Chapter 38

Two-Dimensional Crystallization of the Ca^{2+}-ATPase for Electron Crystallography

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Abstract

Electron crystallography of two-dimensional crystalline arrays is a powerful alternative for the structure determination of membrane proteins. The advantages offered by this technique include a native membrane environment and the ability to closely correlate function and dynamics with crystalline preparations and structural data. Herein, we provide a detailed protocol for the reconstitution and two-dimensional crystallization of the sarcoplasmic reticulum calcium pump (also known as Ca^{2+}-ATPase or SERCA) and its regulatory subunits phospholamban and sarcolipin.

Key words Electron crystallography, Two-dimensional crystals, Sarcoplasmic reticulum, Ca^{2+}-ATPase, Phospholamban, Sarcolipin

1 Introduction

Electron crystallography (EC) has long been recognized as a powerful alternative in the structural biology of macromolecular complexes, with particular relevance for membrane protein structure determination. While EC is complementary to other structural biology techniques such as X-ray crystallography and NMR spectroscopy, it offers the unique advantage of a native, membrane-embedded environment. Nonetheless, there are significant technical challenges in obtaining two-dimensional crystals suitable for EC. And, once suitable crystals are obtained, the structural observations may be limited to low or moderate resolution and there can be a long lag time between the production of crystals and the determination of a three-dimensional structure. Despite these limitations, the origins of EC date back to the 1970s and there have since been numerous high-resolution structures (e.g., bacteriorhodopsin, tubulin, acetylcholine receptor [1–3]) and hundreds of reports of moderate-resolution projection or three-dimensional structures (e.g., [4–7]).

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While structural biology was once the purview of a select group of practitioners worldwide, the techniques have become more generally accessible to researchers interested in their favorite protein targets. As more researchers turn toward structural biology, it is important to have robust means of structure determination and validation, as well as comprehensive protocols for sample preparation. Herein, we provide an experimental protocol for crystallizing the sarcoplasmic reticulum (SR) calcium pump, complete with pitfalls and moments of frustration and serendipity. The SR calcium pump is also known as Ca^{2+}-ATPase or SERCA, and it plays an essential role in muscle contractility. Two-dimensional crystals of SR membranes have been available since the 1980s [8], and the original insights into SERCA structure were uniquely provided by electron cryo-microscopy of well-ordered helical crystals [9, 10]. At 8 Å resolution, the structural detail provided included the domain architecture of SERCA and the localization of the ten transmembrane helices that coordinate calcium. In 2000, the first high resolution structure of SERCA by X-ray crystallography was published [11], and since then there have been more than 50 structures determined in different states of the enzyme (e.g., [12–19]).

In the modern era of structural biology, SERCA has revealed itself to be one of the most well understood proteins, and uniquely amenable to crystallization and structure determination under numerous conditions and conformational states. Herein we focus on a novel two-dimensional crystal form of SERCA in the presence of its regulatory subunits phospholamban and sarcolipin. The crystals were serendipitously discovered in my laboratory ~8 years ago [20], yet we have encountered significant hurdles in the journey from initial crystals to three-dimensional structure determination. Nonetheless, some interesting general principles have resulted from these studies. For two-dimensional crystallization of SERCA, the factors that control membrane reconstitution and crystallization can be considered separately. The crystallization conditions rely on decavanadate, a large polyanion known to promote linear arrays of SERCA molecules. The lipids used in the reconstitution process are chosen based on maximizing the activity of SERCA, yet the lipid mixture also promotes the formation of well ordered two-dimensional crystals. In this case, the lipid mixture includes phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid and the typical outcome is well ordered helical crystals and small crystalline vesicles. Our serendipitous discovery was that we can switch between different crystal lattices and morphologies (helical versus large two-dimensional crystals) in a process that depends on phosphatidic acid and small changes in magnesium concentration. This lipid-dependent switch in crystal behavior may be applicable to other membrane proteins.

2 Materials

Prepare all solutions using ultrapure water (purified deionized water with a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Ensure all waste disposal regulations are followed when disposing waste materials. Carefully read and follow Materials Safety Data Sheets for all reagents used.

2.1 Stock Solutions

All stock and buffer solutions should be prepared at room temperature, filtered using a sterile 0.22 μm filter and stored at 4 °C (unless indicated otherwise).

1. 1.5 mL Eppendorf tubes.
2. 15 mL Falcon tubes.
3. Imidazole buffer: 0.5 M imidazole, pH 7.0. Add about 40 mL of water to a glass beaker with a magnetic stir-bar. Weigh 1.7 g imidazole and transfer to the beaker. Mix and adjust pH with HCl (*see* **Note 1**). Make up to 50 mL with water. Filter and store until use.
4. Potassium chloride (KCl): 2 M KCl. Add about 40 mL of water to a glass beaker with a magnetic stir-bar. Weigh 7.46 g KCl and transfer to the beaker. Mix until fully dissolved. Make up to 50 mL with water. Filter and store until use.
5. Magnesium chloride ($MgCl_2$): 1 M $MgCl_2$. Add about 40 mL of water to a glass beaker with a magnetic stir-bar. Weigh 10.16 g $MgCl_2$ ($6{\cdot}H_2O$) and transfer to the beaker. Mix until fully dissolved. Make up to 50 mL with water. Filter and store until use.
6. Ethylene glycol tetraacetic acid (EGTA): 50 mM EGTA, pH 8.0. Add about 35 mL of water to a glass beaker with a magnetic stir-bar. Weigh 0.95 g EGTA and transfer to the beaker. Mix and adjust pH with NaOH (*see* **Note 2**). Make up to 50 mL with water. Filter and store until use.
7. Sodium azide: 5 % (w/v) sodium azide. Add about 40 mL of water to a glass beaker with a magnetic stir-bar. Weigh 2.5 g sodium azide and transfer to the beaker. Mix until fully dissolved. Make up to 50 mL with water. Store until use (filtration not required).
8. 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer: 0.5 M MOPS, pH 7.0. Add about 40 mL of water to a glass beaker with a magnetic stir-bar. Weigh 5.23 g MOPS and transfer to the beaker. Mix and adjust pH with NaOH (*see* **Note 3**). Make up to 50 mL with water. Filter and store until use (*see* **Note 4**).

9. Calcium chloride ($CaCl_2$): 0.5 M $CaCl_2$. Add about 40 mL of water to a glass beaker with a magnetic stir-bar. Weigh 2.77 g $CaCl_2$ (anhydrous) and transfer to the beaker (*see* **Note 5**). Mix until fully dissolved. Make up to 50 mL with water. Filter and store at room temperature (storage at 4 °C may cause salt precipitation).
10. Dithiothreitol (DTT): 1 M DTT. Add about 8 mL of water to a 15 mL Falcon tube. Weigh 1.54 g DTT and transfer to the tube. Vortex until fully dissolved. Make up to 10 mL with water. The DTT stock solution does not need to be filtered. Transfer 1 mL aliquots into 1.5 mL Eppendorf tubes and store at −20 °C.
11. Glycerol: 80 % (v/v) glycerol. Measure 160 mL of glycerol and transfer it to a glass beaker with a magnetic stir-bar. Make up to 200 mL with water. Transfer to an oversized (greater than 200 mL) screw-top glass bottle and autoclave (*see* **Note 6**). Store at room temperature.

2.2 Reconstitution

1. 12 × 75 mm borosilicate glass disposable culture tubes.
2. 8 × 1 mm (mini) stir-bars.
3. SM2 BioBeads (BioRad): Wash BioBeads in bulk prior to use. Weigh ~5 g BioBeads and transfer to a 50 mL Falcon tube. Add about 30 mL of methanol and gently agitate for 30 min (using a Nutator Mixer, for example). Carefully decant the methanol to waste. Repeat the methanol wash and decant steps. Add about 30 mL of water and gently agitate for 30 min. Carefully decant the water to waste. Repeat the water wash and decant steps twice. Add about 30 mL of 0.05 % sodium azide and store at 4 °C (a 1:100 dilution of the 5 % sodium azide stock will generate the required 0.05 % sodium azide).
4. Detergent—Octaethylene glycol monododecyl ether ($C_{12}E_8$): 10 % (w/v) $C_{12}E_8$. Weigh 0.1 g of solid $C_{12}E_8$ (Barnet Products) and transfer to a 1.5 mL Eppendorf tube. Add 0.92 mL of water. Mix well to dissolve (*see* **Note 7**) and, if necessary, adjust the final volume to 1 mL. Transfer 100 μL aliquots into 1.5 mL Eppendorf tubes and store at −20 °C.
5. Lipids—Egg yolk phosphatidylcholine (EYPC), egg yolk phosphatidic acid (EYPA), and egg yolk phosphatidylethanolamine (EYPE): Chloroform-solubilized lipids can be obtained at concentrations of 25 mg/mL (Avanti Polar Lipids) and stored at −80 °C.
6. Purified phospholamban (PLN) or sarcolipin (SLN): Purify PLN [21] or SLN [22] as described.
7. Purified Ca^{2+}-ATPase: Purify Ca^{2+}-ATPase as described [23].

8. Reconstitution master-mix: Make from stock solutions on the day of reconstitution and store on-ice. To a 1.5 mL Eppendorf tube, add:

   40 μL of 0.5 M imidazole, pH 7.0.

   25 μL of 2 M KCl.

   10 μL of 1 M $MgCl_2$.

   5 μL of 5 % sodium azide.

   Vortex to mix.

9. Storage buffer: Add about 6.5 mL of water to a glass beaker with a magnetic stir-bar. Add:

   400 μL of 0.5 M MOPS, pH 7.0 stock.

   20 μL of 0.5 M $CaCl_2$ stock.

   10 μL of 1 M $MgCl_2$ stock.

   100 μL of 10 % $C_{12}E_8$ stock.

   Adenosine diphosphate (ADP)—weigh 42.7 mg of solid ADP and transfer to the beaker.

   2.5 μL of 1 M DTT stock.

   2.5 mL of 80 % glycerol stock.
   Mix the components well until the solid ADP has dissolved. Adjust the pH of the solution to pH 7.0 (requires ~100 μL of 1 N NaOH; add dropwise until required pH 7.0 is obtained). Make up to 10 mL with water. Transfer 1 mL aliquots into 1.5 mL Eppendorf tubes and store at −20 °C.

10. 50 % sucrose storage buffer: Add about 40 mL of water to a glass beaker with a magnetic stir-bar. Weigh 25 g of sucrose and transfer to the beaker. Add:

    2.0 mL of stock 0.5 M imidazole, pH 7.0.

    2.5 mL of stock 2 M KCl.

    500 μL of stock 5 % sodium azide.
    Mix while gently heating to dissolve the sucrose (bring the total volume up to 50 mL during this process). Store at 4 °C.

2.3 Crystallization

1. 1.5 mL Screw-cap Eppendorf tubes (*see* **Note 8**).
2. Thapsigargin (TG): 1 mg/mL TG stock solution (Sigma-Aldrich). Add 1 mL of absolute ethanol (> 99.5 % v/v) per mg of TG (obtained as a solid film). Mix to dissolve. Transfer 1 mL aliquots to glass vials. Seal vials tightly with polypropylene-lined caps. Store at −80 °C.
3. Sodium orthovanadate (Na_3VO_4): 50 mM Na_3VO_4 stock solution (*see* **Note 9**). Make fresh on the day of crystallization and store on-ice (*see* **Note 10**). Weigh 46 mg of Na_3VO_4 and transfer to a 15 mL Falcon tube. Add 3.5 mL of water. Mix well to dissolve. Adjust the pH to 2.0 or slightly below (*see*

Note 11). Incubate on ice for 30 min. Adjust the pH to 6.5–7.0 (*see* **Note 12**).

4. Crystallization master-mix: Make from stock solutions on the day of crystallization and store on-ice. To a 15 mL Falcon tube, add:

   4.18 mL of water.

   200 μL of 0.5 M imidazole, pH 7.0.

   250 μL of 2 M KCl. 50 μL of 50 mM EGTA.

   20 μL of 5 % sodium azide.

   Vortex to mix.

5. Crystallization buffer: Make from Crystallization master-mix on the day of crystallization and store on-ice. Add 940 μL of Crystallization master-mix to a 1.5 mL Eppendorf tube. Add the required amount of $MgCl_2$ in a 50 μL volume (*see* **Note 13**). For example, to make 40 mM $MgCl_2$ Crystallization buffer, add 40 μL of 1 M $MgCl_2$ stock solution and 10 μL of ice-cold water. Then add the required amount of Na_3VO_4 in a 10 μL volume (*see* **Note 14**).

3 Methods

3.1 Lipid and Co-reconstituted Peptide Preparation

We describe the lipid and co-reconstituted peptide preparations for four reconstitutions, which represents an appropriate number to become familiar with the experimental setup. Once competent and comfortable with the reconstitution procedure, the researcher may choose the number of reconstitutions per experiment and adjust the components as necessary.

1. Calculate the amount of each lipid required for the reconstitution set to determine the volumes of stock lipids to be added to the lipid pool (*see* **Note 15**). The final lipid ratio will be 8 EYPC:1 EYPA:1 EYPE and lipids will be pooled based on a lipid–protein ratio of 1:1, where the protein is represented solely by the Ca^{2+}-ATPase and not the Ca^{2+}-ATPase and co-reconstituted peptide (*see* **Note 16**). Each reconstitution will require 500 μg Ca^{2+}-ATPase and 500 μg of total lipids. Given a lipid weight ratio of 8 EYPC:1 EYPA:1 EYPE, the calculation of EYPA and EYPE required per reconstitution is straightforward at 50 μg (2 μL of the 25 mg/mL stock lipid solutions). The calculation of the EYPC required per reconstitution is not as straightforward and must take into account the volume containing the 500 μg Ca^{2+}-ATPase to be added will include EYPC at a concentration of 0.25 mg/mL (*see* **Note 17**).

For our calculations here, we will be assuming a purified Ca^{2+}-ATPase concentration of 4 mg/mL and, thus, we will use 125 μL from the purified Ca^{2+}-ATPase aliquot for 500 μg. At 0.25 mg/mL EYPC, this aliquot volume will contribute 31.25 μg EYPC to the reconstitution and, to achieve the 400 μg EYPC required per reconstitution, 368.75 μg EYPC must be added. Thus, 14.75 μL of the 25 mg/mL EYPC stock lipid solution is required per reconstitution.

Finally, the volume of each lipid stock to be added per reconstitution is multiplied by 5 for a set of 4 reconstitutions (*see* **Note 15**). Therefore, the final volume calculations for the lipid pool are 73.75 μL EYPC, 10 μL EYPA, and 10 μL EYPE from their respective 25 mg/mL stock lipid solutions. In this example, the final volume of the lipid pool will be 93.75 μL, representing the required lipids for five reconstitutions, and 18.75 μL of the lipid pool will need to be added to each of the four reconstitution tubes.

2. Add 75 μL of a 2:1 chloroform–trifluoroethanol mixture to 100 μg of dried PLN or dried SLN (*see* **Note 18**). One hundred microgram aliquots of purified PLN or SLN should be dried down, labeled, and stored at −80 °C in a (12×75 mm) glass tube to facilitate this step. Vortex vigorously to dissolve the dried PLN or SLN. Repeat this step for a total of four reconstitution tubes.
3. In a (12×75 mm) glass tube, pool the required lipids for the reconstitution set (based on the calculations in **step 1** above). Measure chloroform-solubilized stock lipids (*see* **Note 19**) using a clean gas-tight Hamilton glass syringe of appropriate size (*see* **Notes 20** and **21**).
4. Transfer the required aliquot of the lipid pool (based on the calculations in **step 1**) into each reconstitution tube. Mix the contents of the reconstitution tubes well by vortexing.
5. Dry the contents of the reconstitution tubes under a gentle stream of nitrogen gas while gently vortexing (*see* **Note 22**). The contents of each reconstitution tube should form a thin film at the bottom of the glass tube (Fig. 1). Desiccate the dried lipid and peptide film overnight in a vacuum desiccator (*see* **Note 23**).

3.2 Ca^{2+}-ATPase Reconstitution

For the following reconstitution step, the reconstitution tubes should be kept at room temperature, whereas stock buffers and other reconstitution components should be kept on ice (unless indicated otherwise). In this section, we describe conditions that have provided the most success for Ca^{2+}-ATPase crystallization. However, it is important to note many reconstitution variables, especially the lipids, can be screened to optimize crystallization (Fig. 1).

Co-Reconstitution Screen						
	lipids (wt ratio)				peptide	SERCA
	EYPC	EYPE	EYPA	total		
Highest success	8	1	1	500µg	100µg	500µg
Range for screening	6-8	1-2	1-2	300-600µg	25-150µg	300-500µg

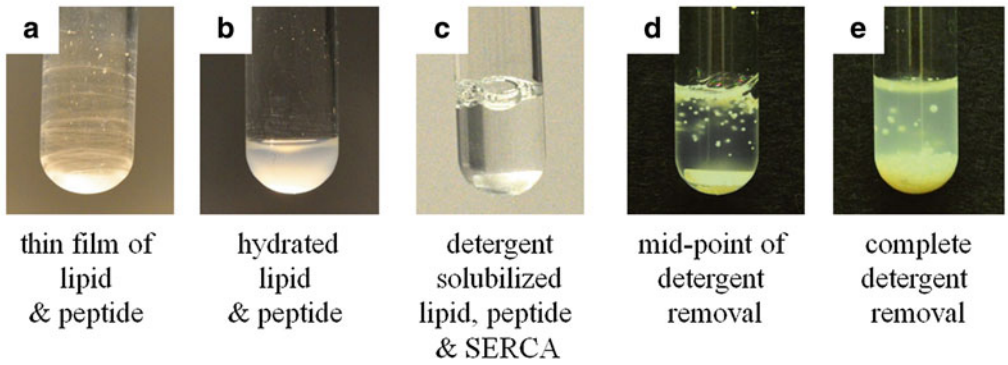

Fig. 1 Key steps in the reconstitution of SERCA into proteoliposomes. The table summarizes the conditions that are typically screened to achieve co-reconstitution of SERCA in the presence of phospholamban or sarcolipin. The steps shown are (**a**) thin film of lipid and peptide on the inside wall of a glass test tube; (**b**) hydrated lipids and peptide; (**c**) detergent-solubilized, mixed-micelle solution containing SERCA, lipids and peptide (the magnetic stir bar is visible at the *bottom* of the test tube); (**d**) mid-point of the detergent removal with SM2 Biobeads (the turbidity indicates the beginning of vesicle formation; the *larger white spheres* are Biobeads); (**e**) complete detergent removal with SM2 Biobeads (this solution contains co-reconstituted proteoliposomes of SERCA and phospholamban or sarcolipin)

1. Add 185 µL of water to each reconstitution tube. Without generating bubbles, gently pipette the water to resuspend the lipid and peptide film. The tube may also be gently vortexed, but use care not to generate bubbles. Also, ensure the contents do not exceed the maximum height of the original dried line of the lipid and peptide film.
2. Incubate the reconstitution tubes with resuspended lipid and peptide at 37 °C for 10 min with gentle vortexing every 2 min.
3. Allow the contents of the reconstitution tubes (hydrated lipid and peptide in Fig.1) to cool to room temperature.
4. Add 7 µL of 10 % $C_{12}E_8$ to each reconstitution tube (*see* **Note 24**).
5. Vigorously vortex each reconstitution tube for 3 min wihtout stopping (*see* **Note 25**).
6. Add 8 µL of Reconstitution master mix to each reconstitution tube. Vortex gently to mix.

7. Add the balance volume of storage buffer to each reconstitution tube (*see* **Note 26**). For this example, we will be adding a balance volume of 175 μL of storage buffer (adding a 125 μL volume of purified Ca^{2+}-ATPase in storage buffer in the next step as we assumed a Ca^{2+}-ATPase concentration of 4 mg/mL). Vortex gently to mix.
8. Add the volume of purified Ca^{2+}-ATPase that contains 500 μg to each reconstitution tube (*see* **Note 27**).
9. Add 8 × 1 mm (mini) stir-bars to each reconstitution tube.
10. Set up a suitable test-tube rack on a magnetic stir-plate and place the reconstitution tubes in the rack. Stir the reconstitutions gently (*see* **Note 27**).
11. Add SM2 BioBeads over a 4-h time-course (*see* **Note 28**).

    First addition: ~1 mg BioBeads (*see* **Note 29**), incubate for 30 min while stirring gently (*see* **Note 30**).

    Second addition: ~1 mg BioBeads and 30 min incubation with stirring.

    Third addition: ~1 mg BioBeads and 30 min incubation with stirring.

    Fourth addition: ~2 mg BioBeads and 30 min incubation with stirring.

    Fifth addition: ~5 mg BioBeads and 60 min incubation with stirring.

    Final addition: ~10 mg BioBeads and 60 min incubation with stirring.

3.3 Ca^{2+}-ATPase Crystallization

We describe the Ca^{2+}-ATPase crystallization procedure for eight samples (four reconstitutions screened at two $MgCl_2$ concentrations), which represents an appropriate number to become familiar with the experimental setup. Once competent and comfortable with the crystallization procedure, the researcher may choose the number of crystallizations per experiment and adjust the components as necessary.

During the crystallization steps, the crystallization tubes should be kept on-ice (unless indicated otherwise). All centrifugation steps are carried out at 4 °C. Rotors and centrifuges should be prechilled to 4 °C prior to use. In this section, we describe screening conditions that have provided the most success for Ca^{2+}-ATPase crystallization for both the thin, helical crystals (5 mM $MgCl_2$Crystal buffer) and the wide, 2D crystals (40 mM $MgCl_2$ Crystal buffer) (Figs. 2 and 3). However, it is important to note that many other crystallization variables can be screened to optimize crystal formation and frequency.

1. Transfer 200 μL of 5 mM $MgCl_2$Crystallization buffer to a screw-cap Eppendorf tube. Repeat for a total of four crystallization tubes.

2D Crystallization Screen (4 °C)

Mg^{2+}	Na_3VO_4	Incubation	Crystal type
5 mM	0.5 mM	3-5 days	Helical
30-50 mM	0.25-0.5 mM	1-6 days	Wide 2D
Mn^{2+}			
5 mM	0.5 mM	3-5 days	Wide 2D
20 mM	0.5 mM	3-5 days	Helical

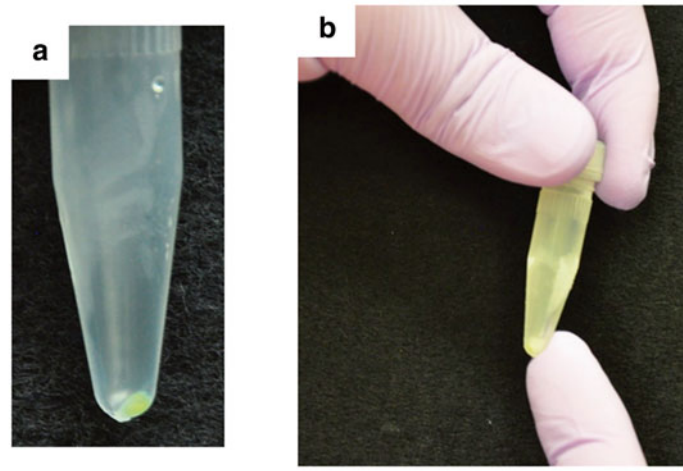

Fig. 2 Key steps in the two-dimensional crystallization of SERCA starting from reconstituted proteoliposomes. The table summarizes the conditions that are typically screened to achieve crystallization of SERCA in the presence of phospholamban or sarcolipin. (**a**) Pellet of the proteoliposomes collected by centrifugation in the presence of decavanadate crystallization buffer. The *yellow color* is indicative of the presence of decavanadate. (**b**) Demonstration of the freeze–thaw cycle that is used to promote proteoliposome fusion. The small proteoliposomes (~0.2 μm diameter) must fuse to form large two-dimensional crystals (0.5 μm wide, 5–50 μm in length). After rapidly freezing in liquid nitrogen, the pellet is thawed between thumb and forefinger. The solution is gently agitated as it begins to melt, and the process is repeated

2. Transfer 200 μL of 40 mM $MgCl_2$Crystallization buffer to a screw-cap Eppendorf tube. Repeat for a total of four crystallization tubes.
3. Transfer 50 μL of each reconstitution to a 5 mM $MgCl_2$ crystallization tube and a 40 mM $MgCl_2$ crystallization tube (*see* **Note 31**). Pipette the mixtures gently up and down to mix.

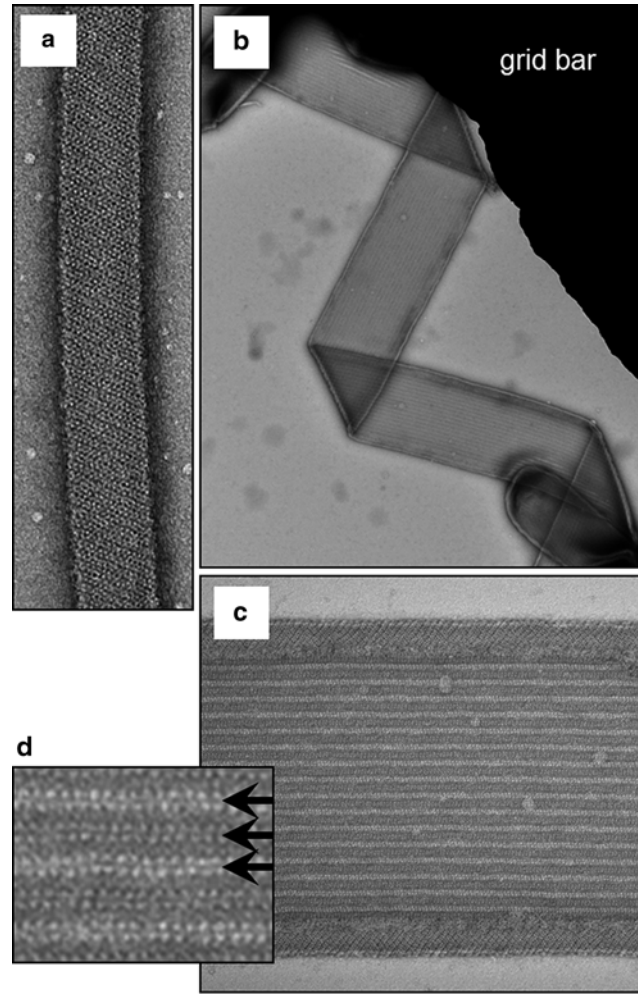

Fig. 3 Two-dimensional crystals of SERCA in the presence of phospholamban (similar crystals are obtained in the presence of sarcolipin). All crystals were imaged by negative-stain electron microscopy. (*A*) A typical helical crystal. The crystal is ~70 nm wide and has p2 symmetry ($a = 57$ Å, $b = 117$ Å, $\gamma = 64$ °). (**B**) A typical two-dimensional crystal. The crystal is ~0.5 μm wide and has $p22_12_1$ symmetry ($a = 350$ Å, $b = 70$ Å, $\gamma = 90$ °). (**C**) Magnified view of a region of a two-dimensional crystal. (**D**) Close-up view of the SERCA dimer ribbons (arrows) that make up the lattice. The *b* axis is horizontal and rigidly held together by decanvanadate. The *a* axis is vertical and highly variable, and this variability represents the major hurdle we have encountered in going from initial crystals to a three-dimensional structure

Repeat for each of the 4 reconstitutions. The remaining reconstitution material that will not be used for the crystallization should be frozen and stored as outlined (*see* **Note 32**).

4. Spin the mixture in the crystallization tubes at $40{,}000 \times g$ for 40 min at 4 °C to pellet the reconstituted proteoliposomes.

5. For each crystallization tube, carefully aspirate the supernatant to waste without disturbing the vesicle pellet.
6. To each crystallization tube, add 20 μL of the appropriate Crystallization buffer on top of the vesicle pellet.
7. To each crystallization tube, add 1 μL of TG to the vesicle pellet (*see* **Note 33**). Tap the screw-cap Eppendorf tube gently to mix its contents without resuspending the vesicle pellet (*see* **Note 34**).
8. Freeze the vesicle pellet and added Crystallization buffer and TG by submerging in liquid nitrogen (*see* **Note 35**).
9. Thaw the frozen vesicle pellet and solution slowly between the thumb and forefinger with gentle mixing just as it thaws (Fig. 2 and *see* **Note 36**).
10. Repeat **steps 8** and **9**.
11. Resuspend the pellet by gently pipetting up and down using a 20 μL pipette tip with the pipette set to 10 μL or less (*see* **Note 37**).
12. Freeze the resuspended vesicle pellet by submerging in liquid nitrogen (*see* **Note 35**).
13. Thaw the frozen resuspension slowly between the thumb and forefinger with gentle mixing just as it thaws (Fig. 2 and *see* **Note 36**).
14. Repeat **steps 12** and **13**.
15. Incubate the crystallization tubes at 4 °C. Also store any remaining master-mix, Crystallization buffers, and Na_3VO_4 solution at 4 °C.

3.4 Crystal Screening by Electron Microscopy

Crystals can be screened after a minimum of 24 h, although crystals are generally best screened after 3–5 days of incubation. To screen the crystals, gently tap the crystallization tubes to resuspend the sedimented material and apply 5 μL of the mixture to a glow-discharged electron microscopy grid for negative-staining. Good crystallization trials identified by negative-stain electron microscopy should have duplicate negative-stain grids prepared, whereas excellent crystallization trials should be prepared for electron cryo-microscopy imaging (*see* **Note 38**). For the successful crystallographer, we have previously provided details of the screening and imaging of crystals by electron microscopy [20, 24, 25], which are beyond the scope of this protocol.

4 Notes

1. Add concentrated HCl (6 N) dropwise to bring the starting pH closer to the required pH, and then add 1 N HCl dropwise to lower the imidazole solution pH to 7.0.

2. EGTA will not go into solution until it nears the required pH. Add concentrated NaOH (10 N) dropwise to bring the starting pH closer to the required pH, and then add 1 N NaOH dropwise to raise the EGTA solution pH to 8.0.
3. Add concentrated NaOH (6 N) dropwise to bring the starting pH closer to the required pH, and then add 1 N NaOH dropwise to raise the MOPS solution pH to 7.0.
4. The 0.5 M MOPS stock solution should be stored in the dark (or wrapped in tinfoil to minimize its exposure to light). The 0.5 M MOPS stock solution should be discarded if it turns yellowish in color and a fresh stock prepared.
5. Dissolving $CaCl_2$ in water is an exothermic reaction and appropriate precautions should be taken. For example, while dissolving the $CaCl_2$ in water, the glass beaker can be placed on ice.
6. Exercise best autoclave practices and take necessary precautions. For example, liquid containers can only be filled to a maximum of one-half to two-thirds capacity, screw-top lids must be loosened to prevent pressure build-up, and there must not be any cracks or vulnerabilities in glass bottles or containers. Autoclave tape, or some other indicator, should be used to ensure that the target temperature has been reached and the autoclave itself should be monitored for operational safety and quality control.
7. It can take several minutes to dissolve the solid $C_{12}E_8$. After sealing the 1.5 mL Eppendorf tube to prevent leakage (use Parafilm M or a screw-cap 1.5 mL Eppendorf), we use a Nutator Mixer to mix the solid detergent and water until dissolution occurs.
8. It is important that the 1.5 mL screw-cap Eppendorf tubes are high quality tubes, such that the tubes will not warp or break during high-speed ($40{,}000 \times g$) centrifugation.
9. The Na_3VO_4 stock solution is specifically prepared to maximize the decameric species of vanadate (decavanadate), which should be apparent as an intense yellow color when the preparation of the Na_3VO_4 stock solution is completed.
10. If performing the crystallization on the same day as the reconstitution, begin preparing the Na_3VO_4 stock solution during one of the last two 60 min BioBead incubations.
11. Add concentrated HCl (6 N) dropwise to bring the starting pH closer to the required pH, and then add 1 N HCl dropwise to lower the Na_3VO_4 stock solution pH to 2.0 or below (a pH of 1.9 is acceptable).
12. The Na_3VO_4 stock solution should be kept on ice during the pH adjustment. Add ice-cold KOH (1 N) dropwise to bring

the pH between 3.0 and 4.0, and then add ice-cold 0.1 N KOH dropwise to bring the Na_3VO_4 stock solution pH between 6.5 and 7.0. Ensure that the pH does not go above 7.0 during this adjustment as it might cause the Na_3VO_4 to precipitate, at which point the Na_3VO_4 preparation needs to be started again.

13. Determining the $MgCl_2$ concentrations to screen is a key decision in the crystallization experiment. Low $MgCl_2$ concentrations (5–10 mM) will generate thin, helical Ca^{2+}-ATPase crystals, whereas higher $MgCl_2$ concentrations (30–50 mM) will generate wide, 2D Ca^{2+}-ATPase crystals. We have previously provided a detailed discussion surrounding this observed Mg^{2+}-dependent switch in Ca^{2+}-ATPase crystal morphology and the potential underlying mechanisms responsible for the switch [24]. We note here that we have had success screening other divalent cations in place of Mg^{2+} in the Crystallization buffer (J.P. Glaves and H.S. Young, unpublished observations). Interestingly, when screening other divalent cations, the different Ca^{2+}-ATPase crystal morphologies did not necessarily have the same concentration-dependence as that observed for Mg^{2+}. As an example, we observed wide, 2D Ca^{2+}-ATPase crystals when screening low concentrations of $MnCl_2$.
14. Ensure that the Crystallization buffer is ice-cold before adding the required amount of Na_3VO_4. Based on our experience, the amount of Na_3VO_4 should only be varied between 0.25 mM and 0.5 mM (5 or 10 μL, respectively, of the 50 mM Na_3VO_4 stock solution added to 1 mL of Crystallization buffer).
15. The lipid pool should include enough lipids for one extra "reconstitution" for every four reconstitutions (i.e., for four reconstitutions, pool lipids necessary for five reconstitutions; for eight reconstitutions, pools lipids for ten reconstitutions; and so on). Including enough lipids in the lipid pool for these extra "reconstitutions" allows for small amounts of evaporation and minor measurement errors that will occur during the reconstitution setup. The purpose of pooling the required lipids prior to their addition to the individual reconstitution tubes is to minimize the number of very small volume measurements and transfers that will be required during the experiment. It also ensures that the individual reconstitutions draw from a lipid pool with an identical overall composition.
16. The lipid ratio and the lipid-to-protein ratio are among the many reconstitution variables that can be screened to optimize 2D crystallization. We have previously described in detail the effects of the lipid ratio, and the type of lipids used, on the reconstitution, activity, and crystallization of the Ca^{2+}-ATPase [26]. Reference to this work should be made for those interested in the important roles of the lipid composition with

respect to critical steps in the Ca^{2+}-ATPase reconstitution and crystallization processes, including vesicle formation and vesicle fusion, as the in-depth discussion of such details is beyond the scope of this chapter. Regarding the lipid-to-protein ratio, we have experienced the most success working with a lipid-to-protein ratio of 1:1 that either excludes the co-reconstituted peptides in the protein calculation (i.e., 500 μg total lipid to 500 μg Ca^{2+}-ATPase) or includes the co-reconstituted peptides in the protein calculation (i.e., 600 μg total lipid to 500 μg Ca^{2+}-ATPase and 100 μg PLN or SLN). In our experience, varying the lipid-to-protein ratio too far beyond these limits leads to inefficient reconstitution, evidenced by an excess of liposomes at higher lipid-to-protein ratios or poor incorporation of the Ca^{2+}-ATPase and co-reconstituted PLN or SLN at lower lipid-to-protein ratios. Less efficient reconstitution leads to poor subsequent crystallization. As such, although this reconstitution variable can be explored, we recommend working near a 1:1 lipid-to-protein ratio.

17. Detergent-solubilized, affinity-purified aliquots of Ca^{2+}-ATPase are stored at −80 °C. When an aliquot is thawed for the first time, we add EYPC to a final concentration of ~0.25 mg/mL in order to stabilize the enzyme and preserve its activity through subsequent freeze–thaw cycles. As an example, our purified Ca^{2+}-ATPase aliquots are 500 μL in volume and we add 12.5 μL of an aqueous 10 mg/mL EYPC stock to each thawed aliquot. Ca^{2+}-ATPase aliquots that have had EYPC added to them are marked to prevent subsequent over-addition of EYPC. An aqueous 10 mg/mL EYPC stock, for this purpose, is prepared by first drying 10 mg of EYPC (400 μL of the 25 mg/mL chloroform-solubilized EYPC stock) in a (12 × 75 mm) glass tube under a gentle nitrogen gas stream while gently vortexing. Drying the stock EYPC in this manner creates a dried EYPC film on the bottom of the glass tube (Fig. 1). The dried EYPC film is then further dried in a vacuum desiccator overnight. Next, the dried EYPC film is resuspended by adding 1 mL of water to the tube and vortexing. Following resuspension, the aqueous EYPC stock is finally transferred, as 100 μL aliquots, into 1.5 mL Eppendorf tubes and stored at −20 °C.

18. Both the thin, helical Ca^{2+}-ATPase crystals and the wide, 2D Ca^{2+}-ATPase crystals can be grown without PLN or SLN (i.e., Ca^{2+}-ATPase alone). It is critical to note, however, that although the presence of PLN or SLN has a negligible effect on the frequency of thin, helical Ca^{2+}-ATPase crystals, the presence of PLN or SLN dramatically increases the frequency of wide, 2D Ca^{2+}-ATPase crystals. As such, we recommend including PLN or SLN as a variable for wide, 2D Ca^{2+}-ATPase

crystal screens. If PLN or SLN is not to be included in the reconstitution step, the 2:1 chloroform–trifluoroethanol mixture does not need to be added to the glass tube. Alternatively, if PLN or SLN will be included in the reconstitution set, the 2:1 chloroform–trifluoroethanol mixture should be added to an empty glass tube for the Ca^{2+}-ATPase-only control, if such a control is also to be included.

19. Perform all steps including the use of chloroform in a fume hood.
20. For washing the Hamilton syringe between lipid stock measurements (or before measuring aliquots from the lipid pool), rinsing the syringe serially using multiple tubes of chloroform is sufficient to prevent contamination of lipid stocks (or the lipid pool) with the previous lipid stock. Be sure the interior of the syringe is cleared of chloroform from the final rinse step and dry before measuring the next lipid aliquot. The Hamilton syringe should be fully cleaned (*see* **Note 21**) after the lipids have been aliquoted for each experiment and prior to storage of the syringe.
21. Hamilton syringes used for measuring chloroform-solubilized lipids can be cleaned by rinsing with chloroform, followed by multiple rinses with deionized water, and a final rinse with acetone. Be sure to expel all remaining cleaning solvents after each rinse step. The interior of the syringe should be cleared of residual acetone and dry before storing the syringe or using the syringe again. Wipe clean the exterior surfaces of the syringe and the needle with a lint-free tissue. See the Hamilton 'Syringe Care and Use Guide' for more details (www.hamiltoncompany.com).
22. First, determine the vortex speed required during the drying step. Vortex the contents of a reconstitution tube and adjust the vortex speed until the top of the contents mixing line reaches a maximum of approximately 1 cm from the bottom of the tube. Second, determine the nitrogen gas flow-rate required during the drying step. We empirically determine this flow-rate by expelling the gas stream on to the back of the hand (use caution during this step and start with a very low flow-rate that can be adjusted in small increments). If the flow-rate causes an indentation of the skin, it is too high. Next, vortex the contents of the tube under the stream of nitrogen gas. The contents of the tube should not be displaced above the original maximum mixing line approximately 1 cm from the bottom of the tube. If the stream of nitrogen gas is displacing the contents above 1 cm from the bottom of the tube, then lower the flow-rate. Drying of the solubilized lipids and peptide to a thin film using this approach should take approximately 5 min per reconstitution tube.

23. The dried lipid and peptide film can be desiccated for a minimum of 2 h, although we recommend allowing desiccation to proceed overnight.
24. The total amount of $C_{12}E_8$ that will be used in the reconstitution is 1000 μg (representing a detergent–lipid mass ratio of 2:1). The addition of $C_{12}E_8$ in this step adds 700 μg to the reconstitution and the remaining 300 μg will be added with the 300 μL total volume of pure Ca^{2+}-ATPase and additional storage buffer that contains $C_{12}E_8$ at a concentration of 1 mg/mL.
25. After the addition of detergent and vortexing, the contents of the reconstitution tube should be fully solubilized and clear (Fig. 1). If the contents are not clear at this point, the addition of very small volumes of 10 % $C_{12}E_8$ (1 μL per addition) can help with solubility, however, do not exceed an additional 3 μL of 10 % $C_{12}E_8$. The addition of buffer components in the Reconstitution master-mix, particularly $MgCl_2$, in the next step might also improve solubility. Unfortunately, poor solubility or the presence of large aggregates at this stage of the reconstitution could indicate problems with the PLN or SLN peptides and might warrant stopping the experiment altogether (prior to the addition of purified Ca^{2+}-ATPase).
26. The total volume of storage buffer to be added to the reconstitution will be 300 μL. After subtracting the volume of storage buffer containing 500 μg of purified Ca^{2+}-ATPase to be added in the next step from the total storage volume of 300 μL, the remaining volume represents the balance volume of storage buffer added in this step.
27. To ensure enzyme stability and activity after the addition of purified Ca^{2+}-ATPase to the reconstitution, care must be taken to avoid vortexing the mixture and generating air bubbles during the remaining experimental steps.
28. Remove excess storage solution from the stock BioBeads before adding them to the reconstitutions. This prevents excessive dilution of reconstitutions from the BioBead storage solution (0.05 % sodium azide in water). We remove the excess by carefully decanting the storage solution to waste and removing the excess solution from the bulk BioBeads using a vacuum trap. Insert a glass Pasteur pipette into tubing attached to a filtering flask capped with a stopper and inserted vacuum line (use caution to prevent breaking the Pasteur pipette when inserting into the tubing). After turning on the vacuum line, insert the Pasteur pipette into a 200 μL pipette tip (much as you would with an air displacement micropipette) and then insert the capped pipette into the bottom of the BioBead storage tube (i.e., to the bottom of the bulk BioBeads). By using the 200 μL pipette tip, you can quickly replace the pipette tip if it

becomes blocked by the very small BioBeads before the excess storage solution is fully removed. At this point, the tube containing the BioBeads should be capped and kept on ice until the BioBead addition is complete, and storage solution can be added again. It is important that the Biobeads do not become dry at any point before additions, as this will prevent them from adsorbing and removing detergent.

29. Rather than attempting to weigh out very small amounts of BioBeads, we use the general rule-of-thumb that four average-sized BioBeads equals ~1 mg.
30. During BioBead additions and incubation, the reconstitution should be stirred very gently such that the BioBeads are moving around in the tube. However, be careful not to introduce air bubbles or break up or damage the Biobeads (*see* also **Note 27**).
31. The amount of reconstituted material added to the crystallization tube is yet another variable that can be screened to optimize crystallization. Given the reconstitution conditions of 500 μg Ca^{2+}-ATPase in an ~500 μL total reconstitution volume, we have had the most success using 50–75 μg of Ca^{2+}-ATPase (50–75 μL of reconstituted material) following the described crystallization protocol.
32. Following this protocol, there will be ~400 μL of material remaining per reconstitution after this step. The remaining reconstitution can be stored long-term for subsequent Ca^{2+}-ATPase activity measurements or future crystallization trials. First, add 267 μL of the stock 50 % sucrose storage buffer to a screw-cap Eppendorf tube (or an appropriate amount of stock 50 % sucrose storage buffer to achieve a final sucrose concentration of 20 %). The sucrose acts to stabilize the reconstituted vesicles during the freeze–thaw process. Second, add the 400 μL of remaining reconstitution. Third, mix the storage buffer and the reconstitution well by gently pipetting up and down. Once fully mixed, the mixture can be flash-frozen in liquid nitrogen by submerging the sealed screw-cap Eppendorf tube. Store at −80 °C.

    The addition of sucrose to the reconstitutions for long-term storage must be addressed when using the vesicles for future crystallization trials, as the sucrose can interfere with the freeze–thaw cycles and vesicle fusion. Also, the dilution of the reconstitution by the addition of the sucrose storage buffer will affect the total volume containing 50–75 μg of Ca^{2+}-ATPase (*see* **Note 31**). To use the frozen reconstituted vesicles in future crystallization trials, the vesicles must first be washed to remove the sucrose. Wash a volume containing 50–75 μg of Ca^{2+}-ATPase by adding it to a screw-cap Eppendorf tube containing 1.2 mL of Crystallization buffer and mixing thoroughly by

gently pipetting up and down. Spin the mixture at 40,000 × *g* for 40 min at 4 °C to pellet the reconstituted vesicles. Carefully aspirate the supernatant to waste without disturbing the vesicle pellet. Add 200 μL of Crystallization buffer to the vesicle pellet and resuspend the pellet by gently pipetting up and down. The crystallization protocol can now proceed as outlined from **step 4** of the Ca^{2+}-ATPase Crystallization section.

33. TG is not required for Ca^{2+}-ATPase crystallization, however, we have had the most success with 2D crystal formation, quality, and frequency using this Ca^{2+}-ATPase inhibitor (Fig. 3). As a crystal-screening variable, TG can be replaced by other Ca^{2+}-ATPase inhibitors, such as cyclopiazonic acid [26], or the crystallization conditions might exclude Ca^{2+}-ATPase inhibitors. Note that this latter crystal screening option of no Ca^{2+}-ATPase inhibitors greatly decreases crystal formation, quality, and frequency.
34. While tapping the screw-cap Eppendorf tube to mix the small volume of Crystallization buffer and added TG, the vesicle pellet may detach from the tube wall. If this occurs, the vesicle pellet will likely remain intact and the proteoliposomes will not disperse at this stage. From experience, we generally consider a vesicle pellet that releases from the tube wall during this step to be a sign of a good reconstitution that will behave and resuspend nicely in the following freeze–thaw steps.
35. When freezing the contents of the crystallization tube, ensure that the liquid volume remains at the bottom of the tube. This can be performed by keeping the crystallization tube upright for the first few seconds of submersion in liquid nitrogen, such that the crystallization volume is at the bottom of the tube (we typically use forceps to submerge and remove the tube, as opposed to simply dropping the tube into liquid nitrogen).
36. To prevent liquid nitrogen burns, use extreme care to ensure that all liquid nitrogen has been gently tapped off of the surface of the crystallization tube before handling. Also, thaw the crystallization tube contents slowly by alternating a few seconds between on-ice and between the thumb and forefinger (to prevent the contents from warming up too much). Gently tap the tube to ensure that the contents are fully thawed (Fig. 2).
37. As with all pipetting in the experiment, do not produce any air bubbles during resuspension. Also, use care to ensure the crystallization tube contents do not warm up too much by alternating between pipetting and placing the tube on-ice. Some pieces of the pellet might not resuspend easily. If some pellet pieces remain after ~1 min of pipetting, it is likely best to discontinue trying to resuspend such pieces and continue to the next step of the experiment.

38. When we prepare frozen-hydrated electron microscopy grids, we often have to dilute the crystals using Crystallization buffer to achieve an optimal amount of material for electron cryo-microscopy screening and imaging. This is why we advise storing the extra Crystallization buffers at 4 °C with the crystallization tubes. As a general procedure, we estimate the number of crystals per grid square during the initial screening by counting the number of crystals on a minimum of 20 grid squares containing "material" (crystals, vesicles, or precipitated material) and then calculating the average number of crystals per grid square [25]. To prepare frozen-hydrated crystal grids, we dilute the crystals using this average as a guide to achieve an approximate frequency of 1 crystal per grid square and immediately apply the diluted crystals to the grid and plunge the grid in liquid ethane.

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