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