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Rapid and simple protein-stability screens: application to membrane proteins

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Approximately 30% of the human genome, and likewise for other genomes, encodes membrane proteins. Also, the majority of known human pharmaceutical targets are membrane proteins. As a consequence, the future success of structure-based drug-design efforts will rely heavily on membrane-protein structural information. While a number of techniques are available to determine the structure of membrane proteins, crystallographic methods (either using two-dimensional or three-dimensional crystals) have been the most productive. Nonetheless, membrane-protein structure determination using crystallographic methods has encountered at least three serious bottlenecks: protein production, purification and crystallization. While a number of crystallization strategies for membrane proteins are available today, they all must ensure that the membrane protein of interest is thermodynamically stable for crystallization to be feasible. Thermodynamic stability is so fundamental to protein crystallization that it is often overlooked experimentally. Here, simple and effective protocols for determining the relative stabilities of membrane proteins using commercially available instruments and reagents are demonstrated. The results demonstrate suitability for the rapid screening of conditions that maximize protein stability using minimal amounts of reagents and protein.

1. Introduction

Information transfer between cells in the human body and between cellular compartments within the cell occurs *via* the membrane. Proteins within the membrane are responsible for regulating the uptake and output of various substances that are required for the wellbeing of the individual cells and cellular regulation throughout the body. Because membrane proteins are situated at the interface between cells and between cellular compartments, it is not surprising that membrane proteins are ideal target molecules for the pharmaceutical industry. Accordingly, while membrane proteins comprise approximately 30% of the proteins within a cell (Wallin & von Heijne, 1998), they account for greater than 50% of known human pharmaceutical targets (Russell & Eggleston, 2000). Furthermore, within the recent genome sequences there are undoubtedly thousands of membrane proteins with unknown function that await discovery as new drug targets.

Success in structure-based design of new drug candidates (Heinemann, 2000; Norin & Sundstrom, 2001; Anderson & Chiplin, 2002; Buchanan *et al.*, 2002) has prompted the development of large-scale proteomic efforts (Service, 2002; Burley & Bonanno, 2003; Gerstein *et al.*, 2003; Kyogoku *et al.*, 2003) which seek to obtain atomic resolution information on proteins rapidly and efficiently. These efforts have developed

high-throughput robotic methods that are capable of screening thousands of crystallization conditions per day (McPherson, 2003). High-throughput methods for protein expression and purification have also been developed (McMullan *et al.*, 2005). While much effort has been devoted to the rapid production, purification and crystallization of target proteins, little effort has been devoted to the systematic determination and optimization of stable conditions for crystallization.

Success or failure of a crystallographic structural project can occur at one of three important steps: protein production, purification, or crystallization (Loll, 2003). Modern X-ray crystallography rarely, if ever, encounters problems when suitably diffracting crystals are available. This is evidenced by the explosion of deposited structures in the PDB (http://www.pdb.org) (Berman *et al.*, 2000) over the past decade. For membrane proteins, the major bottlenecks have yet to be overcome. In particular, the bottleneck of systematically determining conditions that promote the stability of membrane proteins is imperative. Unlike soluble proteins, purified and isolated membrane proteins are in fact a protein–detergent complex, where the detergent micelle surrounding the protein contributes substantially to the thermodynamic stability of the membrane protein itself (Stowell & Rees, 1995). In the case of membrane proteins, simply changing the detergent can result in thermodynamic stability changes that approach the overall stability of the folded state alone (Stowell & Rees, 1995). Such dramatic changes in the overall stability of the membrane protein can severely compromise the ability of a protein to crystallize. Even for soluble proteins, optimization of stable conditions prior to crystallization can mean the difference between the success and failure of a structural study (Weinkauf *et al.*, 2001). In addition, many membrane proteins, such as transporters and ion channels, cannot be assessed for activity in a detergent-soluble state. Hence, stability may be the only means to assess the ability of a given condition to maintain membrane-protein integrity. Alternatively, if a membrane protein has a suitable expression system available, then the protein itself can be systematically mutated to optimize stability (Bowie, 2001). To our knowledge, only one study to optimize conditions for membrane-protein stability has been performed to date (Engel *et al.*, 2002). The reason for this stems from the fact that suitable methods that allow the rapid determination of conditions which are both stabilizing for a given membrane protein and suitable for crystallization are lacking. While future success in membrane-protein structural studies requires systematic approaches to all three of the major bottlenecks, the assessment of membrane-protein stability has been overlooked. Here, we report the development of simple protein-stability assays which utilize the real-time PCR instrumentation that is now commonly available in many laboratories. We have optimized the assay using hen egg-white lysozyme (HEWL) and then applied the assay to four membrane proteins of known structure. For three of these membrane proteins, clear melting transitions could be observed and relative stabilities assigned.

2. Experimental

2.1. Protein samples

Hen egg-white lysozyme (HEWL; catalog No. L-6876) was purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). BtuCD, MscL and MscS from *Escherichia coli* were overexpressed and purified by members of D. C. Rees' laboratory as previously described (Chang *et al.*, 1998; Bass *et al.*, 2002; Locher *et al.*, 2002). Acetylcholine receptor (AChR) was purified from the electric organ of *Torpedo marmorata* as previously described (Tierney *et al.*, 2004). Protein concentrations were determined using the Pierce BCA assay.

2.2. Reporter dyes

A series of fluorescent dyes were tested for their suitability for monitoring the unfolding transitions of membrane proteins. Dye selection was based on the criterion that dye fluorescence should be environmentally sensitive, in that dyes should be nonfluorescent in a hydrophilic environment and fluorescent in a hydrophobic environment. In principle, upon protein unfolding such dyes should partition into exposed hydrophobic regions of the protein and give rise to a fluorescence signal. Dyes that were tested included SYPRO Orange (catalog No. S-6651), NanoOrange (catalog No. N-6666), Pro-Q Amber (catalog No. M33308), 1,8-ANS (catalog No. A-47) and Bis-ANS (catalog No. B-153), all of which were obtained from Molecular Probes Inc. (Eugene, OR, USA).

2.3. Data analysis

All data were downloaded and imported into the *GraphPad Prism* program (*GraphPad Prism* v.4.00 for Windows, GraphPad Software, San Diego, CA, USA). A background curve was subtracted from each data set by fitting the initial data points to a single exponential decay curve in the case where detergent was absent from samples and a double exponential decay curve in the case where detergent was present in the samples. The single exponential decay curve represented a gradual decrease in the background fluorescence as a function of temperature. This background fluorescence decay was a feature that appeared to be inherent to the real-time PCR instrument itself and not the reporter dye, as it was observed not only in samples that contained a reporter dye but also in samples that did not (*e.g.* buffer alone, protein plus buffer, detergent plus buffer *etc.*). In the case where samples contained detergent, in addition to the above decrease in fluorescence intensity, the disassociation as a function of temperature of the reporter dye from free detergent micelles in solution also contributed to the decay in the background fluorescence. For this reason, a double-exponential decay curve was fitted for samples containing detergent.

In order to determine the inflection point of the melting transition and thus more accurately determine the meting temperature, a polynomial function was fitted to the portion of the curve representing the melting transition and subsequently

the first derivative was calculated. Both of these steps were also performed using the *GraphPad Prism* software.

2.4. Assay conditions and instrumentation

All assays were performed using an MJ Research DNA Engine Opticon real-time PCR instrument (catalog No. CFB-3200, Bio-Rad Laboratories, Inc., Waltham, MA, USA). The system, which has a fluorescence-excitation range of 450–495 nm and a fluorescence-detection range of 515–545 nm, can accommodate up to 96 samples in a single run. Assay samples of 25 µl consisted minimally of protein sample and reporter dye, which were aliquoted and mixed in 0.2 ml low-profile eight-tube strips and capped (catalog Nos. TLS-0851 and TCS-0803; Bio-Rad Laboratories, Inc., Waltham, MA, USA). To determine conditions which would yield optimal signal-to-noise ratios, matrices of various protein and dye concentrations were tried for each protein. In some instances, in addition to protein and dye, other components, such as detergents or bis-tris propane or phosphate buffer at various pH values, were included in the assay sample to determine the effects of detergents and pH on protein unfolding. In such cases, detergent concentrations were 1.5× the critical micelle concentration (CMC) and buffer concentrations were 100 m*M*. Assays were performed over a temperature range starting from either 277 or 303 K up to 368 K, with a temperature ramp rate of 1 K min^{-1}.

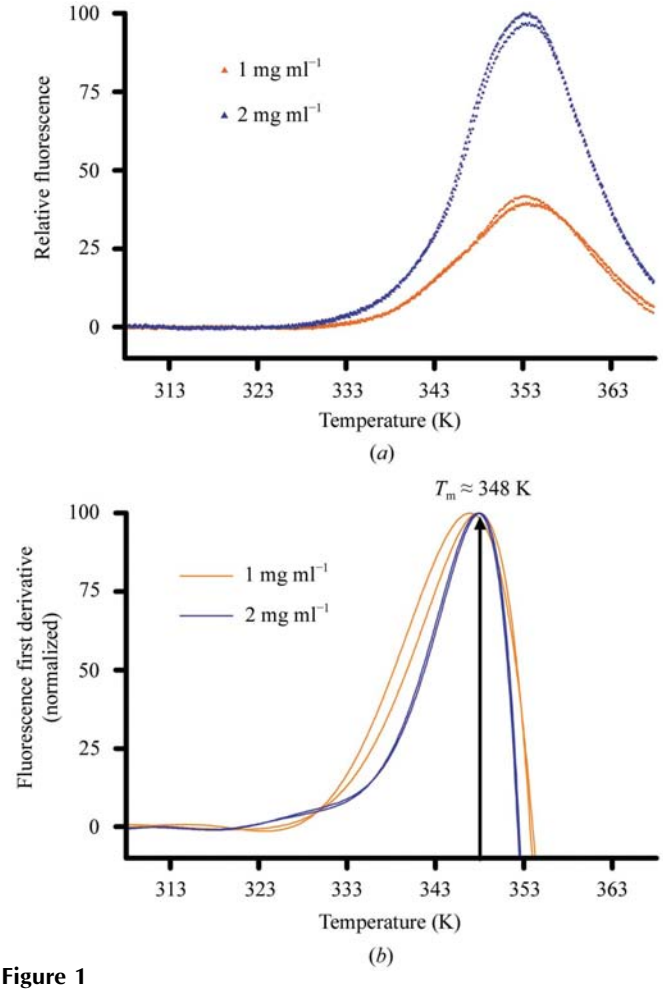

Figure 1
(*a*) Melting curves of HEWL at two different concentrations in 100 m*M* Bis-Tris propane buffer pH 8 monitored using the MJ Research real-time PCR instrument and 1:2500 diluted SYPRO Orange as the reporter dye. Melting curves are of duplicate samples at each concentration, showing the reproducibility of the assay. (*b*) First derivative of the data in (*a*) showing the reproducible determination of the T_m. The total protein used was 25 and 50 µg, respectively.

3. Results

Historically, protein stability has been measured using a variety of techniques. These include differential scanning calorimetry (DSC; Sanchez-Ruiz, 1995), fluorescent dye binding to the unfolded state (Semisotnov *et al.*, 1991; Cardamone & Puri, 1992), circular dichroism (CD; van Mierlo & Steensma, 2000) and nuclear magnetic resonance (NMR) methods (Huyghues-Despointes *et al.*, 2001). Of all these methods, only fluorescence/absorbance can potentially be applied in a high-throughput manner today, such as is performed in a variety of drug-screening techniques, gene chip analysis *etc.* We have taken advantage of commercially available instruments recently developed for real-time quantitative PCR to develop a protein thermal unfolding assay that may aid in optimizing conditions for protein stabilization and ultimately protein crystallization.

3.1. Measuring melting curves to monitor stability changes

For this assay, we used the DNA Engine Opticon real-time PCR instrument from MJ Research Inc. to monitor protein unfolding of the test protein hen egg-white lysozyme (HEWL; Knubovets *et al.*, 1999; $T_m \simeq 348$ K) as well as several membrane proteins. We investigated five different fluorescent dyes purchased from Molecular Probes Inc. (SYPRO Orange, NanoOrange, Pro-Q Amber, ANS and Bis-ANS) for their ability to report on the unfolding transition of these proteins. Fig. 1(*a*) shows typical melting curves for HEWL using the real-time PCR instrument with SYPRO Orange as the reporter dye. Excellent unfolding curves were observed for this test sample. The observed T_m was calculated to be ∼348 K from the first derivative of the unfolding curves, as shown in Fig. 1(*b*), and agrees well with the literature value (Knubovets *et al.*, 1999). These results demonstrate both the reproducibility of the assays as well as provide confidence that the observed fluorescence curves arise from the unfolding of the protein. Because our goal is to ultimately use the assays for membrane proteins, we also conducted control experiments to ensure that transitions do not arise from dye alone or dye in the presence of detergent. Fig. 2 shows typical unfolding curves for HEWL under our assay conditions plotted with control curves using dye alone, dye plus a variety of detergents and protein alone. Melting curves could be obtained using either SYPRO Orange or NanoOrange as the reporter dye. In either case, melting curves are seen only in the presence of both the dye and protein, with the fluorescence signal varying as a function of protein concentration, further supporting the

proposal that the observed transitions indeed arise from protein unfolding. Moreover, as would be expected for an irreversible unfolding transition, subsequent scans of the same sample following the initial scan did not show a pronounced unfolding transition.

3.2. Correlation of crystal diffraction quality and stability

One important goal in developing this assay was to try to improve both the successful crystallization as well as the crystal quality of a protein. To test this assay for pinpointing more promising conditions for crystallization, we tested the stability of HEWL under various pH conditions. HEWL has been crystallized under a variety of conditions in the pH range 4–10. The best diffracting crystals of HEWL were grown at lower pH ranges, typically around pH 4.2–4.7 (http://wwwbmcd.nist.gov:8080/bmcd/bmcd.html and http://www.pdb.org) (Berman *et al.*, 2000; Gilliland *et al.*, 1994). It was next investigated whether this assay could determine the relative stability of HEWL under different pH conditions and to see whether a correlation could be observed between the known diffraction quality of HEWL crystals and the measured stability using this assay. Fig. 3 shows the melting curves and the first-derivative results from a pH screen (pH values 3–8 in one pH-unit increments) of HEWL stability, revealing that the pH values 4, 5 and 8 are among those which yield greatest HEWL stability. Interestingly, this correlates well with the pH conditions under which some of the best diffraction-quality HEWL crystals have been grown [0.93 Å resolution, pH 4.6, PDB code 3lzt (Walsh *et al.*, 1998); 1.72 Å resolution, pH 7.6, PDB code 1lys (Harata, 1994); 1.80 Å resolution, pH 8.0, PDB code 1lkr (Steinrauf, 1998)]. It was also observed that HEWL is least stable at pH 3 and to our knowledge no crystals of HEWL have been reported at pH values below 4.0. This observation is encouraging and suggests the utility of this assay in determining optimal conditions for protein stability and ultimately crystallization and diffraction quality.

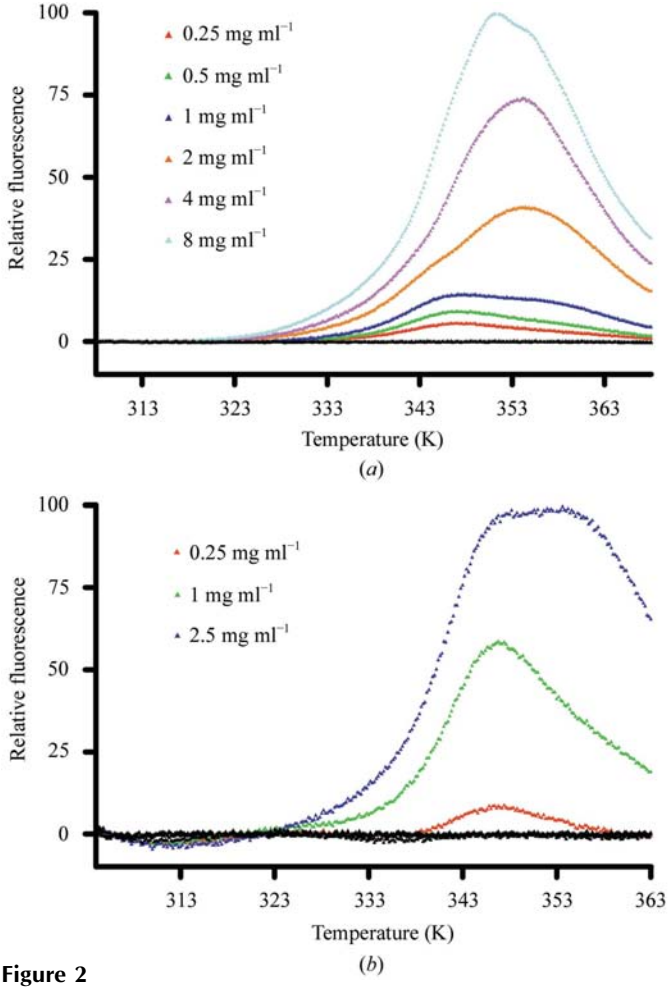

Figure 2
Unfolding curve for HEWL and a series of controls (black) using (*a*) 1:2500 diluted SYPRO Orange and (*b*) 1:125 diluted NanoOrange as the reporter dye. The control curves include detergent alone, protein alone, dye alone, dye plus a variety of detergents and a rescan of the sample. Only in the presence of both protein and reporter dye were evident unfolding curves observed.

3.3. Effect of detergent on protein stability and observation of melting curves

The type of detergent used to extract and solubilize a membrane protein is one of the key factors which must be determined empirically during membrane-protein purification, as it can have a great impact on the stability and as a result the ability of a membrane protein to crystallize. To test whether or not this assay might be applicable towards determining the effect of different detergents on protein stability, we recorded melting curves of HEWL in the presence of either dodecyl-β-D-maltoside (DDM), lauryldimethylamine-*N*-oxide (LDAO) or Zwittergent 3-12, each at 1.5× its critical micelle concentration (CMC). As can be seen in Fig. 4, two of these detergents did indeed have a marked effect on the T_m of HEWL. While the stability of HEWL did not seem to be altered in the presence of DDM ($T_m \simeq 349$ K), it appeared quite adversely affected in the presence of either LDAO ($T_m \simeq 331$ K) or Zwittergent 3-12 ($T_m \simeq 322$ K). This is not overly surprising as these latter detergents are considered destabilizing relative to DDM. Importantly, the detergent did not adversely affect the ability to observe fluorescence changes corresponding to the melting transition of the protein. This observation was encouraging, as it indicated that such an assay could be suitable for membrane proteins, where detergent is a key component.

3.4. Application to membrane proteins

In order to demonstrate the feasibility of this assay for membrane proteins, we utilized the acetylcholine receptor (AChR) from *T. marmorata* (Miyazawa *et al.*, 2003), the ABC-transporter BtuCD from *E. coli* (Locher *et al.*, 2002) and the mechanosensitive channels MscL (Chang *et al.*, 1998) and MscS (Bass *et al.*, 2002) from *E. coli*, the structures of all of which are known. AChR, a neurotransmitter-gated ion channel, consists of 20 transmembrane helices; BtuCD, an ABC transporter which mediates vitamin B_{12} uptake in *E. coli*, also comprises 20 transmembrane helices; MscL and MscS, mechanosensitive channels of large and small conductance, comprise ten and 21 transmembrane helices, respectively. One

concern in applying the assay to membrane proteins was the potential interference that detergent micelles may have on the assay. Because the dyes used in this assay are partially hydrophobic, they are expected to partition into the detergent micelle. Partitioning of the dyes into detergent micelles did indeed appear to occur, as overall fluorescence signals (background plus melting transition) were considerably greater than those in the absence of detergent. However, despite this complication we were able to observe clear melting transitions for three of the membrane proteins studied. Figs. 5(*a*) and 5(*b*) show a series of melting curves for AChR at various concentrations using either SYPRO Orange or NanoOrange as the reporter dye. Fig. 5(*c*) shows a series of melting curves for BtuCD at various concentrations and Fig. 5(*d*) shows melting curves of a pH screen of MscS. However, we were unable to observe an unfolding transition for MscL. Although the background fluorescence was greater owing to the partitioning of the reporter dye into detergent micelles, clear transitions could be observed for these proteins using as little as 5 µg of total protein. Calculation of the melting temperature from the first derivative of the curves gave T_m values of ∼321 K for AChR, ∼325 K for BtuCD and a pH-dependent range of ∼334–336 K for MscS. While no data are available for the thermal stability of BtuCD and MscS, some stability studies on AChR have been performed, which found an approximate T_m of 319 K based on loss of activity (Perez-Ramirez, 1994). This is in good agreement with the measured value using the reporter-dye assay of 321 K.

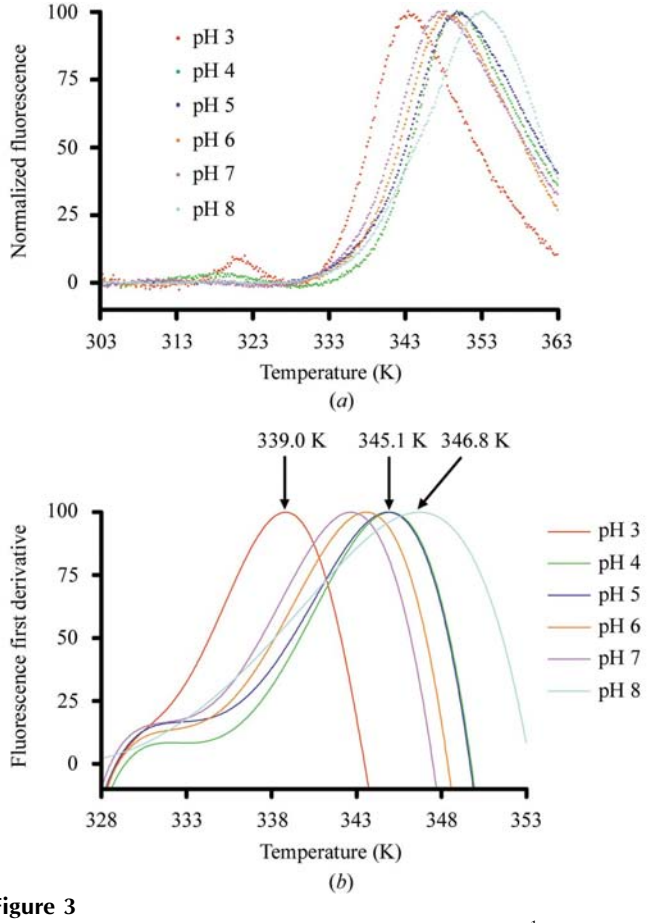

Figure 3
(*a*) Melting curves and (*b*) first derivatives of 2 mg ml^{-1} lysozyme in 100 m*M* Bis-Tris propane at several pH values. 1:2500 diluted SYPRO Orange was used as the reporter dye. Some of the highest melting temperature conditions were observed at pH values of 4–5 ($T_m \simeq 345$ K) and pH 8 ($T_m \simeq 347$ K), which also correspond to the pH range in which the highest diffraction-quality crystals of lysozyme have been grown (http://wwwbmcd.nist.gov:8080/bmcd/bmcd.html and http://www.pdb.org).

4. Discussion

A simple assay for measuring the unfolding transition of membrane proteins by fluorescent-probe binding in combination with real-time PCR instrumentation has been developed. The methods can be applied to a few micrograms of material and rely upon commercially available real-time PCR instrumentation and the commercially available dyes NanoOrange and SYPRO Orange. Although we have not exhaustively screened potentially suitable dyes, we expect similar results to be obtained using other hydrophobic partitioning dyes.

While the results reported above are encouraging and show the feasibility of such an assay, we realise that improvements can be made. One limitation which we encountered involved the DNA Engine Opticon real-time PCR instrument available for use in our assay. While both SYPRO Orange and NanoOrange fluorophores have broad excitation and emission peaks centered about 470 and 570 nm, respectively, the fluorescence-excitation and emission-detection ranges of the DNA Engine Opticon real-time PCR instrument are 450–495 and 515–545 nm, respectively. While the excitation maximum of both dyes lie within the instrument's fluorescence-excitation range, the emission maximum of 570 nm falls outside the detection range of the instrument. While emission fluorescence could still be detected at lower wavelengths, the fluorescence signals capable of being measured were approximately 50% of the maximal signal. Instruments that would be more suitable for use with the SYPRO Orange and NanoOrange dyes include the iQ5, MiniOpticon, DNA Engine

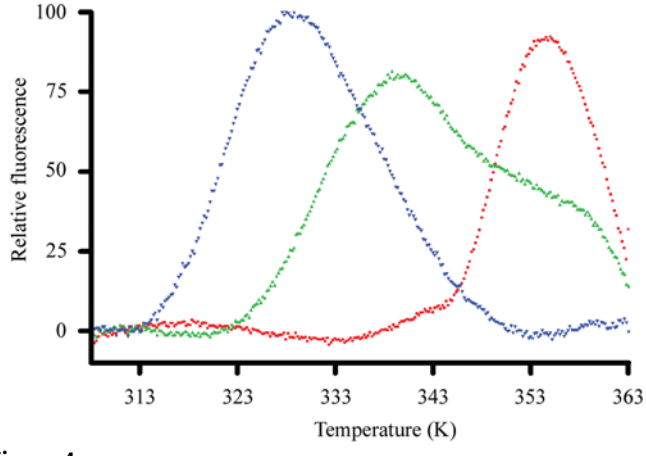

Figure 4
Melting curves of HEWL at 2 mg ml^{-1}, with 1:5000 diluted SYPRO Orange as the reporter dye, in the presence of different detergents: either 0.3 m*M* DDM (red), 3 m*M* LDAO at (green) or 6 m*M* Zwittergent 3-12 (blue), showing the marked effect of detergent choice on protein stability.

Opticon 2 and Chromo 4 Four-Color real-time PCR detector systems (Bio-Rad Laboratories Inc., Waltham, MA, USA), all of which have channel ranges that encompass the excitation and emission maxima of these two dyes. The use of one of these instruments should help to maximize the signal-to-noise ratios of the melting curves.

While the above limitation can be readily addressed, other potential limitations may not be as reparable as they are more inherent to the dyes used in the assay. One of these is the high background fluorescence obtained in the case of membrane proteins as a result of the partitioning of the dye, which is partially hydrophobic, into free detergent micelles and protein–detergent complexes. At high enough detergent concentrations, it is possible that the resulting background fluorescence may become sufficiently large to simply mask protein-unfolding transition signals. Even in the absence of detergent, such complications could still arise. For either soluble or membrane proteins which have solvent-accessible hydrophobic areas in their native folded state, it is plausible for the dye to access and bind to such sites and yield significant background fluorescence signals, giving rise to a situation similar to that of dye binding to detergent micelles.

In our experiments, while background fluorescence was indeed greater for the four membrane proteins tested owing to the partitioning phenomenon, the fact that clear melting transitions were observed for three of the four membrane proteins (AChR, BtuCD and MscS) was encouraging and indicative that this complication is not insurmountable. The fact that we did not observe melting-transition signals for the fourth membrane protein, MscL, might be explained by its different overall structure compared with those of the other three membrane proteins. The structure of MscL differs in that it does not contain large surface-hydrophilic extra-membranous regions, which AChR, BtuCD and MscS all do. Instead, in terms of extra-membranous regions, MscL contains only a periplasmic loop and a short cytoplasmic helix. It seems plausible that a great portion, if not all, of the melting-transition fluorescence signal observed for the three successfully tested membrane proteins would arise from the unfolding of their extra-membranous region(s), as the difference in the hydrophobic dye-accessible surface area presented by these regions pre- and post-denaturation would be likely to be be greater than that of their membrane-spanning regions, which are quite hydrophobic to begin with. If this is indeed so, it may

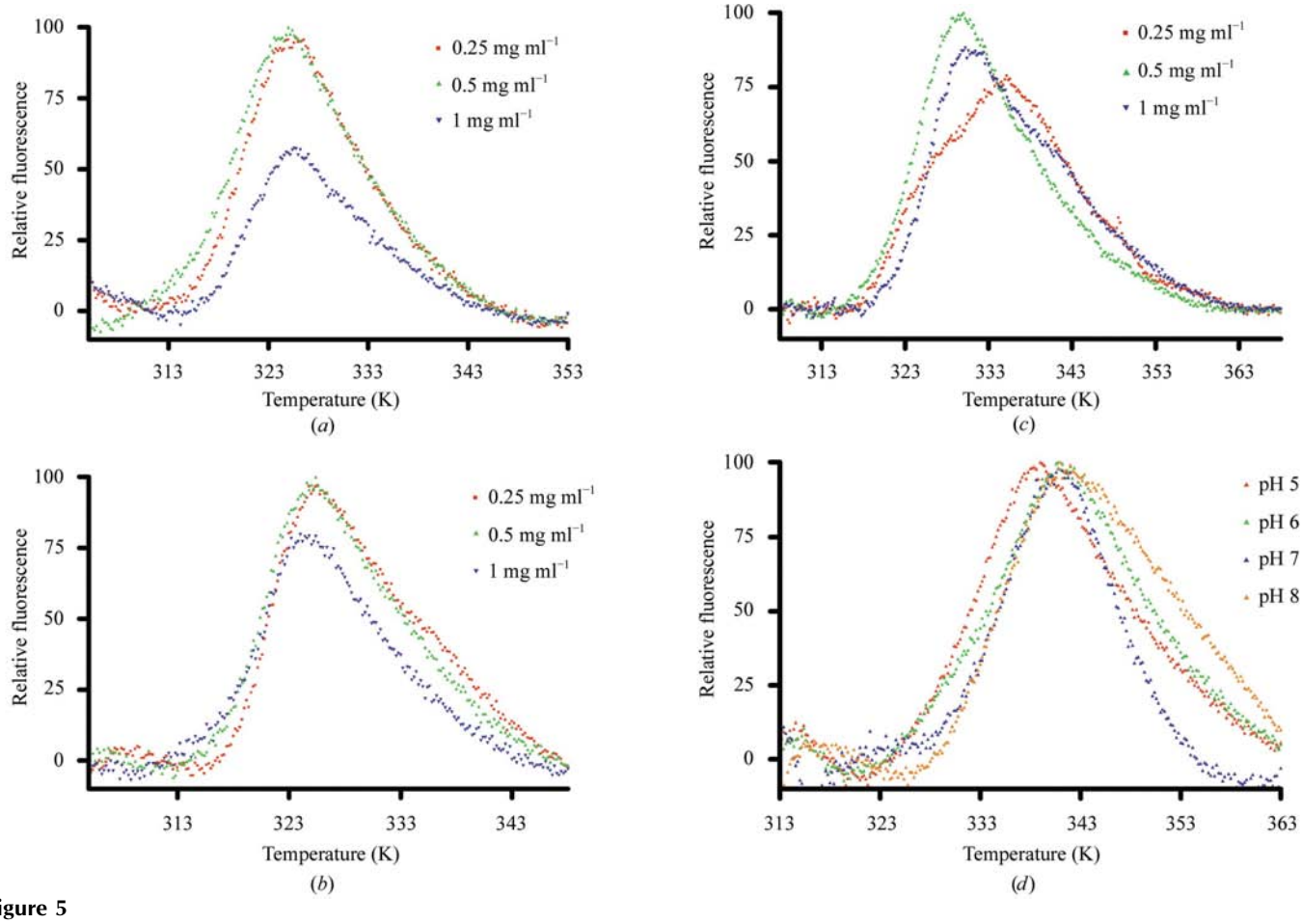

Figure 5
Melting curves for the membrane protein AChR solubilized with Brij-35 measured for three different concentrations using (*a*) 1:1250 diluted SYPRO Orange and (*b*) 1:50 diluted Nano Orange as the reporter dye. (*c*) Melting curves for BtuCD solubilized with LDAO measured for three different concentrations using 1:2500 diluted SYPRO Orange as reporter dye. (*d*) Melting curves for 0.2 mg ml^{-1} MscS solubilized with Fos-choline-14 measured for four different pH values using 1:50 diluted NanoOrange as the reporter dye. These measurements demonstrate the sensitivity and feasibility of using this method for screening optimal conditions for the crystallization of a membrane protein.

be the case that for membrane proteins this assay would be limited to those which contain considerably large extra-membranous regions.

A final caveat on the use of dyes in this assay is the potential effect(s) that a dye may have on the stability of the protein of interest. It is possible that protein–dye interactions could affect, either adversely or otherwise, the stability of the protein and thus alter its melting temperature. Indeed, such concerns regarding protein–dye interactions have been raised previously for the ANS dye (Vanderheeren *et al.*, 1998; Ali *et al.*, 1999; Matulis *et al.*, 1999; Smoot *et al.*, 2001). However, the fact that this assay yielded melting temperatures for HEWL ($T_m \simeq 348$ K) and AChR ($T_m \simeq 321$ K) that are in good agreement with values determined from previous studies seems to suggest that any such effects may not be very pronounced, at least in these two instances. Moreover, in the event that dye binding does indeed affect protein stability, its effect may not be such a critical issue in this case as the goal of this assay is not to measure the absolute melting temperature of a particular protein but rather to provide a basis upon which the relative stabilities of that protein under various conditions can be measured and compared.

In conclusion, the methods developed here should allow a rapid and simple screening of stable conditions for the crystallization of both soluble and membrane proteins. In light of some of the potential limitations discussed above, however, the future goal would be to develop a high-throughput assay which, instead of using external reporter dyes, uses the intrinsic fluorescence from aromatic groups (*i.e.* Phe, Tyr and Trp) of the membrane protein of interest to monitor its unfolding transition. Measuring protein unfolding *via* intrinsic protein fluorescence is well established and would obviate the need for extrinsic dyes, thereby eliminating some of the concerns discussed above. The fact that there is often a prevalence in integral membrane proteins of aromatic residues localized at the membrane–aqueous phase interface (Deisenhofer *et al.*, 1985; Weiss *et al.*, 1991; Chang *et al.*, 1998) further bodes well for the feasibility of such a method. To maintain the high-throughput nature of such an assay, we envision that using a commercially available 96-well microplate real-time PCR instrument would still be viable, although user modification of the instrument with the appropriate UV-light source and detector would be necessary.

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