Manipulating phospholipids for crystallization of a membrane transport protein

Lan Guan*, Irina N. Smirnova*, Gill Verner*, Shushi Nagamoni*, and H. Ronald Kaback*†‡§

Departments of *Physiology, and †Microbiology, Immunology, and Molecular Genetics, and ‡Molecular Biology Institute, University of California, Los Angeles, CA 90095-1662

Contributed by H. Ronald Kaback, December 19, 2005

Crystallization is a major bottleneck to obtaining x-ray structures of membrane proteins. By applying an established crystallization protocol for the lactose permease (LacY) of *Escherichia coli*, a systematic study of the effect of phospholipids (PL) on crystallization of LacY was undertaken. We observe three different crystal forms that diffract to increasingly better resolution in a manner that correlates with the concentration of copurified PL. Consistently, progressive addition of *E. coli* PL to delipidated LacY leads to different crystal forms. Tetragonal crystals are obtained with improved diffraction quality for a stable mutant by carefully adjusting PL content. Furthermore, crystals of good quality from wild-type LacY, a particularly difficult protein, were also obtained by using same approach. Thus, it is likely that manipulation of PL is a good strategy for predominantly hydrophobic membrane proteins like LacY.

lactose permease | membrane protein | structure | bioenergetics | membranes

A large hydrophobic surface with small hydrophilic domains is an intrinsic feature of many transmembrane proteins, as these proteins are largely embedded in the phospholipid (PL) bilayer. Therefore, solubilization, purification, and crystallization are problematic. Relative to soluble proteins (≈30,000 structures in the Protein Data Bank), only a handful of x-ray structures from transmembrane α-helical proteins is available (www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html). Among membrane proteins, a few ion gradient-coupled transport proteins with limited hydrophilic surface are particularly difficult to deal with because of hydrophobicity as well as conformational flexibility, which is required for function. However, major breakthroughs have been achieved recently (1–4, 23).

Purified membrane proteins are protein-detergent complexes that also contain PL, and it has been demonstrated structurally that PL cocrystallize with certain membrane proteins (5). Therefore, it is likely that lattice formation is determined not only by the protein itself but also by the associated detergent and PL. The crucial role of detergent in crystallization of membrane proteins has been well emphasized (6). However, knowledge about the effect of associated PL on crystallization of membrane proteins is limited. It is generally believed that PL are a detrimental contaminant that should be removed as completely as possible. However, it has also been reported recently (7) that crystals of cytochrome b_6f that diffract well can be obtained only by adding PL, which stabilize the intact complex. Successful crystallization of a heterologously expressed rabbit sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase isoform also requires addition of native PL (8). In addition, the amount of residual PL associated with the P_i/glycerol-P antiporter GlpT is important for crystallization (9). In this communication, we describe a systematic study on the effect of PL on crystallization of lactose permease (LacY).

Results

C154G LacY, a mutant that binds ligand as well as wild-type LacY but catalyzes little or no lactose transport (10–12), exhibits hexagonal, orthorhombic, and tetragonal crystals (Fig. 1) by using the same protocol. The hexagonal crystals, which were obtained initially, diffracted to a resolution limit of only ≈5 Å. The orthorhombic crystals diffract much better (resolution limit of ≈3.0 Å with space group $P2_12_12_1$) and provided the first x-ray crystal structure of LacY (1). The orthorhombic crystals usually appear after 1–3 days and exhibit anisotropic diffraction. The tetragonal form diffracts to a resolution limit of ≈2.6 Å with space group $P4_32_12$ (O. Mirza, L.G., G.V., S. Iwata, and H.R.K., unpublished work). Tetragonal crystals usually appear after >2 months under the same conditions and diffract less anisotropically.

It was difficult initially to reproduce tetragonal crystals because they appeared at the late stage of crystallization with low frequency. Systematic analyses of LacY preparations that form the three different crystals demonstrate that PL content is correlated with a particular crystal form (Fig. 1). Preparations containing a high PL content (≈18–25 mol PL per mol LacY) favor formation of orthorhombic crystals. A similar value was reported for GlpT (9). Tetragonal crystals are obtained from preparations containing intermediate PL content (9–16 mol PL per mol LacY), whereas preparations containing low PL content (<8 mol PL per mol LacY) yield hexagonal crystals (Fig. 1).

Manipulation of PL Content. Mutant E325A is specifically defective in all translocation modes that involve net H^+ movement but binds ligand and catalyzes equilibrium exchange and counterflow at least as well as wild type (13). By using the crystallization method described in *Materials and Methods*, E325A LacY crystallizes in the orthorhombic form. To test the effect of PL, membranes containing mutant E325A were first subjected to cholate extraction before solubilization to remove ≈95% of the PL (14). After purification, the preparations contain much less PL (7 mol PL per mol protein), and hexagonal crystals are observed (Table 1). Remarkably, progressive addition of *Escherichia coli* PL alters the crystal form from hexagonal to orthorhombic. Incorporation of PL into LacY-detergent micelles was analyzed by gel filtration chromatography by using FPLC (Fig. 2). Upon addition of PL at increasing concentrations, retention time decreases progressively, in all likelihood because of increasing incorporation of PL into the micelles.

LacY was treated with either low or high concentrations of dodecyl-β-D-maltopyranoside (DDM) before setting up crystallization trials as described in *Materials and Methods*. Consistent with the findings described above, treatment with a high concentration of DDM (0.1%) yields hexagonal, rather than orthorhombic, crystals. Moreover, further purification of LacY by gel filtration yields no crystals or hexagonal crystals

BIOCHEMISTRY

Conflict of interest statement: No conflicts declared.

Abbreviations: DDM, dodecyl-β-D-maltopyranoside; LacY, lactose permease; PL, phospholipid(s).

§To whom correspondence should be addressed at: 675 Charles East Young Drive South, 6720 Macdonald Research Laboratories, Los Angeles, CA 90095-7327. E-mail: rkaback@mednet.ucla.edu.

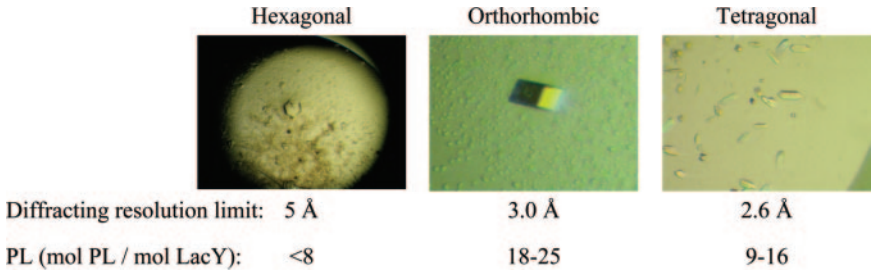

Fig. 1. Correlation of PL content with crystal form and resolution. Crystallization and diffraction were carried out, and PL content was determined as described in *Materials and Methods*. The hexagonal crystal shown is 100 × 100 μm; the orthorhombic crystal is 80 × 100 μm; the tetragonal crystal is 20 × 50 μm.

only. Transition from the orthorhombic to the hexagonal form is probably due to excessive delipidation. With many membrane proteins, a two-step purification by affinity chromatography followed by either ion exchange or gel filtration chromatography is used to obtain purified protein. Clearly, the findings presented here indicate that highly purified preparations may lose PL essential for crystallization of certain membrane proteins.

Another means of manipulating PL content is alteration of the detergent:membrane protein ratio during solubilization. A range of detergent:membrane protein ratios from 1:1 to 3:1 (g:g) was tested, and it was determined that solubilization at a DDM:protein ratio of 2.5:1 (g:g) yields LacY preparations with 10–15 mol PL per mol protein and tetragonal crystals. A data set for the C154G mutant has been obtained to a resolution of 2.95 Å (O. Mirza, L.G., G.V., S. Iwata, and H.R.K., unpublished work).

Crystallization of Wild-Type LacY. Wild-type LacY is unstable and aggregates readily, exhibiting three peaks on gel filtration chromatography. Under various conditions, including those used to crystallize C154G LacY, wild-type LacY either did not crystallize at all or unreproducibly yielded crystals of poor quality. However, by manipulation of PL content, crystals that diffract to a resolution similar to that obtained from orthorhombic crystals of C154G have been obtained.

Discussion

LacY exhibits a strong bias with respect to charge distribution on both sides of membrane. There are 26 charged residues [18 positive (Lys, Arg, and His) and 8 negative (Glu and Asp)] located on the cytoplasmic side of the membrane, whereas only 5 charged residues (3 positive and 2 negative) are located at the periplasmic surface. Clearly, the charge distribution on LacY follows the "positive-inside" rule (15). For both orthorhombic (1) and tetragonal crystals (O. Mirza, L.G., G.V., S. Iwata, and H.R.K., unpublished work), the basic building block is an inverted dimer of LacY, in which the monomers are orientated in opposite directions via direct hydrophobic contacts (Fig. 3). Such a dimer may favor polar interactions through the hydrophilic domains to form crystals because of asymmetric charge distribution. Similar observations were made with GlpT (2, 9) and bovine rhodopsin (16), where both proteins exhibit biased surface-charge distribution and inverted dimers in the asymmetric unit, like LacY. This type of crystal packing is different from typical type I and II crystals (17), where only hydrophobic or hydrophilic contacts, respectively, are involved.

Formation of the inverted dimer is a consequence of nonspecific hydrophobic interactions between the two monomers. By this means, protein–protein interactions replace detergent–PL interactions. It is postulated that excessive PL bound to the protein may hinder formation of inverted dimers, whereas too little PL may lead to a population of heterogeneous dimers. In either case, formation of a crystal lattice is not favored. Thus, PL may play a role in forming identical inverted dimers, and this suggestion may explain why crystallization of LacY and GlpT need a range of PL concentrations.

With the tetragonal crystals, four transmembrane helices (helix III with III′ and helix VI with VI′) pack with pseudo-two-fold symmetry (Fig. 3 *A* and *C*). Strong hydrophobic stacking with at least 18 contacts is observed [Trp-78, Val-85, Pro-89, Phe-93 (helix III), Phe-168, Trp-171, Gly-175, Leu-178, Ile-179, Val-182, Leu-183, and Phe-186 (helix VI)]. Notably, replacement of some of these side chains with Cys [Trp-78 and Phe-93 (helix III), as well as Phe-168, Trp-171, and Phe-186

Table 1. Addition of PL during crystallization

E325A LacY	Addition of *E. coli* PL, mM*	Hexagonal crystals	Orthorhombic crystals
No cholate treatment†		−	+
Cholate treatment‡	No addition	+	−
	0.7	+	+
	6.2	−	+

**E. coli* PL polar extracts were added into the E325A LacY protein with cholate treatment before solubilization. Crystallization was carried out as described.
†The samples without cholate treatment contain 18–25 mol PL per mol LacY.
‡The samples purified from the membrane washed with cholate before solubilization contain 7 mol PL per mol LacY.

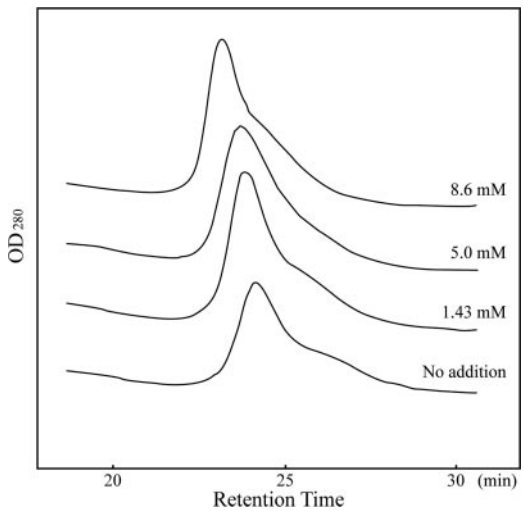

Fig. 2. FPLC gel filtration profile. Each sample containing 80 μg of LacY at a concentration of 8 mg/ml was incubated with given concentrations of PL or DDM for 20 min before chromatography on Superdex 200 10/300 GL at a flow rate of 0.5 ml/min as described in *Materials and Methods*.

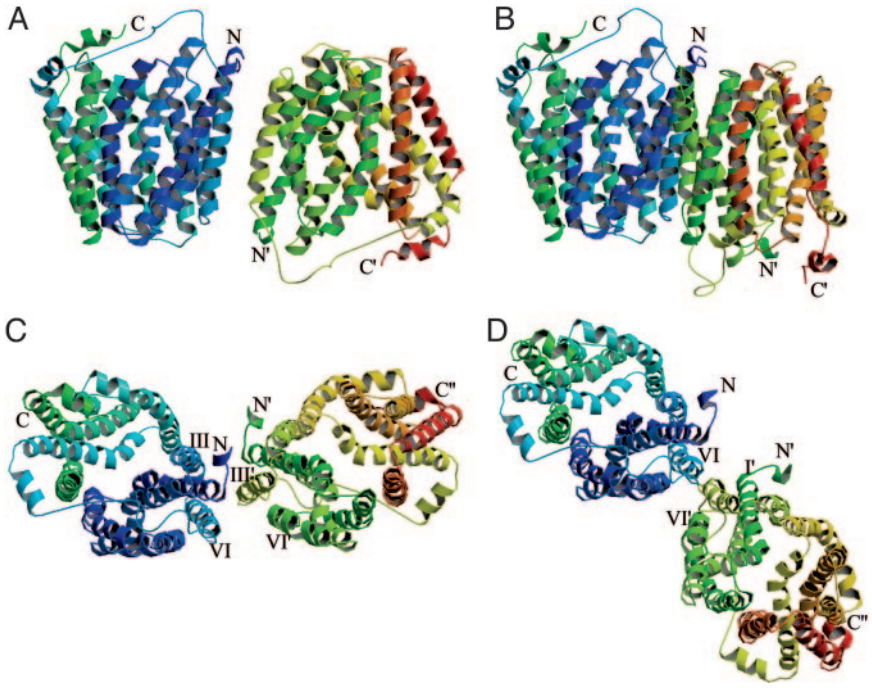

Fig. 3. Inverted dimers of LacY in the crystals. Helical packing between neighboring molecules in the tetragonal (*A* and *C*) (O. Mirza, L.G., G.V., S. Iwata, and H.R.K., unpublished work) and orthorhombic (*B* and *D*) (1) crystals from a side (*A* and *B*) or cytoplasmic view (*C* and *D*). The hydrophobic contacts in the tetragonal crystals (*C*) are between helix III with III′ and helix VI with VI′ and in the orthorhombic crystals (*D*) between helices VI with VI′ plus I′.

(helix VI)] form intermolecular crosslinks in the membrane in the presence of a short homo-bifunctional cross-linking agent (18, 19).

In the orthorhombic crystals, the dimer in the asymmetric unit is formed by interaction between only three helices (VI with VI′ and I′) (Fig. 3 *B* and *D*). The orthorhombic crystals are derived from samples with a significantly higher PL content. The observation is consistent with the idea that excess PL hinders interaction between helices III and formation of the tetragonal crystal lattice (Fig. 3*C*). In addition, less hydrophobic stacking is observed in the orthorhombic crystals. The greater number of hydrophobic contacts in the tetragonal crystals may partly account for better diffraction. Taken together, the findings are consistent with the conclusion that PL plays an important role obtaining LacY crystals, as well as crystal quality (better diffraction).

Whatever the ultimate explanation, manipulation of PL content has led to the crystallization of wild-type LacY, a problem that has been pursued for well over a decade. Thus, these observations, in addition with other findings (2, 16), indicate that manipulation of PL concentration may be useful for obtaining structures of hydrophobic membrane proteins with an asymmetric surface charge distribution.

Materials and Methods

Vector Construction, Expression, and Purification. Expression vector pT7–5/C154G/C$(His)_{10}$ encoding a LacY mutant with Gly in place of Cys-154 with a 10-His tag at C terminus has been described in ref. 1. Plasmid pT7–5/WT-LacY/C$(His)_{10}$ encoding wild-type LacY with a 10-His tag at the C terminus was constructed by restriction fragment replacement. A PstI/XhoI fragment containing a Cys codon at position 154 was ligated into pT7–5/C154G/$(His)_{10}$ predigested with the same enzymes. Plasmid pT7–5/E325A/C$(His)_{10}$ was also generated by a similar approach. Both constructions were confirmed by restriction digestion and DNA sequencing. Cell growth, expression, and purification of LacY were carried out as described (1). The protocol for solubilization was optimized. Briefly, membranes at a protein concentration of 10 mg/ml were solubilized with DDM at a given detergent:protein ratio (wt:wt). As indicated, membranes were extracted with 6% sodium cholate for 30 min on ice and washed twice with 50 mM NaP_i (pH 7.6) with 0.5 mM of 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefabloc, Pentepharm, Basel) before solubilization. All elutes through a cobalt column (BD TALON Superflow resin) were dialyzed overnight against 5 L of buffer with 20 mM Tris/0.01% DDM at a final pH of 7.5, measured on ice, and concentrated with a spin concentration device with 30-kDa cutoff for crystallization trials. As indicated, purified LacY was washed 4 times with 20 mM Tris buffer (pH 7.5) containing either 0.008% or 0.1% DDM (≈1- or 12-fold above the critical micelle concentration) through a spin concentration device with 50-kDa cutoff.

Measurement and Analysis of PL. P_i was released from PL in the purified LacY samples (20) and measured as described (21).

FPLC. Protein samples were analyzed by gel filtration through a Superdex 200 10/300 GL column (Amersham Biosciences) equilibrated with 20 mM Tris·HCl, pH 7.5/50 mM NaCl/0.01% DDM on a FPLC instrument (GE Healthcare).

Crystallization and Diffraction. Crystallization of C154G LacY was carried out as described (1). For the wild-type and E325A LacY, crystallization was performed by using a similar protocol with the following modifications: (*i*) 0.1 M ammonium sulfate was used in place of 0.2 M $CaCl_2$ with the reservoir at a final pH of 7.5, and (*ii*) purified wild-type and E325A LacY were used directly for crystallization trials without chemical modification. All protein samples were centrifuged at 340,000 × *g* for 1 h in a TLA 100.1 rotor to remove the aggregates immediately before setting up hanging drops. *E. coli* PL polar extract (Avanti Polar Lipids; no. 100600) were solubilized with chloroform, dried and solubilized in 0.5%

DDM at a concentration of ≈30 mM, gassed with argon, and stored at −80°C until use. Where indicated, PL were added to the purified protein before crystallization trials.

Crystals of LacY were directly frozen in liquid nitrogen and examined by x-ray diffraction with a synchrotron source at one of the following beamlines: X06SA at the Swiss Light Source in Villagen (Switzerland) or BL 8.2.1 at the Advanced Light Source in the Lawrence Berkeley National Laboratory (Berkeley, CA). Diffraction data were processed by using DENZO and SCALEPACK programs (22).

We thank Jun-Yong Choe (University of California, Los Angeles) for helpful discussions. We also thank Clement Schulz-Briese (Swiss Light Source, Switzerland) and Corie Ralston (Advanced Light Source, Berkeley, CA). This work was supported by National Institutes of Health Grant R01 DK069463.

1. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R. & Iwata, S. (2003) *Science* **301,** 610–615.
2. Huang, Y., Lemieux, M. J., Song, J., Auer, M. & Wang, D. N. (2003) *Science* **301,** 616–620.
3. Hunte, C., Screpanti, E., Venturi, M., Rimon, A., Padan, E. & Michel, H. (2005) *Nature* **435,** 1197–1202.
4. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y. & Gouaux, E. (2005) *Nature* **437,** 215–223.
5. Palsdottir, H. & Hunte, C. (2004) *Biochim. Biophys. Acta* **1666,** 2–18.
6. Ostermeier, C. & Michel, H. (1997) *Curr. Opin. Struct. Biol.* **7,** 697–701.
7. Zhang, H., Kurisu, G., Smith, J. L. & Cramer, W. A. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 5160–5163.
8. Jidenko, M., Nielsen, R. C., Sorensen, T. L., Moller, J. V., le Maire, M., Nissen, P. & Jaxel, C. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 11687–11691.
9. Lemieux, M. J., Song, J., Kim, M. J., Huang, Y., Villa, A., Auer, M., Li, X. D. & Wang, D. N. (2003) *Protein Sci.* **12,** 2748–2756.
10. Menick, D. R., Sarkar, H. K., Poonian, M. S. & Kaback, H. R. (1985) *Biochem. Biophys. Res. Commun.* **132,** 162–170.
11. van Iwaarden, P. R., Driessen, A. J., Lolkema, J. S., Kaback, H. R. & Konings, W. N. (1993) *Biochemistry* **32,** 5419–5424.
12. Smirnova, I. N. & Kaback, H. R. (2003) *Biochemistry* **42,** 3025–3031.
13. Carrasco, N., Puttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D. & Kaback, H. R. (1989) *Biochemistry* **28,** 2533–2539.
14. Padan, E., Schuldiner, S. & Kaback, H. R. (1979) *Biochem. Biophys. Res. Commun.* **91,** 854–861.
15. von Heijne, G. (1989) *Nature* **341,** 456–458.
16. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., *et al.* (2000) *Science* **289,** 739–745.
17. Michel, H. (1991) in *Crystallization of Membrane Proteins*, ed. Michel, H. (CRC, Boca Raton, FL), pp. 73–88.
18. Guan, L., Murphy, F. D. & Kaback, H. R. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 3475–3480.
19. Ermolova, N., Guan, L. & Kaback, H. R. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 10187–10192.
20. Zhou, X. & Arthur, G. (1992) *J. Lipid Res.* **33,** 1233–1236.
21. Baykov, A. A., Evtushenko, O. A. & Avaeva, S. M. (1988) *Anal. Biochem.* **171,** 266–270.
22. Otwinowski, Z. & Minor, W. (1997) in *Methods Enzymol.* **276,** 307–326.
23. Murakami, S., Nakashima, R., Yamashita, E. & Yamaguchi, A. (2002) *Nature* **419,** 587–593.