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4.2. Crystallization of membrane proteins

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4.2.1. Introduction

At the time of writing, the Protein Data Bank contains more than 8000 entries of protein structures. These belong to roughly 1200 sequence-unrelated protein families, which can be classified into ∼350 different folds (Gerstein, 1998). However, all known membrane-protein structures belong to one of a dozen membrane-protein families. Membrane proteins for which the structures have been determined are bacterial photosynthetic reaction centres, porins and other β-strand barrel-forming proteins from the outer membrane of Gram-negative bacteria, bacterial light-harvesting complexes, bacterial and mitochondrial cytochrome *c* oxidase, the cytochrome bc_1 complex from mammalian mitochondria, two cyclooxygenases, squalene cyclase, and two bacterial channel proteins. These structures have been determined by X-ray crystallography. In addition, the structures of two membrane proteins, namely that of bacteriorhodopsin and that of the plant light-harvesting complex II, have been solved by electron crystallography (see Chapter 19.6). Table 4.2.1.1 provides a list of the membrane proteins with known structures. It also contains the key references for the structure descriptions and the crystallization conditions.

It is known from genome sequencing projects that 20–35% of all proteins contain at least one transmembrane segment (Gerstein, 1998), as deduced from the occurrence of stretches of hydrophobic amino acids that are long enough to span the membrane in a helical manner. These numbers may be an underestimate, because β-strand-rich membrane proteins like the porins, or membrane proteins which are only inserted into the membrane, but do not span it ('monotopic membrane proteins'), like cyclooxygenases (prostaglandin-H synthases), cannot be recognized as being membrane proteins by inspecting their amino-acid sequences.

Why do we know so few membrane-protein structures? The first reason is the lack of sufficient amounts of biochemically well characterized, homogeneous and stable membrane-protein preparations. This is especially true for eukaryotic receptors and transporters (we do not know the structures of any of these proteins). For these, a major problem is the lack of efficient expression systems for heterologous membrane-protein production. It is therefore not surprising that most membrane proteins with known structure are either involved in photosynthesis or bioenergetics (they are relatively abundant), or originate from bacterial outer membranes (they are exceptionally stable and can be overproduced). Second, membrane proteins are integrated into membranes. They have two polar surface regions on opposite sides (where they are in contact with the aqueous phases and the polar head groups of the membrane lipids) which are separated by a hydrophobic belt. The latter is in contact with the alkyl chains of the lipids. As a result of this amphipathic nature of their surface, membrane proteins are not soluble in either aqueous or organic solvents. To isolate membrane proteins one first has to prepare the membranes, and then solubilize the membrane proteins by adding an excess of detergent. Detergents consist of a polar or charged head group and a hydrophobic tail. Above a certain concentration, the so-called *critical micellar concentration* (CMC), detergents form *micelles* by association of their hydrophobic tails. These micelles take up lipids. Detergents also bind to the hydrophobic surface of membrane proteins with their hydrophobic tails and form a ring-like detergent micelle surrounding the membrane protein, thus shielding the hydrophobic belt-like surface of the membrane protein from contact with water. This is the reason for their ability to solubilize membrane proteins, although with detergents with large polar head groups it is sometimes difficult to achieve a rapid and complete solubilization. The solubilizate, consisting of these mixed protein–detergent complexes as well as lipid-containing and pure detergent micelles, is then subjected to similar purification procedures as are soluble proteins. Of course, the presence of detergents complicates the purification procedures. The choice of the detergent is critical. The detergent micelles have to replace, and to mimic, the lipid bilayer as perfectly as possible, in order to maintain the stability and activity of the solubilized membrane protein. The solubilization of membrane proteins has been reviewed by Hjelmeland (1990) and the general properties of the detergents used has been reviewed by Neugebauer (1990).

4.2.2. Principles of membrane-protein crystallization

There are two principal types of membrane-protein crystals (Michel, 1983). First, one can think of forming two-dimensional crystals in the planes of the membrane and stacking these two-dimensional crystals in an ordered way with respect to up and down orientation, rotation and translation. This membrane-protein crystal type ('type I') is attractive, because it contains the membrane proteins in their native environment. It should even be possible to study lipid–protein interactions. Crystals of bacteriorhodopsin of this type have been obtained either upon slow removal of the detergent by dialysis at high ionic strength (Henderson & Shotton, 1980), or by a novel approach using lipidic bicontinuous cubic phases (Landau & Rosenbusch, 1996; Pebay-Peyroula *et al.*, 1997; see also below). Alternatively, one can try to crystallize the membrane protein with the detergents still bound in a micellar manner. These crystals are held together *via* polar interactions between the polar surfaces of the membrane proteins. The detergent plays a more passive, but still critical, role. Such 'type II' crystals look very much like crystals of soluble globular proteins. The same crystallization methods and equipment as for soluble globular proteins (see Chapter 4.1) can be used. However, the use of hanging drops is sometimes difficult, because the presence of detergents leads to a lower surface tension of the protein solution. Intermediate forms between type I and type II crystals are feasible, *e.g.* by fusion of detergent micelles.

The use of detergent concentrations just above the CMC of the respective detergent is recommended in order to prevent complications caused by pure detergent micelles. Unfortunately, the CMC is not constant. Normally, the CMC provided by the vendor has been determined in water at room temperature. A compilation of potentially useful detergents, their CMCs and their molecular weights is presented in Table 4.2.2.1. The CMC is generally lower at high ionic strength and at high temperatures. The presence of glycerol and similar compounds, as well as that of chaotropic agents (Midura & Yanagishita, 1995), also influences (decreases) the CMC.

4.2.3. General properties of detergents relevant to membrane-protein crystallization

The presence of detergents sometimes causes problems. The monomeric detergent itself can crystallize, *e.g.* dodecyl-β-D-maltoside at 4 °C in the presence of polyethylene glycol. The detergent crystals might be mistaken for protein crystals. Detergent micelles possess attractive interactions (see Zulauf, 1991). Upon addition of salts or polyethylene glycol, or upon temperature changes, a phase separation may be observed: owing to an increase in these attractive interactions, the detergent micelles 'precipitate', forming a viscous detergent-rich phase and a detergent-depleted

Table 4.2.1.1. *Compilation of membrane proteins with known structures, including crystallization conditions and key references for the structure determinations*

This table is continuously updated and can be inspected at http://www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html. The membrane proteins listed are divided into polytopic membrane proteins from inner membranes of bacteria and mitochondria (*a*), membrane proteins from the outer membrane of Gram-negative bacteria (*b*) and monotopic membrane proteins [(*c*); these are proteins that are only inserted into the membrane, but do not span it]. Within parts (*a*), (*b*) and (*c*) the membrane proteins are listed in chronological order of structure determination.

(*a*) Polytopic membrane proteins from inner membranes of bacteria and mitochondria.

Membrane protein	Crystallization conditions (detergent/additive/ precipitating agent)	Key references (and pdb reference code, if available)
Photosynthetic reaction centre		
from *Rhodopseudomonas viridis*	*N*,*N*-Dimethyldodecylamine-*N*-oxide/heptane-1,2,3-triol/ammonium sulfate	[1], [2] (1PRC), [3], [4] (2PRC, 3PRC, 4PRC, 5PRC, 6PRC, 7PRC)
from *Rhodobacter sphaeroides*	*N*,*N*-Dimethyldodecylamine-*N*-oxide/heptane-1,2,3-triol/polyethylene glycol 4000	[5] (4RCR)
	Octyl-β-D-glucopyranoside/polyethylene glycol 4000	[6] (2RCR)
	N,*N*-Dimethyldodecylamine-*N*-oxide/heptane-1,2,3-triol, dioxane/potassium phosphate	[7] (1PCR)
	Octyl-β-D-glucopyranoside/benzamidine, heptane-1,2,3-triol/polyethylene glycol 4000	[8] (1AIG, 1AIJ)
Bacteriorhodopsin		
from *Halobacterium salinarium*	(Electron crystallography using naturally occuring two-dimensional crystals)	[9] (1BRD), [10] (2BRD), [11] (1AT9)
	(Type I crystal grown in lipidic cubic phases)	[12] (1AP9), [13] (1BRX)
	Octyl-β-D-glucopyranoside/benzamidine/sodium phosphate (epitaxic growth on benzamidine crystals)	[14] (1BRR)
Light-harvesting complex II		
from pea chloroplasts	(Electron crystallography of two-dimensional crystals prepared from Triton X100 solubilized material)	[15]
Light-harvesting complex 2		
from *Rhodopseudomonas acidophila*	Octyl-β-D-glucopyranoside/benzamidine/ phosphate	[16] (1KZU)
from *Rhodospirillum molischianum*	*N*,*N*-dimethylundecylamine-*N*-oxide/heptane-1,2,3-triol/ammonium sulfate	[17] (1LGH)
Cytochrome *c* oxidase		
from *Paracoccus denitrificans*,		
four-subunit enzyme complexed with antibody Fv fragment	Dodecyl-β-D-maltoside/polyethylene glycol monomethylether 2000	[18]
two-subunit enzyme complexed with antibody Fv fragment	Undecyl-β-D-maltoside/polyethylene glycol monomethylether 2000	[19] (1AR1)
from bovine heart mitochondria	Decyl-β-D-maltoside with some residual cholate/polyethylene glycol 4000	[20], [21] (1OCC), [22] (2OCC, 1OCR)
Cytochrome bc_1 complex		
from bovine heart mitochondria	Decanoyl-*N*-methylglucamide or diheptanoyl phosphatidyl choline/polyethylene glycol 4000	[23] (1QRC), [24]
	Octyl-β-D-glucopyranoside/polyethylene gycol 4000	[25]
	Pure dodecyl-β-D-maltoside or mixture with methyl-6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside/polyethylene glycol 4000	[26]
from chicken heart mitochondria	Octyl-β-D-glucopyranoside/polyethylene glycol 4000	[25] (1BCC, 3BCC)
Potassium channel		
from *Streptomyces lividans*	*N*,*N*-Dimethyldodecylamine/polyethylene glycol 400	[27] (1BL8)
Mechanosensitive ion channel		
from *Mycobacterium tuberculosis*	Dodecyl-β-D-maltoside/triethylene glycol	[28]

Table 4.2.1.1. *Compilation of membrane proteins with known structures, including crystallization conditions and key references for the structure determinations* (*cont.*)

(*b*) Membrane proteins from the outer membrane of Gram-negative bacteria and related proteins.

Membrane protein	Crystallization conditions (detergent/additive/ precipitating agent)	Key references (and pdb reference code, if available)
16-Stranded porins		
from *Rhodobacter capsulatus*	Octytetraoxyethylene/polyethylene glycol 600	[29] (2POR)
OmpF and PhoE from *Escherichia coli*	Mixture of *n*-octyl-2-hydroxyethylsulfoxide and octylpolyoxyethylene; or *N*,*N*-dimethyl-decylamine-*N*-oxide/polyethylene glycol 2000	[30] (1OPF, 1PHO), [31]
from *Rhodopseudomonas blastica*	Octyltetraoxyethylene/heptane-1,2,3-triol/ polyethylene glycol 600	[32] (1PRN)
from *Paracoccus denitrificans*	Octyl-β-D-glucoside/polyethylene glycol 600	[33]
18-Stranded porins		
maltoporin from *Escherichia coli*	Mixture of decyl-β-D-maltoside and dodecylnonaoxyethylene/polyethylene glycol 2000	[34] (1MAL)
maltoporin from *Salmonella typhimurium*	Mixture of octyltetraoxyethylene and *N*,*N*-dimethylhexylamine-*N*-oxide/polyethylene glycol 1500	[35] (1MPR, 2MPR)
sucrose-specific ScrY porin from *Salmonella typhimurium*	Mixture of octyl-β-D-glucopyranoside and *N*,*N*-dimethylhexylamine-*N*-oxide/polyethylene glycol 2000	[36] (1AOS, 1AOT)
α-Haemolysin		
from *Staphylococcus aureus*	Octyl-β-D-glucopyranoside/ammonium sulfate, polyethylene glycol monomethylether 5000	[37] (7AHL)
Eight-stranded β-barrel membrane anchor		
OmpA fragment from *Escherichia coli*	Not yet available	[38] (1BXW)
22-Stranded receptors		
FhuA from *Escherichia coli*	*N*,*N*-Dimethyldecylamine-*N*-oxide/inositol/ polyethylene glycol monomethylether 2000	[39] (1FCP, 2FCP)
	n-Octyl-2-hydroxyethylsulfoxide/polyethylene glycol 2000	[40] (1BY3, 1BY5)
ferric enterobacterin receptor (FepA) from *Escherichia coli*	*N*,*N*-dimethyldodecylamine-*N*-oxide/heptane-1,2,3-triol/polyethylene glycol 1000	[41] (1FEP)

(*c*) Proteins inserted into, but not crossing the membrane ('monotopic membrane proteins').

Membrane protein	Crystallization conditions (detergent/additive/ precipitating agent)	Key references (and pdb reference code, if available)
Prostaglandin H_2 synthase 1		
(cyclooxygenase 1) from sheep	Octyl-β-D-glucopyranoside/polyethylene glycol 4000	[42] (1PRH)
Cyclooxygenase 2		
from mouse	Octyl-β-D-glucopyranoside/polyethylene glycol monomethylether 550	[43] (1CX2, 3PGH, 4COX, 5COX, 6COX)
from man	Octylpentaoxyethylene/polyethylene glycol 4000	[44]
Squalene cyclase		
from *Alicyclobacillus acidocaldarius*	Octyltetraoxyethylene/sodium citrate	[45] (1SQC)

References: [1] Diesenhofer *et al*. (1985); [2] Diesenhofer *et al*. (1995); [3] Lancaster & Michel (1997); [4] Lancaster & Michel (1999); [5] Allen *et al*. (1987); [6] Chang *et al*. (1991); [7] Ermler *et al*. (1994); [8] Stowell *et al*. (1997); [9] Henderson *et al*. (1990); [10] Grigorieff *et al*. (1996); [11] Kimura *et al*. (1997); [12] Pebay-Peyroula *et al*. (1997); [13] Luecke *et al*. (1998); [14] Essen *et al*. (1998); [15] Kühlbrandt *et al*. (1994); [16] McDermott *et al*. (1995); [17] Koepke *et al*. (1996); [18] Iwata *et al*. (1995); [19] Ostermeier *et al*. (1997); [20] Tsukihara *et al*. (1995); [21] Tsukihara *et al*. (1996); [22] Yoshikawa *et al*. (1998); [23] Xia *et al*. (1997); [24] Kim *et al*. (1998); [25] Zhang *et al*. (1998); [26] Iwata *et al*. (1998); [27] Doyle *et al*. (1998); [28] Chang *et al*. (1998); [29] Weiss *et al*. (1991); [30] Cowan *et al*. (1992); [31] Cowan *et al*. (1995); [32] Kreusch *et al*. (1994); [33] Hirsch *et al*. (1997); [34] Schirmer *et al*. (1995); [35] Meyer *et al*. (1997); [36] Forst *et al*. (1998); [37] Song *et al*. (1996); [38] Pautsch & Schulz (1998); [39] Ferguson *et al*. (1998); [40] Locher *et al*. (1998); [41] Buchanan *et al*. (1999); [42] Picot *et al*. (1994); [43] Kurumbail *et al*. (1996); [44] Luong *et al*. (1996); [45] Wendt *et al*. (1997).

Table 4.2.2.1. *Potentially useful detergents for membrane-protein crystallizations with molecular weights and CMCs [in water, from Michel (1991) or as provided by the vendor]*

The lengths of the alkyl or alkanoyl side chains are given as C_6 to C_{16}.

Detergent	Molecular weight	CMC (m*M*)
Alkyl dihydroxypropyl sulfoxide		
C_8	238	20.6
N,*N*-Dimethylalkylamine-*N*-oxides		
C_8	173	162
C_9	187	50
C_{10}	201	20
C_{12}	229	1–2
n-Dodecyl-*N*,*N*-dimethylglycine (zwitterionic)	271	1.5
N-Alkyl-β-D-glucopyranosides		
C_6	264	250
C_7	278	79
C_8	292	30
C_9	306	6.5
C_{10}	320	2.6
n-Alkanoyl-*N*-hydroxyethylglucamides ('HEGA-*n*')		
C_8	351	109
C_9	365	39
C_{10}	379	7.0
C_{11}	393	1.4
Alkyl hydroxyethyl sulfoxide		
C_8	222	15.8
n-Alkyl-β-D-maltosides		
C_6	426	210
C_8	454	19.5
C_9	468	6
C_{10}	483	1.8
C_{11}	497	0.6
C_{12}	511	0.17
C_{13}	525	0.033
C_{14}	539	0.01
C_{16}	567	0.006
cyclohexyl-C_1	438	340
cyclohexyl-C_2	452	120
cyclohexyl-C_3	466	34.5
cyclohexyl-C_4	480	7.6
cyclohexyl-C_5	494	2.4
cyclohexyl-C_6	508	0.56
cyclohexyl-C_7	522	0.19
n-Alkanoyl-*N*-methylglucamides ('MEGA-*n*')		
C_8	321	79
C_9	335	25
C_{10}	349	6
Methyl-6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside ('HECAMEG')	335	19.5
n-Alkylphosphocholines (zwitterionic)		
C_8	295	114
C_9	309	39.5
C_{10}	323	11
C_{12}	315	1.5
C_{14}	379	0.12
C_{16}	407	0.013
Polyoxyethylene monoalkylethers (C_nE_m)		
C_8E_4	306	7.9
C_8E_5	350	7.1
$C_{10}E_6$	422	0.9
$C_{12}E_8$	538	0.071
n-Alkanoylsucrose		
C_{10}	497	2.5
C_{12}	525	0.3
n-Alkyl-β-D-thioglucopyranosides		
C_7	294	29
C_8	308	9
C_9	322	2.9
C_{10}	336	0.9
n-Alkyl-β-D-thiomaltopyranosides		
C_8	471	8.5
C_9	485	3.2
C_{10}	499	0.9
C_{11}	513	0.21
C_{12}	527	0.05

aqueous phase. The membrane proteins are found exclusively in the viscous phase and crystals – if formed – are difficult to handle. Some detergents, *e.g.* those with polyoxyethylene head groups, undergo a phase separation at higher temperatures. This phenomenon has been used to separate solubilized membrane proteins, which are found in the detergent-rich phase, from the water-soluble proteins. The latter are concentrated in the detergent-depleted phase (Bordier, 1981). Other detergents, *e.g.* octyl-β-D-glucopyranoside, show this phase separation at lower temperatures. Therefore, if phase separation causes problems, a change of the crystallization temperature may help.

The polar head groups of the detergents influence their usage in many ways. One would like to have a small polar head group, because the head group 'covers' the part of the protein's polar surface that is adjacent to the hydrophobic surface belt. The bigger the head group the more of the polar surface is covered and unavailable for the polar interactions needed to form the crystal lattice. Unfortunately, detergents with small polar head groups are rather denaturing. Detergents with charged head groups cannot be used, but detergents with zwitterionic head groups, *e.g.* sulfobetaines, can be tried with more stable proteins. The head group of a very successful detergent, *N*,*N*-dimethyldodecylamine-*N*-oxide, is of zwitterionic nature. I estimate that it can only be used with about 20% of all membrane proteins. Detergents with sugar residues as head groups have been used successfully. Octyl-β-D-glucopyranoside also tends to be denaturing. The lifetime of many membrane proteins can be prolonged by a factor of three by the use of nonyl-β-D-glucopyranoside instead of the shorter homologue. Such behaviour is observed within each series of homologous detergents; an increase in the alkyl chain by one methylene group leads to an increase in stability by a factor of three, an increase by two methylene groups leads to an increase in stability by a factor of about ten. Unfortunately, decyl-β-D-glucopyranoside is too insoluble to be used as detergent. For less stable membrane proteins, alkylmaltoside detergents or alkanoylsucrose detergents have to be tried. There is one special problem when using alkyl-β-D-

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*-dimethyl-alkylamine-*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.

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