Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Membrane's Eleven: heavy-atom derivatives of membrane-protein crystals

J. Preben Morth,[a] Thomas Lykke-Møller Sørensen[b] and Poul Nissen[a]*

[a]Center for Structural Biology, Department of Molecular Biology, University of Aarhus, Gustav Wieds Vej 10C, DK-8000 Aarhus C, Denmark, and [b]Diamond Light Source Ltd, Rutherford Appleton Laboratory, Chilton, England

Correspondence e-mail: pn@mb.au.dk

Received 11 April 2006
Accepted 20 June 2006

A database has been assembled of heavy-atom derivatives used in the structure determination of membrane proteins. The database can serve as a guide to the design of experiments in the search for heavy-atom derivatives of new membrane-protein crystals. The database pinpoints organomercurials, platinum(II) and trimethyllead compounds as being particularly useful. On the other hand, lanthanide and uranyl compounds are poorly represented, which may be a consequence of these compounds having aggressive effects in crystal-soaking procedures. Furthermore, the database highlights the variety of methods applied in the preparation of heavy-atom-derivatized crystals and in phasing. Cocrystallization can be further exploited. Phases have predominantly been obtained by SIRAS/MIRAS methods rather than SAD/MAD in recent structure determinations.

© 2006 International Union of Crystallography
Printed in Denmark – all rights reserved

1. Introduction

Membrane proteins represent an important frontier in structural biology because of their great importance in molecular biology combined with the difficulties associated with the determination of their crystal structures. Since the pioneering work by Michel and coworkers on the photosynthetic reaction centre more than 20 y ago (Deisenhofer *et al.*, 1984), a total of only about 40 unique structures of α-helical inner membrane proteins have been determined. Similarly, about 20 structures of β-barrel structures from bacterial outer membranes have been structurally characterized. In total, membrane proteins constitute about 0.2% of PDB entries. Compared with their significant abundance in genomes, typically 20–30% of open reading frames, there is obviously a strong deficit.

One primary reason for the low number of membrane protein crystal structures is limited amounts of protein for crystallization studies because of low expression levels. The use of overexpression systems has only recently been successfully applied to the crystallization and structure determination of eukaryotic membrane proteins (Jidenko *et al.*, 2005; Long *et al.*, 2005; Törnroth-Horsefield *et al.*, 2006) and in most instances eukaryotic membrane proteins have been obtained from native tissue. An increased use of advanced expression systems in the future can be predicted, but in these instances, as for native tissue from higher eukaryotes, SeMet protein may not be an option. With a limited number of known membrane-protein structures available and a growing demand for model-independent phasing in structure determination, it is furthermore unlikely that molecular replacement will be a general option. We are therefore left with the use of classical heavy-atom derivatives prepared by soaking or cocrystallization for experimental

phasing of membrane proteins and this will probably remain the case for many years.

2. Membrane proteins and their crystals

A main characteristic of membrane proteins from a structural biology point of view is that they have evolved to accommodate three different environments: the lipophilic membrane, the outer aqueous environment and the inner aqueous enviroment. Thus, membrane proteins contain lipophilic surfaces as well as hydrophilic surfaces to a variable degree depending on the number and nature of the membrane-spanning segments (α-helices or β-strands) and the presence of large loops or domains on either side of the membrane. The very different nature of these distinctive surfaces requires special care with the buffer conditions and the optimal buffer for one may not be optimal for the other, a typical example being the detergent, which is required for solubilization of the lipophilic transmembrane domain but which may at the same time impose destabilizing effects on water-soluble domains. Also, physiological differences in, for example, the pH and oxidation level of the aqueous environments on either side of a membrane are impossible to mimic in a single buffer used for a detergent-solubilized membrane protein.

Looking at membrane-protein crystal packing, there is a reoccurring tendency of the lipophilic and hydrophilic regions to partition in specific layers and directions of the crystal packing (Sørensen *et al.*, 2006). The lipophilic regions are shielded by detergent molecules and therefore these layers and directions of the crystal packing tend to be less ordered, resulting in weak and anisotropic diffraction properties.

There are thus several important reasons for the huge membrane-protein deficit in the PDB: (i) membrane proteins are generally difficult to obtain in large quantities, (ii) they are difficult to stabilize in a solubilized and functional form outside the membrane, (iii) they are typically very difficult to crystallize and (iv) their crystals are often fragile and diffract rather poorly and anisotropically.

3. Heavy-atom derivatives of membrane proteins

A number of studies have approached the general use and preparation of heavy-atom derivatives of protein crystals (Garman & Murray, 2003; Islam *et al.*, 1998). A range of internet servers also provide access to databases that may be helpful in making good decisions on which elements and compounds to aim for in a heavy-atom soak or cocrystallization strategy (http://hatodas.harima.riken.go.jp/; http://skuld.bmsc.washington.edu/scatter/AS_periodic.html; http://www.sbg.bio.ic.ac.uk/had/heavyatom.html). A shortlist of compounds known as the 'Magic Seven' [$HgCl_2$, K_2HgI_4, PCMB, K_2PtCl_4, $KAu(CN)_2$, $UO_2(O_2CCH_3)_2$, $K_3UO_2F_5$] has previously been pointed out by Boggon & Shapiro (2000) as having particularly frequent usage in protein crystallography.

Frequently updated databases are also available of membrane proteins of known structure, including useful details of crystallization conditions (http://www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html) and with direct links to PDB entries and key references (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html and http://www.lipidat.chemistry.ohio-state.edu/MPDB/index.asp).

It might be thought that once well diffracting crystals of a membrane protein are available the challenges of determining its structure would be similar to those of a 'normal' protein crystal. However, this is not entirely the case and in our search for heavy-atom derivatives of Ca^{2+}-ATPase crystals (Sørensen *et al.*, 2004) we realised that specific trends for membrane proteins were apparent. In particular, the handling of the crystals and the applicability of different heavy-atom compounds exhibit specific characteristics and challenges in the case of α-helical membrane proteins.

We have therefore decided to see if proper data mining of reported uses of heavy-atom derivatives of membrane-protein crystals will allow us to document a specific shortlist of prime candidate compounds for membrane proteins. Furthermore, a synopsis of the methods and strategies used in the derivatization, data collection and phasing of membrane-protein crystals will be useful as guide in attempts to determine crystal structures of unknown membrane proteins.

4. A database of membrane-protein structures determined using heavy-atom compounds

4.1. Structure determinations forming the database

Our database, including references and PDB codes for associated structure determinations, is available at http://www.bioxray.dk/~premo/MEMBRANES11.html.

Even though the total number of membrane-protein structures is very small compared with that of soluble proteins, the database of heavy-atom derivatives is still substantial. We have thus far been able to assemble a database of 73 reports of membrane-protein structure determinations encompassing a total of 153 examples of successful heavy-atom (HA) derivatives. Of this total, a subset of 38 structures were of α-helical membrane proteins solved by classical use of HA compounds to obtain derivative crystals (97 individual uses of compounds). We find this subset to represent the most intriguing subject for further analysis. Our analysis is quantitated against individual cases of structure determination to avoid the bias of similar compounds gaining several counts from a single case of structure determination. Furthermore, we have not made any attempts to differentiate between derivatives of major and minor impact in phasing since published details were often too incomplete to make such distinctions.

4.2. The α-helical *versus* β-barrel membrane proteins

We readily observed that α-helical and β-barrel structures show rather different statistics for HA derivatives.

The β-barrel membrane proteins (23 cases of HA-based phasing) are often easily expressed and purified and they represent rather stable protein structures from bacterial outer

membranes. Indeed, they exhibit a prominent use of SeMet incorporation for SAD/MAD phasing (52%, 12 cases) and a rather large spread in the specific types of heavy-atom compounds used in HA soaking and cocrystallization experiments, with platinates being the most frequent (Fig. 1*a*).

The α-helical membrane proteins (50 cases of HA-based phasing) exhibit a strong prevalence of mercurials and platinates being used for phasing (Fig. 1*b*). It is also worth noting that only 16% of the structures (eight cases) were determined on the basis of SeMet incorporation alone for SAD/MAD phasing. In another four cases structures were determined exclusively by SAD/MAD phasing exploiting intrinsically bound metal centres (Fe and Cu). When such opportunities are recognized, a rather well defined rationale in the experimental strategy towards phasing is available. We will therefore focus our attention on the remaining 38 cases of α-helical membrane-protein structures where derivatives obtained by crystal soaking or cocrystallization with HA compounds were critical in the structure-determination process.

Interestingly, a large fraction of these cases represent proteins purified from *Escherichia coli* expression systems which in principle would allow for SeMet incorporation. Yet, only ten out of 27 structures of α-helical membrane proteins expressed in *E. coli* include the use of SeMet protein.. The

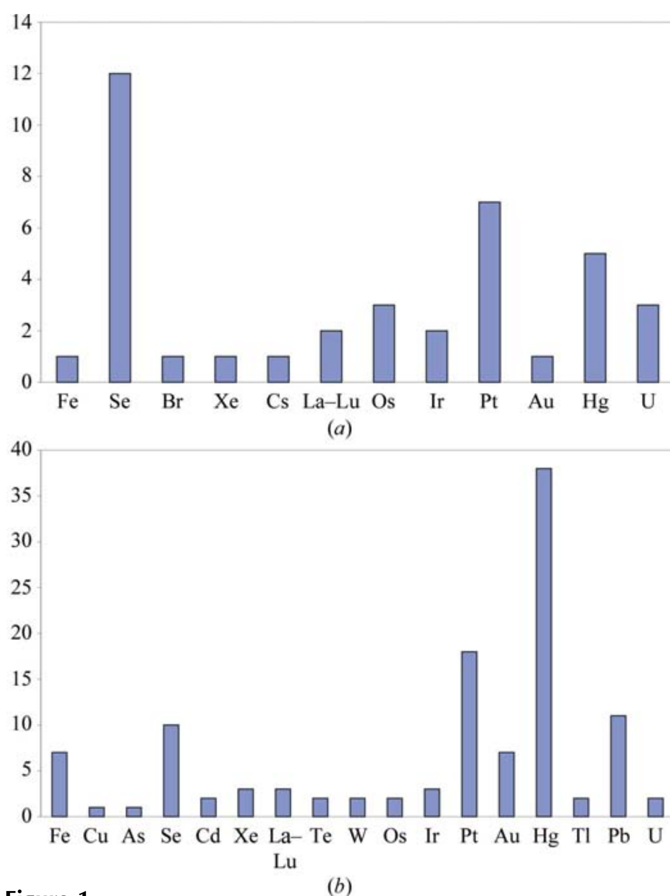

Figure 1
Histogram showing the uses of various elements in heavy-atom derivatives in (*a*) β-barrel membrane proteins and (*b*) α-helical membrane proteins. Tabulations can be found at http://www.bioxray.dk/~premo/MEMBRANES11.html.

Table 1
Membrane's Eleven: a table of 11 suggested compounds for use in primary attempts to identify a heavy-atom derivative of an α-helical membrane protein.

Category	Success score (structure determinations)	Suggested compounds for membrane proteins
Organomercurials	50% (19)	MeHgOAc, EMTS, PCMB
Platinum(II)	34% (13)	K_2PtCl_4, $K_2Pt(NO_2)_4$, orange Pt
Trimethyllead	18% (7)	TMLA
Au(I)	13% (5)	$KAu(CN)_2$
Os/Ir	11% (4)	$OsCl_3$, Na_3IrCl_6
Lanthanides	8% (3)	$YbCl_3$ (cocrystallization)
HA cluster	8% (3)	Ta_6Br_{12}

probable reason is to be found in the difficulty with which membrane proteins are expressed at all in *E. coli* inner membranes. This may have the consequence that the additional stress of using SeMet medium leads to poor yields and unstable protein preparations, as observed for example for the FadL protein (van den Berg *et al.*, 2004). Also, the rather weak anomalous signal from Se may be difficult to detect in data sets of impaired quality, as is unfortunately often the case for α-helical membrane protein crystals. This point is also reflected in the maximum resolution of data sets used for phasing when comparing α-helical and β-barrel membrane proteins: about two-thirds of the β-barrel proteins have been phased with data equal to or better than 3 Å resolution, while this is only the case for one-third of the α-helical proteins.

4.3. Frequently used heavy-atom compounds for α-helical membrane proteins

Table 1 summarizes our major findings from analysis of the 38 structure determinations of α-helical membrane proteins obtained by the use of classical HA derivatives. Further statistics can be found at the website of our database (http://www.bioxray.dk/~premo/statistics.htm).

As also seen in Fig. 1(*b*), mercurials are clearly top-ranking, with use in 60% of cases (23 structures). Organomercurials as a group clearly dominate, having been used alone in 50% of cases (19 structures). Analysis of the type of organomercurials used further highlight methylmercurials and ethylmercurials (acetate, chloride, thiosalicylate and phosphate salts), which have been successfully used in 37% of cases (14 structures). We believe that organomercurials dominate so strongly owing to a large abundance of Cys residues and extracellular/luminal disulfide bonds in α-helical membrane proteins. Popular mercurials such as $HgCl_2$, $Hg(O_2CCH_3)_2$ and K_2HgI_4 may therefore react too aggressively, whereas methyl/ethyl functionalities modulate reactivity and selectivity to appropriate levels while at the same time allowing the compounds to partition in both hydrophilic and lipophilic environments.

Platinum, in particular Pt^{II} compounds such as K_2PtCl_4, orange platinum [platinum(II) tertpyridine chloride] and PIP [di-μ-iodo-bis(ethylene-diamine)-di-platinum(II) nitrate] take a clear second place in our counting statistics, with succesful

applications in 34% of solved structures (13 cases). Trimethyllead acetate (as a group that also includes triethyllead acetate and trimethyllead chloride) ranks third and was used in 18% of structure determinations (seven cases). Interestingly, trimethyllead acetate is rather selective for hydrophobic sites, as demonstrated in the Ca^{2+}-ATPase Ca_2E1-AMPPCP form (Sørensen *et al.*, 2004), where three out of five major sites were located in the transmembrane region (Fig. 2). This suggests a general applicability for membrane proteins, in accordance with the observed trend of organomercurials.

Au^I compounds, in particular $KAu(CN)_2$, appear as a rather significant group in fourth place (13%, four cases), followed by Os and Ir compounds treated as a single group (11%, four cases). Os and Ir provide strong anomalous scattering properties and identification of a derivative will be highly useful. Xe was also used in four cases and thus would also qualify as a candidate, but only in one case was it used for phasing and in that case with K_2PtCl_4 having produced the major derivative (Prince *et al.*, 2002). In the remaining three cases (Andrade *et al.*, 2005; Chang *et al.*, 1998; Schubert *et al.*, 1997) Xe was used to aid the HA-substructure refinement and phase combination through cross-phased difference Fourier analysis.

Another interesting observation is the low representation of commonly tested HA compounds, in particular those of the lanthanide series and uranylates (8%, three cases). We suspect these compounds to disrupt the diffraction properties of membrane proteins. In the case of Ca^{2+}-ATPase (Sørensen *et al.*, 2004), we observed that three-dimensional crystals soaked with various lanthanides appeared to be unaffected when inspected under the light microscope, yet that diffraction nevertheless vanished. With plate-like crystals of membrane proteins, we have observed severe bending when adding lanthanides, in some cases making the crystal curl almost 360°. This is in contrast to the common shattering of crystals when a HA compound is applied. One explanation could be the potent cationic character causing lanthanides (and also uranyl compounds) to react aggressively with the negatively charged phosphate groups of phospholipids partitioned in layers of the crystal packing, thereby causing mechanical stress and disorder in the crystal.

5. Preparation of HA-derivative crystals of membrane proteins

5.1. Soaking of membrane-protein crystals

HA compounds can be introduced to react with the protein by two means: either the protein is pretreated and cocrystallized with the compound or the compound is introduced by the soaking of crystals. Both methods have their advantages and disadvantages.

The advantage of soaking is that many different conditions and compounds can easily be tested in parallel if the supply of crystals is plentiful. With the careful use of identical buffer conditions, isomorphism among crystals may also be attained. A particular problem for such procedures is that many (if not most) membrane proteins are crystallized as a protein–lipid–detergent complex, which can be difficult to stabilize outside of the mothor liquor. In such cases, specific approaches may be applied, such as the use of an artificial mothor liquor saturated with protein. This was critical for soaking experiments of Ca^{2+}-ATPase in the Ca_2E1-AMPPCP form (Sørensen *et al.*, 2004). Another possibility is to mix the crystallization drop with a drop of HA solution in crystallization buffer which has been pre-equilibrated against the same reservoir by vapour diffusion. Osmotic shock effects may thus be prevented upon mixing. We have found this procedure to be helpful in ongoing experiments. Finally, the HA compound can be added directly to the drop in the solid form and left to dissolve and react over days or weeks. In all of the above cases the presence of protein in solution must be taken into account and the site-to-HA ratio be considered carefully. Protein precipitation may also cause problems for subsequent crystal mounting. In severe cases the precipitated protein may be removed as a skin using fine tools.

As mentioned earlier, membrane proteins are often adapted to both oxidative and reductive environments, which are normally separated by the membrane. This may impose

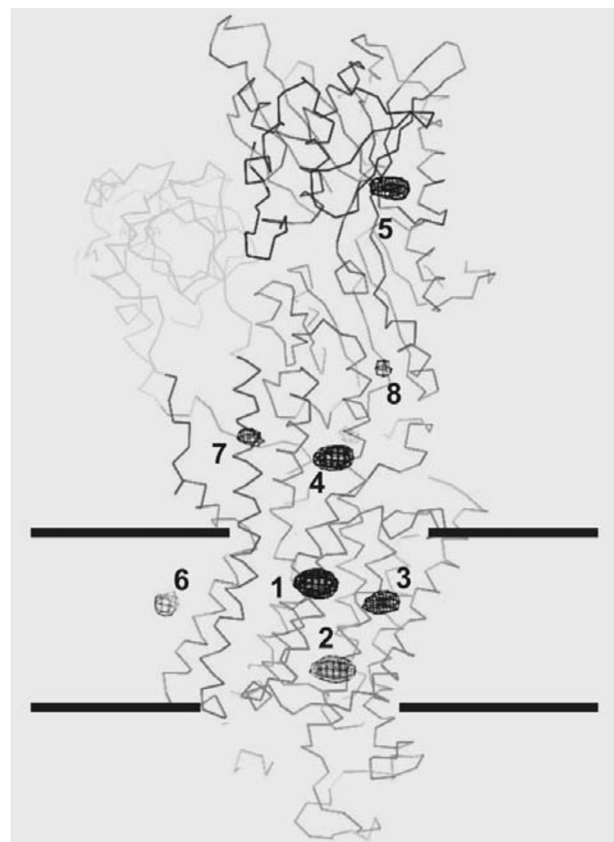

Figure 2
Structural representation showing the anomalous difference Fourier map (based on model phases) of a trimethyllead acetate derivative (TMLA) of Ca^{2+}-ATPase from sarcoplasmic reticulum in the Ca_2E1-AMPPCP form (Sørensen *et al.*, 2004). The protein is shown as a C^α trace and the map is displayed at a 6σ level, highlighting five major and three minor sites (1–5 and 6–8, respectively). Note the presence of three major sites in the transmembrane region. Most sites were associated with Cys residues. The crystal was soaked at 10 mM concentration for 3 d. A similar soak at 1 mM concentration for 24 h produced on a single major site (site 4).

problems when a single environment is enforced during crystallographic studies. This is also a serious issue for HA derivatization, as the use of sulfides such as β-mercapto-ethanol and dithiotreitol will severely interfere with many classes of HA compounds, such as those of Hg, Pb, Au and Pt. If stabilizing solutions can be used, then exchange for a non-reduced buffer is possible. Also, the use of alternative reductants such as TCEP [tris(2-carboxyethyl)phosphine hydrochloride] which do not react with soft cations may be a solution.

Considering the HA concentration and time range of soaking, there seem to be no specific rules. It would seem that a 1–5 m*M* concentration combined with an overnight soak is a convenient starting point. A scatter plot of soaking time *versus* derivative concentration (Hg,Pt and Pb) is given at http://www.bioxray.dk/~premo/statistics.htm.

5.2. Cocrystallization of membrane protein with heavy-atom compounds

An alternative to soaking procedures is to approach derivatization by cocrystallization. This was successfully used to obtain $KAu(CN)_2$ and TlCl derivatives of aquaporin 1 crystals that diffracted to high resolution (Sui *et al.*, 2001) and was a critical part of a crystal-improvement scheme of the mechanosensitive channel MscL (Chang *et al.*, 1998). Letting reaction with HA compounds take place prior to solubilization from the membrane may even be considered. Membrane proteins are generally more stable in the membrane-embedded form, which may prevent unfavourable reactions at secondary sites. Referring to the discussion of reduced *versus* oxidized environments separated by the membrane, pretreatment in the membrane-embedded form may also allow specific reaction from one side. Cocrystallization should also be considered for HA compounds that appear to react efficiently with soaked crystals but at the same distort their diffraction properties. In particular, we predict that lanthanides (*e.g.* $YbCl_3$) would perform better for α-helical membrane proteins when used in this way, thus offering opportunities to obtain derivatives with exceptional anomalous signals.

6. Data collection

6.1. Identification of derivative crystals

Identification of a potential HA derivative represents an important experimental challenge and it must be carefully approached to improve efficiency. Typically, this will now take place at a synchrotron and depending on end-station and beamline design, different strategies are possible.

A tunable beamline combined with convenient and swift procedures for wavelength changes and fluorescence measurements allows a first-line screening of HA-treated crystals by observation of specific X-ray absorption edges in the crystal. Data collection at the wavelength of maximum anomalous signal can then also be performed. If, however, the change of wavelength is too time-consuming, a compromise on wavelength for a batch of different crystals should be made to ensure a significant anomalous signal in all cases. This will typically be at an energy above any of the relevant absorption edges of the elements in question.

Promising heavy-atom derivatives may be identified by significant anomalous differences in the diffraction data. It is therefore advisable to let the first 20–30° of data collection pass an axis of symmetry to readily obtain redundancy in Friedel pairs. Depending on the speed of data collection at the given X-ray source, it may be more efficient to collect full data sets right away and then later analyse their potential as derivatives.

In all the commonly used data-processing programs it is possible to detect an anomalous signal during scaling. In *SCALEPACK* (Otwinowski & Minor, 1997), a χ^2 test upon merging of the Friedel mates is recommended. In *XDS* (Kabsch, 1993) the ratio S_norm/S_anom will indicate the presence of an anomalous scatterer and likewise an anomalous correlation (anom CC) is derived in *SCALA* (Evans, 1993). The detected signal should decline gradually with increasing resolution regardles of the program used and only significant signals should be considered if site identification is to be performed without prior phase information.

In recent years, most data sets from membrane-protein crystals have been collected at third-generation synchrotron sources. Poorly diffracting membrane-protein crystals often call for little or no attenuation of the beam or extended exposure times to exploit the full potential of resolution in the diffraction. However, radiation damage then becomes a serious issue and will become particularly evident when data are being collected at the absorption edges of heavy atoms in a derivatized crystal. High-quality data are critical for an unambiguous identification of derivatives and subsequent site refinement and phasing. It is therefore better to accept a somewhat lower resolution in the data by appropriate attenuation of the beam and/or reduced exposure times than to push resolution limits. Merging incomplete higher resolution data sets from several crystals is possible, but must be part of later optimization attempts.

With efficient procedures in place, it will be possible to screen a significant amount of HA-treated crystals in a few hours at the synchrotron, followed by optimized data collection on the promising targets.

6.2. Phasing

Going back to the database, we have observed that phasing was based on isomorphous replacement (eventually with anomalous scattering) in 31 of the 38 cases where HA derivatives were prepared by soaking or cocrystallization. Only in seven cases was an exclusive use of SAD/MAD phasing employed. If SIRAS/MIRAS phasing is aimed for upon identification of a derivative, then time must also be devoted to obtaining a native data set with an optimal level of isomorphism to the derivative crystal. The use of crystals from the same batch or even the same drop combined with a consistent use of identical buffers, conditions and crystal-

handling procedures are the important issues when isomorphous native crystals are required. For further improvement of the site identification, back-soaking the crystals should also be considered. Generally, back-soaking is performed to decrease the amount of heavy metals in the mother liquor, thereby intensifying the vectors between the bound HAs and removing weakly bound HAs. However, the back-soaking procedure does introduce additional crystal handling that could lead to non-isomorphism between the native and derivative crystals. In the case of the light-harvesting complex (LH2; McDermott *et al.*, 1995) the highest isomorphism between data sets was observed between the soaked and the back-soaked crystals, which eased the following initial site identification (Prince *et al.*, 1999).

7. Conclusion

The use of heavy-atom derivatization is likely to continue to be a significant part of membrane-protein crystallography. Either native tissues and cells or sophisticated expression systems will be used in many instances and such preparations may not be compatible with SeMet incorporation. Furthermore, a consistent use of experimental phases is a priority in contemporary and future crystallography. Molecular replacement can nevertheless play an important role in heavy-atom site identification by difference Fourier analysis. We find that a systematic focus on specific compounds and methods may improve the chances and speed of identifying appropriate heavy-atom derivatives for structure determination of the α-helical membrane proteins. We report the formation of an updated database on heavy-atom derivatives of membrane-proteins crystals and we suggest a list of 'Membrane's Eleven' that in most cases may serve as a help in identifying putative derivatives in a first-line approach. We also advocate a wide use of cocrystallization attempts.

References

Andrade, S. L. A., Dickmanns, A., Ficner, R. & Einsle, O. (2005). *Proc. Natl Acad. Sci. USA*, **102**, 14994–14999.

Berg, B. van den, Black, P. N., Clemons, W. M. Jr & Rapoport, T. A. (2004). *Science*, **304**, 1506–1509.

Boggon, T. J. & Shapiro, L. (2000). *Structure*, **8**, R143–R149.

Chang, G., Spencer, R. H., Lee, A. T., Barclay, M. T. & Rees, D. C. (1998). *Science*, **282**, 2220–2226.

Deisenhofer, J., Epp, O., Miki, K., Huber, R. & Michel, H. (1984). *J. Mol. Biol.* **180**, 385–398.

Evans, P. R. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 114–122. Warrington: Daresbury Laboratory.

Garman, E. & Murray, J. W. (2003). *Acta Cryst.* D**59**, 1903–1913.

Islam, S. A., Carvin, D., Sternberg, M. J. & Blundell, T. L. (1998). *Acta Cryst.* D**54**, 1199–1206.

Jidenko, M., Nielsen, R. C., Sørensen, T. L., Møller, J. V., le Maire, M., Nissen, P. & Jaxel, C. (2005). *Proc. Natl Acad. Sci. USA*, **102**, 11687–11691.

Kabsch, W. (1993). *J. Appl. Cryst.* **26**, 795–800.

Long, S. B., Campbell, E. B. & Mackinnon, R. (2005). *Science*, **309**, 897–903.

McDermott, G., Prince, S. M., Freer, A. A., Hawthornthwaite-Lawless, A. M., Papiz, M. Z., Cogdell, R. J. & Isaacs, N. W. (1995) *Nature (London)*, **374**, 517–521.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Prince, M. S., Achtman, M. & Derrick, J. P. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 3417–3421.

Prince, S. M., McDermott, G., Freer, A. A., Papiz, M. Z., Lawless, A. M., Cogdell, R. J. & Isaacs, N. W. (1999). *Acta Cryst.* D**55**, 1428–1431.

Schubert, W.-D., Klukas, O., Krauss, N., Saenger, W., Fromme, P. & Witt, H. T. (1997). *J. Mol. Biol.* **272**, 741–769.

Sui, H., Han, B. G., Lee, J. K., Walian, P. & Jap, B. K. (2001). *Nature (London)*, **414**, 872–878.

Sørensen, T. L., Møller, J. V. & Nissen, P. (2004). *Science*, **304**, 1672–1675.

Sørensen, T. L., Olesen, C., Jensen, A. L., Møller, J. V. & Nissen, P. (2006). In the press.

Törnroth-Horsefield, S., Wang, Y., Hedfalk, K., Johanson, U., Karlsson, M., Tajkhorshid, E., Neutze, R. & Kjellbom, P. (2006). *Nature (London)*, **439**, 688–694.