Oils used in microbatch crystallization do not remove a detergent from the drops they cover

**Thomas R. M. Barends and
Bauke W. Dijkstra***

Laboratory of Biophysical Chemistry,
University of Groningen, Nijenborgh 4,
9747 AG Groningen, The Netherlands

Correspondence e-mail: bauke@chem.rug.nl

In microbatch crystallizations, a small volume of protein solution is placed in contact with a large volume of oil. Detergents present in the water phase may be expected to migrate into the oil phase, which could have effects on the protein in solution. A new method is described in which detergent partitioning into the oil can be checked. The accuracy of this method is sufficiently high to estimate even very low partitioning coefficients. The measurements indicate that in the case of dodecyl maltoside, no significant loss of detergent will occur.

1. Introduction

Recent years have seen remarkable success in the field of membrane-protein structure elucidation. Among the most notable structures are ion channels such as the potassium channel KvAP (Jiang *et al.*, 2003) and transporters such as the vitamin B_{12} transporter BtuCD (Locher *et al.*, 2002), the phospholipid flippase MsbA (Chang & Roth, 2001) and the multidrug transporter AcrB (Murakami *et al.*, 2002). As witnessed by some of these articles, an enormous volume of work was needed to crystallize these membrane proteins, often involving the cloning and expression of several related proteins from different organisms (Locher *et al.*, 2002; Chang & Roth, 2001; Jiang *et al.*, 2003) and the setting up of thousands of crystallization experiments. One complicating factor is the amphiphilicity of membrane proteins, which necessitates the use of detergents in all but a few special cases (Nollert *et al.*, 1999; Chiu *et al.*, 2000; Landau & Rosenbusch, 1996). These detergents add extra parameters to the already highly multidimensional problem of discovering suitable crystallization conditions, as illustrated by the strong dependence of the success rate on the nature of the detergent (Ostermeier & Michel, 1997).

To increase the success rate of membrane-protein crystallization experiments, research into membrane-protein crystallization thus remains useful and important, encompassing efforts as diverse as automation, investigations of the physical chemistry of detergent solutions (Rosenbusch, 1990; Thiyagarajan & Tiede, 1994; Littrell *et al.*, 2000), detergent development (McQuade *et al.*, 2000; Yu *et al.*, 2000) and method evaluation. Amongst the methods being evaluated, the microbatch (Chayen,

1997) and modified microbatch methods (D'Arcy *et al.*, 1996) are especially interesting. These methods involve the mixing of drops of a protein solution with precipitants under a layer of a hydrophobic oil. In the case of membrane proteins, it may seem imprudent to subject a hydrophobic membrane protein to such treatment for fear of denaturation of the protein by the oil and/or diffusion of the detergent into the oil. Indeed, a heptane/water partitioning coefficient of the detergent octaethyleneglycol dodecylether ($C_{12}E_8$) of 13.7 ± 1.9 was reported by le Maire *et al.* (1987), indicating a large preference of this detergent for migration into the organic phase.

It has, however, proven possible to crystallize membrane proteins under oil, such as for example chlorophyll-binding protein 43 (Chayen, 1998) and ATPase F_1c_{10} complex (Stock *et al.*, 1999). Indeed, it was suggested that crystallization may be stimulated by desorption of the detergent from the protein drop by the oil (Chayen, 1998) forcing the protein out of solution. Thus, microbatch crystallization could be highly suited to membrane proteins.

To investigate the partioning of detergents into oils, an accurate method is required that can measure an oil/water partioning coefficient of as low as 5×10^{-4}. To illustrate this, an estimate of the detergent loss can be made for a typical microbatch setup of 50 1 µl drops containing $1\%(w/v)$ of detergent in contact with 7000 µl of oil in the standard Terazaki plate usually used for such experiments. In case of an oil/water partitioning coefficient of only 5×10^{-4}, the equilibrium amount of detergent in the drops can be calculated to be $0.9\%(w/v)$. Such a partitioning coefficient would thus translate into a loss of detergent in the drops of as much as 10% of the total detergent. The loss

of such a large portion of the detergent may indeed lead to a mild precipitant-like effect and assist crystallization or may be deleterious to the protein.

Recently, Loll *et al.* (2003) have incubated solutions of the detergents octyl glucoside and fos-choline-12 with oils typically used in microbatch crystallization and, under normal mixing conditions, found no statistically significant decrease of the amount of either detergent in the aqueous phase. However, in their method, Loll and co-workers investigated a system consisting of 2 ml of oil and 1 ml of aqueous detergent solution, which does not represent a standard microbatch experiment as described above.

To complement the results obtained by Loll and coworkers, we have investigated the amount of detergent migrating into the oil phase using an alternative method. In this method, a radiolabelled detergent is used to follow detergent partitioning. Radioactively labelled detergents have previously been used for the determination of detergent binding by membrane proteins (Møller & le Maire, 1993; le Maire *et al.*, 2000). In the present case, the detergent n-dodecyl-β-D-maltopyranoside was investigated. This detergent has been used successfully in at least seven membrane-protein crystallizations (Chang *et al.*, 1998; Iwata *et al.*, 1998; Ostermeier *et al.*, 1995; Koronakis *et al.*, 2000; Stock *et al.*, 1999; Lancaster *et al.*, 1999; Murakami *et al.*, 2002) and is a well known representative of the class of alkyl maltoside detergents which are used in a large number of membrane-protein crystallizations. The behaviour of this detergent was investigated with two standard oils used in protein crystallization, paraffin oil and 'Al's oil', a 1:1 mixture of paraffin and low-viscosity silicone oil, which allows slow evaporation of the protein drops it covers (D'Arcy *et al.*, 1996).

2. Methods

Unlabelled n-dodecyl-β-D-maltopyranoside (dodecyl maltoside, 'Anagrade' quality) was obtained from Anatrace (Maumee, USA). ^{14}C-labelled dodecyl maltoside was a generous gift from Professor Marc le Maire, CNRS, Gif-sur-Yvette, France. All work with this compound was performed using the correct safety precautions in a radioisotopes laboratory. Paraffin oil 'dünnflüssig' was obtained from Merck (Darmstadt, Germany). Dow Corning silicone fluid (1cSt) was purchased from BDH Laboratory Supplies (Poole, UK). 'Al's oil' was prepared by thoroughly mixing equal volumes of

paraffin and silicone oil. Given the large amount of detergent that may bind to a membrane protein (up to 400% by weight) (Møller & le Maire, 1993) and the protein concentrations used in crystallization (up to 10 mg ml^{-1}), a detergent concentration around 1%(w/v) was chosen as representative for a crystallization experiment.

To obtain an aqueous solution of radiolabelled dodecyl maltoside, 60 µl of the stock solution in ethanol was carefully allowed to evaporate at room temperature. The residue was dissolved in 60 µl water and the radioactivity was measured. No significant loss of activity was found.

To 20 µl of this solution, 80 µl of a 1% aqueous solution of unlabelled dodecyl maltoside was added, resulting in a total detergent concentration of around 0.9%. 5 µl drops of this solution were then placed at the bottom of 1.5 ml Eppendorf cups containing 100 µl of either paraffin or Al's oil. This resulted in a total activity of >200 000 counts min^{-1} per drop. All samples were prepared at least in triplicate. The cups were left at room temperature. After 2 and 7 d, the top 50 µl of each oil layer and the 25 µl directly thereunder were harvested with a pipette and separately counted in a liquid scintillation counter.

3. Results and discussion

Measured activities ranged from 500 ± 50 to 2000 ± 100 counts min^{-1} per 50 µl, with background activities in the oils of 30 ± 10 counts min^{-1} per 50 µl. Since a migration of 1% of the total activity from the drop into the oil would lead to an activity of >1000 counts min^{-1} per 50 µl oil, such a small effect can be measured accurately. With the volumes employed, such a detergent partitioning would correspond to a partitioning coefficient of 5×10^{-4}.

After 2 d, the bottom 25 µl of oil contained almost twice as much radioactivity per unit volume as the top 50 µl in both the paraffin and Al's oil cases. In total, without taking this gradient into consideration, 1% of the total activity had diffused into the oil layer in the Al's oil case and 0.7% in the paraffin oil case. After 7 d, these amounts had become 1% in each case, but the activity gradients had disappeared.

To test whether the activity measured in the oil was caused by dodecyl maltoside or by a radioactive contaminant with a high preference for the oil phase, experiments were conducted in which after one, two and three weeks the top 50 µl of the oil layer was removed for counting and replaced with fresh oil. These experiments clearly showed

that no new equilibrium was established after the first removal of oil. Thus, the radioactivity measured was caused by a radiolabelled contaminant of the dodecyl maltoside stock which has a high affinity for the apolar oil phase. This contaminant could possibly be [1-^{14}C]-dodecanol, which was used in the synthesis of the labelled dodecyl maltoside (Kragh-Hansen *et al.*, 1993).

The current method allows us to estimate an upper limit for the partitioning coefficients. Because no radioactivity owing to dodecyl maltoside could be measured and the method employed here can measure detergent losses corresponding to low (5×10^{-4}) partitioning coefficients, it may be expected that the partitioning coefficients are less than this value. Furthermore, the experiments described constitute a worst-case scenario: in a real crystallization experiment, the presence of a membrane protein in the drops will shift the equilibrium towards keeping even more detergent in the drops. Thus, dodecyl maltoside partitioning into the oil in a standard microbatch experiment will not lead to a dramatic loss of dodecyl maltoside from the drop. Given these observations, we conclude that dodecyl maltoside may be safely used in microbatch experiments and urge crystallographers to include the microbatch method in the standard array of crystallization techniques for membrane proteins.

References

Chang, G. & Roth, C. B. (2001). *Science*, **293**, 1793–1800.
Chang, G., Spencer, R. H., Lee, A. T., Barclay, M. T. & Rees, D. C. (1998). *Science*, **282**, 2220–2226.
Chayen, N. E. (1997). *Structure*, **5**, 1269–1274.
Chayen, N. E. (1998). *Acta Cryst.* D**54**, 8–15.
Chiu, M. L., Nollert, P., Loewen, M. C., Belrhali, H., Pebay-Peroula, E., Rosenbusch, J. P. & Landau, E. M. (2000). *Acta Cryst.* D**56**, 781–784.
D'Arcy, A., Elmore, C., Stihle, M. & Johnston, J. E. (1996). *J. Cryst. Growth*, **168**, 175–180.
Iwata, S., Lee, J. W., Okada, K., Kyongwon Lee, J., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S. & Jap, B. K. (1998). *Science*, **281**, 64–71.
Jiang, Y., Lee, A., Chen, J., Ruta, V., Cadene, M., Chait, B. T. & MacKinnon, R. (2003). *Nature (London)*, **423**, 33–41.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B. & Hughes, C. (2000). *Nature (London)*, **405**, 914–919.

Kragh-Hansen, U., le Maire, M., Nöel, J.-P., Gulik-Krzywicki, T. & Møller, J. V. (1993). *Biochemistry*, **32**, 1648–1656.

Lancaster, C. R. D., Kröger, A., Auer, M. & Michel, H. (1999). *Nature (London)*, **402**, 377–385.

Landau, E. M. & Rosenbusch, J. (1996). *Proc. Natl Acad. Sci. USA*, **93**, 14532–14535.

Littrell, K., Urban, V., Tiede, D. & Thiyagarajan, P. (2000). *J. Appl. Cryst.* **33**, 577–581.

Locher, K. P., Lee, A. T. & Rees, D. C. (2002). *Science*, **296**, 1091–1098.

Loll, P. J., Tretiakova, A. & Soderblom, E. (2003). *Acta Cryst.* D**59**, 1114–1116.

McQuade, D. T., Quinn, M. A., Yu, S. M., Polans, A. S., Krebs, M. P. & Gellman, S. H. (2000). *Angew. Chem. Int. Ed.* **39**, 758–761,659.

Maire, M. le, Champeil, P. & Møller, J. (2000). *Biochim. Biophys. Acta*, **1508**, 86–111.

Maire, M. le, Møller, J. & Champeil, P. (1987). *Biochemistry*, **26**, 4803–4810.

Møller, J. V. & le Maire, M. (1993). *J. Biol. Chem.* **286**, 18659–18672.

Murakami, S., Nakashima, R., Yamashita, E. & Yamaguchi, A. (2002). *Nature (London)*, **419**, 587–593.

Nollert, P., Royant, A., Pebay-Peyroula, E. & Landau, E. M. (1999). *FEBS Lett.* **457**, 205–208.

Ostermeier, C., Essen, L. O. & Michel, H. (1995). *Proteins*, **21**, 74–77.

Ostermeier, C. & Michel, H. (1997). *Curr. Opin. Struct. Biol.* **7**, 697–701.

Rosenbusch, J. P. (1990). *J. Struct. Biol.* **104**, 134–138.

Stock, D., Leslie, A. G. W. & Walker, J. E. (1999). *Science*, **286**, 1700–1705.

Thiyagarajan, P. & Tiede, D. M. (1994). *J. Phys. Chem.* **98**, 10343–10351.

Yu, S., McQuade, D., Hackenberger, M. Q., Krebs, M., Polans, A. & Gellman, S. (2000). *Protein Sci.* **9**, 2518–2527.