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Self-interaction chromatography as a tool for optimizing conditions for membrane protein crystallization

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The second virial coefficient, or B value, is a measurement of how well a protein interacts with itself in solution. These interactions can lead to protein crystallization or precipitation, depending on their strength, with a narrow range of B values (the 'crystallization slot') being known to promote crystallization. A convenient method of determining the B value is by self-interaction chromatography. This paper describes how the light-harvesting complex 1–reaction centre core complex from *Allochromatium vinosum* yielded single straight-edged crystals after iterative cycles of self-interaction chromatography and crystallization. This process allowed the rapid screening of small molecules and detergents as crystallization additives. Here, a description is given of how self-interaction chromatography has been utilized to improve the crystallization conditions of a membrane protein.

1. Introduction

Membrane proteins are involved in a wide variety of processes in the cell, such as photosynthesis, respiration, transport of ions and nutrients, signal transduction and cell–cell recognition. They are also important drug targets, being targeted by more than 60% of prescription drugs. However, membrane protein structures are severely underrepresented in the Protein Data Bank (Berman *et al.*, 2000). The difficulties that are encountered in obtaining membrane protein crystals are caused by many factors, including the expression of suitable quantities of pure and stable protein. Although the main principles for crystallization are similar for membrane and soluble proteins, membrane proteins are more difficult to crystallize owing to their amphipathic nature (the transmembrane portion is highly hydrophobic and the extra/intracellular regions are hydrophilic). To overcome this problem, a detergent is generally required to cover the exposed transmembrane regions and to solubilize the purified membrane protein *via* the formation of a protein–detergent complex. Crystallization conditions must therefore take into consideration the properties of the detergent as well as of the protein *per se*. Furthermore, the solvent-exposed portions of the protein often contain loops that may be flexible and unstable, introducing further difficulties for the formation of a stable crystalline lattice. For a recent review of the importance of detergents in the crystallization of membrane proteins, see Privé (2007).

As part of a long-term research project, we are interested in crystallizing and determining the three-dimensional structure of the membrane protein light-harvesting complex 1–reaction centre (LH1-RC) from *Allochromatium vinosum*. This protein has been subjected to a number of commercially available crystallization screens, but thus far these attempts have not

yielded crystals. By combining standard crystal screening methods with determination of second virial coefficients (also known as the B values) in the presence of promising precipitants and additives, we have been able to produce single well defined crystals of LH1-RC from *A. vinosum*.

1.1. The second virial coefficient, B

The B value is a measurement of how well a protein interacts with other copies of itself in solution (*i.e.* self-interactions). These interactions depend on a number of variables such as temperature, pH and ionic strength and are correlated with protein solubility (for more in-depth discussions about B and protein solubility, see Chiang *et al.*, 1997; George & Wilson, 1994; Guo *et al.*, 1999; Haas *et al.*, 1999). By altering these parameters protein–protein interactions are affected and these changes can be quantitatively monitored *via* B-value measurements. Hence, by mixing the protein solution with varying amounts of precipitants and additives normally used in a crystallization screen, the B value for each condition can be determined. A narrow range of B values (between -1×10^{-4} and -8×10^{-4} mol ml g^{-2}), known as the 'crystallization slot', has been shown to promote crystallization (George & Wilson, 1994). The solvent conditions must be poor enough (*i.e.* slightly negative B values) to promote the formation of nucleation sites, but not excessive (*i.e.* more negative B values) such that the conditions promote the formation of amorphous aggregate (George & Wilson, 1994). If good solvent, or high protein solubility, conditions are chosen (*i.e.* B is positive), it is likely that very high protein concentrations in the range 100–300 mg ml^{-1} would be required to cause interactions that favour nucleation (Guo *et al.*, 1999).

The crystallization slot represents a probability zone for protein crystallization. While working with solution conditions within the slot greatly enhances the chances of a successful crystallization trial, it does not guarantee it. However, working outside the slot is likely to lead to an unsuccessful result.

Several methods have been used to characterize B for different proteins. Static light-scattering (SLS) is a well established method, but methods including small-angle X-ray or neutron scattering, membrane osmometry and sedimentation-equilibrium studies have all been utilized (Ducruix *et al.*, 1996; Porschel & Damaschun, 1977; Receveur *et al.*, 1998; Velev *et al.*, 1998; Behlke & Ristau, 1999; Haynes *et al.*, 1992; Schaink & Smit, 2000). These methods suffer from several disadvantages, including a requirement for moderate to large amounts of protein, excessive time needed to perform each experiment and the requirement of a skilled operator. A rapid and cost-effective method that requires small amounts of protein is needed if the B value is to be used as a general method for identifying potential crystallization conditions.

1.2. Self-interaction chromatography (SIC)

One approach to rapidly characterize the B value is self-interaction chromatography (SIC). SIC involves binding a small but known amount of the protein of interest onto chromatographic particles, such as commercially available amino or formyl beads, and then packing these into a column. The same protein in solution is loaded onto the column and the retention volume, which reflects the average strength of the interaction, is monitored (Patro & Przybycien, 1996). As the protein column can be connected to an ordinary high-performance liquid-chromatography (HPLC) machine, the volume of the column as well as the volume of the sample can be kept small (on a microlitre scale), which will also reduce the experiment time. Determination of the B value does not require measurements to be performed at protein concentrations near the saturation point, which further reduces the amount of sample needed (Chiang *et al.*, 1997; George & Wilson, 1994). The absorbance at 280 nm (A_{280}) is monitored to determine the retention volume of the protein in solution. The retention volume from the column, V_r, can be used to calculate the B value when expressed as a function of the retention factor, k', where

$$k' = \frac{V_r - V_0}{V_0}$$

and

$$B_{22} = B_{HS} - \frac{k'}{\rho_s \varphi},$$

where V_0 is the dead volume of the column, B_{HS} is the excluded volume, or hard-sphere contribution (= $2/3\pi d^3$ for spheres), ρ_s is the amount of protein immobilized per unit surface area and φ is the phase ratio, or the accessible surface area per mobile phase volume. These quantities can all be measured or calculated (Tessier, Vandrey *et al.*, 2002; Tessier, Lenhoff *et al.*, 2002). Altering the solvent conditions of the protein sample loaded onto the column will cause differences in the protein–protein interactions and therefore affect the retention volume. The measured B values for the protein in different solvents are used to identify and adjust conditions within the 'crystallization slot'. This was demonstrated successfully by Tessier *et al.* (2003), who used the B value determined by SIC to identify suitable solvent conditions for the crystallization of ribonuclease A (RNase A).

1.3. Membrane proteins and B values

Previous work by Hitscherich *et al.* (2000) using SLS showed that the crystallization conditions of the outer membrane protein OmpF had B values that fell within a similar 'crystallization slot' as those of soluble proteins. Additionally, Berger *et al.* (2005, 2006) have used SIC to measure the B values of bacteriorhodopsin solubilized in octyl β-D-glucoside (β-OG) using a number of precipitants and amphiphiles. This approach has allowed an increased understanding of the role of the detergent and amphiphiles during crystallization.

Here, we describe how we optimized the crystallization conditions of a membrane protein by improving the B value using SIC in combination with in-house crystallization screens that allowed us to proceed from poorly formed crystals with multiple lattices to single crystals.

2. Experimental procedures

2.1. Protein production and purification

The purple bacterium *A. vinosum* was cultured in rich medium (Bose, 1963) and grown anaerobically at 303 K with bright illumination. Cells were harvested after 3 d growth and were stored at 253 K until required. Cell pellets were homogenized in 20 m*M* Tris pH 8.0, 10 mg $MgCl_2$ and a little DNAse was added; the cells were then broken by passage through a French press. The lysate was cleared by centrifugation at 8000*g* for 30 min, followed by 90 min centrifugation at 100 000*g* to harvest the membranes. The membranes were adjusted to an OD at 850 nm of 50 cm^{-1} and solubilized in 20 m*M* Tris pH 8.0, 2% *n*-dodecyl D-maltoside (DDM; Anatrace) for 4 h at 277 K. LH1-RC was separated from the peripheral antenna complex and other membrane proteins by overnight sucrose-gradient centrifugation (100 000*g*). The purified sample was loaded onto a DE52 column and eluted with 150 m*M* NaCl, 20 m*M* Tris pH 8.0, 0.04% DDM (buffer *A*), in which the protein was kept throughout the experiment unless otherwise stated. The integrity of the samples was determined by measuring the absorbance spectra of the samples from 250 to 950 nm. The protein was concentrated in a Centricon (100 000 molecular-weight cutoff); when required, detergents were also exchanged using these.

2.2. Measuring the protein concentration

The protein concentration was measured quantitatively by determining the amounts of amino acids in the sample that was applied to the beads (in milligrams) and that remained in the pooled samples washed off the beads after binding. The two samples (applied to and washed off the beads) were treated with formic acid and dried before being subjected to hydrolysis in 6 *N* HCl/0.1% phenol at 383 K for 24 h. The samples were then diluted in aminoethyl-cysteine buffer and injected into a Hitachi L-8 900 amino-acid analyzer. The output from the amino-acid analyzer was used to calculate the total protein concentration in each sample. This quantification was performed at the University of California (UC) Davis Molecular Structure Facility. The difference between the protein concentrations of the two samples was then assumed to be equivalent to the amount of protein bound to the resin.

2.3. Self-interaction chromatography

It should be noted that in this paper we refer to the second virial coefficient as '*B*', although other designations such as A_2 and B_{22} are often used in the literature. The second virial coefficient represents the total contributions to the thermodynamic non-ideality in a dilute protein solution, but it is the two-body protein–protein interactions that are likely to dominate the total interactions (hence the term B_{22}). Thus, for the purposes of simplicity we will refer to the measured second virial coefficient as the *B* value, acknowledging the significance of the B_{22} designation.

LH1-RC was bound to AF-amino-650M beads (Tosoh Bioscience) using the method described by Valente *et al.* (2005). 100 µl protein-bound resin was used to create a column consisting of Teflon FEP tubing with an inner diameter of 0.7 mm and a length of 18 cm, resulting in a final column volume of 69 µl. The full method for packing the column is described by Johnson *et al.* (2009). A 'dead' column consisting of unbound resin was used as a control to determine the interactions between the protein in solution and the resin. The void volume of the column with covalently bound LH1-RC was measured by loading acetone instead of protein onto the

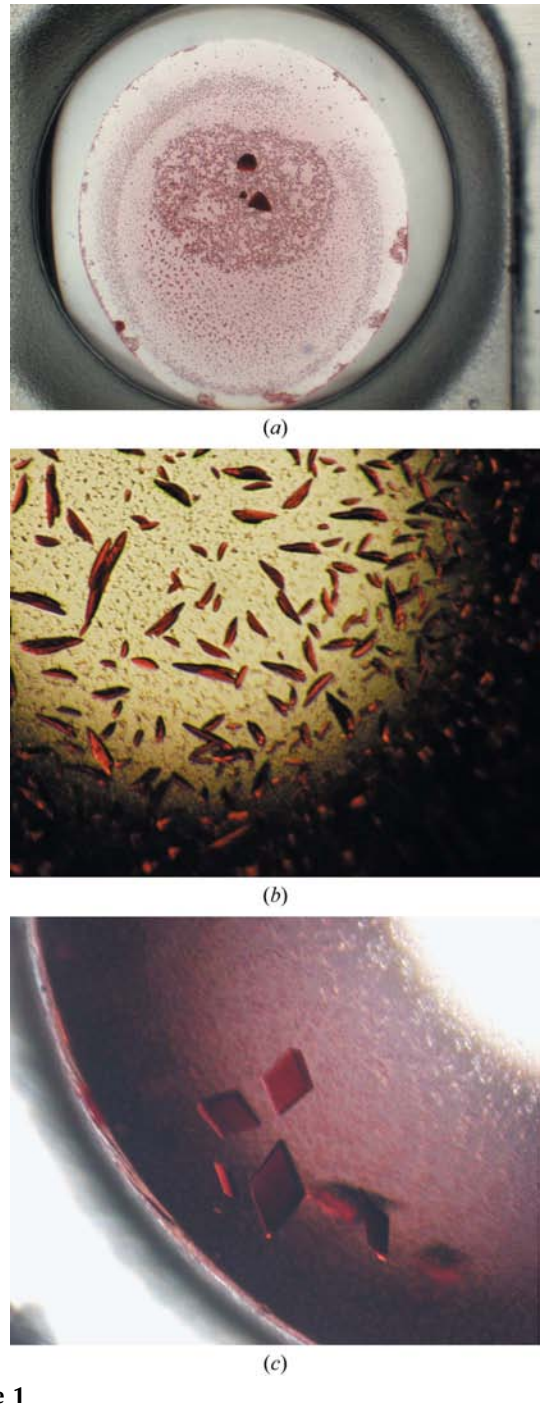

Figure 1
Images of (*a*) crystals from condition 1 from the MemSys screen, (*b*) crystals from condition 2 from the in-house screen at CBSE and (*c*) final crystals in β-OG and with 3% heptanetriol in condition 2.

column and monitoring the volume at which acetone first appears in the eluent (7.2 µl). To ensure that acetone did not interact with the protein on the column, the elution volume was compared with that of acetone applied to a 'dead' column (no protein bound to the media) and was found to be similar. Before each run the column was pre-equilibrated in the solutions to be tested. The volume of the loaded sample was 1 µl and the column was run at 1 µl s^{-1}. Each measurement was performed in triplicate and the *B* values were calculated as an average of the three measurements. Protein was typically loaded onto the column in either 100% buffer *A*, 75% buffer *A* or 50% buffer *A*. The remaining volume was made up of buffer *B*, as described below. The column integrity was continually monitored based on the colour of the protein bound to the column, the column operating pressure (maintaining identical elution rates) and by periodic injections of acetone instead of protein to control for alterations in the stationary phase protein. B_{HS} (the hard-sphere contribution of the column) was estimated based on the molecular weight of the protein (B_{HS} = 1.5 × 10^{-18} Da Å^{-3} for an approximate molecular weight of 322 kDa) and the phase ratio was estimated by plotting the molecular weight of LH1-RC onto a standard curve (φ = 2.8). The presence of detergents was not taken into consideration as the amount bound to the protein is likely to vary and any attempt at estimation would be highly inaccurate.

2.4. Protein crystallization

Initial crystal screens were performed with the MemSys screen (Molecular Dimensions) and in-house screens (L. A. Nagy & L. J. DeLucas, unpublished data) using sitting-drop vapour diffusion at 288 K with protein and reservoir solutions in a 1:1 ratio. The protein was maintained in buffer *A* unless otherwise specified and was kept at a concentration of about 10 mg ml^{-1}, as discussed below.

3. Results

3.1. Determining the protein concentration

Determination of the concentration of protein bound to the resin is required to accurately determine the *B* value (Tessier, Lenhoff *et al.*, 2002). Direct measurement of the protein concentration of the LH1-RC complex presents some difficulties as its extinction coefficient is unknown and the presence of carotenoids hinders accurate bicinchoninic acid

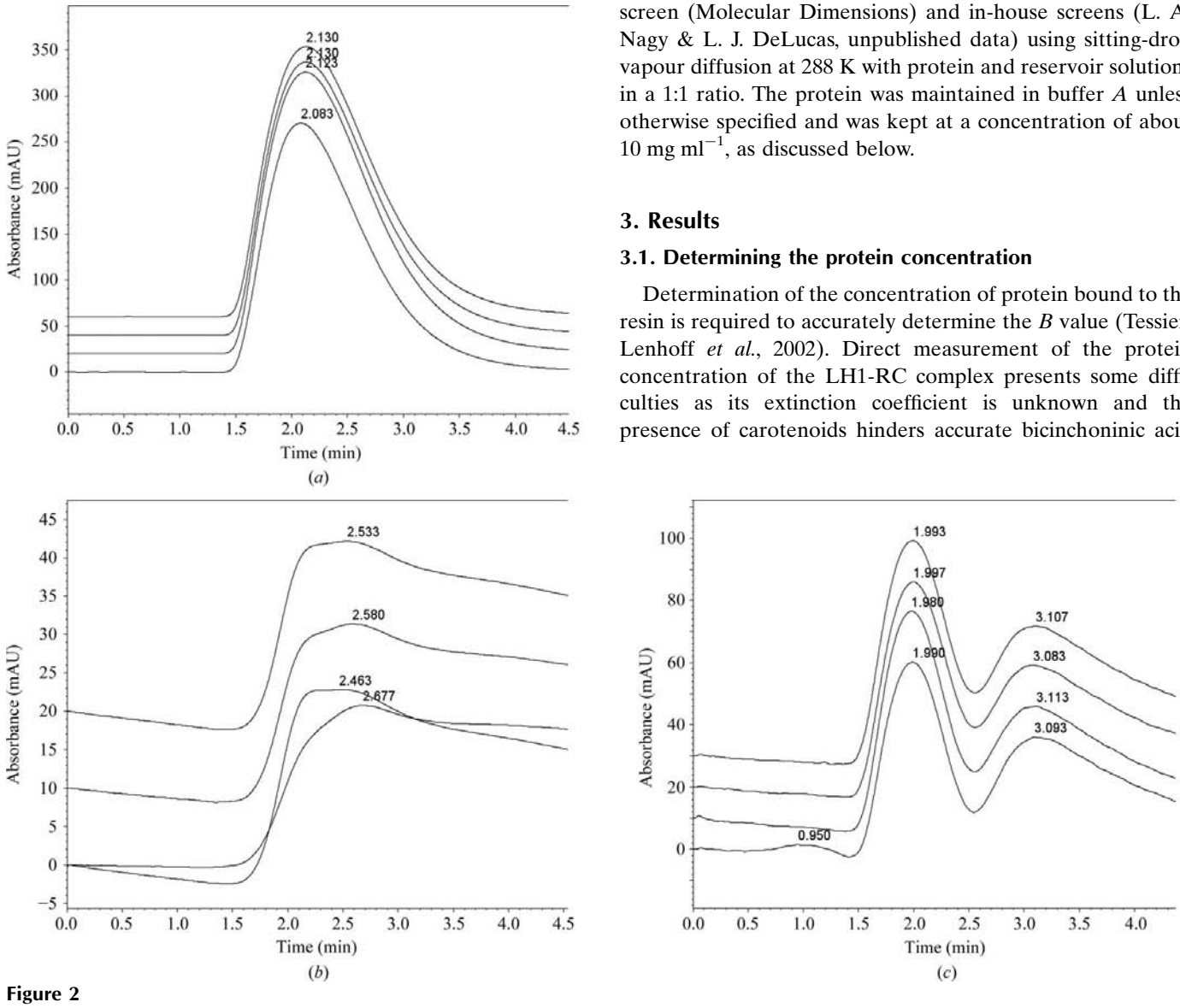

Figure 2
Traces from throughout the experiment; each section shows the experiment performed in triplicate, from which three samples were chosen to give an average *B* value. (*a*) Results from protein in buffer *A* alone (20 m*M* Tris pH 8, 150 m*M* NaCl, 0.04% DDM). (*b*) Results from condition 1 (100 m*M* NaCl, 12% PEG 2000) mixed with protein in buffer *A*. Although the peak has shifted, giving a *B* value within the 'crystallization slot', it is clear from the chromatogram that the sample is precipitating. (*c*) Results from final condition 2 (100 m*M* bicine pH 8.3, 10% PEG 750 MME, 356 m*M* sodium acetate, 4% dioxane, 20 m*M* KSCN, 50 m*M* arginine) mixed with protein in buffer *A* with 1% β-OG replacing the DDM. This condition yielded single crystals. The two peaks are discussed in the text.

assay (BCA) measurements since they absorb at the wavelength used by this assay. Therefore, the amount of protein present in the sample before binding to the beads as well as the residual (protein that did not bind) present in the wash was quantified by total amino-acid analysis. The difference between the two samples provides an accurate estimate of the quantity of protein bound to the resin. A total of 3.86 mg protein was added to the beads and 2.79 mg was found in the wash volume (data not shown). The difference between these two amounts gives a concentration of 10.7 mg ml^{-1} protein bound to the beads.

3.2. Initial conditions

The retention volume of the protein was measured in buffer *A* (150 m*M* NaCl, 20 m*M* Tris pH 8.0 with 0.04% DDM, unless otherwise stated) and the *B* value was calculated to be 2.6×10^{-5} mol ml g^{-2}, which is outside the 'crystallization slot' (some of the *B* values determined in this study are shown in Fig. 3).

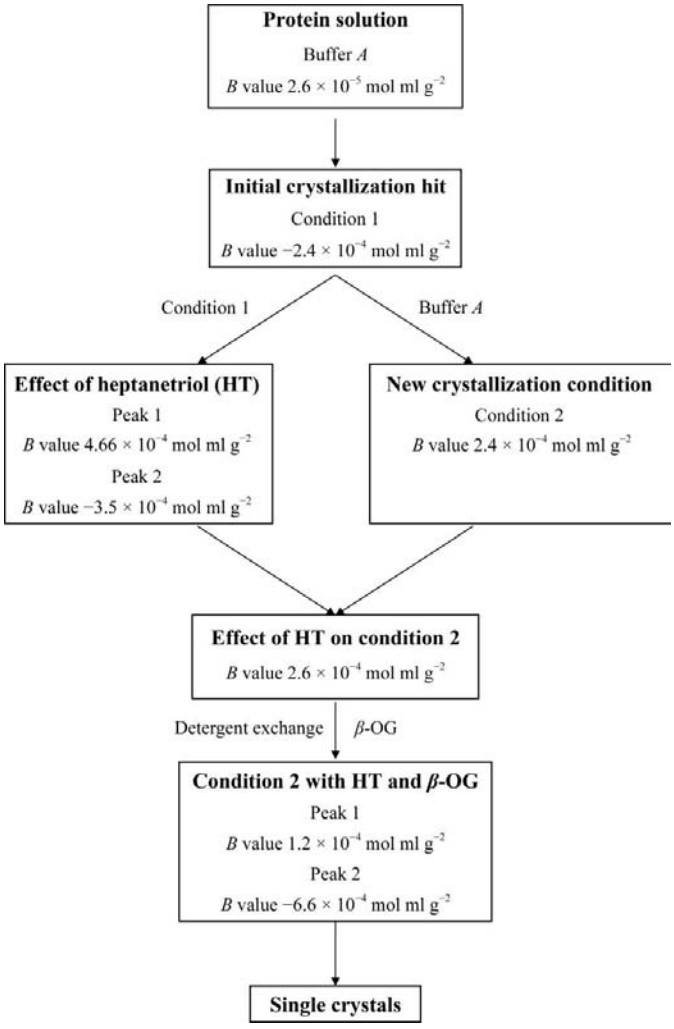

Figure 3
Schematic of the process used to develop the initial crystallization conditions to single regular crystals and the *B* values determined throughout the experiment. The concentration of detergents was 0.04% DDM or 1% *β*-OG. Buffer *A* consisted of 20 m*M* Tris pH 8, 150 m*M* NaCl. Condition 1 consisted of 100 m*M* NaCl, 12% PEG 2000 and condition 2 consisted of 100 m*M* bicine pH 8.3, 10% PEG 750 MME, 356 m*M* sodium acetate, 4% dioxane, 20 m*M* KSCN, 50 m*M* arginine. The additive which improved conditions was heptanetriol (HT). The separation of some conditions into peaks 1 and 2 are described in the text.

Initial crystal trials using commercial screens yielded a single hit with 12% polyethylene glycol (PEG) 2000 and 100 m*M* NaCl as the reservoir solution (used as buffer *B* in the initial experiment, with 0.04% DDM added to avoid any precipitation caused by the detergent concentration dropping below the CMC). The crystals were of poor morphology, with no straight edges, and diffracted to below 25 Å resolution (Fig. 1). However, these conditions were deemed to be appropriate starting conditions for SIC experiments. Keeping the protein in buffer *A*, the column was equilibrated in varying amounts of buffer *B* (containing detergent throughout) and the SIC experiments were repeated. This caused the *B* value to change from the previous positive value to -2.4×10^{-4} mol ml g^{-2}, which is within the 'crystallization slot', at a concentration of 100% buffer *B*. However, the trace from the HPLC showing the retention volume *versus* the absorbance indicates that the peak was not uniform, suggesting that the protein may be precipitating (Fig. 2).

To determine whether the protein's behaviour in solution could be improved, several small molecules were added to the crystallization conditions and the SIC experiments were repeated. The small molecules used were glutamate, arginine, trehalose and 1,2,3-heptanetriol, as these compounds have previously been shown to have an impact on protein solubility and physical stability (*i.e.* nonspecific aggregation; Lu *et al.*, 2008). The addition of 3% heptanetriol produced two distinct populations, where the first peak eluted in the void volume and a second peak eluted at increased volume (*i.e.* longer retention time), resulting in a *B* value of -3.5×10^{-4}. This is also within the 'crystallization slot'. The addition of arginine (50 or 100 m*M*) or glutamate (25 or 50 m*M*) made no difference to the profile of the plot from the SIC experiment, whereas the addition of trehalose increased protein precipitation.

3.3. Improving conditions by iterative use of SIC and crystallography

While the initial SIC experiments were being performed, new crystallization trials were prepared using an in-house crystal screen that was developed at the Center for Biophysical Sciences and Engineering (CBSE; L. A. Nagy & L. J. DeLucas, unpublished data). These screens yielded long crystalline needles with few or no sharp edges. The crystals were not single and were unsuitable for diffraction studies. The crystals were found using a reservoir consisting of 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.1, 0.356 *M* NaCl, 20% polyethylene glycol monomethyl ether 750 (PEG 750 MME), 3% ethylene glycol, 20 m*M* potassium thiocyanate (KSCN), 50 m*M* arginine. An additional screen was prepared in an attempt to optimize these conditions.

As the initial SIC experiments showed that the presence of heptanetriol (HT) split the protein into two populations, one

of which was within the 'crystallization slot', the optimized crystal screens were prepared in duplicate with 0 and 3% heptanetriol added to the protein solution (buffer *A*). In particular, one hit from this optimized screen showed a marked difference based on the presence or absence of heptanetriol in the protein solution. When the protein was screened against a reservoir solution consisting of 0.1 *M* bicine pH 8.3, 10% PEG 750 MME, 0.356 *M* sodium acetate, 4% dioxane, 20 m*M* KSCN, 50 m*M* arginine, crystals grown in the presence of heptanetriol were fewer and larger than those grown in the absence of heptanetriol.

To understand how the amphiphile was affecting the crystallization results, this condition was evaluated using SIC. Again, when analyzing the plot from the SIC experiment it appeared that the 1,2,3-heptanetriol split the peak into a major peak representing non-interacting species (a more positive *B* value outside the slot) and an extended shoulder of more closely interacting protein. To further try to understand and improve the behaviour of the protein, DDM was replaced with three different detergents [0.1% lauryldimethylamine *N*-oxide (LDAO), 25 m*M* octyl β-D-glucoside (β-OG) and 40 m*M* HEGA-9], with and without 3% heptanetriol in the protein solution. The use of β-OG instead of DDM gave two separate peaks from the SIC column, the first of which was close to the void volume and the second of which had a *B* value of -6.6×10^{-4} mol ml g^{-2}, which is in the middle of the 'crystallization slot' and thus where most proteins tend to produce optimum crystals (Fig. 2). HEGA-9 showed no difference from DDM, whereas LDAO appeared to degrade the protein covalently attached to the column. The final conditions with β-OG (150 m*M* NaCl, 20 m*M* Tris pH 8.0, 25 m*M* β-OG, 3% heptanetriol) were prepared in a 1:1 ratio with the reservoir (0.1 *M* bicine pH 8.3, 10% PEG 750 MME, 0.356 *M* sodium acetate, 4% dioxane, 20 m*M* KSCN, 50 m*M* arginine). These conditions gave reasonably sized rhombohedral crystals with straight edges (Fig. 1*c*). It was noticed that these crystallization conditions also resulted in a proportion of the sample exhibiting phase separation. This phase-separated portion may well correlate with that fraction of the sample that elutes in the void volume in the SIC experiments.

4. Discussion

In comparison to soluble proteins, crystallizing membrane proteins is a difficult task owing in part to the existence of the protein as a part of a larger protein–detergent micelle complex. Using SIC in tandem with crystal screens allows a number of conditions to be rapidly evaluated and provides a more analytical approach to deciphering the effect of amphiphiles and additives.

Using an iterative process, cycling between SIC and standard crystallization methods, enabled us to quickly identify a suitable amphiphile, 1,2,3-heptanetriol, from a number of candidates by determining the differences in *B* values. By monitoring these changes, conditions which were far away from the 'crystallization slot' could be disregarded and conditions with values within the slot could be fine-tuned in the crystallization screens. Similarly, different detergents can be screened quickly. Using SIC as part of the screening helped to minimize the number of conditions screened to find the final optimum conditions that yielded higher quality crystals. A schematic that illustrates the workflow, as well as highlighting some of the measured *B* values, is presented in Fig. 3.

The presence of what appears to be two populations of our single protein, as judged by SIC, is interesting. Based on our knowledge of the structure of LH1-RC from other species, these two populations are not likely to be caused by conformational changes. It is also not likely that there are different oligomeric states, as the protein has been tested on size-exclusion chromatography and elutes as a single peak (data not shown). We therefore believe that the two populations, one which is prone to crystallization and one which interacts minimally with itself, are caused by differences in the consistency of the protein–detergent micelle complex. As the injected samples are small (1 µl), it is not possible using our current setup to determine whether the two populations can be separated or if they exist in equilibrium. This requires further study.

As the protein in 0.1% LDAO was loaded onto the column, the matrix of the column started to change colour from a dark purple, caused by the pigmentation of the protein, to a pale pink and finally to white. We can only assume that the combination of binding the protein to the column and exposing it to LDAO somehow causes the complex to denature. Interestingly, this protein is stable in solution in the presence of LDAO. Similar results were observed when RC-LH1 from *Rhodopseudomonas palustris* in LDAO was bound to a similar column.

It is clear from the results described here that not all detergents will be suitable for use in SIC experiments. This limits the areas of crystallization 'space' that can be explored using our suggested iterative process of SIC and crystallization screening. However, using this method, which only requires small amounts of protein while enabling quantification of *B* values, provides an especially promising approach for determining optimum crystallization conditions for membrane proteins, which are often only available in small quantities.

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