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Engineering the Lac Permease for Purification and Crystallization

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The lactose permease is being used as a model system for the rational redesign of a membrane protein with the goal of increasing the likelihood of crystallization. Various modifications to the protein have been added for the purposes of purification, stability, and potential for crystallization. The addition of six consecutive histidines at the C-terminus of the protein allows for the rapid purification by nickel-chelate chromatography, and the insertion of an entire protein domain into one of the inner cytoplasmic loops of the permease gives the resulting protein a larger hydrophilic surface area. The increase in polar surface area makes the fusion protein easier to handle and more likely to crystallize. In particular, the introduction of cytochrome *b*562 of *E. coli* into the central hydrophilic domain of the lac permease results in a fusion protein with the transport activity of the permease and the visible absorbance spectrum of the cytochrome. The "red permease" is very easy to monitor through the steps of expression, purification, concentration, and crystallization.

KEY WORDS: Active transport; membrane protein; crystallization; affinity purification; protein engineering; carrier domain.

INTRODUCTION

The techniques used in the crystallization of membrane proteins are not radically different from those used with soluble proteins. Once a suitable sample is available, screening for crystallization conditions is usually done with the traditional methods of vapor diffusion or equilibrium dialysis. A major difference, however, is the detergent requirement of the protein. This adds an additional level of complexity to the system, since the particle being crystallized is a protein–detergent complex (PDC), whose behavior is more complex than that of a soluble protein (Michel, 1990; Garavito, 1990). Solutions of pure detergents alone can exhibit a staggering array of behaviors (Zulauf, 1990), and when one further considers a PDC in the presence of free detergent, precipitants, salts, buffers and additives, it becomes clear why such systems are extremely difficult to characterize. For this reason, it is important to start with the best possible sample. As with all proteins, the issues of purity, stability, and monodispersity have to be met with stringent standards before crystallization can have a reasonable chance of success. This is especially true of membrane proteins in which each of these items is notoriously difficult. Efforts must be focused to meet these requirements before a protein can even be considered as a serious candidate for crystallization. Conditions must be found in which the solubilized protein is stable and functional. The importance of protein chemistry is paramount, and molecular biology techniques can play a vital role in producing protein suitable for crystallization.

The use of protein engineering tools for the crystallization of soluble proteins has a long history, and examples include the truncation of C or N terminal ends of proteins, the isolation of single domains in a

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multidomain protein, and changes of single residues to affect the solubility and surface properties of a protein. As a whole, less use has been made of molecular biology techniques with membrane proteins. The principal reason for this is that membrane proteins are often not easily expressed in heterologous systems, and the native organism may not be suitable for transformation with artificially manipulated genes. Membranes can be very different from one organism to another, resulting in expression problems in heterologous hosts. Some membrane proteins are posttranslationally modified, and many occur as complex multimers or have complicated cofactor requirements (especially the respiratory complexes). These factors often make it difficult to manipulate membrane proteins at the genetic level, and even when it is possible to do so, the yields are often disappointingly low.

An integral membrane protein from *E. coli*, the lactose (lac) permease, has several advantages in this respect. Its native host is ideal for genetic manipulations, and strains lacking the genomic lacY gene are readily available, providing a blank background. Activity can be reconstituted in the host by transformation with a plasmid carrying the lacY gene, so that it is relatively easy to functionally characterize engineered mutant forms. The permease is a monomer (see Sahin-Tóth *et al.*, 1994), and it is one of the very few membrane proteins which can be overexpressed, purified to homogeneity, and reconstituted with full activity in proteoliposomes. The lac permease has been extensively manipulated with point mutations, insertions, deletions, and fusions (Kaback *et al.*, 1994). It is one of the most well-characterized transporters, representing an important class of proteins that occur in essentially all biological membranes which catalyze the passage of specific solutes across membranes. The basic mechanism of active transport remains unknown, largely because of a lack of structural information about this type of protein. This minireview will consider the methods that we have been using in our attempts to crystallize the lac permease, and will be limited to the protein engineering aspects of the work.

PURIFICATION TAGS

Since purity is so central to success in crystallization, the use of affinity tags for purification can have a major impact on the outcome of a project. Many affinity methods are now available, and more are continually being developed, and so an exhaustive discussion is beyond the scope of this discussion. Most of these methods have been developed for soluble proteins, and in general, they seem to be adaptable to solubilized membrane proteins since the detergents used are usually quite mild. In fact, the presence of the solubilizing detergent may reduce nonspecific binding to the column and actually improve the performance of some of these methods. It is important to note that with membrane proteins, the purity of the final preparation may be difficult to define since one has to consider not only contaminating proteins, but also the presence of endogenous lipids and impurities in the detergents. Two affinity purification methods which have been used with the lac permease are discussed below.

Monomeric Avidin

This system makes use of the high avidin/biotin specificity. The key is to add a recognition target to the protein of interest so that it gets biotinylated *in vivo* by endogenous biotin ligase. Once tagged, the protein will bind to avidin. Although biotin binding to the native tetrameric form of avidin is essentially irreversible, the affinity to monomeric avidin is several orders of magnitude weaker, allowing for reversible binding.

Cronan (1990) has defined a 100 amino acid domain from the oxaloacetate decarboxylase from *Klebsiella pneumoniae* which contains a single lysine which can be recognized by the *E. coli* biotin ligase. This biotin acceptor domain has been fused with the lac permease at various positions, including the N-terminus, the C-terminus, and the middle cytoplasmic loop, and the chimeras are biotinylated *in vivo*. The fusion proteins are active in lactose transport, and can be reversibly bound to a monomeric avidin column. The proteins thus purified show only a single band on a silver-stained gel (Consler *et al.*, 1993). When using this method to purify the large quantities of protein necessary for crystallization trials, it was found that only 20–30% of the permease is actually recovered from the column because only a portion of the protein is biotinylated. The regulation of biotinylation activity in *E. coli* is complex (Cronan 1989), and presumably the overexpression of the permease fusion overwhelms the system. Thus, only a fraction of the overexpressed protein can be recovered by this method as much of the recombinant protein washes out from the column without binding. Yields of purified permease on the

order of a few milligrams are possible from 50 g of cell paste. Currently, we are investigating both *in vivo* and *in vitro* methods to increase the degree of biotinylation in this system in order to increase the final yield of protein (Chapman-Smith *et al.*, 1994).

Ni-NTA

This method has recently gained wide popularity because of its ease of use and its wide range of applications. The target protein is modified by the addition of a short run of consecutive histidines, which imparts it with an affinity to metals (Hochuli *et al.*, 1988; Le Grice *et al.*, 1990; Janknecht *et al.*, 1991). The engineered protein can be purified by metal chelate chromatography, most commonly on a Ni^{2+}-nitrilotriacetic acid (Ni-NTA) column, and this method has been used to purify chloroplast triose phosphate translocator from yeast internal membranes (Loddenkötter *et al.*, 1993). We have used the Ni-NTA system from Quiagen, although other commercial sources of metal chelate resin are available. The addition of six histidines to the C-terminus of the lac permease works well in this system, and the presence of these additional amino acids has no deleterious effects on the permease activity or expression. The bound protein can be eluted by either lowering the pH of the column buffer to below the pK_a of histidine, or by competitive elution with imidazole or free histidine. Lac permease prepared in this way still contains some impurities, and a second column is then used to yield the final preparation. The strategy for the second column is based on the rationale that most of the impurity proteins from the Ni-NTA column result from ionic binding to the resin, and thus should also have affinity to DEAE resins. Attempts at washing the Ni-NTA column with high salt buffers did not significantly reduce the presence of these impurities. Instead, the final buffer for the Ni-NTA column was designed to elute the permease at a precise ionic strength so that the solution can be directly used in DEAE chromatography, as described below.

After induction and growth, cells are harvested and membranes are prepared (Viitanen *et al.* 1986). Proteins are extracted by the addition of solid dodecyl maltoside to a membrane suspension to a final concentration of 4%. The extract is centrifuged to remove remaining unsolubilized material, and the supernatant is then mixed with 2–4 ml of pre-equilibrated Ni-NTA resin at 4° for 2 h. The slurry is then poured into a column and washed consecutively with a wash buffer (50 mM Hepes, pH 7.2, 200 mM NaCl, 0.1% dodecyl maltoside, and 10% glycerol), and equilibration buffer (Buffer A: 50 mM Hepes, pH 7.2, 0.02% dodecyl maltoside, and 10% glycerol)), a pre-elution buffer (Buffer A + 5 mM histidine), and a final elution buffer (Buffer A + 100 mM histidine). The permease elutes as a sharp peak upon the addition of the last buffer.

The peak fractions are pooled and applied to a pre-equilibrated DEAE column. The permease does not bind under these conditions, but most of the impurities remain bound to the column, so that the purified permease is recovered in the run-through fractions. From 25 g of cell paste, the final yield of purified permease is typically 10 mg.

MODIFICATIONS FOR STABILITY AND CRYSTALLIZATION

The forces stabilizing crystals of membrane proteins are similar to those of soluble proteins. These involve mostly polar contacts between hydrophilic parts of the protein surface. The regions of the protein normally exposed to the membrane are shielded by detergent in the solubilized protein, and these regions contribute very little to the order of the lattice. The detergent molecules themselves are found as a discrete phase within the crystal, and are highly dynamic and not well ordered (Roth *et al.*, 1989, 1991) (Fig. 1).

One general aspect of protein crystallization that is universally recognized is that rigid, stable proteins are much more likely to crystallize than proteins that are internally flexible or have dynamic surfaces. Thus, the detergent torus that surrounds a solubilized membrane protein is a poor region for forming the polar, directed intermolecular contacts required of a crystal lattice. As a consequence, proteins that have large extramembranous domains are more likely to crystallize than proteins that are mostly buried in the membrane, since the former have more surface area with the potential to form favorable lattice contacts. It is noteworthy, however, that the porins can form high-quality crystals, yet they are almost completely buried in the membrane (Weiss *et al.*, 1991; Cowan *et al.*, 1992). Here, a major contributing factor is the exceptionally stable nature of this class of proteins. Weiss and Schulz (1992) have analyzed the crystal packing of the *Rhodobacter capsulatus* porin in which strong, polar contacts are made between the protruding protein surfaces.

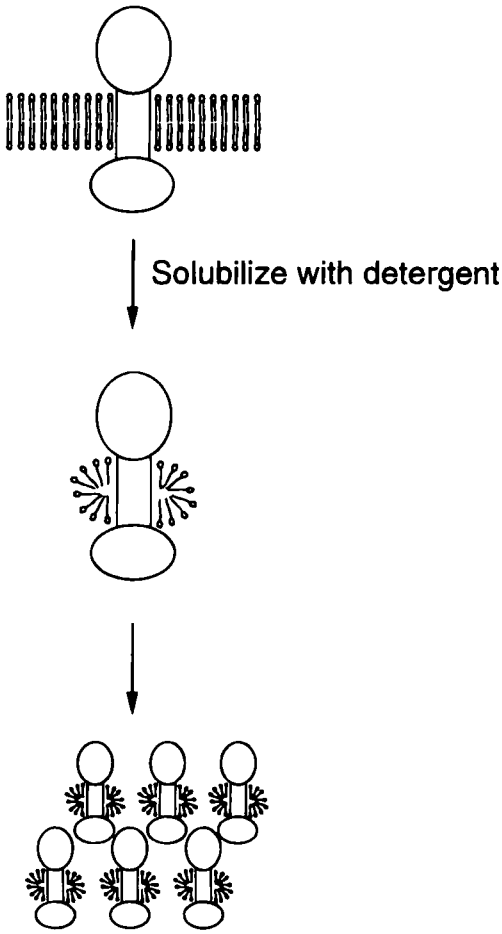

Fig. 1. Steps in the crystallization of a membrane protein containing large hydrophilic domains, from the inital membrane bound protein, to the detergent-solubilized PDC, to the crystal lattice. The final lattice is of the type II in the nomenclature of Michel (1983).

From these considerations, it is clear that the lac permease is a poor candidate for crystallization, since it is neither exceptionally stable, nor does it have a domain outside of the membrane. Like many other transporters in this class, the topology of the lac permease is quite simple: it is made up of 12 consecutive transmembrane helices which are joined by short polar loops. At a very simple level, the permease can be envisioned as a simple cylinder made up of a bundle of the 12 aligned helices. The side of the cylinder is made up from the lipid-exposed surfaces of the outer helices, and the two ends are made up from the polar loops. If we extend this picture to the detergent-solubilized PDC, the surface of the particle is dominated by the dynamic, micellular nature of the bound detergent, leaving very little hydrophilic surface available to form protein–protein contacts. Thus, there is little chance for long-range order as the molecules are driven out of solution in a crystallization experiment.

One way to overcome these problems is to add an extra polar domain to the permease, by either forming a noncovalent complex with another protein, or by engineering a fusion protein. The formation of a complex can be achieved with a monoclonal antibody, which is an approach that has met with success in the crystallization of an unstable soluble protein (the human immunodeficiency virus type I reverse transcriptase; Jacobo-Molina *et al.*, 1991) and of a membrane protein (the cytochrome *c* oxidase from *Paracoccus dentrificans*; Iwata *et al.*, 1995). A significant contact surface area between the two proteins ensures that the complex behaves as a single rigid particle. However, binding proteins with the required affinity may not be available.

The fusion protein approach has generally been regarded as unfavorable since the linked proteins are usually joined head-to-tail, forming a two-domain particle connected by a flexible tether. This arrangement is inherently flexible and internally very dynamic—features that are not conducive to crystallization.

A variation of the fusion design is being used with the permease in an attempt to minimize the flexibility problem. The basic strategy is to introduce a protein domain into an *internal* position of the permease, so that *two* linkages are formed between the partners. The presence of two short linkers reduce the number of internal degrees of freedom of the fusion protein relative to that of conventional head-to-tail fusions. In this context, the soluble protein introduced into the membrane protein is called a "carrier" domain (Privé *et al.*, 1994). The use of an internal site for the introduction of a carrier restricts the choice of a fusion partner to proteins that have their N and C termini close together in space at the protein surface to avoid distortion of either protein (Fig. 2). Recently, Russell (1994) has identified several naturally occurring examples of this type of domain insertion in soluble proteins.

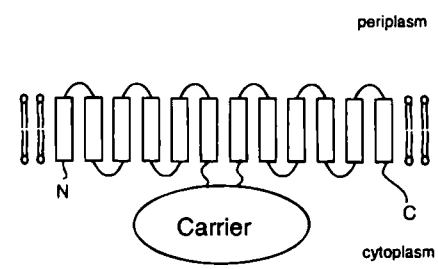

Fig. 2. Schematic of a fusion between the lac permease and a soluble carrier domain.

The desirable features of a carrier include the following:

- Single domain monomer
- Crystallizable, with the structure available in the PDB
- C and N termini close together at the surface of the protein
- No disulfide bonds
- Good expression in *E. coli*
- Large
- Bonus features: color, enzymatic activity and/or affinity purification

It is possible to engineer many variants of carrier fusions that meet these criteria. This opens the possibility of screening many different fusion proteins for the ability to form crystals, so that the nature of the protein itself becomes a parameter to be optimized in the crystallization search.

FUSION WITH CYTOCHROME b_{562}

Cytochrome b_{562} is a protein that meets many of the requirements of a carrier domain. It is a stable structure that folds as a four α-helix bundle with its C and N termini close together in space (Mathews *et al.*, 1979). The sequence of the gene is available (Trower, 1993), and the protein can be overexpressed in *E. coli* (Nikkila *et al.*, 1991). Cytochrome b_{562} has a distinctive visible spectrum making it easy to monitor the protein in both crude and pure solutions (Itagaki and Hagar, 1966). It is a monomer, and it contains no cysteine residues. Crystals of the cytochrome diffract to 1.4 Å (PDB entry 256B). One potential weak point is that cytochrome b_{562} is made up of only 106 residues, so that its carrier effect may not be large enough with the 417-residue lac permease.

A fusion protein with cytochrome b_{562} spliced into the central hydrophilic loop (L6) of the lac permease has wild-type lactose transport activity. Furthermore, the fusion protein has a visible spectrum identical to that of authentic cytochrome b_{562} in both the oxidized and reduced forms (data presented in Privé *et al.*, 1994) Since the protein is fully functional in active transport, the sites responsible for the activity are not significantly perturbed. Also, protoporphyrin IX is quantitatively and correctly loaded onto the cytochrome moiety of the fusion protein. Thus, both partners are properly folded and there is minimal distortion of the two domains.

Two versions of the "red permease" have been produced: one contains the biotinylation domain at the C terminus, and the other has a 6His C-terminal tag. The latter form, lacY/L6cyt/H6, is well expressed and readily purified by Ni-NTA chromatography as described above. Relative to the protein lacking the cytochrome carrier, the protein is more stable in solution and can be concentrated to higher levels (up to 5 mg/ml), fulfilling one of the design goals of this construct.

The red color of this protein has many useful applications, ranging from the estimation of expression levels during cell growth to the monitoring of crystallization experiments. For example, Fig. 3 shows the time course of expression during cell growth. From these data, the optimum production of protein occurs at 5–7 h of growth (late log phase), and older cells in stationary phase contain significantly less intact fusion protein. Thus, cell growth can be monitored for the optimal harvest time. Another use of the red protein is in following the course of chromatography, from the initial binding step to the final elution. Also, the spectrum of the final sample is used to measure the concentration and purity of the protein prior to the crystallization experiments. Color also aids in monitor-

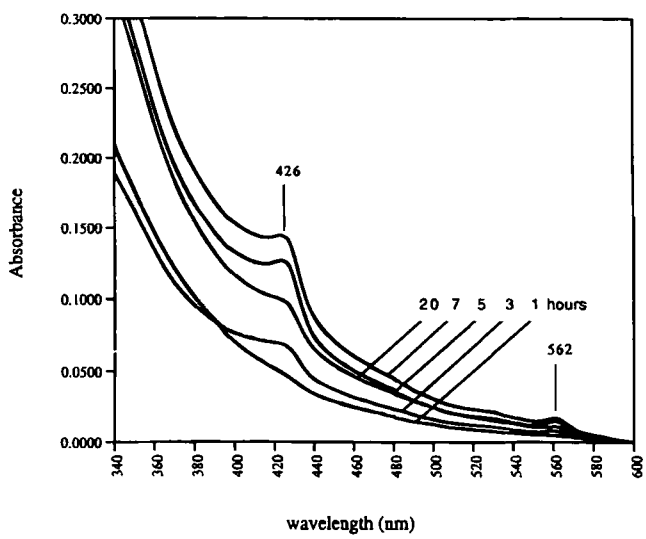

Fig. 3. Spectra of the supernatant from sonicated cells expressing lacY/L6cyt/H6, sampled at 1, 3, 5, 7, and 20 h of growth. Expression was induced at early log phase. The spectra are adjusted to A600 = 0.0. In similar spectra taken from uninduced cells, only a weak shoulder is seen at ~410 nm throughout the growth period (due to endogenous cytochromes) and no bump is seen at 562 nm. The spectra are measured in the presence of 1 mM dithiothreitol and reflect the reduced form of cytochrome b_{562}, which has a Soret absorption at 427 nm and an α absorption at 562 nm.

ing the state of the protein in the crystallization droplet. Colorless crystals can immediately be identified as either salt or detergent, and it is readily apparent whether the protein is still in solution, has precipitated, crystallized, or has denatured. To date, the most promising results have been red oily droplets, some of which possibly show edgelike features. Future plans include the continued search for crystallization conditions for lacY/L6cyt/H6, as well as the construction of lac permease fusions with other carrier domains.

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