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General qPCR and Plate Reader Methods for Rapid Optimization of Membrane Protein Purification and Crystallization Using Thermostability Assays

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Abstract

This unit describes rapid and generally applicable methods to identify conditions that stabilize membrane proteins using temperature-based denaturation measurements as a proxy for target time-dependent stability. Recent developments with thiol-reactive dyes sensitive to the unmasking of cysteine residues upon protein unfolding have allowed for routine application of thermostability assays to systematically evaluate the stability of membrane protein preparations after various purification procedures. Test conditions can include different lipid cocktails, lipid-detergent micelles, pH, salts, osmolytes, and potential active-site ligands. Identification and use of conditions that stabilize the structure have proven successful in enabling the structure determination of numerous families of membrane proteins that otherwise were intractable.

Keywords

thermostability; membrane proteins; dye-based assay

INTRODUCTION

There are predicted to be 6718 membrane proteins in the human genome (Niesen et al., 2007), making up 23% of the human genome, of which 2925 are unique integral α-helical transmembrane proteins that cross the membrane with two or more helices (Pieper et al., 2013). Membrane proteins account for 60% of current drug targets (Sanders and Myers, 2004), but their structure determination lags far behind that of soluble proteins. This lag is due to a number of reasons, including a greater difficulty in expressing, purifying, and crystallizing membrane proteins, as well as unstable and aggregation-prone samples and structural flexibility. A number of advances have gained recent success by focusing on stabilization of membrane proteins. One noted approach is to stabilize proteins in a more native-like environment with lipid mesophase crystallization (Caffrey and Cherezov, 2009), crystallization in bicelles (Ujwal and Bowie, 2011), and the use of facial amphiphiles (Zhang et al., 2007). Another approach is the use of fusion tags, especially in loops located between transmembrane helices that can stabilize the membrane protein and make crystallization contacts. Thermostability optimization can also provide a useful proxy for

identifying stabilizing conditions at purification temperatures with the overall goal of stabilizing the protein and compensating for loss of the lipid environment being similar.

Membrane Protein Purification for Structural Studies

Despite advances in nanoscale crystallization protocols, structural techniques still often require large amounts of material over the course of a successful structure determination, which is often difficult to obtain because of the problems detailed above.

X-ray crystallography and several other biophysical techniques usually require milligrams of very highly pure membrane protein for successful analysis. Heterologous overexpression of membrane proteins in *Escherichia coli* (Sahdev et al., 2008), *Lacto-coccus*, *Saccharomyces cerevisiae* (Hays et al., 2010), *Pichia pastoris* (Ramon and Marin, 2011), *Sf*9 (Possee, 1997), and human embryonic kidney (HEK; Chaudhary et al., 2012) cells have been successfully used to determine membrane protein structures, as have various native sources. Usually, purification proceeds from isolated membranes. Briefly, after cell harvesting and lysis in an appropriate lysis buffer, cell debris is removed with a 10,000 × *g* centrifugation for 10 min. Following debris clearance the lysate is centrifuged at 100,000 × *g* for 1 to 1.5 hr to isolate membranes. Often, proteins are purified from these isolated membranes and captured using some variant of affinity chromatography (Hays et al., 2010). Normally, this is followed by a single polishing purification step such as reverse affinity chromatography (where the original tag used for purification is removed via proteolysis then passed through the original affinity matrix, leaving only untagged protein), or size-exclusion chromatography. Once suitable criteria for purity (usually >95% as judged by SDS-PAGE) and homogeneity as judged by monodispersity on a size-exclusion column are met, further purification steps are normally not employed, to avoid excessive sample loss. Typically, target proteins are often concentrated to at least ~5 mg/ml for crystallization trials (McPherson, 1982), which can be problematic with certain integral membrane targets because of the increased likelihood of aggregation.

Thermostability Assays

Alteration of thermostability profiles has been successfully used to improve every step in the structure determination pipeline, including extraction, purification, and crystal optimization (Ericsson et al., 2006). While direct relationships between thermostability and crystallization probability are difficult to establish, a rough heuristic estimate concluded that identification of stabilizing conditions of soluble proteins correlates to a doubled propensity to crystallize in common crystallization screens (Ericsson et al., 2006). A caveat is that it is unclear whether optimization of buffer conditions limits the conformational flexibility sometimes required to obtain a suitable conformation for crystallization. In these cases, thermostability assays may be considered a guide to vary multiple parameters for crystallization trials. In many cases, thermostability optimization can be useful where instability limits the production of protein before crystallization trials. Buffer optimization following thermostability assays has assisted in the structure determination of multiple types of integral membrane proteins including GPCRs (Cherezov et al., 2007), transporters (Mancusso et al., 2011; Pedersen et al., 2013; Waight et al., 2013), and ion channels (Hattori et al., 2012).

A number of approaches can be employed to obtain thermostability profiles, and each varies as to how the protein is heat-denatured and the specific detection method used to measure protein unfolding. They all are similar in that they maintain the basic premise of identifying a baseline melting temperature and screen variations of purification conditions to improve the stability of the target with the assumption that the behavior during purification will likewise be improved by using similar changes.

Biophysical Properties as Proxies for Melting

Protein denaturation can be assayed in a number of ways, but special considerations must be taken into account when working with membrane proteins because of their hydrophobic environment. Melting curves of soluble proteins are most commonly performed with a dye such as SYPRO orange (Niesen et al., 2007) with fluorescent properties that change when exposed to an environment with a different dielectric constant, in this case that of the solubilizing buffer and that of interior hydrophobic core of a soluble protein. As the protein unfolds, internal hydrophobic residues become exposed, yielding a signal that can be measured as a proxy for unfolding. The use of stabilizing detergents and lipid-containing samples, as well as the hydrophobic makeup of integral membrane proteins preclude the use of dyes sensitive to the hydrophobicity of a sample. The identification of dyes that react with specific thiol groups (from cysteines) that become exposed upon unfolding (Ayers et al., 1986; Alexandrov et al., 2008) overcame this limitation and these dyes are now routinely used with integral membrane proteins. The lower relative availability of thiol groups in proteins relative to other potential active groups such as amines and carboxylates make cysteine the preferred target for reactive dyes. Furthermore, cysteines have a propensity to be located at the interface of packing helices in integral membrane proteins, making them a sensitive reporter for the unfolding of integral membrane proteins (Alexandrov et al., 2008). Other assays that exploit the natural behavior of an integral membrane protein, such as ligand binding, have also been successfully used to measure protein unfolding (Serrano-Vega et al., 2008). Finally, dynamic light scattering, isothermal calorimetry, and circular dichroism all provide widely used biophysical tools compatible with integral membrane proteins and are widely used to assay unfolding.

The use of thiol-reactive dyes requires that all the surface-exposed cysteine residues first be blocked with an acetylating agent such as iodoacetamide to avoid a high background signal (Alexandrov et al., 2008). Upon unfolding during the assay, internal cysteine residues become exposed and conjugated to the thiol-reactive dyes that are often coumarin-based, such as 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM; Alexandrov et al., 2008; Vedadi et al., 2006) (Fig. 29.11.1). Non-dye-based melting assays can also provide useful thermostability data, such as those using differential right-angle light scattering (Senisterra et al., 2006; Vedadi et al., 2006) to measure scattering from larger aggregated assemblies upon denaturation. However, these assays require specialized instrumentation.

There is great advantage to conducting thermal assays in a multi-well high-throughput format. New dyes that take advantage of the accessibility of standard qPCR machines can be used, since the instruments are already equipped to run thermal cycles in multi-well plate formats. Commercial qPCR instruments commonly contain filters for ~500 nm to 750 nm.

The emission of the CPM dye, however, does not overlap well with the filters that are a standard part of many instruments. Thus, if using qPCR machines, it is important to try to use a dye with fluorescent properties that match the limits of the wavelength ranges available in a given instrument. Recently, a CPM-like dye, called the “Membrane Protein PTS dye” system, was created by Life Technologies (available by special order, cat. no. 4488971), which is easily adapted to feed directly in to the qPCR detector systems.

Successful thermostabilizing conditions will typically shift the melting temperature upwards by 4°C to >20°C. As a useful rough empirical estimate, a >4°C shift in melting temperature corresponds to binding of a ligand with a K_d of approximately <1 μM (Vedadi et al., 2006). Reliable affinity constants are obtainable from thermostability assays, but require a determination of unfolding enthalpy to calculate (Matulis et al., 2005).

Thermostability can be used to screen for ligands of any protein, and can form the basis for screening drug target proteins using ligand-based and fragment-based libraries.

Types of Thermostability Assays

Thermostability can either be measured using melting temperature (T_m), by steadily increasing the temperature (ramp method; Basic Protocol 1), or using isothermal analysis (Basic Protocol 2) by keeping the temperature steady at a high but not completely unfolding temperature and measuring target half-life (i.e., the time before half the sample becomes unfolded; Sonoda et al., 2011). Additional information can be obtained by following loss of A_{280} or GFP fluorescence signal with size-exclusion chromatography analysis upon partial (50%) heat denaturation. Each method has pros and cons making them beneficial and complementary in different circumstances.

Analysis at a single melting temperature to identify thermostabilizing buffer conditions or ligands has the advantage of providing a physically meaningful number, the T_m, over other methods. By measuring unfolding enthalpy (ΔH) with isothermal calorimetry, unfolding temperatures can be correlated to binding affinity, providing a useful way to identify ligands or ligand fragments that bind an active site or other allosteric binding site. Relative changes in melting temperatures can also provide relative ligand affinities in the absence of enthalpy measures and are also useful for identifying the most meaningful conditions for stabilizing a given protein. Finally, sensitivity to unfolding of multiple domains or binding partners allows for the stabilization of complicated proteins or assemblies, processes which are masked with other techniques.

Cons of the temperature ramp unfolding method can include difficult-to-interpret curves, especially in cases with especially unstable integral membrane protein that unfolds below the experimental range of the assay. It is estimated that 33% of soluble proteins yield uninterpretable results in thermostability assays (Dupeux et al., 2011), a number which is likely higher for integral membrane proteins.

Isothermal melts can be very useful in cases where protein instability precludes identifying a reasonable T_m from temperature ramp unfolding data. Even in cases with partial unfolding prior to experimental analysis, melting half-life data can give useful trends of low

temperatures that can then be employed to stabilize a given protein and identify reasonable starting conditions for purification.

Cons of the isothermal method include inability to differentiate melting of multiple domains, as everything is represented in a single, global half-life. In addition, screening for potential ligands is difficult, since identifying a saturating point for ligand concentration is problematic.

Pros to the size-exclusion method include the ability to assay stabilizing conditions in relation to both amounts of denatured protein and to protein behavior as judged by a protein profile. SEC traces can serve as a useful measure of sample homogeneity, which can be of utmost importance for crystallization trials. Identification of stabilizing conditions that also improve sample homogeneity provide a highly useful way of identifying purification conditions suitable to initiate crystal trials, especially since sample homogeneity can be a large problem with integral membrane proteins.

Cons of the SEC denaturation assay relate to the low throughput and long analysis times. Since each run requires a separate SEC run, an average of 1 condition per hour can be tested. Multi-channel SEC systems with different buffers, or small-volume SEC columns, can be useful in these cases to improve throughput.

RAMP METHOD TO MEASURE MEMBRANE PROTEIN THERMOSTABILITY USING EITHER CPM DYE OR PTS DYE IN HIGH-THROUGHPUT FORMAT

The ramp method of protein thermostability analysis is useful because it provides a physically meaningful number, the apparent melting temperature (T_m), which can be altered with different conditions. It has a particularly interesting use in identifying tight binding ligands or inhibitors, possibly for identifying novel small molecules such as substrates or inhibitors, which is a common use for soluble proteins in the pharmaceutical industry. The assay can be performed on spectroscopic instruments with heat blocks or could be carried out in a higher-throughput format in multi-well plates on standard qPCR instruments (Fig. 29.11.2).

Materials

Isolated membranes for protein purification (see above, Membrane Protein Purification for Structural Studies)

Iodoacetamide

CPM dye (Life Technologies)

Dimethyl sulfoxide (DMSO)

PTS dye (Life Technologies)

Test buffer (e.g., test conditions varying pH, salt, osmolytes, lipid, and ligands)

Method for concentrating protein (e.g., spin concentrator, stir cell)

Method for determining protein concentration (e.g., BCA assay, A280 absorbance)

qPCR instrument or cuvette fluorometer

Aluminum foil

Software (GraphPad/Prism from GraphPad Software, Excel/Gnu plot)

Prepare the protein sample

1. Isolate membranes using standard membrane protein purification protocols (e.g., Hays et al., 2010). In the detergent solubilization step, add 1 mg iodoacetamide per 1 ml of membrane suspension and stir vigorously (can be concurrent with solubilization).

   This obviates the need for a reducing agent and is compatible with crystallization and structure determination.

2. Prepare the purified sample under desired purification conditions and concentrate protein to >0.1 mg/ml in preparation for the assay.

   A size-exclusion step is not necessary for the protocol, and omitting it can conserve protein if yields are very low.

   Purification buffer should be selected to ensure compatibility with the thermostability assay, namely the exclusion of reducing agents such as DTT or 2-ME. TCEP is a suitable alternative compatible with most coumarin dye-based assays. To ensure the lowest variability of buffer pH with changes in temperature, a buffer with thermally constant pKa, such as sodium phosphate, can be selected if desired.

Determine membrane protein thermostability

3 Prepare the appropriate dye for the assay; here the CPM procedure with a cuvette fluorometer is described adapted from Alexandrov et al. (2008). Dissolve CPM dye into DMSO at 4 mg/ml.

   The stock solution can be frozen up to a few months at −80°C. The dye should be kept encased in aluminum foil to prevent photobleaching and should be stored in single-use aliquots. It can be beneficial to consider using a control protein of known melting behavior to control for dye quality. In the alternate microplate approach, either the CPM dye mixture can be used if an instrument with appropriate optical filters is available, or the membrane protein PTS dye (Life Technologies) can be used with most qPCR instruments. Membrane protein PTS is sold as a 1:1000 (v/v) mixture with DMSO.

4 Mix CPM dye stock into a 1:40 stock (v/v) from the original 4 mg/ml solution into the protein purification buffer (with detergent) for a final concentration of 0.04 mg/ml.

   The membrane protein PTS dye should be diluted to 1:20 (v/v) final concentration.

5 For the cuvette fluorometer method, add 10 µl of 1:40 CPM dye mixture to 120 µl of test protein diluted in test buffer to attain a total volume of 130 µl. Proceed quickly at this step, especially with unstable proteins, to eliminate background. For the high-throughput microplate method, mix protein and test buffer for final volume of 20 to 50 µl.

Appropriate final concentration of protein will vary for each target but will range between 0.025 and 0.5 mg/ml. It is recommended to test a range of concentrations prior to assaying different test conditions, to establish the minimal protein concentration that will give melting curves with a clear transition and to ensure that the measurement is not outside of the linear window of detection for a given instrument.

6 Set up the software for a thermal assay, before adding protein, with a temperature ramp beginning at 20° to 30°C and ending at 80° to 90°C over a period of 25 min to 2 hr, depending on instrumentation. Set excitation and emission wavelengths at 387-nm excitation/463-nm emission for the CPM assay and 450-nm excitation/500-nM emission for the membrane protein PTS assay.

Practical experience has shown that longer ramp protocol times can lead to qualitatively better curves in some cases and should be considered if curve quality needs improvement.

Analyze the data

7 Visually inspect each curve to identify outliers (i.e., flat curves, curves lacking a defined melting transition, curves denoting bubbles as evidenced by an extremely rapid jump in fluorescent signal; Fig. 29.11.2). Select a region with a pronounced transition for analysis.

8 Analyze the melting temperature with appropriate software (e.g., GraphPad/ Prism from Graphpad Software, San Diego, CA/ Excel/ Gnu plot) to identify the midpoint of the transition with an analysis of the 1st derivative or fit to a Boltzmnn two-state sigmoid model (see Support Protocol).

Melting curves may not show increasing or decreasing traces near the beginning or end of the curves, due to competing effects such as protein aggregation. Normally, a reasonable endpoint can still be determined; however, visual inspection of $T_m$selection is necessary to ensure that an adequate transition is determined, and is recommended for each condition assayed.

ISOTHERMAL METHOD TO DETERMINE MEMBRANE PROTEIN THERMOSTABILITY USING CPM OR PTS DYE IN qPCR FORMAT

An alternative to creating a melting curve is to use an isothermal melt where temperature is held steady at a denaturing temperature and the half-life is measured under differing test conditions, pH, ligands, etc. (Sonoda et al., 2011). Data fitting to an exponential decay equation can yield consistent half-life values independent of normalization, offering a robust

measure of target stability. As in the case of the temperature ramp, the experiment is best performed in triplicate when possible, and benefits from a high-throughput approach. The isothermal method has several advantages over temperature ramps in the case of protein that is extremely unstable (i.e., a melting temperature around room temperature) where a proper melting curve is unavailable. The isothermal method will work on any temperature-controlled fluorescent plate reader with appropriately selectable wavelength.

Careful consideration must be paid to determining the temperature used in the isothermal method. Empirical evidence from personal experience has shown that 40°C is a good starting point; however, the method should be tested with a control prior to assaying samples. Following this information, the melting temperature can be adjusted upward in the case of insufficient melting (i.e., melts with a half-life > 2 hr) or downward in the case of overly fast melting that precludes reliable fit of the data (i.e., melts with a half-life < 5 min).

Materials

Isolated membranes for protein purification

Iodoacetamide

CPM dye (Life Technologies)

Dimethyl sulfoxide (DMSO)

Test buffer or ligand solutions (varying pH, salt, lipid, and/or ligands)

Method for concentrating protein (e.g., Millipore spin filter, stir cell)

Method for determining protein concentration (BCA assay)

Aluminum foil

Multichannel pipets

Temperature-controlled fluorescent plate reader with selectable wavelength

Software (GraphPad/Prism/Gnuplot/Excel)

Prepare the protein sample

1. Isolate the membranes using standard membrane protein purification protocols. In the detergent solubilization step, add a molar excess of iodoacetamide to cysteine residues, empirically 1 mg iodoacetamide per 1 ml of membrane suspension (5.4 mM final, which is 5000× molar ratio over protein at ~0.1 to 1 μM concentration, has been determined to work well with the assay) and stir vigorously (can be concurrent with solubilization).

   This obviates the need for a reducing agent and is compatible with crystallization and structure determination.

2. Prepare purified sample as normal, concentrate protein to >0.1 mg/ml final concentration as determined by BCA assay or A_{280} absorbance.

A size-exclusion step is not necessary for the protocol, and omitting it can conserve protein if yields are very low. Generally, protein concentration for the assay must be selected empirically to identify a concentration that produces an interpretable signal. A final concentration of 0.05 mg/ml protein will typically yield useful denaturation curves; however, a test analysis with a range from 0.01 to 0.5 mg/ml may be useful to determine the optimal protein concentration for the assay.

Measure thermostability

3 Dissolve CPM dye in DMSO at 4 mg/ml. Stock solution can be frozen up to a few months at −80°C. Keep the dye vials encased in aluminum foil to prevent photobleaching.

4 Add dye mixture to the plate with a multichannel pipet (for a 100-µl total reaction, add 2.5 µl).

5 Add appropriate test buffer/ligand solutions to the dye mixture. Add protein last, bringing the total volume to 100 µl.

It is critical that the test buffer contain detergent to help keep protein stable.

6 At this step, move rapidly especially for unstable proteins to eliminate background. Set the excitation wavelength at 387 nm, and 463 nm for emission.

7 Add protein and mix by pipetting up and down, being careful not to introduce bubbles.

Analyze the data

8 Visually inspect each curve to identify outliers (i.e., flat curves, uninterruptable curves).

9 Analyze melting temperature with Excel or GraphPad/Prism/Gnuplot and fit to an exponential decay curve [$F(x) = a * \exp(-x/b) + c$] to calculate half-life $t½$ [$t½ = b * \ln(2)$] (Fig. 29.11.3) (see Support Protocol).

SIZE-EXCLUSION CHROMATOGRAPHY (SEC) PROFILE-BASED THERMOSTABILITY MEASUREMENT OF MEMBRANE PROTEINS

A third method for assaying thermostability involves measuring the changes to a size-exclusion profile of a target protein before and after heating in the presence of various test buffer or other conditions (Hattori et al., 2012; Mancusso et al., 2011). Although providing much lower throughput, this method has advantages over plate-based methods in that it can also be used to measure sample heterogeneity and requires no special equipment.

Materials

Membrane protein sample

Test buffers—including variations of pH, salt, lipids, and ligands

FPLC instrument with A_{280} optical absorbance detector

In-line fluorometer (for GFP detection assay)

Size exclusion chromatography column (e.g., GE Superdex S200, Shodex, GE Sephacryl column, or TSK-3000)

Heat block

0.22-μm filter *or* centrifuge filter

Prepare protein sample

1. Protein can either be conjugated to green fluorescent protein (GFP) as part of a fusion construct or assayed with A_{280} absorbance.

*Note the GFP-conjugated protein requires special equipment, notably an in-line fluorometer for a size-exclusion system, but can be performed with much lower amounts of material. For design of the fusion construct, special consideration should be given to design of the GFP fusion construct. A suitable construct design will include an N-terminal placement of the GFP following a several-amino-acid linker (Glycine-Serine repeats are a suitable choice). N-terminal placement of GFP also has the added advantage as a marker for protein production. GFP will not fold properly as an N-terminal construct without proper folding and membrane insertion of the target protein (*Alexandrov et al., 2008*), providing a sensitive marker for proper protein production. GFP use is applicable in a wide range of temperatures owing to its high melting temperature of 83°C (*Stepanenko et al., 2008*). Molecular concentrations as low as 10 μg/mol of expressed membrane protein-GFP fusions have been detected reliably. In general, the notion is that the target protein will unfold at a certain melting temperature, but the GFP at another, generally much higher temperature (*Stepanenko et al., 2008*). Thus, the first transition will generally signal unfolding of the target. Isolate membranes as described above (see Membrane Protein Purification for Structural Studies) using standard membrane protein purification protocols*.

Perform thermostability assay

2 Separate the protein sample into multiple equal aliquots to be tested.

The number of samples will depend on the number of desired test conditions.

3 Perform control SEC injection with unheated sample to obtain base peak height.

4 Perform heat denaturation in a standard heat block. After heating, filter or centrifuge the sample and rerun the SEC.

Denaturation requires identification of a temperature and time sufficient to lose one half of the peak height on subsequent SEC analysis.

It may be necessary to perform a few different incubations for different durations and at different temperatures to determine appropriate conditions. A reasonable starting point is 50 °C for 10 min.

5 Incubate the remaining samples at the determined test conditions and determine sample height recovery for each.

Analyze data

6 Analyze the peak heights and profiles (see Support Protocol).

Ideally, good thermostabilizing conditions will stabilize close to 100% of the original, unincubated peak height and retain or improve heterogeneity as judged by peak profile.

DATA PROCESSING: IMPLEMENTING THERMOSTABILITY RESULTS AND TRENDS

Fluorescence intensity data points versus temperature output from the qPCR instrument can be processed with standard analytic packages including GNUPLOT, GraphPad Prism, or Excel. Alternatively, dedicated software packages, including the protein thermostability software from Life Technologies, can be utilized. After visual inspection of unfolding curves to ensure lack of mitigating factors such as sharp peaks from bubbles, the curves can be analyzed for either a first-derivative function or fit to a two-state Boltzmann model. Equations 29.11.1-29.11.4 describe the Boltzmann equation and its use in determining T_m (adapted from Matulis et al., 2005). The Boltzmann two-state model fits the Boltzmann equation (Eq. 29.11.1) to the entire fluorescence intensity curve to solve for T_m.

$$F(x)=a_U+\frac{a_F-a_U}{1+e^{-\Delta_U G_{(T)}/RT}} \quad \text{Equation 29.11.1}$$

where a_U is the maximum curve asymptote value of unfolded protein output as either photon counts or normalized fluorescent units, a_F is output as either photon counts or normalized fluorescent units is the minimum curve asymptote value of folded protein, and x_0 is the curve inflection point (i.e., the T_m). Normally, the asymptotic value for unfolded and fully folded protein is difficult to determine because of nonzero slope in these areas of the curves. Therefore, they must be corrected by modeling them as linear functions:

$$y_{F(T)}=y_{F,T_m}+m_F\,(T-T_m) \quad \text{Equation 29.11.2}$$

$$y_{U(T)}=y_{U,T_m}+m_U\,(T-T_m) \quad \text{Equation 29.11.3}$$

where y_{F,T_m} is the fluorescence intensity of folded protein, $y_{U,\,T_m}$ the fluorescence intensity of unfolded protein, and m_F and m_U are linear slopes of the folded and unfolded segments of the curve. To utilize Equation 29.11.1 to solve for T_m, the Gibbs free energy term must be expressed in terms of T_m using Equation 29.11.4:

$$\Delta_U G_{(T)} = \Delta_U H_{(T)} - T\Delta_U S_{(T)} = \Delta_U H_{T_r} + \Delta_U C_P (T - T_R) - \left[\Delta_U S_{T_r} + \Delta_U C_P \ln\left(\frac{T}{T_r}\right)\right] \quad \text{Equation 29.11.4}$$

where $\Delta_U H_{(T)}$, $T\Delta_U S_{(T)}$, and $\Delta_U C_P$ are the enthalpy, entropy, and heat capacity, respectively, of unfolding at a given temperature T_r, chosen here to be near the transition melting temperature T_m. Incorporating Equations 29.11.2 and 29.11.3 into Equation 29.11.4 yields the final relationship (Equation 29.11.5):

$$F(x) = y_{F,T_m} + m_F (T - T_m) + \frac{y_{U,T_m} - y_{F,T_m} + (m_U - m_F)(T - T_m)}{1 + e^{-\left[\Delta_U H_{T_r} + \Delta_U C_P (T - T_R) - \Delta_U S_{T_r} + \Delta_U C_P \ln\left(\frac{T}{T_r}\right)\right]/RT}} \quad \text{Equation 29.11.5}$$

Solving for the parameters y_{F,T_m}, y_{U,T_m}, m_U, $\Delta_U H_{T_r}$, T_m using a least square residual method will identify T_m. For more information and a derivation of Equations 29.11.1-29.11.4, see Matulis et al. (2005).

A first derivative of the fluorescence intensity can also be calculated (Eq. 29.11.6) and provides a more straightforward identification of the T_m, which is taken to be the peak of the first derivative plot.

$$F(x) = \frac{y_P}{d(x)} \quad \text{Equation 29.11.6}$$

where y_p is fluorescence intensity.

Both the Boltzmann method and first-derivative method can be used for melting temperature assessment. For ease of calculation and the added benefit of visual verification of transition temperatures, the first derivative method is preferable and sufficient in simple cases where a melting temperature is assessed in multiple conditions. The Boltzmann equation may be used when a more detailed thermodynamic model is needed (i.e., when Gibbs Free Energies need to be calculated).

For isothermal melts, data should be fit to a single exponential decay model (Equation 29.11.7) where:

$$F(x) = ae^{-x/b} + c \quad \text{Equation 29.11.7}$$

to calculate half-life (Eq. 29.11.8):

$$t\frac{1}{2} = b \times ln(2) \quad \text{Equation 29.11.8}$$

and the half-time calculated as described in Basic Protocol 1.

COMMENTARY

Background Information

Difficulties arise at every stage of membrane protein crystallography, including expression, membrane extraction, purification, and crystallization. Many problems encountered while working with membrane proteins are due to the inherent instability of extracting a highly hydrophobic protein from a lipid environment into a detergent micelle, as well as the inherent flexibility of membrane proteins partially arising from the loss of lateral pressure upon extraction (Alexandrov et al., 2008). A large amount of effort is normally put into creating more stable preparations as a way to improve membrane protein behavior, resulting in higher amounts of extracted protein, less aggregation, sharper size-exclusion chromatography (SEC) profiles, increased protein concentrations, and improved crystallization behavior.

Critical Parameters

For these studies, the protein should be purified to at least <95% purity. All membrane protein purification and handling procedures should be performed without delay to minimize time-dependent degradation, and all solutions and plates should be kept on ice. Lipids or osmolytes such as glycerol can be used to stabilize especially aggregation-prone material since they are compatible with thermostability assays. Additionally, care should be taken with pipetting into 96-well plates to minimize the formation of bubbles, which can collapse and cause spikes in fluorescence data and complicate data analysis, and which are of special concern due to the presence of detergent in the purification conditions.

Troubleshooting

Table 29.11.1 describes some commonly encountered problems when performing the protocols described in this unit, along with explanations of likely causes and recommendations for overcoming or avoiding these problems.

Anticipated Results

Many proteins purified following thermostability optimization show improvements in solubility, aggregation behavior, and heterogeneity as judged by SEC on columns appropriate for membrane proteins, including Superdex, Sephacryl, and TSK. In one example, a eukaryotic intra-membrane protease was identified with very high expression yields but with extreme difficulty in purification (T. Tomasiak, unpub. observ.). Final purified protein was difficult to concentrate above 10 mg/ml, showed a large void peak on size exclusion, and visibly aggregated overnight at 4°C. Thermostability testing determined that a lower pH, increased ionic strength, and addition of osmolytes would optimally stabilize the protease. Individual testing of each variable identified the pH and salt as being the most crucial. Following optimization in the new conditions, the protein SEC profile improved dramatically; it could be concentrated to 30 mg/ml and could be stored for several days at 4°C.

Most thermostabilizing conditions are best utilized as a guide for changing purification conditions to improve protein behavior. Trends in pH, salt concentration, lipids, detergents

and amphiphiles, osmolyte concentration, and ligand identity should be examined and implemented on a small scale to ensure that observed trends are also found during purification. For example, many integral membrane proteins tend to be maximally stabilized with a buffer pH slightly lower than neutral (6.0-6.5); a buffer optimized to these results might work best at that pH or one in between the starting pH and target. It is often advantageous to test multiple variables, one at a time, on a small scale to identify the least invasive changes. An optimal way to do this is via the high throughput qPCR approach of testing multiple conditions at once in multiple replicates on the same sample.

In combination with thermostability optimization of purification conditions, alterations to expression conditions, such as using different host growth strains, lowering growth temperatures, and varying levels of inducing agent, have become standard practice in optimizing protein production. No evidence the authors are aware of has linked growth conditions with the apparent stability of integral membrane proteins.

Time Considerations

Overall, assay preparation time can take 1 to 3 days for sample purification, ~30 min to 1 hr for assay preparation, and 20 min to 2 hr for assay measurement. This method can be powerful in screening many possible conditions and can benefit protein structural characterization with front-end time prior to extensive purification.

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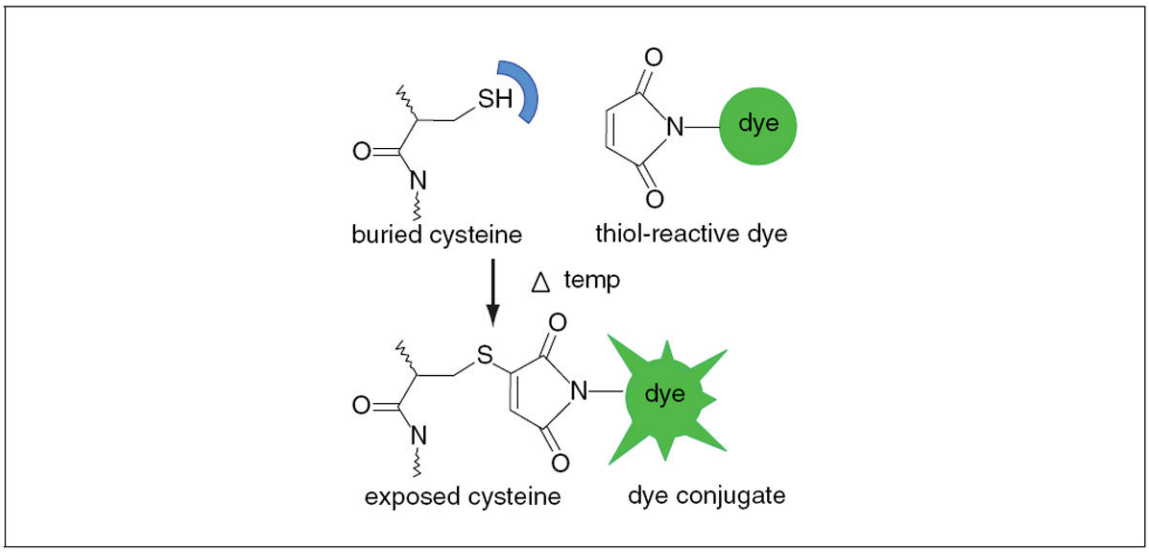

Figure 29.11.1.

Diagram of measurement of protein unfolding upon reacting with a thiol-sensitive dye. A buried thiol group on cysteine is not available for reaction with the thiol-reactive dye in a properly folded integral membrane protein. Upon heating and protein unfolding, the exposed cysteine reacts with the electrophilic dye to create a conjugate with altered fluorescent properties that can be followed and used as a proxy for protein unfolding.

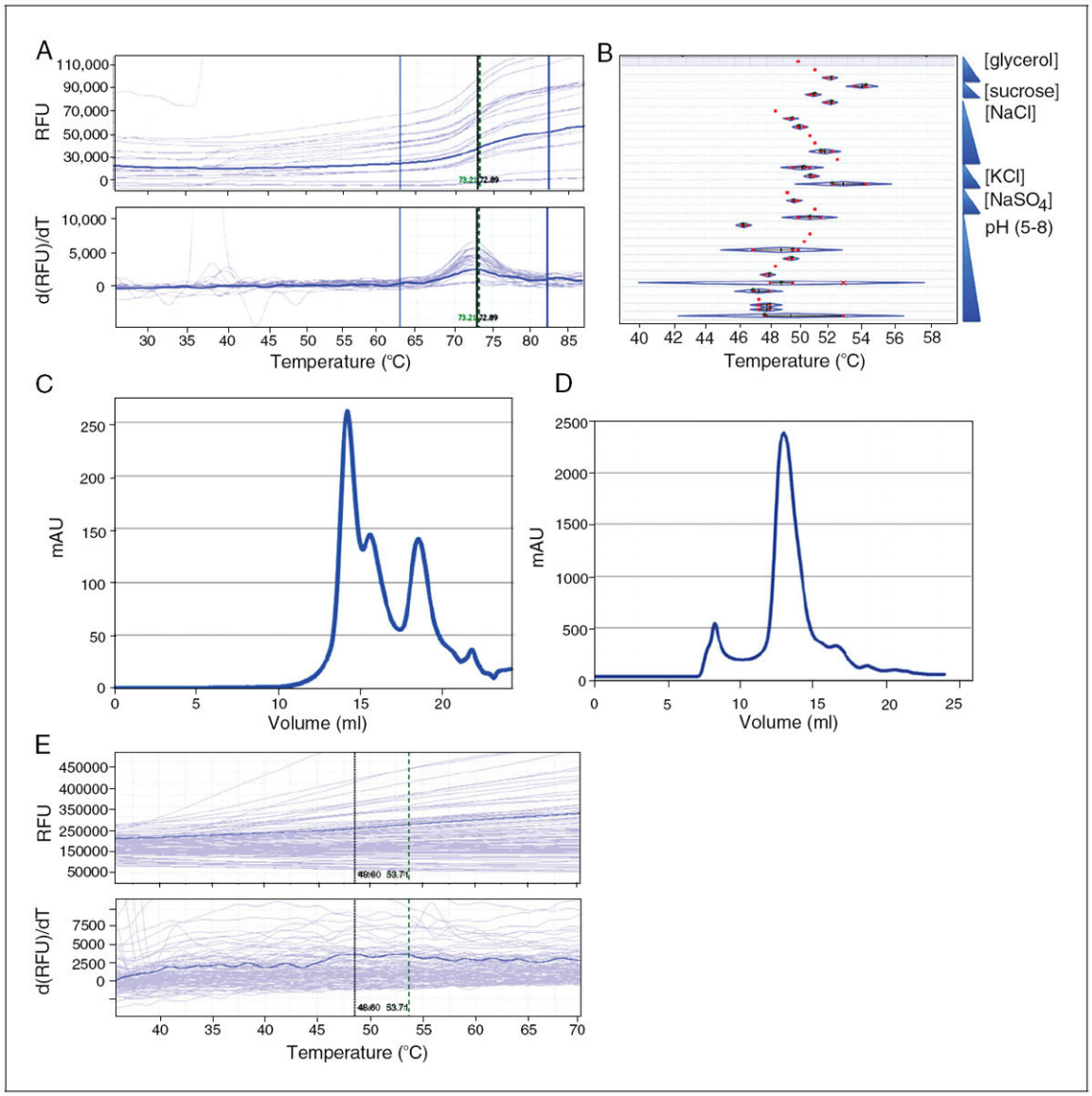

Figure 29.11.2.

Melting curve examples with thermal denaturation analysis. (**A**) Denaturation profile of a human cation channel on the Viia7 instrument (Life Technologies). (**B**) Statistical summary slide of various test conditions on a eukaryotic intramembrane protease. Experiments were performed in triplicate for each condition. Red dots indicate individual replicates, the vertical black bar represents the mean, and the blue diamonds represent 95% confidence interval of melting temperature. The Red X's signify an uninterruptable melting curve that was omitted from the final analysis. (**C**) Size-exclusion chromatography profile of a eukaryotic intramembrane protease prior to buffer optimization with thermostability testing. Note the large aggregate peak. (**D**) Purification of a eukaryotic intramembrane protease following thermostability optimization. A concomitant reduction of pH from 7.5 to 6.5 and increase of NaCl concentration from 150 mM to 400 mM resulted in a narrower profile as judged by SEC with larger amounts of extracted protein, likely due to prevention of partial

protein unfolding leading to denaturation and aggregation. (**E**) Example of an uninterpretable protein melting curve on the Viia7 instrument (Life Technologies) and analyzed with the Protein Thermal Shift (PTS) software (Life Technologies). For the color version of this figure go to http://www.currentprotocols.com/protocol/ps2911.

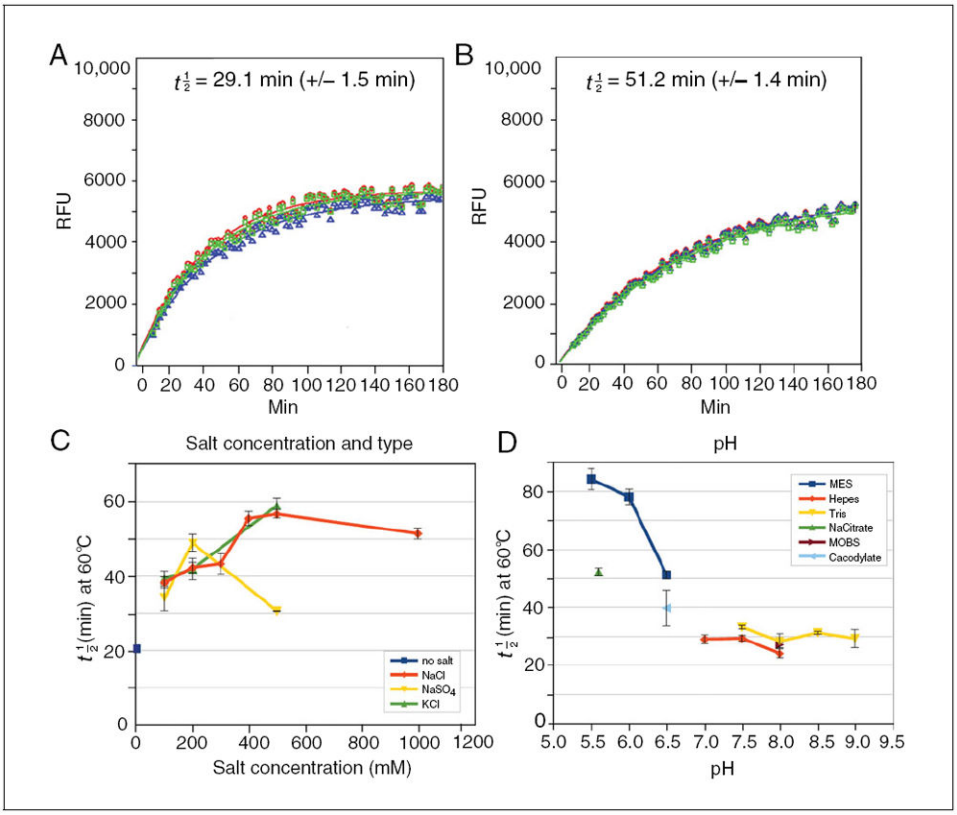

Figure 29.11.3.

Isothermal melt with analysis. (**A**) Isothermal denaturation curve of the phosphate transporter PiPT at pH 7.0. (**B**) Isothermal denaturation curve of PiPT at pH 6.5. (**C**) Analysis of isothermal half-lives of PiPT melted in varying salt conditions. (**D**) Analysis of isothermal half-lives of PiPT analyzed in buffers of different pH.

Table 29.11.1

Troubleshooting

Common problems	Possible cause	Solution
Weak, absent signal	Incorrect selection of experimental wavelength, expired dye	Ensure correct selection of filter
		Use single-use aliquots of carefully stored dye
Sharp profile spikes	Bubbles in solution	Carefully pipet well solution, perform plate centrifugation
Difficult-to-interpret curves, multiple peaks	Unstable protein sample	Use lower starting temperatures Extend ramp times
		Perform isothermal melt
		Add osmolyte to stabilize protein Perform careful visual identification of transition