Structure
Ways & Means

Microscale Fluorescent Thermal Stability Assay for Membrane Proteins

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SUMMARY

Systematic efforts to understand membrane protein stability under a variety of different solution conditions are not widely available for membrane proteins, mainly due to technical problems stemming from the presence of detergents necessary to keep the proteins in the solubilized state and the background that such detergents usually generate during biophysical characterization. In this report, we introduce an efficient microscale fluorescent stability screen using the thiol-specific fluorochrome N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) for stability profiling of membrane proteins under different solution and ligand conditions. The screen uses the chemical reactivity of the native cysteines embedded in the protein interior as a sensor for the overall integrity of the folded state. The thermal information gained by thorough investigation of the protein stability landscape can be effectively used to guide purification and biophysical characterization efforts including crystallization. To evaluate the method, three different protein families were analyzed, including the Apelin G protein-coupled receptor (APJ).

INTRODUCTION

Crystallization of membrane proteins remains a formidable challenge. Although eukaryotic expression and purification methods are appearing that allow for the generation of milligram quantities (Hanson et al., 2007; Lundstrom, 2006; Midgett and Madden, 2007), achieving stability with these molecules is perhaps the most difficult hurdle to overcome. Purification necessitates a release of the membrane protein from the lipid bilayer by detergent solubilization, a process during which hydrophobic surfaces of the protein are coated with surfactant monomers to form a protein-detergent complex (PDC). However, the detergent belt formed around the protein is a poor replacement for the lipid bilayer, as much of the lateral pressure exerted on the protein by the surrounding lipids is lost. Thus, solubilization of membrane proteins often results in destabilization, unfolding and subsequent aggregation. Analysis of the Protein Data Bank indicates that high stability is a prominent feature of integral membrane proteins of known atomic structure (Rosenbusch, 2001). The optimization of stability, in addition to other biophysical properties such as homogeneity and solubility, is likely to be predictive of successful crystallization. Precrystallization screening based on stability can substantially increase the crystallization success rate, and its utility for soluble proteins has already been demonstrated successfully (Malawski et al., 2006). Recently, fluorescent dye-based thermal stability assays for soluble proteins have gained popularity as a simple and rapid way to analyze the stability of crystallization targets in a high-throughput manner (Ericsson et al., 2006; Pantoliano et al., 2001; Vedadi et al., 2006). While extraordinarily efficient with soluble proteins, these methods are not easily applied to membrane proteins, since the presence of detergents and the highly hydrophobic nature of the proteins substantially increases the background fluorescence of the assay, and in many cases practically masks the melting transitions (Yeh et al., 2006).

For integral membrane proteins like transporters, ion-channels, and G protein-coupled receptors (GPCRs) that are very challenging to assay for activity in a detergent-soluble state, stability and cooperative unfolding can be an advantageous measure to assess the quality of the membrane protein samples under various buffer, detergent, or ligand conditions. Systematically determining conditions that promote the stability of membrane proteins is thus imperative. Historically, structural changes of proteins during heating have been studied by various analytical techniques, such as sedimentation velocity, differential scanning calorimetry (DSC), dynamic light scattering (DLS), circular dichroism spectroscopy (CD), UV/VIS spectroscopy, electrophoresis, fluorescence, and NMR. However, these techniques often require large amounts of material, are difficult to adapt to a high-throughput process, and often suffer from poor signal-to-noise ratios due to a high background from the detergents.

We have developed an efficient fluorescence-based thermal stability assay applicable to both soluble and membrane proteins, yet particularly suitable for integral membrane proteins with limited hydrophilic regions such as GPCRs. The proposed assay utilizes the accessibility of the native cysteine residues to covalent modification as readout for the unfolding process. The temperature-induced unfolding renders the cysteine residues embedded in the protein interior solvent-exposed and, thus, modifiable with a diffusable fluorescent probe. The highly reactive thiol-specific fluorochrome N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM), is well suited to the assay because it is practically nonfluorescent in its unbound form (Ayers et al., 1986). Due to their high packing value, cysteines are frequently located at helix-helix interaction sites (Eilers et al., 2002) and, therefore, are ideal sensors for the overall integrity of the membrane protein structure.

To demonstrate the utility of this method, we have evaluated proteins from three distinctly different protein families using

this assay. β-lactoglobulin (β-LG), a model lipophilic protein with a well-understood thermal behavior (Roefs and De Kruif, 1994) is used here as a control to validate the assay. The applicability of the method is further demonstrated for stability studies of the monotopic membrane protein fatty acid amide hydrolase (FAAH), whose structure has been solved (Bracey et al., 2002), as well as of the human apelin (APJ) receptor, an integral membrane protein from the rhodopsin-like GPCR family. GPCRs represent a large family of integral membrane proteins involved in signal transduction and are of enormous medical importance (Fredriksson et al., 2003; Takeda et al., 2002).

RESULTS

CPM Method Validation

β-LG is a dimer at physiological pH. Each monomer is composed of 162 amino acid residues and contains two disulfide bonds and a single free cysteine (Figure 1A). A simplified model of β-LG unfolding consisting of two steps, denaturation and subsequent aggregation, has been described previously (Roefs and De Kruif, 1994). Samples containing increasing amounts of β-LG were heated under controlled conditions in the presence of CPM and the changes in fluorescence were monitored (Figure 1B). Unfolding curves were observed for all β-LG samples, but not buffer-only and dye-only controls. The unfolding transitions could be described by a simple two-state model, including the native (folded) and the denatured state, representing the lower and upper plateaus in the melting curves, respectively. As expected, the fluorescent signal at the end of the melting transition (upper plateau) is proportional to the starting concentration of β-LG. The calculated T_m of 75°C –76°C is consistent with published data obtained under similar conditions (low ionic strength and neutral pH). Thus, we conclude that the CPM dye can be successfully used as a probe to monitor the accessibility of the cysteine residues exposed during the unfolding process.

FAAH, a dimeric monotopic membrane protein that degrades neuromodulatory fatty acid amides and esters (Patricelli et al., 1998), is a good test case for the CPM stability assay as it contains fourteen cysteines (Figure 1C), and is therefore representative of a more complex system than β-LG. Additionally, the unfolding transition of FAAH as a function of the concentration of the chemical denaturant guanidinium hydrochloride and of the applied hydrostatic pressure has been reported, and methyl arachidonyl fluorophosphonate (MAFP), a covalent irreversible inhibitor of FAAH activity, has been shown to enhance the stability of the dimer by ~2 kcal/mol when compared to the unbound form (Mei et al., 2007). We analyzed the thermal stability of unbound FAAH and FAAH covalently modified with MAFP using the CPM stability assay (Figure 1D). Consistent with the reported results, MAFP binding stabilized FAAH by increasing its T_m by 12°C. Intriguingly, the only crystal structure of FAAH solved to date is from FAAH complexed to MAFP (Bracey et al., 2002), and attempts to crystallize the apo form have been unsuccessful. The results obtained with FAAH further validate the CPM method as a measure of protein stability.

The APJ receptor is an integral membrane protein and belongs to the peptide subfamily of class A GPCRs. Members of the GPCR superfamily share a common membrane topology, with an extracellular N terminus, a cytoplasmic C terminus, and seven transmembrane helices (TM). The APJ receptor is expressed in various tissues and is involved in a broad range of physiological functions, such as blood pressure modulation, regulation of the adipoinsular axis, food intake, water balance, and thermoregulation (Lee et al., 2006; Sorli et al., 2006). Apelin, the endogenous ligand of the APJ receptor, is one of the most potent positive cardiac inotropes (Lee et al., 2005). The APJ receptor construct used in this study contains four cysteines in the extracellular loops which are predicted to form two disulfide bonds, six unpaired cysteines in the TM domains, and three cysteines in the C-terminal tail, two of which are potential sites for palmitoylation based on sequence similarity with other GPCRs (Figure 1E). Purified APJ receptor solubilized in n-dodecyl-β-D-maltoside (DDM) (Figure S1, see the Supplemental Data available with this article online) was heated in the presence of CPM. A clear and reproducible melting transition was observed at 43°C (Figure 1F). The standard deviation in the calculated T_m was less than 2°C for a specific APJ receptor preparation (n = 5–8), yet varied by as much as 4°C between preparations (n = 30). Not surprisingly, the native fold of the APJ receptor could be destabilized by the addition of the chemical denaturant urea, which causes a pronounced shift in the melting transition of the protein to a lower temperature (Figure 1F).

APJ Receptor Stability: Effect of Ionic Strength

To further our understanding of GPCR stability, we examined the APJ receptor purified in DDM, diluted in a series of buffers containing increasing concentrations of various salts, and exposed to CPM. As the salt concentration is increased, the APJ receptor melting transitions shift to higher temperatures (Figure 2A). This salt-induced stabilization of the detergent-solubilized receptor is observed in the presence of all tested salts. However, the nature of the ions had a very pronounced effect on the extent of the stabilization, with NaCl being most efficient in stabilizing the APJ receptor (Figure 2B). Clear unfolding transitions could not be observed with some salts (e.g., potassium nitrate) at low concentration, implying that under these conditions the APJ receptor may be highly prone to aggregation, a phenomenon that we observed during the development of the purification procedure.

APJ Receptor Stability: Effect of pH and Buffer System

The pH as well as the chemical nature of the buffer components can have a very pronounced effect on the stability of a protein. The stability of the APJ receptor was tested in a series of buffer systems obtained from the Molecular Dimensions Crystal Clear I crystallization kit and Hampton Research StockOption buffer sets. The APJ receptor exhibited clear melting transitions in a broad pH range between 4.0 and 8.5. A composite plot of calculated T_m values as a function of pH for a set of commercially available buffers is shown in Figure 2C. Additionally, APJ receptor melting curves generated in a series of citrate buffers with increasing pH are presented in Figure 2D. Representative melting curves of APJ receptor for all buffer series used in Figure 2C, as well as thermal denaturation profiles of the control protein β-LG at different pH are included in Figure S2. As the buffer pH is lowered, a reduction in the fluorescence intensity of the upper plateau of the melting curves is typically observed. This may be in

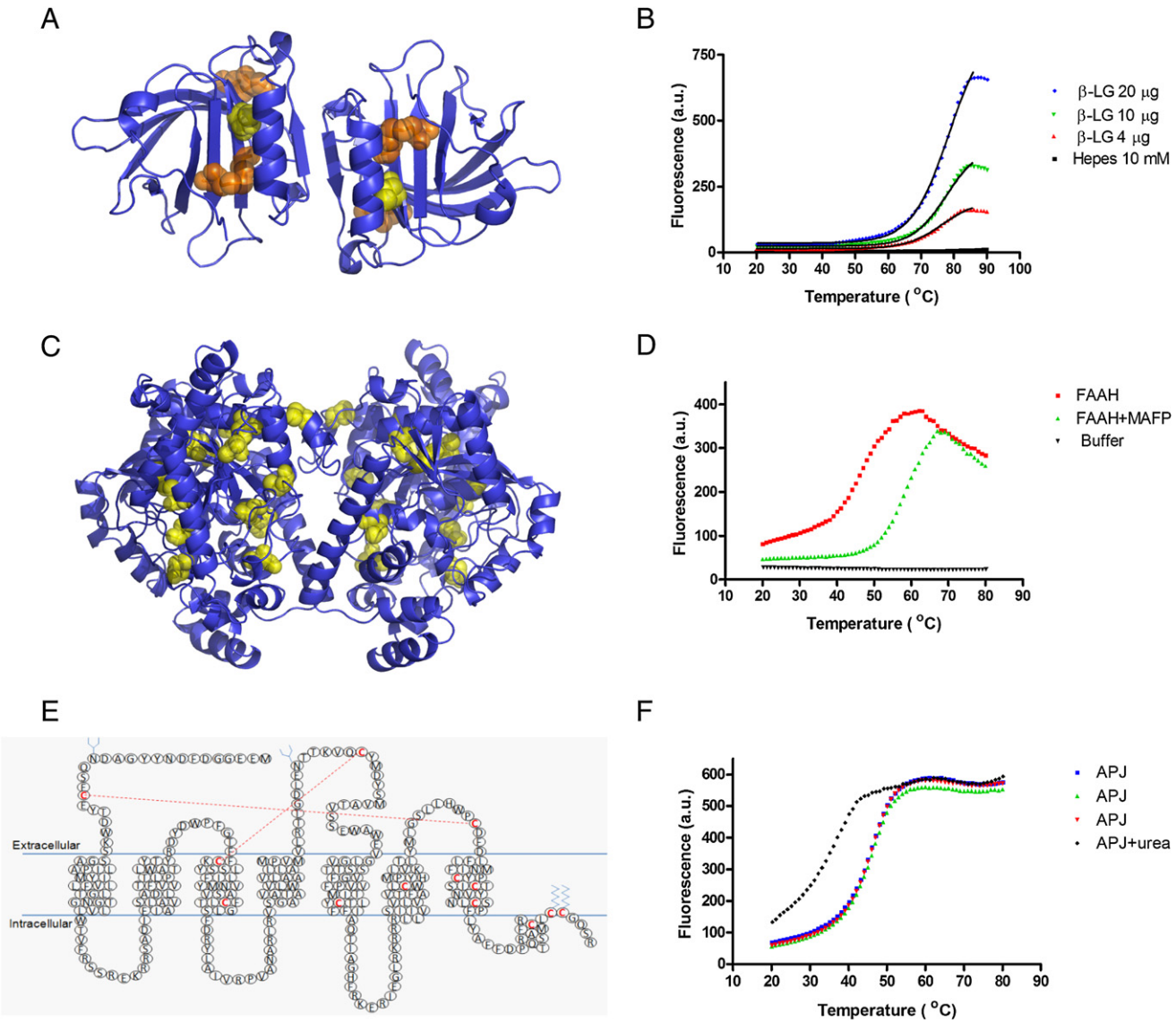

Figure 1. Thermal Stability Profiles of β-LG, FAAH, and APJ Receptor

(A) Structure of β-LG (PDB accession code: 1B0O) with paired Cys residues shown in brown and free Cys residues highlighted in yellow.

(B) Representative melting curves of different amounts of β-LG in 10 mM HEPES (pH 7.5). Melting temperatures T_m were determined by fitting the curves to a Boltzmann sigmoidal equation (see Experimental Procedures) and are as follows: 4 μg β-LG, T_m = 75°C; 10 μg β-LG, T_m = 76°C; 20 μg β-LG, T_m = 76°C. The fitted curves are shown as black lines.

(C) Structure of FAAH (PDB accession code: 1MT5) with free Cys residues highlighted in yellow.

(D) Representative melting curves of 10 μg unbound FAAH (T_m = 46°C) or FAAH complexed with the covalent inhibitor MAFP (T_m = 58°C) in 400 mM NaCl, 10% glycerol, 20 mM HEPES (pH 7.5), 0.1% DDM.

(E) Snake plot diagram of APJ receptor. Cysteine residues are depicted in red. Red lines represent disulfide bond formation. Potential N-linked sugars in the extracellular loops (Y) and putative palmitoylation sites in the C terminus are also indicated.

(F) Melting curves of 8 μg APJ receptor in reference buffer (400 mM NaCl, 10% glycerol, 20 mM HEPES [pH 7.5], 0.1% DDM) and in reference buffer supplemented with 2 M urea (black diamonds). Three replicate samples (calculated T_m values are: 44.8°C, 44.7°C, and 44.9°C) are shown to demonstrate reproducibility.

part due to the decreased reactivity of the CPM dye at pH lower than 6.0. Alternatively, increased aggregation of the unfolded protein at lower pH could also cause a reduction of the CPM fluorescence. Moreover, at pH below 5.0 changes in the shape of the melting curves are noticed, mainly influencing the slope of the unfolding transition.

Taken together these results indicate that while the APJ receptor is stable over a wide range of pH conditions, it is somewhat more stable at lower pH. The chemical nature of the buffer appeared to have a significant effect on the melting transition of the APJ receptor, with acetate and citrate buffers being the most stabilizing, while Tris-based buffers were slightly destabilizing.

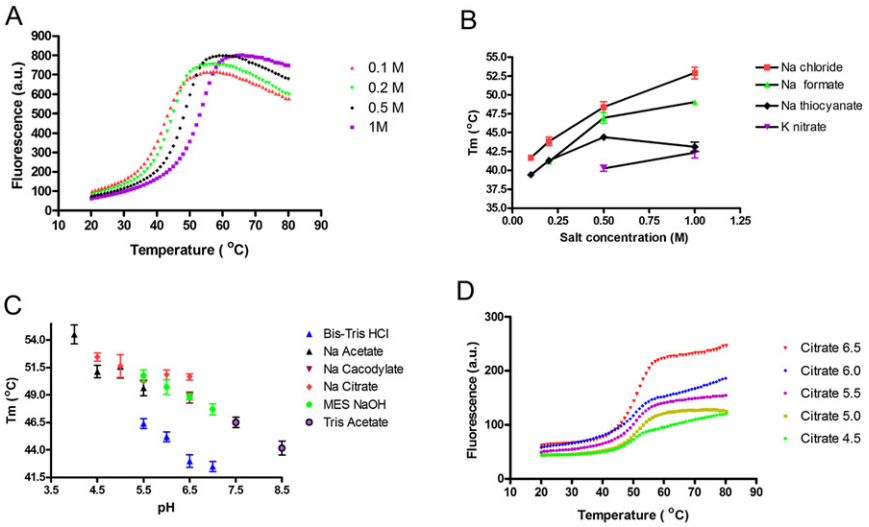

Figure 2. Effects of Solution Variables on APJ Receptor Thermal Stability

(A) Effect of NaCl on APJ receptor thermal stability. Representative melting curves of 10 μg APJ receptor in 0.1% DDM in buffer (20 mM HEPES [pH 7.5], 10% glycerol) containing 0.1, 0.2, 0.5, and 1 M NaCl. Calculated T_m values in order of increasing salt concentration are: 41°C, 43°C, 47°C, and 51°C.

(B) Effects of salt type and concentration: a composite plot of calculated T_m versus concentration for various salts.

(C) Effect of buffer composition and pH: a composite plot of calculated T_m versus pH for various buffer systems. All buffers were used at 100 mM. Data points are means of at least duplicate, in most case triplicate samples, with error bars indicating SEM.

(D) Effect of pH on APJ receptor thermal stability. Representative melting curve of 4 μg of APJ in Na citrate buffers (100 mM Na citrate, 10% glycerol and 400 mM NaCl) of various pH. Calculated T_m values are: citrate pH 4.5, 52°C; pH 5.0, 51°C; pH 5.5, 50°C; pH 6.0, 50°C; and pH 6.5, 50°C.

APJ Receptor Stability: Effect of Additives

Small molecule ligands and additives represent a diverse group of compounds that interact with proteins: salts, sugars, detergents, organics, and cofactors, and are known to affect the biophysical properties of proteins. We tested the effects of dozens of additives from the commercially available additive screen (Hampton Research) on APJ receptor stability. In general, additives were diluted 10-fold from the supplied stocks. Results obtained with a subset of the compounds, mostly sugars and salts, are presented in Figure 3A. The unfolding profiles illustrate the commonly observed changes in the melting transitions of the APJ receptor relative to our standard condition (400 mM NaCl, 10% glycerol, 20 mM HEPES [pH 7.5], 0.1% DDM). Salt additives at low concentration (~100 mM) appear to influence the T_m of the APJ receptor in two different ways. In the case of sodium citrate, the onset of the melting transition remains the same as that of the reference sample, yet the maximum fluorescent intensity (representing the end of the unfolding process) is higher than the reference. In the case of sodium malonate, the onset of the melting transition is moved to higher temperatures, with an additional upward shift in the upper plateau of the melting curve. In both cases, however, the calculated T_m is shifted to higher temperatures. One possible explanation is that sodium citrate may simply be suppressing the aggregation of the unfolded protein, rather than having a true stabilizing effect. In the case of sugars, with the exception of sucrose, the melting profile of the APJ receptor is mostly unaffected. However, a small but consistent increase in the maximum of the fluorescent signal is observed. Additionally, glycerol was identified as an additive which stabilizes the detergent-solubilized APJ receptor in a concentration-dependent manner (Figure 3B).

APJ Receptor Stability: Effect of Detergents and Small-Molecule Amphiphiles

Specifically for membrane proteins, identification of surfactants is very important, since the chemical nature of the detergents can exert a profound effect on the stability of the PDC (Odahara, 2004). We used the CPM method to characterize the stability of the APJ receptor in various detergents as well as in the presence of small-molecule amphiphiles. APJ receptor samples were diluted 20-fold in buffer containing the indicated concentration of a tested detergent, incubated on ice for 20 min to allow exchange of detergents, and heated in the presence of CPM (Figure 4A). Detergents with a high critical micelle concentration (CMC) were used at ~1–2 times their CMC, while detergents with a low CMC were present at 0.1%. We observed that all of the tested detergents were less stabilizing than DDM. This result was not unexpected, as similar trends have been observed for other membrane proteins such as rhodopsin (De Grip, 1982), lactose permease (Engel et al., 2002), and diacyl glycerol kinase (Zhou and Bowie, 2000). Interestingly, LDAO, which has a low CMC and an alkyl chain similar to DDM, is highly destabilizing with respect to the APJ receptor, possibly due to its zwitterionic nature (Michel, 1983). While maltoside detergents are usually mild and are able to maintain the stability of proteins, they also form large micelles, a property that could hinder crystallization (Michel, 1983). Small-molecule amphiphiles have been used to effectively reduce the size of the detergent micelles (Gast et al., 1994; Rosenow et al., 2001). Thus, we tested the effect of alkane polyols on the apparent stability of DDM-solubilized APJ receptor. The results for 1-methyl-2,4-pentadiol, 1,2,3-heptanetriol, 1,2-hexanediol, and 1,6-hexanediol are presented in Figure 4B. The addition of any of the four polyols rendered the APJ receptor more susceptible to modification with CPM at lower temperatures than DDM alone.

DISCUSSION

The proposed fluorescent assay extends the array of available biochemical tools for analysis of membrane protein stability. The CPM stability assay has several important advantages over existing methods. It is highly sensitive, with less than 10 μg

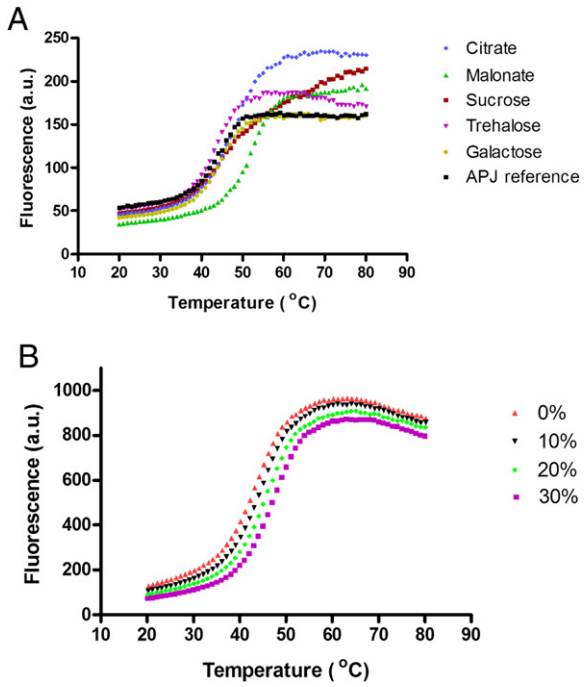

Figure 3. Effect of Selected Additives on APJ Receptor Thermal Stability

(A) Representative melting curves of 4 μg APJ receptor in 0.1% DDM in reference buffer supplemented with the indicated additives: 100 mM Na citrate (pH 7.5; T_m = 48°C), 100 mM Na malonate (pH 7.5; T_m = 52°C), 3% sucrose (T_m = 46°C), 3% trehalose (T_m = 43°C), and 3% galactose (T_m = 44°C). The melting curve obtained in the presence of reference buffer is in black (T_m = 43°C).

(B) Representative melting curves of 10 μg of APJ receptor in reference buffer containing increasing glycerol concentrations. Calculated T_m values in order of increasing glycerol concentration are: 43°C, 44°C, 45°C, and 47°C. All reported T_m values are means of at least two samples.

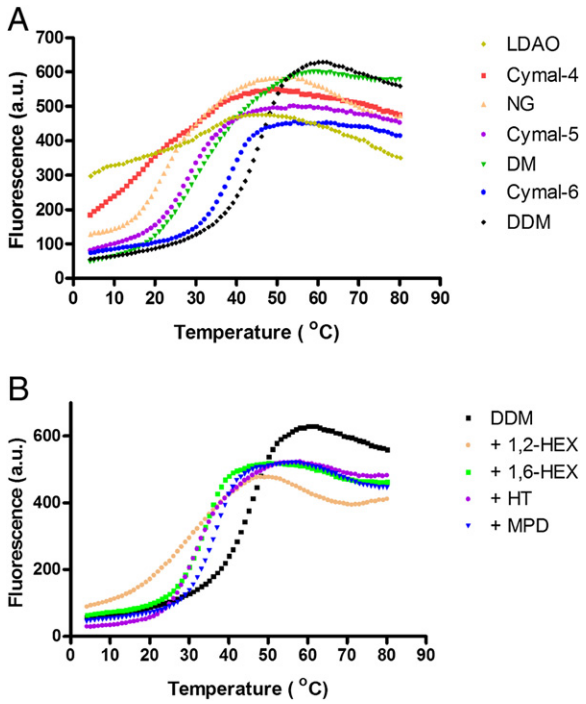

Figure 4. Effect of Detergents and Small-Molecule Amphiphiles on APJ Receptor Thermal Stability

(A) Representative melting curves of 8 μg APJ receptor in various detergents. DDM-solubilized APJ receptor was diluted into buffer (20 mM HEPES [pH 7.5], 100 mM Ammonium citrate, 10% glycerol) containing an excess of the indicated detergents. Protein samples were incubated on ice for 20 min prior to exposure to CPM dye and subsequently transferred to cuvettes prechilled to 4°C. Concentrations and calculated T_m values for the used detergents are: 0.1% LDAO, T_m = N.D.; 0.75% Cymal4, T_m = 22°C; 0.25% NG, T_m = 24°C; 0.5% Cymal5, T_m = 28°C; 0.2% DM, T_m = 32°C; 0.1% Cymal6, T_m = 37°C; and 0.1% DDM, T_m = 44°C.

(B) Thermal denaturation profiles of APJ receptor in buffer (20 mM HEPES [pH 7.5], 100 mM Ammonium citrate, 10% glycerol) containing 0.1% DDM and additionally supplemented with 5% of the following amphiphiles: 1,2-Hexanediol (1,2-HEX), T_m = 29°C; 1,6-Hexanediol (1,6-HEX), T_m = 33°C; 1,2,3-Heptanetriol (HT), T_m = 33°C; 1-Methyl-2,4-pentadiol (MPD), T_m = 36°C. The melting curve obtained in the presence of reference buffer and DDM only is in black. All reported T_m values are means of at least two samples.

of total protein required per sample, it is easy to implement and is very reproducible. Importantly, the assay is compatible with a wide range of detergents and detergent concentrations, and performs with a high signal-to-noise ratio for membrane proteins with limited hydrophilic extramembraneous domains such as GPCRs. Alternative dyes considered, such as sypro-orange (Thermofluor), were not as useful due to a high background signal which masks the unfolding transition of the detergent-solubilized proteins (Yeh et al., 2006). To our knowledge, the only report of a successful Thermofluor analysis of a GPCR-like protein is that of bacteriorhodopsin, a thermophilic protein with covalently bound retinal (Pantoliano et al., 2001).

Some limitations of the method are the decreased rate of CPM-thiol formation at lower pH, a decreased chemical selectivity of the CPM dye at pH above 8.0, and fluorescence quenching in the presence of certain salts (like bromides). Care should be taken to eliminate reducing agents such as DTT and β-mercaptoethanol from the protein preparations as they react with the CPM dye. Additionally, as with other dye-based methods, it is possible that protein-dye interactions adversely affect the stability of the studied protein and its melting transition. However, the favorable correlation of the observed melting temperatures for β-LG and FAAH with the published values suggests that such effects may not be very pronounced. Moreover, the usefulness of this method is in obtaining relative stability values under various conditions, and not determination of absolute unfolding temperature values. Thus far, the assay has been implemented using a Cary Eclipse spectrofluorometer, which allows the simultaneous analysis of only four samples, yet in principle this assay could be adapted to a high-throughput format using real-time PCR machines or temperature-controlled plate readers with suitable excitation and emission filters. As a useful extension of the proposed assay, we have successfully used the CPM dye to generate isothermal denaturation profiles of several other GPCRs. This assay format precludes determination of T_m, but rather the protein stability under certain solution conditions at a fixed temperature is measured as a rate of unfolding, and thus provides complementary information. In summary, given the limited dataset we have at present, the results from the two formats of the CPM assay seem to be in good agreement.

An additional limitation of this approach is in the lack of available free thiol residues located inside of membrane protein cores. A detailed analysis of the PDB indicate that 66% of membrane proteins with known structures contain one or several thiols and 91% of the present cysteine residues are buried in the protein interior. In cases where no free thiol exists, cysteine residues can be engineered into different sites if a stability assay is considered critical.

Another potential concern with the CPM assay is when working with proteins containing cysteine residues in unstructured regions. This class of proteins will still be amenable to analysis, however, we expect higher fluorescence intensity in the lower plateau of the melting curves, reflecting the possible derivatization of all accessible cysteines with the CPM dye without any heating. Any additional cysteine residues protected in the protein core yet made accessible to the CPM dye in the unfolded state would be expected to be modified in a temperature-dependent fashion and thus to generate a clear melting transition.

APJ Receptor Stability Analysis—A Representative GPCR

Membrane proteins, like GPCRs, ions channels, and transporters, are highly dynamic molecules known to exist in at least two, if not multiple conformations. This inherent conformational flexibility, which is conducive to their functional role in the lipid bilayer, is further increased upon solubilization in detergents. The increased dynamics of the molecules in aqueous solution stems from the loss of lateral pressure (Marsh, 1996), as well as a reduction in the magnitude of the forces holding the structure together due to an increase in the dielectric constant of the medium embedding the proteins. The latter effect is especially important for ionic interactions that are much weaker in water-based solutions compared to the membrane bilayer. Additionally, detergents with a high CMC, proven especially useful for three-dimensional crystal formation, assemble into dynamic micelles that allow substantial ''breathing'' of the protein and consequently a transient exposure of the hydrophobic surfaces of the protein to water (Rosenbusch, 2001). In general, an inverse relationship between protein dynamics and thermal stability is observed for soluble proteins (Tsai et al., 2001). Overall, the above mentioned effects can lead to substantial destabilization of the native state of a membrane protein and, in the worst cases, to aggregation. Fortunately, some of the problems encountered when working with membrane proteins can be remedied, to some extent, by a judicious choice of detergents and solution conditions that promote protein stability.

Our analysis of APJ receptor behavior under various solution conditions underscores the importance of several factors for preserving the thermal stability of this membrane protein in the solubilized state, especially salt type and concentration, glycerol concentrations, as well as detergent type. We believe that the lessons learned from the APJ receptor will likely be applicable to other GPCRs. It is important to keep in mind that the effect of the studied compounds on the stability of detergent-solubilized APJ receptor reflects the combined effects on the protein itself, as well as on the detergent micelle encircling the protein.

One of the major conclusions of the stability screening of the APJ receptor is that its stability in detergents is strongly influenced by the solution's ionic strength. In general, salts that do not have specific binding sites increase protein thermal stability of both soluble and membrane proteins, essentially through their effect on the water structure manifested by an increase in the surface tension (Lin and Timasheff, 1996). This likely increases the strength of the hydrophobic interactions and additionally causes exclusion of water from the interior of the protein. The differential stability provided by salts of different combinations indicates that the contribution of both the cations and anions depends on their position in the Hofmeister series. Several GPCRs are known to contain sodium binding sites located in cytoplasmic parts of the TM helices (Oliveira et al., 2007; Seifert and Wenzel-Seifert, 2002). The saturation of these putative sodium sites would be expected to shift the conformational equilibrium of GPCRs toward the inactive state which, in principle, should be more stable. Additionally, salts are known to affect the properties of the detergent micelles, leading in general to an increase in the micelle size with the rise of the salt concentration (Berger et al., 2005, 2006; Molina-Bolivar et al., 2006, 2007). The salt-induced effects for the nonionic sugar-based detergent n-nonyl-β-D-glucoside (NG) have been reported to follow the Hofmeister series for both cations and anions (Ericsson et al., 2004). In summary, the salt driven stabilization of the APJ receptor could be explained by the cumulative effect on the membrane protein structure itself, through increased surface tension and exclusion of water, on one hand, and on the micelle size, on the other.

Glycerol, like many polyols, stabilizes the native structure of proteins and is used as a cosolvent to preserve the activity of enzymes in aqueous solutions. As an osmolyte, it is strongly excluded from the protein domain and favors its hydration (Gekko and Timasheff, 1981). Importantly, glycerol reduces the conformational flexibility of loops, both in soluble and membrane proteins, by inhibiting the local thermal backbone dynamics, thus, favoring the most compact structure of the protein (Tsai et al., 2000; Weinkauf et al., 2001; Xia et al., 2004). Additionally, glycerol reduces the dielectric constant of the medium affecting the strength of the electrostatic interactions. Clearly glycerol affects protein structure in multiple ways, and further experiments will be needed to understand how this compound brings about stabilization of the APJ receptor.

The choice of detergent is of paramount importance for the stability of any membrane protein. In addition to its influence on monodispersity, the detergent micelle surrounding the protein contributes substantially to the overall thermodynamic stability of the PDC. A first approximation of the magnitude of this effect for nonionic surfactants is proportional to the standard free energy of micelle formation, ΔG^{O}_{mic}, and is given by the equation $\Delta G^{O}_{mic} \sim RT\ln\chi_{cmc}$, where χ_{cmc} is the mole fraction of surfactant at the CMC, R is the gas constant, and T is the absolute temperature (Stowell and Rees, 1995). In general, within a certain class of detergents, those with low CMC are expected to be more stabilizing. We utilized the CPM assay to rank detergents with respect to APJ receptor stability and observed that the effect of the detergent on the apparent T_m of the PDC is a complex function of the alkyl chain length, as well as the head group chemistry. Maltoside-based detergents seem the best at preserving the structure of the APJ receptor, and not surprisingly we have empirically selected DDM during the development of the APJ receptor purification procedure. Data obtained from detergent screening could be used to rapidly

eliminate surfactants which grossly alter the fold or stability of the protein. In the case of the APJ receptor, severely destabilizing detergents include LDAO, Cymal4, and TRIPAO and are easily recognized by the marked increase in the susceptibility to modification with the CPM dye at 4°C, as well as by the decrease in the steepness of the observed melting transition. Additionally, calculated T_m values could be used to guide the choice of a temperature range for setting crystallization trials. For example, in the case of the APJ receptor, the use of NG at room temperature is not warranted, as the receptor unfolds with a T_m of 24°C. However, NG could be included as a surfactant in crystallization experiments conducted at 4°C –10°C, a temperature range where the protein is more stable. Conversely, DDM, the most stabilizing detergent for the APJ receptor, could be utilized to set up trials encompassing the entire crystallization range from 4°C –25°C.

GPCR Unfolding: Mechanistic Aspects of the CPM Stability Assay

The overall fluorescent signal in the CPM stability assay is formed as a sum of several processes. Thiol-CPM adduct formation is the major reaction which positively contributes to the rise of the fluorescent intensity, while protein aggregation taking place simultaneously or shortly after the protein denatures effectively reduces the maximally attainable fluorescent signal. Secondary processes which affect the overall shape of the unfolding transitions are: modification of amino groups as well as side chains of amino acids other than cysteines, thiol-CPM adduct hydrolysis, and the decrease in quantum yield with increase of temperature.

The unfolding curves for the APJ receptor could be described by a simple two state model: the native (folded) and the denatured states. The melting transition displays substantial cooperativity and suggests that unfolding proceeds through the concerted loss of multiple interactions. It is a general observation for α-helical membrane proteins that unfolding reactions proceed with only limited changes in secondary structure (Brouillette et al., 1987; Grinberg et al., 2001; Stowell and Rees, 1995). The unfolded state is best described by the concept of a loose helical bundle, a state which displays an almost complete preservation of the membrane spanning helices, but a dramatic loss of tertiary contacts between these helices and between the helices and the bound chromophore, as is the case for rhodopsin in its inactive state (Vogel and Siebert, 2002a, 2002b).

The proposed stability method based on global cysteine accessibility in principle provides a general readout, similar to the tryptophan fluorescence, for the overall integrity of the membrane proteins. At the same time, the structural information gained from the assay is limited by the precise positions of the thiol residues in the primary sequence of the studied proteins. For example, most of the unpaired cysteines in the APJ receptor are clustered in the C-terminal half of the protein, TM helices 5-8 and the C terminus, with only a single thiol located in TM3 in the N-terminal portion of the protein (Figure 1E). Thus, in the CPM assay, regions devoid of cysteine residues are practically invisible for direct analysis.

Data accumulated from numerous studies indicate that GPCRs can be viewed as a combination of two separate blocks of helices, block 1 includes TM-1-5 and block 2 includes TM-6-7-8. TM-1 through TM-4 appear to form a relatively rigid core, which does not move substantially during functional activation of the GPCRs. In contrast, TM-6 and TM-7 are highly mobile and activation leads to a vertical see-saw movement around the highly conserved prolines with the simultaneous movement of the intracellular segments of TM-6 and to a lesser extent TM-7 away from the center of the receptor (Schwartz et al., 2006). In terms of mobility, TM-5 occupies an intermediate position between the N- and C-terminal helices and is thought to be flexible in its extracellular portion, yet relatively rigid in the intracellular part. This idea has been further substantiated by reconstitution experiments. In fact, for the β_2 adrenergic receptor (β_2AR) helices 1-5 and helices 6-7 behave as independent folding domains that can be expressed on separate plasmids and assemble to form a functional "split" receptor (Kobilka et al., 1988). Intracellular loop 3, connecting the N- and C-terminal parts is known to be highly flexible, and as a hotspot of instability in GPCRs will presumably be the first to unfold (Hubbell et al., 2003; Jaakola et al., 2005). Given this model, it is conceivable that the CPM assay with the APJ receptor allows us to monitor the separation of TM-6-7 and the C terminus away from the rest of the APJ receptor, a process that could bear some mechanistic similarities with the functional activation of the receptor. Additional work with other GPCRs with unpaired cysteines at different positions will be needed to prove or disprove this model.

The APJ receptor contains six unpaired cysteines in the TM helices, and any of these residues could be modified by the CPM dye as the protein is heat-denatured. The data we have currently accumulated does not allow us to determine if all or only a subset of them are derivatized. The availability of APJ mutants with only a single unpaired cysteine residue or with a subset of engineered cysteine residues would be useful in determining if any of the cysteine positions are being preferentially attacked by the dye and positioned in a highly unstable region of the receptor. Alternatively, mass spectroscopy could be used to identify modified cysteine residues.

We envision that the CPM dye gains access to the TM-embedded thiols by direct transfer from the solvent penetrating the spaces between the TM helices during thermal denaturation. Alternatively, given the lipophilic nature of the dye it is conceivable that the dye would partition, at least to some extent, into the detergent belt around the receptor and eventually find its way to the TM helices to ultimately modify the cysteine residues facing the lipid bilayer. In detergents like DDM, which form large and relatively stable micelles, the first mode of attack is probably the major route for thiol modification. In detergents which form smaller or highly dynamic micelles, as well as in the presence of amphiphiles modifying the biophysical properties of the micelles, the second mode of attack probably contributes substantially to the overall signal of the CPM assay and the measured T_m.

Conclusions

Identifying solution formulations that augment membrane protein stability has the potential to dramatically reduce or at least simplify the complexity in the biophysical characterization of membrane proteins. Optimization of the target stability would additionally lead to improved yields during purification, as well as to identification of optimal buffer formulations for concentrating the protein prior to crystallization trials. As a generic method,

the CPM stability assay could be used to guide the development of purification procedures for membrane proteins with unknown function or for which no functional assay is available. Additionally, the CPM stability assay could aid in the search for new ligands through screening of compound libraries or stability analysis of protein variants engineered via random or rational mutagenesis.

EXPERIMENTAL PROCEDURES

Protein Samples

β-LG was purchased from Sigma. Purification procedures for FAAH and APJ receptor, as well as protocols for Semliki forest virus (SFV) viral stock generation and expression are described in the Supplemental Experimental Procedures.

CPM Assay Conditions

CPM dye was obtained from Invitrogen and dissolved in DMSO (Sigma) at 4 mg/ml. This stock solution was kept at −80°C. Prior to use the dye stock is diluted 1:40 in dye dilution buffer (20 mM HEPES [pH 7.5], 200 mM NaCl, 0.025% DDM), incubated for 5 min at room temperature, and is used immediately while protected from light to reduce photobleaching. In general, optimal dilution of the CPM dye should be determined empirically for each tested protein. The thermal denaturation assay was performed in a total volume of 130 μl. The tested protein (1–20 μg) was diluted in the appropriate buffer to a final volume of 120 μl. After an incubation period (usually 5 min at room temperature unless indicated otherwise), included to allow equilibration of the protein with the buffer components, 10 μl of the diluted dye was added and thoroughly mixed with the protein. The reaction mixture was transferred within a 5 min period to a sub-micro quartz fluorometer cuvette (Starna Cells, Inc., Atascadero, CA) and heated in a controlled way with a ramp rate of 2°C/min in a Cary Eclipse spectrofluorometer. The excitation wavelength was set at 387 nm, while the emission wavelength was 463 nm. Assays were performed over a temperature range starting from either 4 or 20°C and ending at 80 or 90°C.

As a maleimide-based thiol-reactive probe, CPM has an optimal pH between 6 and 8. At a pH lower than 6, the rate of CPM-thiol adduct formation decreases, while at a pH above 8 the chemical selectivity of the probe is reduced, and CPM may react with primary amines. Additionally, above pH 8 the rate of CPM-adduct hydrolysis increases substantially and this reaction can compete significantly with thiol modification (Invitrogen, 2006). The slower rate of adduct formation can be partly compensated for by increasing the concentration of the CPM dye in the reaction (data not shown).

Data Analysis

All data were processed with GraphPad Prism program (GraphPadPrism v.4.00 for Windows, Graphpad Sofware, San Diego, CA, USA). In order to determine the inflection point of the melting curves, which was assumed to equal the melting temperature (T_m), a Bolzmann sigmoidal equation was fitted to the raw data. In case the fluorescent signal showed considerable fluctuation at high temperature, the top plateau of the unfolding profile was constrained and made equal to the maximally attainable fluorescent reading immediately following the steep melting transition. The background fluorescence in the presence of buffer components and dye only rarely exceeds 40 a.u. at temperatures below 60°C. Raw data were used directly for the fitting algorithm because it was determined that the fluorescent background correction has little effect on the fitted values of T_m.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, two figures, and Supplemental References and can be found with this article online at http://www.structure.org/cgi/content/full/16/3/351/DC1/.

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