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Development of a Thermofluor assay for stability determination of membrane proteins using the Na^+/H^+ antiporter NhaA and cytochrome *c* oxidase

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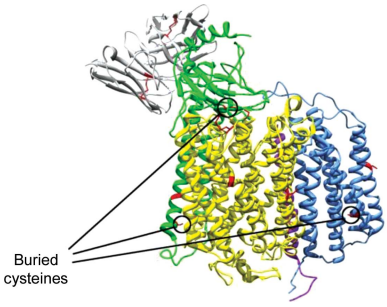
Crystallization of membrane proteins is very laborious and time-consuming, yielding well diffracting crystals in only a minority of projects. Therefore, a rapid and easy method is required to optimize the conditions for initial crystallization trials. The Thermofluor assay has been developed as such a tool. However, its applicability to membrane proteins is still limited because either large hydrophilic extramembranous regions or cysteine residues are required for the available dyes to bind and therefore act as reporters in this assay. No probe has been characterized to discriminate between the hydrophobic surfaces of detergent micelles, folded and detergent-covered membrane proteins and denatured membrane proteins. Of the four dyes tested, the two dyes 1-anilinonaphthalene-8-sulfonic acid (ANS) and SYPRO Orange were systematically screened for compatibility with five detergents commonly used in the crystallization of membrane proteins. ANS showed the weakest interactions with all of the detergents screened. It was possible to determine the melting temperature of the sodium ion/proton antiporter NhaA, a small membrane protein without large hydrophilic domains, over a broad pH range using ANS. Furthermore, cytochrome *c* oxidase (C*c*O) was used to apply the method to a four-subunit membrane protein complex. It was possible to obtain preliminary information on the temperature-dependent denaturation of this complex using the dye ANS. Application of the dye 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) to C*c*O in the Thermofluor assay enabled the determination of the melting temperatures of distinct subunits of the complex.

1. Introduction

The Thermofluor assay or differential scanning fluorimetry is a very widespread method to determine the melting temperatures of soluble proteins. Usually, a hydrophobic and environmentally sensitive fluorescent dye is added to the protein of interest. During the experiment, the fluorescence signal is monitored while a temperature gradient is applied to the sample. With increasing temperature the protein unfolds and provides hydrophobic patches for dye interaction. Binding of the dye leads to an increase in the fluorescence quantum yield of the dye and henceforth an increase in fluorescence (see Supplementary Fig. S1). After aggregation of the denatured protein the fluorescence signal decreases again owing to a smaller interaction surface for the dye. The interaction of the dye with the denaturing protein leads to a sigmoidal curve progression (see Supplementary Fig. S1). The inflexion point of the slope is the temperature at which half of the protein is denatured and the fluorescent dye is bound to it. This



temperature is defined as the apparent melting temperature T_m (Boivin *et al.*, 2013; Senisterra & Finerty, 2009; Dupeux *et al.*, 2011; Kopec & Schneider, 2011; Niesen *et al.*, 2007).

Since 2006, the Thermofluor assay has been used as a tool to optimize the initial conditions for crystallization studies by screening for buffer conditions and additives that promote thermal stabilization of the protein. A stabilization of more than 4°C was observed for 50% of 221 proteins in a high-throughput approach. 100 proteins were crystallized with a ligand, and 20 of these ligands were only identified by Thermofluor screening (Vedadi *et al.*, 2006). The Thermofluor assay has also been applied to protein–protein complexes. Kopec & Schneider (2011) obtained melting temperatures for 29 out of 31 protein complexes with the dye SYPRO Orange (SO). These curves showed a single transition consistent with a two-state model of thermal denaturation. Such a curve shape would be expected if the complex starts to disassemble at temperatures above the melting temperature of its individual components. The complex is enhanced in stability compared with the individual proteins.

Thermal stability screening is even more desirable when membrane proteins are the targets of structural studies, where usually only small amounts of sample are available. Yeh *et al.* (2006) reported a high background fluorescence signal for this method when detergent was present in the sample owing to interaction of the dye with the amphiphilic detergent molecules. However, SO and NanoOrange (NO) have successfully been used to determine the melting temperatures of membrane proteins with large hydrophilic extramembranous regions. For smaller membrane proteins without large hydrophilic regions melting temperatures were not obtained owing to the diminished difference in hydrophobicity of the native and denatured states of the protein (Yeh *et al.*, 2006; Kean *et al.*, 2008). To expand the use of the Thermofluor assay to membrane proteins without interference from the detergent, Alexandrov *et al.* (2008) used the fluorescent dye 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM), which binds covalently to thiol groups, becoming exposed upon denaturation. This assay is very useful if the protein contains at least one cysteine hidden in the membrane-protein core. Alexandrov and coworkers claimed that such a hidden cysteine is present in about 60% of all membrane proteins. The Thermofluor assay in combination with CPM is usable in the pH range 6–8 based on the nature of the coupling reaction.

This study extends and facilitates the use of the Thermofluor assay for membrane proteins, in particular for those without large hydrophilic domains.

Fluorescent probes that bind to hydrophobic surfaces could be used to determine the melting temperatures of proteins without cysteines and cover a broad pH range. The remaining problem is that detergent micelles, detergent-covered folded membrane proteins and denatured membrane proteins offer only surfaces that are mostly lipophilic. Therefore, we address the following questions. Are there dyes and detergents which both interact in a less pronounced manner? If so, do these dyes interact more strongly with the unfolded membrane protein than with the detergent-solubilized native protein? We tested the dyes SO, ANS, NO, Nile red (NR) and in addition CPM to answer these questions.

2. Materials and methods

2.1. Dyes and detergents

The following dyes were used as probes as in prior studies. SYPRO Orange (SO; Lo *et al.*, 2004; Yeh *et al.*, 2006; Niesen *et al.*, 2007), 1-anilinonaphthalene-8-sulfonic acid (ANS; Pantoliano *et al.*, 2001; Niesen *et al.*, 2007), Nile red (NR; Niesen *et al.*, 2007), NanoOrange (NO; Yeh *et al.*, 2006) and 7-diethylamino-3-(4′-maleimidylphenyl)-4-methlycoumarin (CPM; Alexandrov *et al.*, 2008; Sonoda *et al.*, 2011) were purchased from Invitrogen (Carlsbad, California, USA). The dyes SO and NO were obtained as 5000× stock solutions of unknown concentration. These were diluted in the assay and the final concentration is given as × stock solution (*e.g.* 20× SO is a 250-fold dilution of the original dye). The detergents *n*-dodecyl-β-D-maltoside (DDM), *n*-octyl-β-D-glucopyranoside (OG), *n*-dodecylphosphocholine (FOS12), *n*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide (LDAO) and octaethylene glycol monododecyl ether (C12E8) were obtained from Glycon (Luckenwalde, Germany), Affymetrix (Santa Clara, California, USA) and Sigma–Aldrich (Steinheim, Germany).

2.2. Proteins

Chicken egg-white lysozyme was purchased from Hampton Research (Aliso Viejo, California, USA). C*c*O from *Paracoccus denitrificans* (strain ATCC 13543) was affinity-purified using a Strep-tagged Fv fragment from a monoclonal antibody as described previously (Kleymann *et al.*, 1995). Recombinant His_6-tagged NhaA from *Escherichia coli* (RK20/pAXH/pIQ) was overexpressed and purified as described by Hunte *et al.* (2005). C*c*O and NhaA were purified in buffers containing DDM. For the Thermofluor assay, stock solutions of 40 µ*M* C*c*O in 10 m*M* potassium phosphate buffer pH 8, 0.2 m*M* EDTA, 0.01%(*w*/*v*) DDM and of 200 µ*M* NhaA in 20 m*M* HEPES pH 7.5, 100 m*M* KCl, 5 m*M* $MgCl_2$, 10%(*w*/*v*) sucrose, 0.01%(*w*/*v*) DDM were prepared.

2.3. Thermofluor assay

The Thermofluor assay was carried out using a Rotor-Gene Q (Qiagen, Hilden, Germany) with a temperature gradient from 30 to 95°C and a heating rate of 12°C min^{-1}. The Rotor-Gene Q contains a rotor with 36 sample tubes spinning at 4000 rev min^{-1}. Heating the rotor chamber with coils and cooling with an air flow allows very precise temperature adjustment and uniform air circulation on all tubes. Thermal denaturation of the protein samples was indirectly measured by monitoring the fluorescence change based on the binding of the dye to the denatured protein. The dyes are listed to have the following excitation and emission wavelengths: SO, 470/570 nm; ANS, 350/480 nm; NO, 470/570 nm; NR, 552/636 nm; CPM, 384/470 nm. Information on the excitation and emission wavelengths was taken from the manufacturer's webpage (http://www.lifetechnologies.com) and those for ANS from

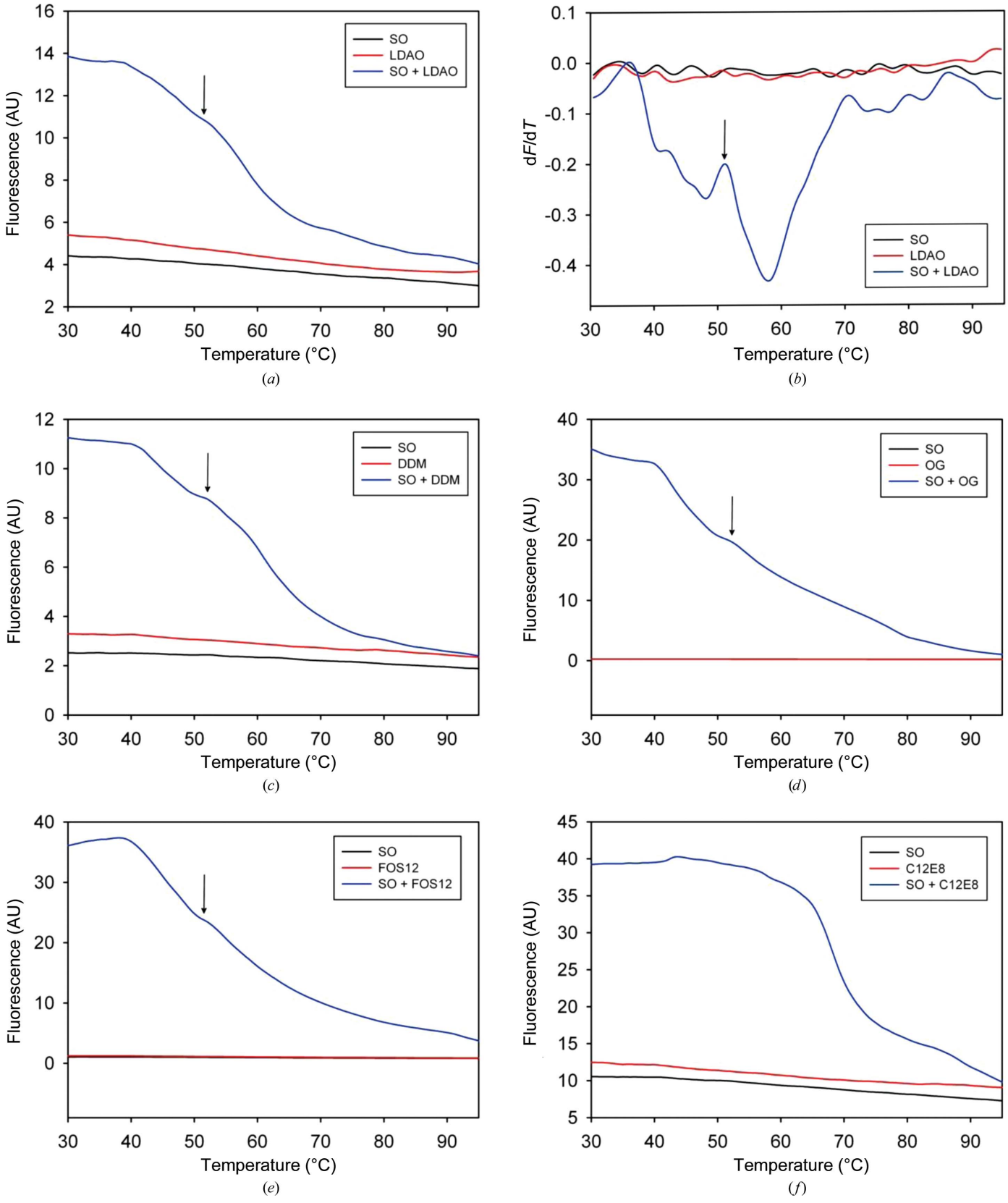


Figure 1
(*a*) Raw data for an experiment with 1.1 CMC LDAO and SO in blue with two controls of dye in buffer and LDAO in buffer in black and red, respectively (gain levels are listed in Supplementary Table S1). The fluorescence intensity of the detergent-containing sample decreases considerably with increasing temperature. The controls remain constant. Fluorescence changes resulting in the described artefact signal are marked by an arrow. (*b*) First derivative of the data shown in (*a*). The experiment with LDAO shows a local maximum in the first derivative at approximately 50°C (arrow). (*c*–*f*) Experiments performed as in (*a*) with (*c*) DDM, (*d*) OG, (*e*) FOS12 and (*f*) C12E8.

Kirk *et al.* (1996). The emission wavelength of ANS is blue-shifted by about 60 nm when the dye is transferred from an aqueous environment to a hydrophobic environment and the quantum yield is increased from 0.004 to 0.98 (Stryer, 1965). For each dye, different fluorescence filter settings were tested (green, 470/510 nm; yellow, 530/557 nm; orange, 585/610 nm; red, 625/660 nm; magenta, 680/712 nm; HRM, 460/510 nm). The excitation and emission filters of all channels can be used in pairs as appropriate. For all dyes used, the high-resolution melt channel (HRM) worked best. The manufacturer reported a higher excitation intensity for this channel at a wavelength of 460 nm compared with all other excitation sources. The gain level providing the highest fluorescence signal but remaining below 30% of the detector maximum was used. The detector gain varied from a minimum sensitivity of −10 to a maximum sensitivity of 10; increasing the gain by one unit resulted in a doubling of the fluorescence intensity. Data analysis was performed using the *Rotor-Gene Q Series* software. This tool calculates the first derivative *versus* temperature and allows the T_m of an experiment to be easily read out. The raw data and the first derivative were exported for visualization with *SigmaPlot* 10.0.

Experiments with lysozyme were carried out with 5 µ*M* lysozyme in 100 m*M* HEPES pH 7.5, 100 m*M* NaCl (HEPES buffer) with varying dye concentrations (1× to 50× SO; 0.3–4 m*M* ANS; 1 µ*M* to 4 m*M* NR; 1× to 100× NO). 20× SO or 800 µ*M* ANS were used to screen different detergents, varying the detergent concentrations between 0.1 CMC (critical micelle concentration) and 5.0 CMC in HEPES buffer.

For the experiments with NhaA and ANS the protein and dye were diluted in HEPES buffer with 0.01%(*w*/*v*) DDM to final concentrations of 20 µ*M* and 1 m*M*, respectively, in a reaction volume of 20 µl. The influence of different pH values on the T_m of NhaA was measured with 75 m*M* NaCl, 0.01%(*w*/*v*) DDM and 75 m*M* sodium acetate pH 5.0, MES pH 6.0, HEPES pH 7.0, Bicine pH 8.0 or CHES pH 9.0. Experiments with NhaA and SO were carried out using final concentrations of 5 µ*M* NhaA and 20× SO in HEPES buffer with 0.01%(*w*/*v*) DDM.

For experiments with C*c*O and CPM the protein and dye were diluted in HEPES buffer containing 0.01%(*w*/*v*) DDM to final concentrations of 0.84 and 380 µ*M*, respectively, in a reaction volume of 26 µl. Measurements with C*c*O and ANS were performed with C*c*O concentrations from 1 to 4 µ*M* C*c*O and 0.5 to 4 m*M* ANS in HEPES buffer containing 0.01%(*w*/*v*) DDM.

2.4. Calorimetry

The thermal stability of C*c*O and NhaA was determined using a VP capillary differential scanning calorimeter (GE Healthcare, Buckinghamshire, England). The thermal stability of C*c*O and NhaA was determined with protein concentrations of 5 mg ml^{-1} in phosphate buffer and 3 mg ml^{-1} in HEPES buffer, respectively, each containing 0.01%(*w*/*v*) DDM. The samples were scanned at a scan rate of 1.5°C min^{-1} in low-feedback mode. Data analysis was performed with *Origin* 7 (Additive GmbH, Friedrichsdorf, Germany).

3. Results

3.1. Initial tests with the dyes

The dyes NO, NR, SO and ANS were chosen because they have been previously applied in Thermofluor assays. All four dyes were used separately with lysozyme to demonstrate functionality in our setup. Protein and dye concentrations were varied to find the optimal parameters. Melting curves for lysozyme were obtained with SO, ANS (Supplementary Fig. S1) and NO. However, the intensity of the fluorescence signal using NO was too low. The most applicable conditions for the Thermofluor assay exceeded the NO tolerance level of 20 m*M* for NaCl (Life Technologies, catalogue No. N-6666). The detector gain used was at its maximum and the fluorescence signal potentially indicating protein denaturation was not reproducible. In contrast, NR fluorescence exhibited a strong background signal that was dependent on the change in temperature as indicated by controls with dye and buffer only. No working filter combinations were present when using NR at the described concentrations and detergents. Therefore, the dyes NO and NR were excluded from further measurements.

For all measurements a heating rate of 12°C min^{-1} was chosen. Chicken egg-white lysozyme was used to assess the deviation in T_m applying different heating rates. Five heating rates were chosen between 0.1 and 12°C min^{-1}. The melting temperatures differed only by 1.25°C in total (data not shown) and the lowest melting temperature was observed at 1°C min^{-1}, showing that the influence on the observed T_m was negligible.

3.2. Detergent tests

The detergents DDM, OG, FOS12, LDAO and C12E8 were used at 1.1 CMC in combination with the dyes ANS and SO. Both dyes showed a decreasing fluorescence signal for all detergents with increasing temperature in the experiments. One obvious difference between the dyes is the gain level used to record the fluorescence signal. The gain levels used for SO varied between −3 for OG and 4.7 for C12E8. For ANS, the gain levels used varied between 4 for LDAO and 6 for DDM (see Supplementary Table S1 and Fig. 1). The high gain levels for ANS point to a rather weak interaction between the dye and the detergents. This feature is important for establishing the assay with detergents and membrane proteins.

An increase in the detergent concentration leads to an increased initial fluorescence intensity. The shape of the fluorescence signal *versus* temperature plot varies for the different detergents. These differences possibly arise from interactions of the dyes with the detergent micelles that change with temperature (Pinaki & Blume, 2001). When the first derivative of the different experiments is plotted, a consistent maximum at about 50°C is found in all measurements. This maximum varies and shifts slightly depending on the detergent used. The 50°C signal was first observed in

detergent controls. The integrated area of this signal increases with increasing detergent concentrations, which is exemplarily shown for DDM and ANS in Supplementary Fig. S2. This signal seems to be an artefact that depends on the detergent concentration and temperature screening. More precisely, it arises from a retardation of the heating process, since at about 50°C the instrument switches to another heating coil (Qiagen, personal communication).

3.3. Tests with detergent and soluble protein

Both the SO and ANS dyes were used to test whether differentiation between the detergent and protein components of the fluorescence signal in the Thermofluor assay is possible. Fig. 2 shows the fluorescence intensity (Fig. 2*a*) and the corresponding first derivative (Fig. 2*b*) for lysozyme with SO. The controls SO, DDM and DDM + lysozyme did not show a considerable signal in the assay. In contrast, SO + DDM showed a decrease in fluorescence intensity resulting in a maximum visible in the first derivative at about 50°C as described above. The experiment with SO + DDM + lysozyme showed a very similar result for the first derivative.

The same set of controls and experiment was performed for the dye ANS. The results are illustrated in Figs. 2(*c*) and 2(*d*). The controls ANS, DDM and DDM + lysozyme again did not show a considerable signal. The control ANS + DDM showed a decreasing fluorescence intensity and a maximum visible in the first derivative at about 50°C. The sample with ANS + DDM + lysozyme showed a similar fluorescence signal as ANS + DDM with an additional initially increasing and then

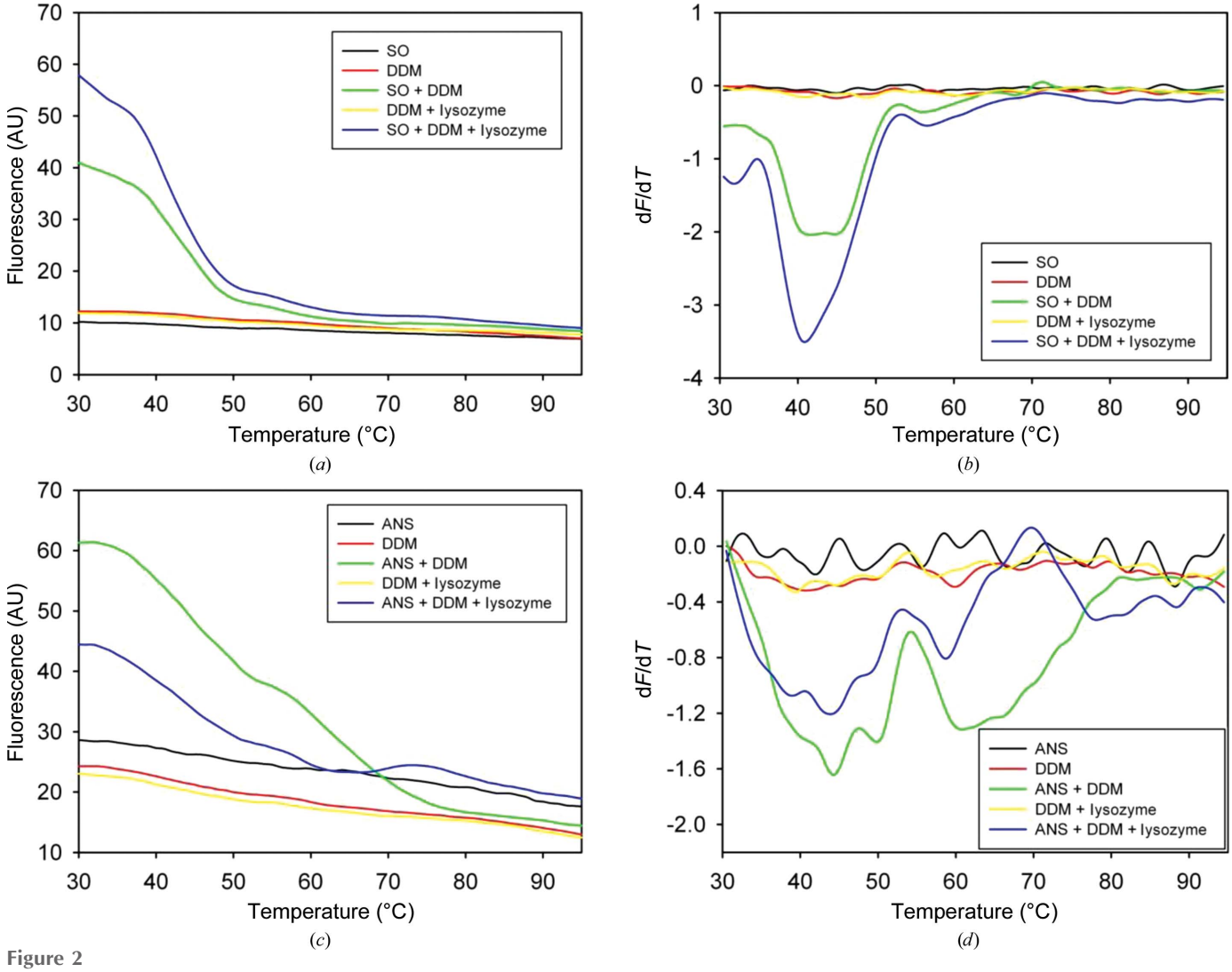


Figure 2
(*a*) Raw data for a lysozyme and detergent (1.1 CMC DDM) experiment with SO in blue and four controls of dye in black, DDM in red, SO + DDM in green and DDM + lysozyme in yellow (gain 4). The fluorescence readout for the controls without detergent is low and almost constant. The sample with dye, detergent and lysozyme and the control with dye and detergent show a rapidly decreasing signal from 30 to 50°C. (*b*) First derivative of the data shown in (*a*). The detergent control of SO + DDM shows a local maximum at about 50°C. The experiment with SO + DDM + lysozyme also shows a local maximum at about 50°C. (*c*) The same experiment as in (*a*) but with ANS instead of SO. The fluorescence signal for the controls without detergent decreases slightly. The sample and the control with detergent decrease from 30 to 60°C and from 30 to 70°C, respectively. (*d*) First derivative of the data shown in (*c*). The control with ANS and DDM shows a local maximum at about 50°C, whereas the sample with ANS + DDM + lysozyme shows an additional maximum at 70°C. This result is in absolute agreement with the T_m of lysozyme determined without detergent (Supplementary Fig. S1).

decreasing signal from 65 to 80°C. The first derivative showed a local maximum at about 50°C and a maximum at 70°C. A T_m of 70°C for lysozyme had also been determined as the melting temperature in the initial tests lacking detergent (Supplementary Fig. S1). However, the signal-to-noise ratio in this experiment was still inadequate and was hard to reproduce.

As a setup containing lysozyme and detergent cannot replace and reflect the conditions of an experiment with a membrane protein, both dyes were used to check their practicability for membrane proteins.

3.4. Application to the membrane protein NhaA

The sodium ion/proton antiporter from *E. coli* NhaA was used to assess the feasibility of the Thermofluor assay for membrane proteins. NhaA is a dimer consisting of 12 transmembrane helices and a β-sheet that contributes to the dimer interface of the transporter. NhaA has a compact transmembrane core and no large hydrophilic domains. It has been intensively studied and two crystal structures at 3.5 Å resolution are available for the protein at acidic pH (PDB entries 1zcd and 4au5; Hunte *et al.*, 2005, D. Drew, O. Beckstein, C. Lee, S. Yashiro, M. S. P. Sansom, S. Iwata & A. D. Cameron, unpublished work). NhaA is inactive below pH 6.5 and its rate of activity increases drastically with pH, reaching a maximum at pH 8.5 (Padan, 2014). To date, no detailed structural information on NhaA in the active state is available. Measurements of the thermal stability of NhaA at different pH values could be advantageous for determining the structure of the antiporter in the active state.

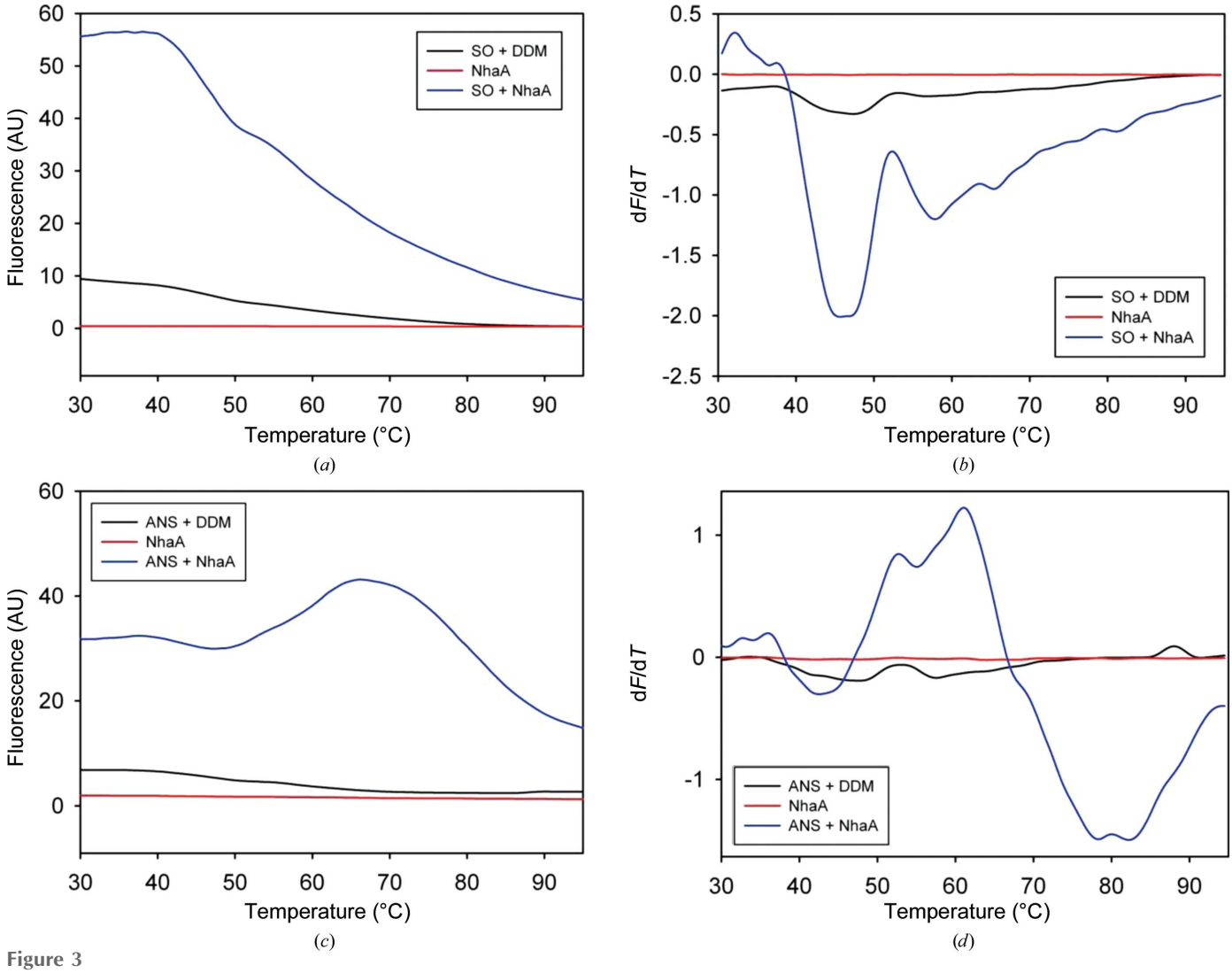


Figure 3
(*a*) Raw data for an experiment with SO and NhaA. The controls are SO + DDM and NhaA in DDM-containing buffer and are shown in black and red, respectively. The sample SO + NhaA is shown in blue (gain −1.7). The fluorescence intensity of the detergent control decreases slightly from 30 to 80°C, whereas the fluorescence intensity of the sample decreases from 45 to 95°C. (*b*) First derivative of the data shown in (*a*). The detergent control and the sample both show a local maximum at about 50°C, which can be assigned to an artefact signal only. (*c*) Raw data for an experiment with NhaA and ANS in blue with two controls of dye in detergent buffer and NhaA in detergent buffer in black and red, respectively (gain 1.3). The fluorescence signal of both controls remains almost flat. The readout for the NhaA-containing sample shows an increase in fluorescence from 50 to 67°C and a decrease in the signal to 95°C. (*d*) First derivative of the data shown in (*c*). The NhaA-containing sample shows a clear T_m of 61.5°C. The 50°C artifact signal is a local maximum which can be distinguished from the melting temperature. The control also shows the local maximum at about 50°C.

In trials with SO and NhaA, a decreasing fluorescence intensity was observed for SO + DDM, with a higher intensity for SO + DDM + NhaA. The first derivatives only show a maximum at about 50°C that has been described as an artefact signal above (Figs. 3*a* and 3*b*). The dye SO therefore could not be used to establish a Thermofluor assay for this membrane protein with DDM as detergent.

Experiments with NhaA and ANS were carried out to test this dye. Fig. 3(*c*) shows the fluorescence signal *versus* temperature for the controls ANS + DDM and DDM + NhaA and for the experiment ANS + DDM + NhaA and the corresponding first derivatives. The raw data for DDM + NhaA showed no increase in fluorescence intensity. A slight decrease in fluorescence for ANS + DDM is visible between 40 and 70°C. This decrease yields a maximum in the first derivative at approximately 52°C corresponding to the described artefact signal. A roughly three times more intense initial fluorescence signal is observed for the sample consisting of ANS + DDM + NhaA. The signal intensity increases steadily from 30 AU at 50°C to 40 AU at 67°C and decreases to 20 AU at 95°C. The high fluorescence intensity might reflect interaction between ANS and NhaA that is already present at the start of the experiment, suggesting a rather large hydrophobic interaction between ANS and the native NhaA. However, the signal shows a clear increase and decrease with rising temperature. This behaviour leads to a maximum and therefore a T_m of 61.1 ± 0.4°C in the first derivative of the experiment (Fig. 3*d*). A DSC measurement for NhaA was performed to independently determine the melting temperature. Fig. 4(*d*) shows the DSC results, with a clear transition temperature at 63.7°C.

The next step was to examine the thermal stability of NhaA in the pH range from pH 5 to 9. Fig. 4 shows the fluorescence signal (Fig. 4*a*) and the corresponding first derivative (Fig. 4*b*) for the controls and measurements from pH 5 to 9 with NhaA. Increments of one pH unit were chosen. The results of at least three measurements are plotted in Fig. 4(*c*). The melting temperatures determined vary between 66.4 ± 0.6°C at pH 6 and 52°C at pH 9. Because of the artefact signal the observed melting temperatures at about 52°C can only be evaluated in a qualitative way. The true melting temperatures for pH 8 and 9 are below 61.5°C (pH 7.5; Fig. 3*c*) and above 52°C.

3.5. Application of the Thermofluor assay to a four-subunit membrane protein

After successful application of the assay to a homodimeric membrane protein, the method was further tested on a membrane protein complex. C*c*O from *P. denitrificans* is a well studied membrane protein consisting of four protein subunits

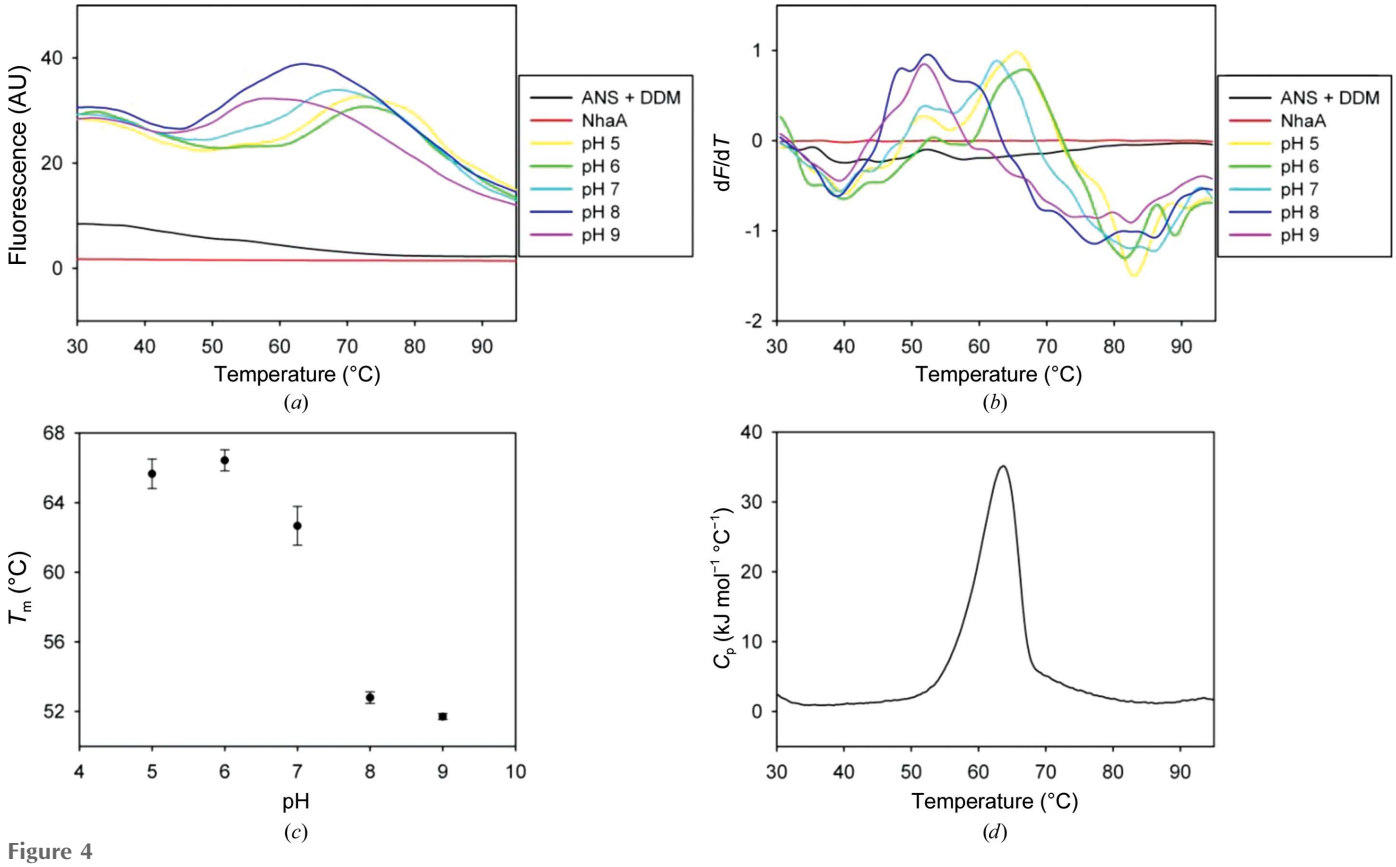


Figure 4
(*a*) Raw data for experiments with NhaA and ANS at different pH values in yellow, green, light blue, blue and magenta with two controls of dye in detergent buffer and NhaA in detergent buffer in black and red, respectively (gain 1.3). (*b*) First derivative of the data shown in (*a*). NhaA shows melting temperatures between 66 and 52°C. (*c*) Diagram of averaged melting temperatures at different pH values. Standard deviations were calculated for at least three measurements. (*d*) Data for a DSC experiment with NhaA. A single transition temperature at 63.7°C was found at a pH of 7.5.

with a known structure (Iwata *et al.*, 1995; Harrenga & Michel, 1999; Koepke *et al.*, 2009; PDB entries 1qle and 3hb3). C*c*O is one of the terminal enzymes of the respiratory chain and catalyses the reduction of molecular oxygen to water, contributing to the formation of an electrochemical proton gradient across the membrane.

Measurements were performed with C*c*O and ANS (Figs. 5*a* and 5*b*). Again, the fluorescence intensity of ANS and DDM decreases, showing the artefact signal at 50°C in the first derivative. The measurement with ANS + DDM + C*c*O, however, shows a more slowly decreasing fluorescence intensity from 60 to 75°C. The first derivative of this signal shows a maximum at 70°C. The signal-to-noise ratio obtained is insufficient to probe the membrane protein complex with this dye only. Therefore, measurements were performed with CPM to compare and consolidate the result for C*c*O. CPM interacts with the unfolded protein by forming a covalent bond to cysteine residues; therefore, it is insensitive to detergents. Figs. 5(*c*) and 5(*d*) show the fluorescence intensity and the corresponding first derivatives. The covalent attachment of CPM results in a fluorescence increase when the temperature is raised from 45 to 55°C and a steeper increase from 65 to 75°C. The first derivative shows two broad maxima at 47–49°C and 69°C.

The observed melting temperature profile in our DSC measurement (Fig. 5*f*) corresponds well to previously published data (Haltia *et al.*, 1994; Hilbers *et al.*, 2013). The T_m values of Hilbers and coworkers and our results are similar,

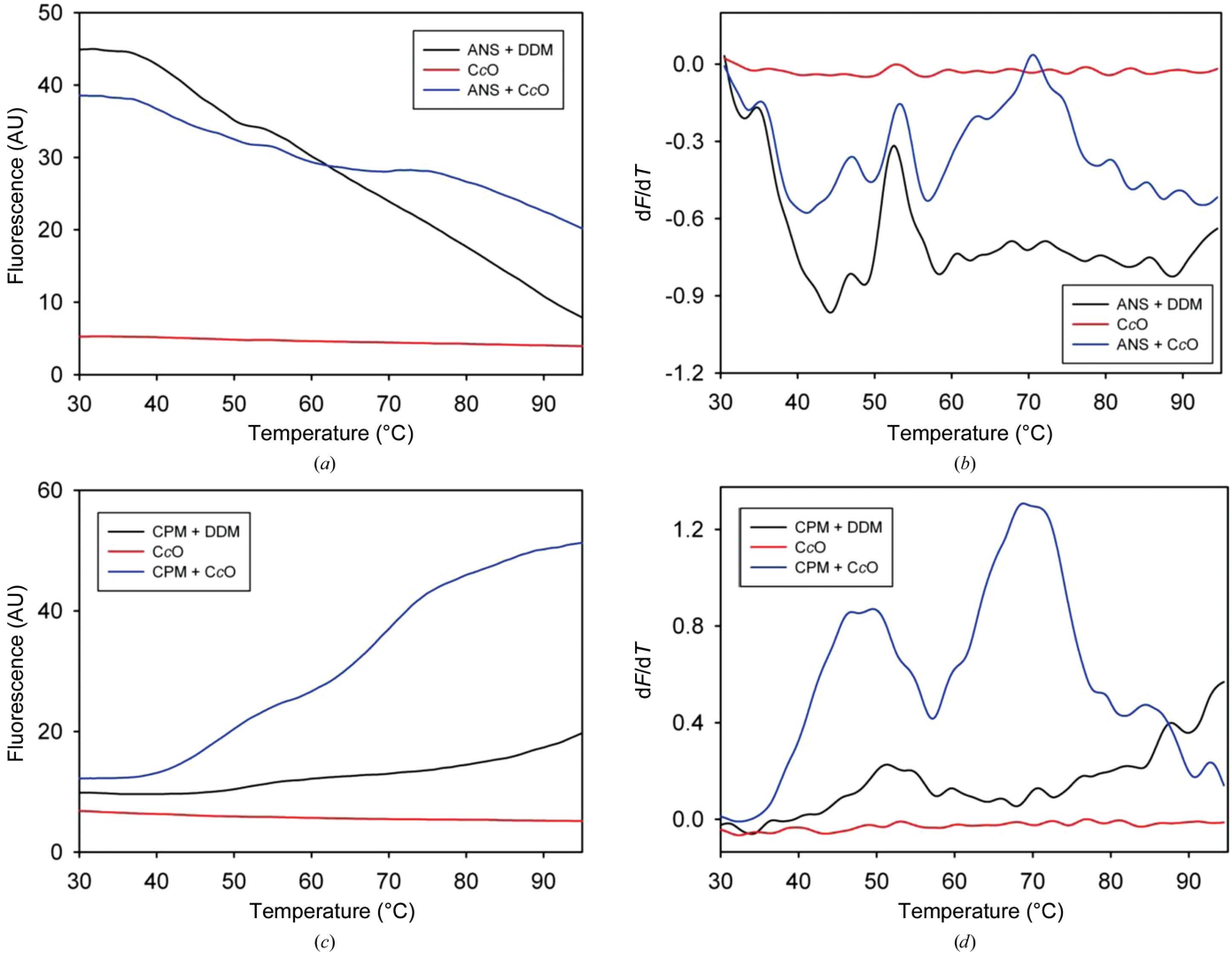


Figure 5
(*a*) Raw data for a C*c*O experiment with ANS (blue) with two controls of dye in detergent buffer and C*c*O in detergent buffer in black and red, respectively (gain 2.7). The fluorescence signal for the detergent control decreases from 30 to 95°C. The signal for the sample containing ANS and C*c*O also decreases from 30 to 60°C, reaches a plateau at about 70°C and decreases further at 95°C. (*b*) First derivative of the data shown in (*a*). A local maximum at about 50°C can be observed for the detergent control and the sample. Additionally, a maximum can be observed for ANS and C*c*O at 70.5°C. The signal-to-noise ratio monitored in this experiment is insufficient. (*c*) Raw data for a C*c*O experiment with CPM in blue with the two controls of dye in detergent buffer and C*c*O in detergent buffer in black and red, respectively (gain 4). The fluorescence for CPM and detergent increases slightly owing to hydrolysis of the dye at higher temperatures. The signal of the sample increases in a biphasic manner from 45 to 55°C and from 65 to 75°C. (*d*) First derivative of the data shown in (*c*). C*c*O shows two distinct peak maxima for T_m. T_{m1} is in the range 46.8–49.5°C and T_{m2} is at 68.8°C.

with values of 43°C for the thermal denaturation or dissociation of subunits III and IV, 50°C for the Fv fragment and 61°C for subunits I and II. Haltia and coworkers determined T_m values of 45°C for subunit III and 67.5°C for subunits I and II.

4. Discussion

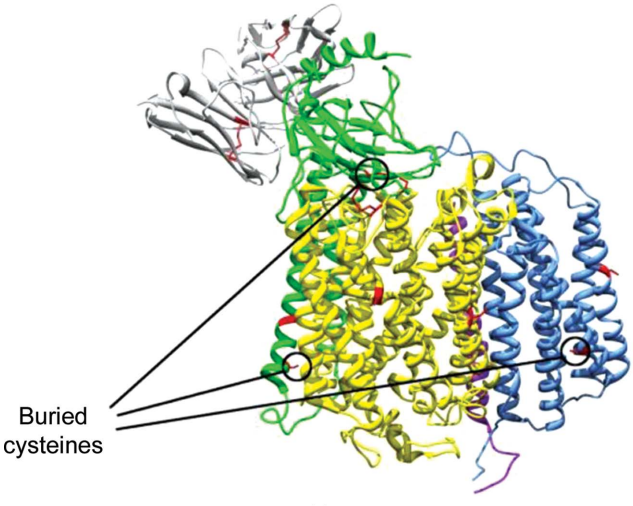
The Thermofluor experiments in this study were carried out using a Rotor-Gene Q, which was initially developed as a real-time PCR cycler. The Rotor-Gene Q incorporates a rotor containing the sample tubes. This setup allows very fast and precise heating and cooling that is uniform for all tubes. Another important feature is the 90° angle formed between the source of the incident beam and the detector. These are the major differences compared with a standard PCR cycler working with 96-well or 384-well plates. This setup with a small sample volume of 20–30 µl and the effective and uniform heating of the samples also allowed a heating rate that was eight times faster compared with the calorimetric measurements used as a reference. In this study, lysozyme was used to assess the deviation in T_m applying different heating rates. Five heating rates were chosen between 0.1 and 12°C min^{-1}. The melting temperatures differed only by 1.25°C in total. Nevertheless, in the case of membrane proteins the thermal transition is irreversible and T_m is sensitive to the parameters of the experiment. However, we choose DSC as a well established method for determination of the thermal stability of membrane proteins. This approach gives us the possibility of comparing the melting temperature profiles of the Thermofluor assay using an unbiased method.

As a starting point, the utility of the Thermofluor assay for membrane proteins with several fluorescent probes was tested with several detergents with regard to their ability to interact specifically with the hydrophobic core of a denatured membrane protein. While the interaction with the correctly folded membrane protein with surrounding detergent molecules or with detergent micelles was simultaneously unfavoured.

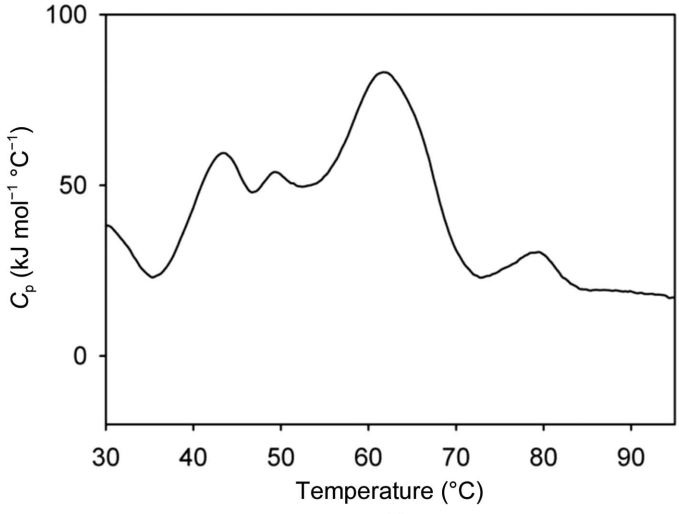
All five tested detergents used showed high background fluorescence when combined with ANS or SO. A further investigation of the ANS data indicated a weaker interaction with the detergents since the gain levels used were usually higher (an increase of about one gain unit doubles the fluorescence intensity). This observation could also partly be caused by the selection of the excitation and emission wavelengths. The excitation wavelength of 460 nm matches that of SO (470/570 nm) but not the emission wavelength of 510 nm, while the opposite is the case for ANS (350/480 nm).

The dyes used showed varying fluorescence profiles with the different detergents when the temperature was changed. These profiles are related to the interactions between the fluorescent dyes and the detergent molecules. As similar fluorescence profiles were observed with the dyes ANS and SO, the profiles might point to the intrinsic properties of each detergent. The observed fluorescence profile in experiments with C12E8 is quite different compared with the other four tested detergents and might be related to the different polar head group of C12E8, which is large and shaped like a polymer, compared with the other detergents. From the experiments above it is concluded that the use of different detergents in the Thermofluor assay seems to be possible. This result also points to the possibility of using the Thermofluor assay to describe the properties of detergents and/or lipids. Nevertheless, the detergent concentration should be as low as possible to reduce the background signal of the measurement.

NhaA was used to evaluate the Thermofluor assay for small membrane proteins without large hydrophilic domains. SO did not yield any results. This observation might correlate well



(*e*)



(*f*)

Figure 5 (continued)
(*e*) Ribbon diagram of C*c*O with an Fv fragment (PDB entry 1qle). Subunits I, II, III, IV are coloured yellow, green, blue and purple, respectively. The Fv fragment is coloured grey. All cysteines are shown in red with side chains as sticks. Cysteines that become accessible during denaturation are highlighted with circles and arrows. (*f*) Data for a differential scanning calorimetry (DSC) experiment with CcO. The transition temperatures of the complex are 43°C (dissociation of subunits III and IV) for the first maximum, 50°C for the second maximum (denaturation of Fv) and 61°C for the third maximum (dissociation or denaturation of subunits I and II).

with the findings of Yeh *et al.* (2006) and Kean *et al.* (2008). They observed melting transitions of large membrane proteins with large hydrophilic extramembranous regions with SO but not for a rather small membrane protein such as NhaA with no additional hydrophilic regions. SO is not as useful as ANS for differentiating between hydrophobic environments of detergent micelles, folded detergent-covered membrane proteins and denatured membrane proteins under our experimental conditions.

ANS, however, showed a more prominent fluorescence signal with NhaA, which resulted in a strong maximum in the first derivative. The artefact signal and the melting transition are not well separated, but the maxima can be easily distinguished. ANS was applied to a pH screen in a pH range from pH 5 to 9. It was shown that NhaA is most stable in its inactive state at pH 6 (T_m = 66.4 ± 0.6°C). These results correlate well with the results from Fourier-transform infrared spectroscopy (FTIR) measurements, in which a transition of the α-helical protein content was observed at 63°C (pH 6.2; Dzafic *et al.*, 2009), and the transition temperature profile determined by DSC measurements (T_m = 63.7°C at pH 7.5; Fig. 4*d*)

The observed melting temperatures of about 52°C at pH 8 and 9 can only be evaluated qualitatively because the signals overlap with the artefact signal. This feature is a general problem in Thermofluor assays using the Rotor-Gene Q. The problem could be overcome by using a different thermal cycler heated with a Peltier cooler. Another way of probing the temperature range close to 52°C using the current setup might be by employing isothermal denaturation (ITD; Epps *et al.*, 2001; Senisterra *et al.*, 2010). In connection with the Rotor-Gene Q this would mean no change of the heating coil and no artefact signal. At present, we are setting up an ITD assay to address the described problem.

Application of the Thermofluor assay to membrane protein complexes is an even more challenging task. C*c*O from *P. denitrificans* was used as a model protein complex with four protein subunits. Utilizing ANS allowed the acquisition of temperature-dependent changes in fluorescence intensity, yielding a T_m of 70.5°C for C*c*O. The signal-to-noise ratio of the experiment is inadequate, but a first hint is provided regarding the melting temperature of the complex. However, usage of the dye CPM allowed the melting temperature of the complex to be traced more successfully. Additionally to the signal probed with ANS, a second transition was observed with a T_{m1} at 46.8–49.5°C and a T_{m2} at around 69°C. CPM can be used to probe individual subunits of a complex if they contain cysteine residues buried in the protein core. This was true for the C*c*O complex, in which subunit III contains one cysteine and subunit II contains two cysteines which should be accessible to maleimide coupling after denaturation (Fig. 5*e*). The transition temperature profiles of several DSC measurements (Haltia *et al.*, 1994; Hilbers *et al.*, 2013) are in agreement with the Thermofluor assay data. The increased T_m values in the DSC measurements of Haltia and coworkers compared with our results and those of Hilbers and coworkers might be caused by different reaction conditions. The transition temperatures of the FTIR measurements of Haltia and coworkers are somewhat lower than the calorimetric scans owing to the much slower heating rate in the FTIR experiments.

Summarizing, if the target membrane protein or membrane protein complex does not have large hydrophilic domains, then the most versatile dye for Thermofluor assays to determine T_m values is CPM because it has minimal interactions with detergents. However, its primary limitation is that this dye requires free cysteines that are initially buried in the core of the protein and are exposed upon denaturation. In contrast, these studies also showed that for a representative small membrane protein lacking cysteines, for example NhaA, ANS is particularly useful, especially if broad pH screening is desired. The chemical properties of ANS favour an interaction with denatured membrane proteins over folded membrane proteins covered by detergent molecules and detergent micelles. This feature makes ANS attractive for stability studies of membrane proteins that do not contain cysteines, which corresponds to about 40% of all membrane proteins (Alexandrov *et al.*, 2008). Regardless of the dye chosen, the detergent concentration should be as low as possible in order to minimize the background signal for screening the stability of membrane proteins.

5. Conclusions

In this work, we showed on the basis of NhaA and the four-subunit C*c*O that the Thermofluor assay is applicable to membrane proteins without large hydrophilic domains. It is a versatile tool to rapidly screen different conditions before crystallization using only small amounts of protein. The dye ANS offers the possibility of broad-range pH screening without the need for cysteine residues. CPM can be used, for example, when screening of different salts or detergents is desired at pH values between 6 and 8.

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