glucopyranosides or alkyl-β-D-maltosides as detergents: the commercially available detergents are often 'contaminated' with the α-anomers in varying, sometimes substantial, concentrations. The α-anomers are much less soluble, and appear to prevent crystallization. In the case of photosystem I from a thermophilic cyanobacterium, it has been reported that for a 2% α-anomer content in dodecyl-β-D-maltoside preparations no crystals can be obtained, with a 0.5–2% content the diffraction of the crystals is anisotropic with a reduction in resolution to 5–6 Å, whereas diffraction to better than 3.5 Å resolution in all directions is observed when the content of the α-anomer is below 0.1% (Fromme & Witt, 1998). The percentage of α-anomer can be determined using NMR spectroscopy or high-performance liquid chromatography. During ageing of detergent solutions, a conversion from the β-anomer to the α-anomer is expected. Therefore, ageing of detergent solutions has to be prevented.

The detergents that have been successfully used to crystallize membrane proteins can also be found in Table 4.2.1.1. The possibilities for developing new detergents for membrane-protein crystallization have not been exhausted. There is a need for new detergents, *e.g.* detergents with head groups with sizes between glucose and maltose are still missing!

It has been observed that crystals of the photosynthetic reaction centre from the purple bacterium *Rhodopseudomonas viridis* could be obtained when *N*,*N*-dimethyldodecylamine-*N*-oxide is used as detergent, but not when *N*,*N*-dimethyldecylamine-*N*-oxide is used. Even crystals formed with the dodecyl homologue lost their order when soaked in a buffer containing the decyl homologue. These observations were found to have an obvious explanation when the location of the detergent molecules bound to the protein was determined using neutron crystallography (Roth *et al.*, 1989); the detergent molecules surrounding neighbouring photosynthetic reaction centres in the crystal lattice are in contact. It is likely that attractive interactions between neighbouring protein-bound detergent micelles contribute to the stability of the crystal lattice. Particularly striking (see Table 4.2.3.1) is the dependence of the crystal quality on the alkyl-chain length in the case of the two-subunit cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans.* Well ordered crystals were obtained with undecyl-β-D-maltoside, but not with the decyl and dodecyl homologues. Table 4.2.3.1 also lists the names of important vendors of detergents.

Table 4.2.3.1. *Summary of the results of attempts to crystallize the two-subunit cytochrome c oxidase from the soil bacterium Paracoccus denitrificans using different detergents (after Ostermeier et al., 1997)*

The resolutions of the crystals obtained are given in parentheses. Abbreviations: C_{12}: dodecyl; C_{11}: undecyl; C_{10}: decyl; C_9: nonyl; CYMAL-6: (cyclohexyl)hexyl-β-D-maltoside; CYMAL-5: (cyclohexyl)pentyl-β-D-maltoside. x in E_x is the number of oxyethylene units in the alkylpolyoxyethylene detergents. Suppliers: A: Anatrace (Maumee, OH); B: Biomol; C: Calbiochem; F: Fluka.

Detergent	Supplier	Crystals?
C_{12}-β-D-maltoside	B	Yes (8 Å)
C_{11}-β-D-maltoside	B	Yes (2.5 Å)
C_{10}-β-D-maltoside	B	No
CYMAL-6	A	Yes (2.6 Å)
CYMAL-5	A	No
Dodecylsucrose	C	No
Decylsucrose	C	No
C_9-β-D-glucoside	C	No
$C_{12}E_8$	F	Yes (> 8 Å)
$C_{12}E_6$	F	No
$C_{12}E_5$	F	No
$C_{10}E_6$	F	No
$C_{10}E_5$	F	No

4.2.4. The 'small amphiphile concept'

From the arguments and observations presented above, it is evident that the size and shape of the detergent micelle are very important in membrane-protein crystallization. Detergent micelles can be made smaller (and their curvatures changed) when small amphiphilic molecules like heptane-1,2,3-triol are added (Timmins *et al.*, 1991; Gast *et al.*, 1994). These compounds form mixed micelles with detergents. When 10% (*w*/*v*) heptane-1,2,3-triol is added to 1% solutions of *N*,*N*-dimethyldodecylamine-*N*-oxide in water, the number of detergent molecules per micelle decreases from 69 to 34, ~23 heptane-1,2,3-triol molecules are incorporated and the radius of the micelle is reduced from 20.9 to 16.9 Å (Timmins *et al.*, 1994). This so-called 'small amphiphile concept' has been used successfully to crystallize bacterial photosynthetic reaction centres (Michel, 1982, 1983; Buchanan *et al.*, 1993), bacterial light-harvesting complexes (Michel, 1991; Koepke *et al.*, 1996) and other membrane proteins (see Table 4.2.1.1). The light-harvesting complexes from the purple bacterium *Rhodospirillum molischianum* yield an astonishing number of different crystal forms, but only one diffracts to high resolution. A large amount of heptane-1,2,3-triol had to be added to obtain this crystal form. As a result, heptane-1,2,3-triol itself reached supersaturation during crystallization of the protein. Normally, the protein crystallizes first and heptane-1,2,3-triol later. When heptane-1,2,3-triol crystals form, the protein crystals crack and eventually redissolve. A likely explanation is that the concentration of solubilized heptane-1,2,3-triol is reduced when it forms crystals and the mixed detergent–heptane-1,2,3-triol micelles lose heptane-1,2,3-triol and take up detergent molecules from solution. The micelles expand and the crystals crack. This behaviour caused severe problems when searching for heavy-atom derivatives. Small amphiphiles work well with the *N*,*N*-dimethylalkylamine-*N*-oxides and alkylglucopyranosides as detergents, but not with alkylmaltosides. The most successful small amphiphiles are heptane-1,2,3-triol and benzamidine.

4.2.5. Membrane-protein crystallization with the help of antibody Fv fragments

The number of detergents that can be tried is limited for rather unstable membrane proteins. For instance, the four-subunit cytochrome *c* oxidase from *P. denitrificans* is sufficiently stable only with dodecyl-β-D-maltoside as detergent. When other detergents are employed, subunits III and IV and some lipids are removed from subunits I and II. These lipids might contribute to the binding of the protein subunits III and IV to the central subunits I and II. Therefore, the size of the detergent micelles can not be varied by using different detergents. In order to obtain crystals, the size of the extramembraneous part of this important enzyme was enlarged by the binding of Fv fragments of monoclonal antibodies. For this purpose, monoclonal antibodies against the four-subunit cytochrome *c* oxidase were generated using the classical hybridoma technique. Then hybridoma cell lines producing conformation-specific antibodies were selected (such antibodies react positively in enzyme-linked immunosorbent assays, but negatively in Western blot assays). The cDNA strands coding for the respective VL and VH genes were cloned and expressed in *Escherichia coli.* Binding of conformation-specific antibody fragments can be expected to lead to a more homogeneous protein preparation. The first Fv fragment worked, and well ordered crystals of the four-subunit and

two-subunit cytochrome *c* oxidases were obtained (Ostermeier *et al.*, 1995, 1997). As an important additional advantage of this approach, an affinity tag can be fused to the recombinant antibody Fv fragment and used for efficient isolation. The affinity tag can then be used to purify the complex of antibody fragment and membrane protein rapidly and with a high yield (Kleymann *et al.*, 1995). Because Fv fragment cocrystallization also worked with the yeast cytochrome bc_1 complex, again with the first and only conformation-specific antibody Fv fragment tried (Hunte *et al.*, 2000), we are rather confident about this method for the future. The usefulness of single-chain Fv fragments, which can be obtained by the phage-display technique, has not been investigated, because the linker region joining the VL and VH chains must be expected to be flexible. Such flexibility would induce inhomogeneity and reduce the chance of obtaining crystals.

4.2.6. Membrane-protein crystallization using cubic bicontinuous lipidic phases

Landau & Rosenbusch (1996) introduced the use of bicontinuous cubic phases of lipids for membrane-protein crystallization. In such phases, the lipid forms a single, curved, continuous three-dimensional bilayer [see Lindblom & Rilfors (1989) for a review]. One can incorporate membrane proteins into such a bilayer, as demonstrated with octyl-β-D-glucopyranoside-solubilized monomeric bacteriorhodopsin. The three-dimensional bilayer network serves as a matrix for crystallization. The membrane protein can diffuse through the bilayer, but is also able to establish polar contacts in the third dimension. Landau & Rosenbusch demonstrated that bacteriorhodopsin forms small, well ordered three-dimensional crystals. The X-ray data indicate that the same two-dimensional crystals are present as formed by bacteriorhodopsin in its native environment (the purple membrane). These membranes are now stacked in the third dimension in a well ordered manner. Therefore, these crystals belong to type I.

The method has the conceptual problem that the growing three-dimensional crystal has to disrupt and displace the cubic lipidic phase. Nevertheless, it is hoped that this method can also be used for membrane proteins that do not have a strong tendency to form two-dimensional crystals spontaneously. In particular, this method appears to be the only chance for crystallizing those membrane proteins that are unstable in the absence of added lipids.

4.2.7. General recommendations

When trying to crystallize a membrane protein the first, and most important, task is to obtain a pure, stable and homogeneous preparation of the membrane protein under investigation. Unfortunately, general methods for producing substantial amounts of membrane proteins using heterologous expression systems in a native membrane environment do not exist, nor is refolding of membrane proteins from inclusion bodies well established. Once a pure and homogeneous preparation of a membrane protein has been obtained, its stability in various detergents has to be investigated at different pH values. Frequently, a sharp optimum pH for stability is observed with detergents of shorter alkyl-chain length. In crystallization attempts, the usual parameters (nature of the precipitant, buffer, pH, addition of inhibitors and/or substrates, see Chapter 4.1) should be varied. The most important variable, however, is the detergent. Detergents can be exchanged most conveniently by binding the membrane protein to column materials (*e.g.* ion-exchange resins, hydroxyapatite, affinity matrices), washing with a buffer containing the new detergent at concentrations above the CMC under non-eluting conditions, and then eluting the bound membrane protein with a buffer containing the new detergent. The completeness of the detergent exchange should be checked. For this purpose, the use of ^{14}C- or ^{3}H-labelled detergents is recommended. Washing with about 20 column volumes is frequently required for a complete detergent exchange. It is more difficult to exchange a detergent with a low CMC (this means long alkyl chains) against a detergent with a high CMC (shorter alkyl chains). The detergent can often be exchanged during a final step in the purification. Less satisfying methods for detergent exchange are molecular-sieve chromatography, ultracentrifugation or repeated ultrafiltration and dilution. If the amount of membrane protein is limited, it is advisable to restrict the usual parameters to a set of, *e.g.*, 48 standard combinations with respect to precipitating agent, pH, buffer *etc.*, but to try *all* available detergents. If crystals of insufficient quality or size are obtained, trying the antibody Fv fragment approach is highly recommended. Alternatives are to use a different source (species) for the membrane protein under investigation, or to remove flexible parts of the membrane protein by proteolytic digestion. I am convinced that 50% of all membrane proteins will yield well ordered, three-dimensional crystals within five years, once the problem of obtaining a pure, stable and homogeneous preparation has been solved.

references