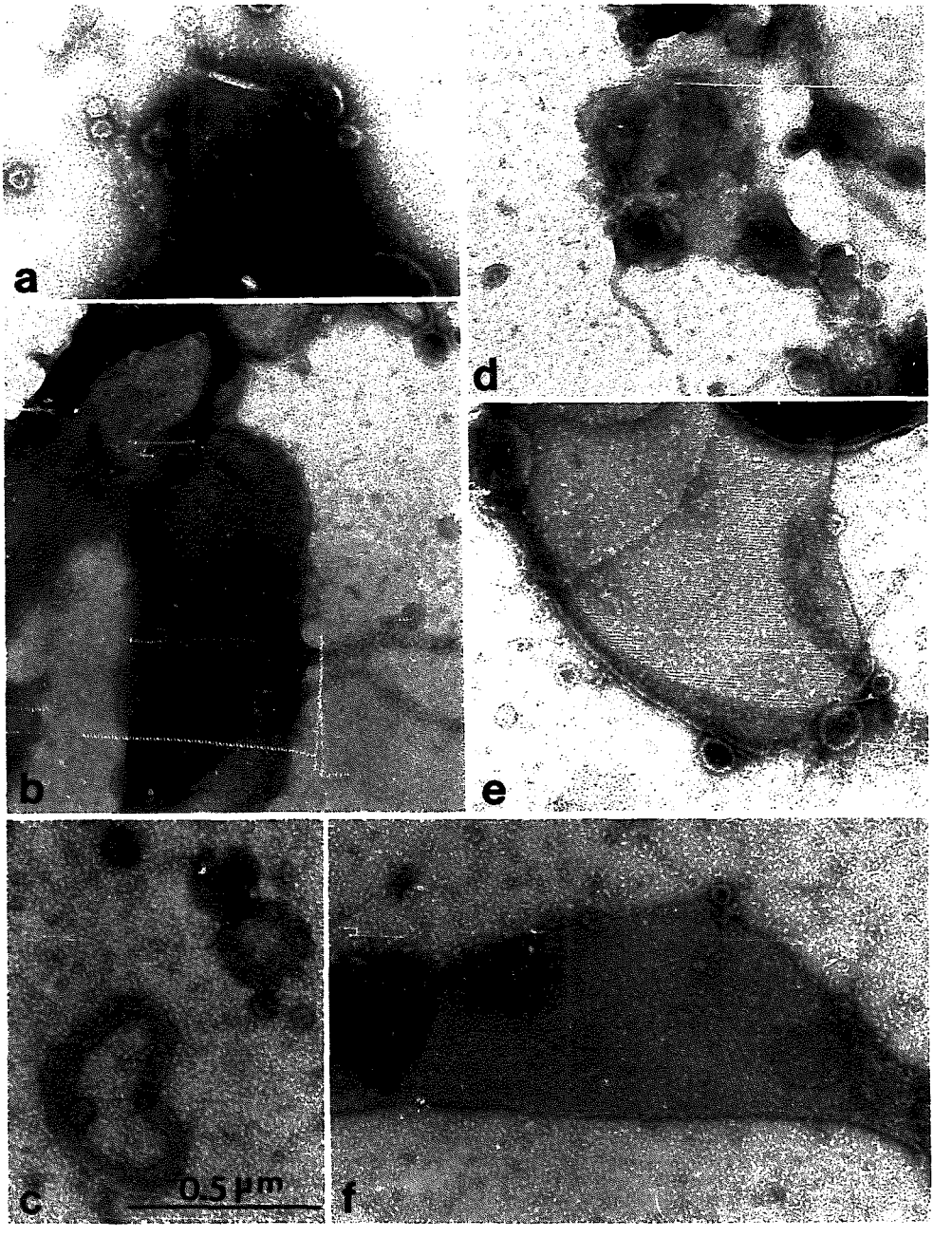


Fig. 3. Crystallization of detergent solubilized SR in the presence of 0.8 M sodium propionate at protein concentrations of 0.12 to 0.5 mg/ml. (a) and (b) show clear evidence of the formation of single layers of crystalline sheets. One side of the sheets is smooth and it belongs to an extended necklace-like structure, the side away from the necklace is rough. Occasionally, folded over regions of the sheets are also seen (arrows). A low power image of a necklace (c), after four months of crystallization; one of the crystals (arrowhead) is shown enlarged in (d). Even the stacked crystalline layers retain the necklace-like structure after four months of crystallization. At least two layers are present in this crystal (d). (a). (b) and (d) are at the same magnification. Computed diffraction pattern shown in (a) reveals systematic absences characteristic of the 2-sided plane group c12.


a
b
c
0.5 µm
d
e
f

occurred upon raising the concentration of acetate or propionate to 0.8 M. Electron microscopy of these preparations revealed crystals devoid of the extensive stacking observed at 0.4 M (Fig. 2d and f), and which were considerably larger in lateral extent. Crystals formed in 0.8 M ammonium acetate (Fig. 2d) extended to about 1 μm in diameter. However, aggregated patches of smaller crystals frequently appeared. Crystallization in 0.8 M sodium propionate resulted in a greater number of large crystalline sheets, many of which were over a micron in size (Fig. 2f). Stacking of individual sheets to form multilayered 3-D crystals was also less prominent in sodium propionate. Of all the crystallizations undertaken in various salts, at different concentrations, 0.8 M sodium propionate seemed to give rise to the largest crystalline sheets with minimum stacking of individual sheets.

Effect of protein concentration on detergent solubilized SR crystallization

Initial efforts to crystallize detergent solubilized SR in 0.8 M sodium propionate were undertaken at the more standard protein concentration of 2 mg/ml [1]. However, crystalline sheets with unusual morphology were obtained by lowering the protein concentration by 4–20-fold. Within a few days after the initiation of crystallization, even a protein concentration of 0.5 mg/ml seemed to favor the growth of single layers of crystalline sheets of calcium ATPase. A characteristic feature of these sheets in their early stages of formation was their 'necklace-like' appearance (Fig. 3a). The crystals always formed a sharp boundary on the inside of the nekclace that was parallel to the (1,1) crystal planes, whereas the other sides of the crystals were rounded or irregular. Sheets up to 1 μm in size were seen at these low protein concentrations. However, after a month or more of incubation stacked layers became prominent (Fig. 3c and d), yet still retained the necklace morphology observed initially.

Comparison of DOC-purified calcium-ATPase in KCl and sodium propionate

Deoxycholate purified calcium-ATPase was crystallized in KCl and sodium propionate over a range of ionic concentrations (0.1, 0.5 and 1.0 M). Crystals formed under these conditions using purified Ca^{2+}-ATPase seem to differ somewhat from those crystallized using native SR membranes. In general, for a given set of solution conditions and a given protein concentration, the crystals obtained using purified ATPase were larger and had less stacking. Whereas using native SR extensive pancake-like structures predominate, such structures are less frequently seen using purified enzyme. Instead, at up to 0.5 M concentration of either KCl or sodium propionate few multilayered crystals were observed in the first week of crystallization. Individual sheets were seen to aggregate with time irrespective of the ionic conditions (Fig. 4a, b, d and e). However, at 1 M KCl concentration, no crystals of purified calcium-ATPase were seen (Fig. 4c). Whereas, at corresponding concentration of sodium propionate two-dimensional sheets with little stacking appeared (Fig. 4f).

As expected, the purity of the enzyme seemed to govern crystal size. Larger crystalline sheets, up to 3 μm in size, could be obtained by using purified calcium-ATPase at low protein concentrations (0.1 to 0.5 mg/ml) in 0.8 M sodium propionate. These large sheets are probably not single layers. Multiple layers often appear as step-like gradations of density in a micrograph of a multi-layered crystal. In general, based upon magnitude and the optical density step in the micrographs at the crystal border, the number of layers appeared to be less with purified calcium-ATPase than were obtained with detergent solubilized SR. Furthermore, judging from the lattice lines at the boundary of a layer and the prominence of moire fringes in the images of crystals from purified ATPase, the individual layers tend to stack with better registration than that found in detergent solubilized SR (Fig. 5).

Embedding and sectioning of ATPase crystals

To investigate the number of crystalline layers present, the crystals grown from detergent solubilized SR were pelleted, fixed and embedded for microtomy. Thin sections viewed in the electron microscope revealed extensive lamellae in which the crystals were embedded (Fig. 6). The lamellae were usually less than 40 Å in thickness and were far more prominent than the crystals. Whether stacking of the lamellae leads to stacking of the crystals or vice-versa is not known at this time.

Projection map of the single layered crystalline sheet

Images of single layered crystalline sheets of Ca^{2+}-ATPase were used to compute the projected filtered image. In previous work [14] it was not possible to compute this projection map because of the unregistered stacking of these crystals. To obtain an averaged

Fig. 4. Crystallization of purified calcium-ATPase in the presence of different strengths of potassium chloride and sodium propionate. (a–c) correspond to 0.1, 0.5 and 1.0 M KCl and (d–f) correspond to 0.1, 0.5 and 1.0 M sodium propionate, respectively. Protein concentration was 0.5 mg/ml and the ratio of protein to detergent (Brij 36T) was 1 : 4. Aggregation of small crystals and their stacking could be seen for KCl and sodium propionate both, for concentrations upto 0.5 M. At 1 M concentration of KCl, no crystals were observed (c), but in comparable sodium propionate, large sheets predominate (f).

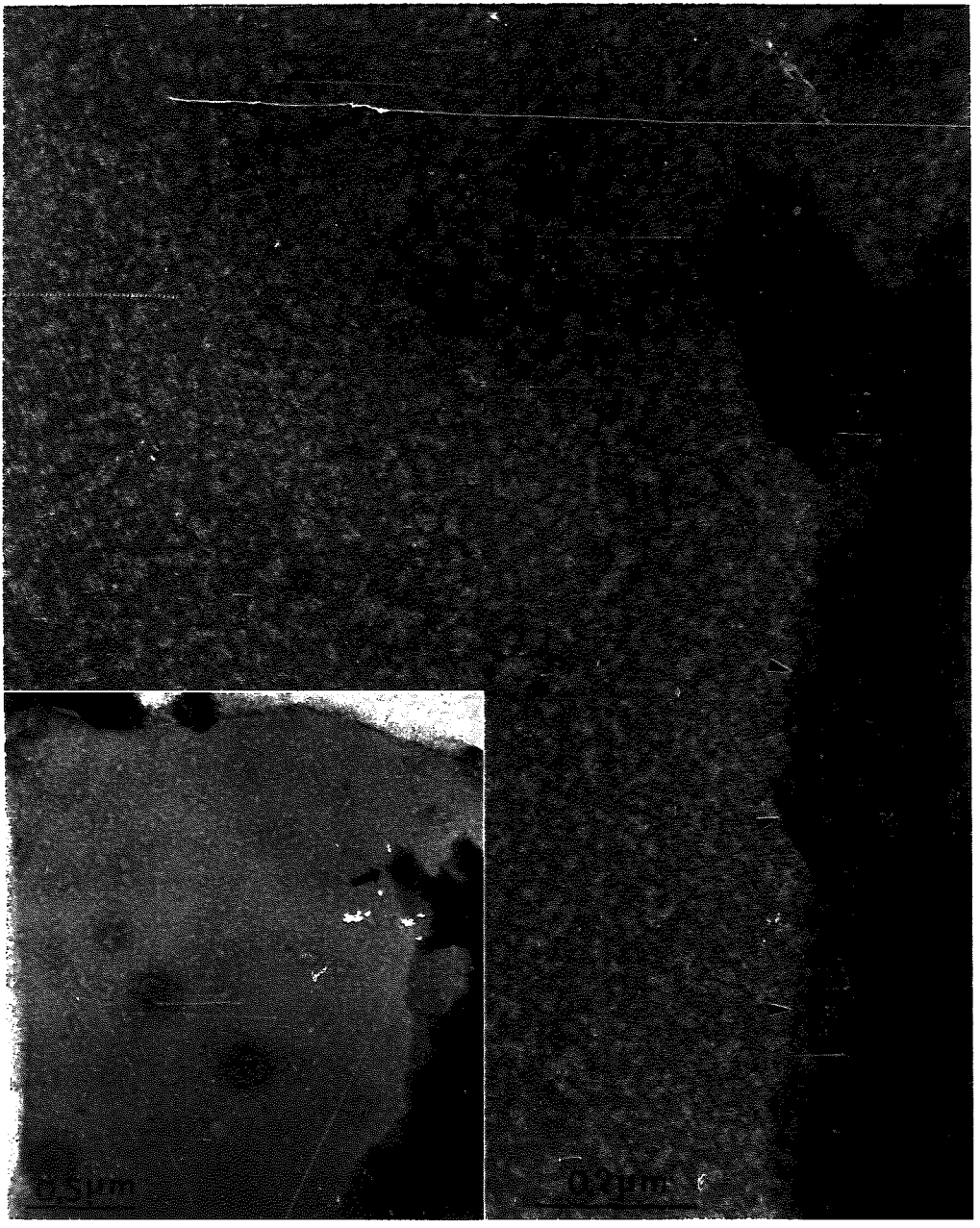


Fig. 5. Large crystalline sheets, up to 3 μm in size, were obtained from purified calcium-ATPase in 0.8 M sodium propionate. The inset shows the entire crystal, the indicated portion (arrow) of which has been enlarged to highlight the two layers (arrowheads) which seem to stack in register.

TABLE I

Structure factors derived from negatively stained single layer crystals

H	K	Amplitude	Phase degrees	Residual degrees
2	0	11558.4	180.0	10
4	0	1329.2	0.0	6.3
6	0	311.9	0.0	16
8	0	75.3	0.0	15.8
1	1	8478.2	138.8	8.1
3	1	1148.3	−90.0	18
5	1	306.0	172.8	47
0	2	767.8	144.8	16
2	2	196.3	−0.5	36
4	2	111.2	−149.0	36
6	2	67.4	−37.1	39
1	3	41.6	44.0	39
5	3	25.6	59.3	52

projection of the single layer in negative stain, a total of seven images, similar in appearance to the one shown in Fig. 3a, were processed. The average unweighted phase residual for the seven merged images against the unsymmetrized average was 25°. Rotating the images 180° about the *Z* axis approximately doubled the residual while flipping the image 180° about the *b* axis changed the residuals by less than one degree. These are exactly the expected changes for the two-sided plane group c12.

The projection map of the single layer produced from the symmetrized data of Table I reveals a zig-zag ribbon of stain exclusion parallel to the *b* axis of the unit cell (Fig. 7). The peaks within the ribbons represent the centers of mass of the individual ATPase molecules that extend alternately out of both sides of the lipid-detergent phase [14,15]. The 2-sided plane group c12 contains two pairs of ATPase molecules of which one pair is located in the center of the unit cell and the other pair located at the corner. The ATPase molecules within each pair are related by 2-fold axis at *0* and *a/2*. The ribbons themselves are centered on 2-fold screw axes at *a/4* and *3a/4*. The densities connecting the ribbons are more prominent in the projection of the single layer than they are in the projection of multilayered sheets [15]. Presumably, this difference represents the accumulation of stain between the layers of the 3-D crystal that is not present in the single layer crystals.

Discussion

Since the first reports of crystals of Ca^{2+}-ATPase from detergent-solubilized SR appeared [13], there have been several studies investigating the conditions under which crystals form. Pikula et al. [1] studied in detail the conditions required for long term stabilization of detergent solubilized Ca^{2+}-ATPase that are necessary for growing crystals. 49 detergents were tested for crystallization potential with the finding that optimal crystalli-

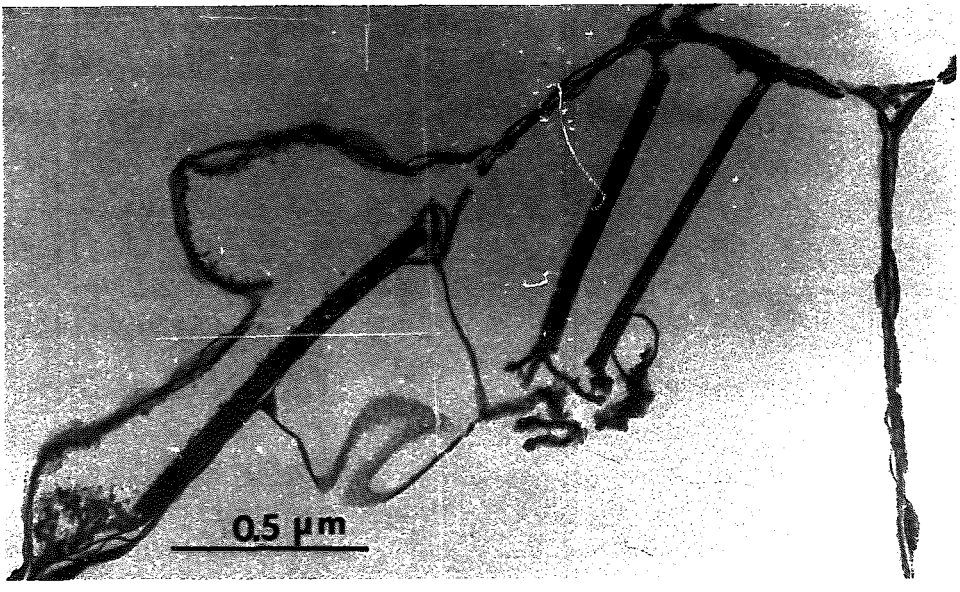


Fig. 6. Thin sections of pelleted crystals reveal crystals three to four layers in thickness (arrowheads) embedded in what are apparently lipid-detergent lamellae of ≈ 30 Å thickness (arrows). It is presumably the presence of these lamellae that leads to formation of the necklace-like structures such as that shown in Fig. 3a. Each of the three crystals shown here appears to belong to its own lamella.

zation occurred with non-ionic detergents of the polyoxyethylene glycol ether class, such as $C_{12}E_8$ and Brij 36T. In addition, high calcium concentrations and the presence of polyalcohols, such as glycerol, were found to be essential for long term stabilization and crystal growth.

Stokes and Green [15] have recently reported conditions that lead to production of 3-D crystals of Ca^{2+}-ATPase. They investigated the effect of detergent : lipid : protein on crystallization as well as the effect of different enzyme purification procedures. Their major finding was that by varying the detergent to lipid ratio they could promote in-plane crystal growth at the expense of stacking in the third dimension. Stacking was not completely eliminated, but growth of crystals in the third dimension was ordered. Despite their 3-D nature, the crystals obtained by Stokes and Green are of thickness and lateral extent suitable for electron crystallography and from them a projection map to 6 Å resolution has been obtained [22].

Our efforts reported here began with finding conditions that disturbed the aggregation of the 2-D crystalline sheets in the third dimension thereby leading to the production of single layer crystals. Starting with the conditions of Pikula et al. [1] we found solution treatments that disrupted multilayered crystals and then tested them for crystallization potential. High salt concentrations disrupted preformed stacks without disturbing the order within the individual sheets. The disruption of preformed stacks seems to have only a slight dependence on the salt composition and appears to be mainly a function of ionic strength. The most significant finding was that acetate and propionate salts promoted the in-plane growth of the crystals to a much greater degree than the more standard KCl medium used by previous investigators and that the stacking pattern depended upon the concentration of the salt. Moreover, propionate salts promoted in-plane growth better than acetate or formate salts, suggesting that the small hydrocarbon chain has some as yet unidentified role in contributing to the in-plane growth of the crystals.

Decreasing protein concentration in crystallization medium also had an effect on crystal stacking. Single layered sheets were predominantly obtained upon lowering protein concentration to about 0.1–0.5 mg/ml. This is to be expected, since at low protein concentration there would be fewer encounters between the two-dimensional crystalline sheets.

The use of organic salts to promote crystallization has rarely been reported in the literature. Kelders et al. [23] used sodium formate as a precipitant in the crystallization of thermitase by vapor diffusion. They observed that the size of crystals depended upon the concentration of sodium formate, with large crystals being obtained using 40–60% sodium formate. At lower formate concentrations crystals were very small, whereas at higher concentrations no crystals were observed. Janson et al. [24] have also used formates and acetates in the presence of PEG 6000 for the crystallization of glutamine synthetase by vapor diffusion. In this instance the crystals formed in the presence of organic anions were either too small or unstable for characterization by X-ray crystallography. These reports de-

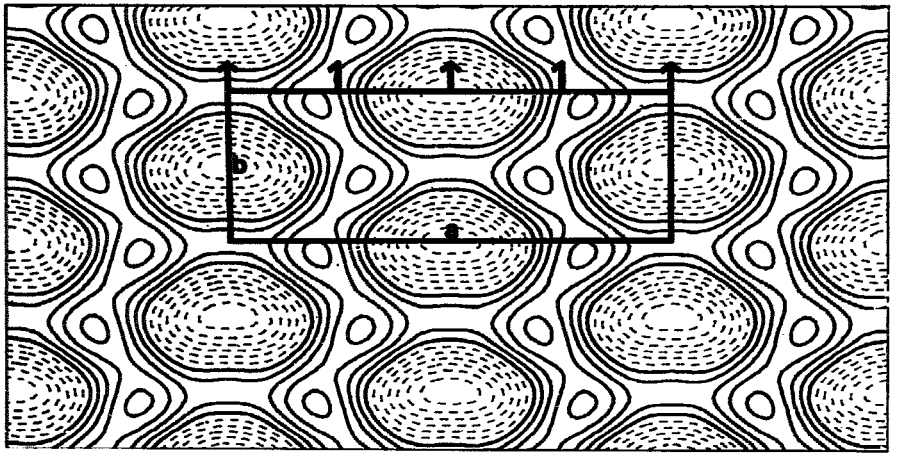


Fig. 7. Averaged filtered image of a single layer of crystals (similar to the one shown in Fig. 3a) of calcium-ATPase. The solid lines represent the stain-excluding regions corresponding to protein molecules and the dashed regions correspond to regions of stain accumulation. The unit cell of dimensions $a = 160.2$ Å, $b = 52.9$ Å with an included angle of 90° is drawn in dark solid lines. The data from these single layer crystalline sheets preserved in uranyl acetate stain extended to a resolution of about 18 Å.

scribed efforts to produce crystals suitable for X-ray diffraction, whereas the crystallization requirements for electron crystallography are very different. Taurine at 0.12 M concentration has been used in the 2-D crystallization of CP47-D1-D2-cytochrome *b*-559 photosystem II complex [25]. However, we know of no applications previous to ours that used organic salts in high concentration for crystallizing membrane proteins into 2-D sheets.

The mechanism whereby propionate promotes in-plane growth at the expense of stacking is not clear, but some observations may be pertinent. In general, high concentrations of salts reduce the solubility of proteins and it is this effect that is primarily of use in protein crystallization. In solution, the solutes and the protein together compete for the ambient water molecules [26]. The presence of high ionic strength decreases the availability of water molecules for the protein. According to Tanford [27] the water molecules surrounding an ion are firmly bonded to one another and less labile than they are in the absence of ion. This dehydration increases the thermodynamic activity of the protein, which in turn decreases its solubility [28]. However, all salts are not equally effective as protein precipitants. For example, high ionic strength alone does not explain the differences in crystallization that we observed between, for example, KCl and organic salts. Even amongst organic salts we noticed differences between formate, acetate and propionate ions. This would indicate that the type of charged species is just as important as its concentration.

Arakawa and Timasheff [29] have compared the solubility of lysozyme and bovine serum albumin in sodium acetate and sodium chloride at 1 M concentration. They observed, as expected, that the solubility of these proteins decreases with increasing salt concentration. However, protein solubility was greater in sodium chloride compared to that in sodium acetate. Or, in other words, sodium acetate was a more effective protein precipitant than sodium chloride at the same concentration. This implies that sodium acetate is more efficient than sodium chloride at removing ambient water. Possibly sodium acetate is a better protein precipitant because potential hydrophobic interactions between acetate and water can remove more ambient water than the interactions that occur in sodium chloride. If this trend can be extrapolated, propionate ions may even further decrease protein solubility and hence promote greater hydrophobic interactions. At pH 6, sodium propionate is almost 93% ionized and could, therefore, cause extensive structuring of water molecules around the charged anion.

For integral membrane proteins, there are the additional hydrophobic interactions that may play an important role in crystallization into 2-D sheets. The in-plane crystallization probably involves both hydrophilic interactions between ATPase molecules in the aqueous phase and hydrophobic interactions within the lipid-detergent phase. It is possible, but not proven, that organic salts affect the 2-D crystallization by promoting the incorporation of the protein into the 2-D phase, within which crystallization is facilitated because the protein molecules have fewer degrees of freedom. This hypothesis is supported by the high degree of crystallization even at very low protein concentrations. Based upon the above arguments, organic anions would promote the 2-D crystallization because they are more efficient at binding the ambient water, thereby promoting hydrophobic interactions between the protein, lipid and detergent.

The stacking of the 2-D sheets seems to involve a different effect than the promotion of in-plane crystal growth. The stacking of the 2-D sheets requires interaction between the 'head' of the calcium-ATPase molecules which protrudes out of the lipid-detergent phase. These interactions between individual sheets in the multi-layered crystal may involve both hydrophobic and hydrophilic interactions [11]. Presumably the relative importance of hydrophobic and hydrophilic interactions for stakcing will be dependent upon the types of amino acid residues present in this region. What seems clear, however, is that stacking is favored at low ionic strengths. It seems likely that interactions between sheets involve primarily charged interactions because of the disrupting effect of high ionic strength on the stacking. High ionic strength, therefore, seems to promote formation of 2-D sheets for two reasons: it not only shields the charged residues causing decreased polar interactions between 2-D sheets, but it also renders the water molecules less labile thereby facilitating hydrophobic interactions.

In conclusion, we have shown that organic anions affect the stacking of crystalline sheets of calcium-ATPase molecules. Sodium propionate at 0.8 M strength seems to be most effective in producing large sheets with minimal stacking. Under suitable conditions (low protein concentration) it is even possible to get predominently single layers of crystalline sheets. To our knowledge, the role of organic anions in the 2-D crystallization of membrane proteins has not so far been studied and it is possible that organic anions may have a similar effect on the stacking pattern and in-plane growth of other membrane protein crystals which tend to form 3-D stacks.

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