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Biochimica et Biophysica Acta 1564 (2002) 47–56

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Stability of the lactose permease in detergent solutions

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Received 26 February 2002; accepted 7 March 2002

Abstract

Protein stability, as measured by irreversible protein aggregation, is one of the central difficulties in the handling of detergent-solubilized membrane proteins. We present a quantitative analysis of the stability of the *Escherichia coli* lactose (lac) permease and a series of lac permease fusion proteins containing an insertion of cytochrome$_{b562}$, T4 lysozyme or β-lactamase in the central hydrophilic loop of the permease. The stability of the proteins was evaluated under a variety of storage conditions by both a qualitative SDS-PAGE assay and by a quantitative hplc assay. Long-chain maltoside detergents were more effective at maintaining purified protein in solution than detergents with smaller head groups and/or shorter alkyl tails. A full factorial experiment established that the proteins were insensitive to sodium chloride concentrations, but greatly stabilized by glycerol, low temperature and the combination of glycerol and low temperature. The accurate quantitation of the protein by absorbance spectroscopy required exclusion of all contact with clarified polypropylene or polyvinyl chloride (PVC) materials. Although some of the fusion proteins were more prone to aggregation than the wild-type permease, the stability of a fusion protein containing a cytochrome$_{b562}$ insertion was indistinguishable from that of native lac permease.

Keywords: Lactose permease; Protein stability; Membrane protein crystallization; Fusion protein; Glycerol

1. Introduction

A fundamental challenge in the crystallization of membrane proteins is the preparation of a sample suitable for crystallization trials. Because crystallization requires a purified sample, a target protein is typically solubilized from the membrane-associated state with a detergent prior to chromatographic procedures. The purified protein–detergent complex (PDC) is then used directly in 3D crystallization trials, or reconstituted into a bilayer system for either 2D crystallization or 3D crystallization via lipidic cubic phases [1–5]. Although membrane proteins are usually very stable in a lipidic environment [6,7], the properties of the protein in the detergent-solubilized state are often less favorable [8]. Difficulties include the solubility, stability and purity of the PDC, and the accurate characterization of these parameters. While a preparation of protein that is pure, stable and concentrated is an important prerequisite in any protein crystallization experiment, these objectives are inherently more difficult to achieve in the case of a PDC because the properties of both the protein and detergent components of the complex, as well as their interactions, affect the behavior of the system.

One class of membrane proteins that has been especially difficult to study at the structural level are the proteins involved in secondary transport. To date, the highest resolution structures of transporters have been determined by electron crystallography on 2D crystals: the mannitol transporter enzyme II determined to 5 Å in projection [9], and NhaA, determined to 4 Å resolution in projection [10] and to 7 and 14 Å in a 3D reconstruction [11]. Many of the transporters have features that make them challenging

Abbreviations: OG, *n*-octyl-β-D-glucoside; DDM, *n*-dodecyl-β-D-maltoside; OM, *n*-octyl-β-D-maltoside; DM, *n*-decyl-β-D-maltoside; C-HEGA-11®, cyclo-undecanoyl-*N*-hydroxyethylglucamide; C12E8, octaethyleneglycol-mono-*n*-dodecyl-ether; LDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide; MEGA-9, *n*-nonanoyl-*N*-hydroxyethylglucamide; PDC, protein–detergent complex; IPTG, isopropyl β-D-thiogalactoside; R_S, Stokes radius; SE-HPLC, size exclusion high pressure liquid chromatography; DEHP, di(2-ethylhexyl)phthalate; PVC, polyvinyl chloride; DBS, dibenzylidene sorbitol

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0005-2736/02/$ - see front matter
PII: S0005-2736(02)00397-8

targets for crystallization: they typically have only small extramembranous surface areas and also exhibit a high degree of internal flexibility [12–16]. Our goal is to prepare samples that are well behaved in solution, in that they remain nonaggregated and monodisperse for long periods of storage. Proteins that readily aggregate in solution are, in general, not suitable for biophysical studies. For the purposes of this paper, we use aggregation as a convenient and relevant measure of the kinetic stability of the protein. Bowie and co-workers have used enzymatic activity to study the stability of the integral membrane protein diacylglycerol kinase in detergent solutions [17,18], but direct activity measurements are not possible for solubilized transporter and channel proteins.

We have used the lactose (lac) permease from *Escherichia coli* as a model system to investigate the properties of a delicate α-helical membrane protein in the detergent-solubilized state. The lac permease is a monomeric protein consisting of 12 transmembrane α-helices connected by short hydrophilic loops, and catalyses the 1:1 coupled symport of protons and β-galactosides (reviewed in Refs. [19,20]). A battery of techniques that combine molecular biology with various biochemical and biophysical approaches have culminated in a packing model and a reaction mechanism [21], but the direct visualization of the protein at the atomic level has resisted all efforts to date. In the accompanying paper [22], we describe the design and expression of a set of fusion proteins containing soluble "carrier" domains inserted into various loop positions of the lac permease. The design goal of these fusion proteins was to increase the polar surface area of the transporter in order to favor crystallization through protein–protein interactions between the carrier domains. The insertion of the carrier domains into loop positions of the permease with minimal length N and C linkers ensures an internally rigid particle, another feature expected to increase the potential for crystallization. Stable, soluble proteins with their N and C termini close together in space and at the surface of the protein are chosen as carriers since these can be readily inserted into internal positions of the target membrane protein. We have identified *E. coli* cytochrome$_{b562}$, *E. coli* flavodoxin and bacteriophage T4 lysozyme as carrier domains that can be readily inserted into the central hydrophilic loop ("L6") of the permease without affecting the expression or activity of the transporter [22–24]. These fusion proteins are named L6_cyt_N2C6, L6_fla_N5C5 and L6_lyso_N1C1, respectively, where "L6" refers to the central hydrophilic loop position of the permease, and the "N*x*C*y*" designation refers to the number of linker residues present at the C and N termini of the internal carrier domain. Although these proteins are well behaved in their native membranes, the next step is to characterize their properties in purified solutions in order to judge their suitability for crystallization trials.

The optimization of conditions for stabilizing a protein is not unlike the search for crystallization conditions: there are many possible conditions to test, and a quantitative analysis of the influence of each factor is not usually done. We were inspired by the work of Carter and co-workers, who have applied rigorous statistical methods to the problem of analyzing complex interactions between factors in protein crystallization trials [25,26]. Here, in the case of protein aggregation, we consider the presence or absence of three different carrier proteins and a variety of storage conditions as variables that affect the stability of the lac permease in a full-factorial experiment.

2. Experimental procedures

2.1. Materials

N,*N*-Dimethyldodecylamine-*N*-oxide (LDAO) was obtained from Fluka, nonanoyl-*N*-hydroxyethylglucamide (MEGA-9) from Calbiochem, *n*-octyl-β-D-maltoside (OM), *n*-octyl-β-D-glucoside (OG), *n*-decyl-β-D-maltoside (DM), *n*-dodecyl-β-D-maltoside (DDM), cyclo-undecanoyl-*N*-hydroxyethylglucamide (C-HEGA-11®) and CHAPS were purchased from Anatrace (Maumee, OH). Ni–NTA resin was from Quiagen. All other materials were reagent grade and obtained from commercial sources.

2.2. Protein expression and purification

The plasmids encoding the lac permease with a decahistidine C-terminal tag (referred to here as the wild-type permease), and the three carrier fusion proteins L6_cyt_N2C6, L6_fla_N1C1 and L6_lyso_N5C5, are described in Ref. [22]. *E. coli* BL21 cells expressing wild-type lac permease and fusion proteins were grown in Terrific Broth [27], harvested and lysed with a French press. Total membranes were isolated by ultracentrifugation and inner membranes were prepared by ultracentrifugation of the total membranes in 25–50% sucrose gradients [28]. The inner membranes were resuspended in 40 mM sodium phosphate buffer, pH 8.0, 85 mM NaCl, 10% glycerol, and membrane proteins were extracted by the addition of 3% (w/v) DDM at 4 °C for 30 min followed by ultracentrifugation. The supernatant was mixed with washed Ni–NTA resin (13 ml extract/ml resin as a 50% slurry) for 12 h. The resin was then poured into a column and washed successively with buffer A (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20% glycerol, 0.2% DDM), buffer B (50 mM sodium phosphate pH 8.0, 200 mM NaCl, 20% glycerol, 0.02% DDM), buffer C (50 mM sodium phosphate pH 6.3, 200 mM NaCl, 20% glycerol, 0.01% DDM, 40 mM histidine) and eluted with buffer D (100 mM sodium acetate pH 4.5, 200 mM NaCl, 20% glycerol, 0.01% DDM). The purified protein solution was simultaneously concentrated and dialysed in a Spectrum ProDiCon filtration system (Spectrum Medical Industries) with buffer E (50 mM sodium phosphate pH 7.5, 200 mM NaCl, 20% glycerol, 0.01% DDM). The protein concentra-

tion was determined by the BCA assay (Pierce) using BSA as a standard with modifications to remove the detergent and other interfering substances [29].

2.3. Stability in detergent solutions by SDS-PAGE

Freshly purified L6_cyt_N2C6 in DDM was concentrated to 0.65 mg/ml. The actual DDM concentration in the protein solution was 0.2% (w/v) as measured by the method described by Dubois et al. [30], which corresponds to approximately 3 DDM micelle equivalents per protein monomer. Equal volumes of buffer or 10% w/v stock solutions of the test detergents MEGA-9, OM, OG, C-HEGA-11®, CHAPS, DM, LDAO, DDM and C12E8 were added to the protein solutions. These solutions were stored at room temperature and sampled after 3 days by mixing aliquots with SDS sample buffer. These samples were mixed briefly at room temperature and loaded directly onto 15% SDS-PAGE gels. Protein was visualized by Coomassie Blue or silver staining.

2.4. Extraction of UV light-absorbing substances

Several common storage containers and materials were tested for extraction of UV absorbing substances by detergent solutions: (1) 1.5-ml clarified polypropylene microcentrifuge tubes (Rose Scientific), (2) Tygon® R3603 laboratory tubing (Saint-Gobain Performance Plastics), (3) glass scintillation vials (VWR), (4) disposable 13×100 mm borosilicate culture tubes (Fisher), (5) 14-ml polystyrene culture tubes (Falcon), (6) 15-ml polypropylene screwcap tubes (Falcon) and (7) 2-ml general purpose borosilicate glass sample vials with screwcaps (Kimble). One set of containers was used as provided by the manufacturer, and a second set was rinsed three times with ethanol and air-dried before use. A 1.5% (w/v) DDM solution was freshly prepared in water and an absorption spectrum was measured; 1.0 ml of this solution was added to each test container and spectra of the stored solutions were taken after 3 days.

2.5. Size exclusion high pressure liquid chromatography (SE-HPLC) stability assay

We measured the time-dependent aggregation of wild-type lac permease, L6_cyt_N2C6, L6_fla_N5C5 and L6_lyso_N1C1 under native conditions by SE-HPLC. Proteins were purified as described above, except that buffers C, D and E used in the affinity chromatography did not contain any sodium chloride or glycerol. The protein was concentrated to 23 μmol/l (approximately 1 mg/ml, depending on the particular fusion protein) and Vitamin B_{12} was added to a final concentration of 0.1 mg/ml as an internal calibration standard. The protein solution was then divided into four samples (A to D), and to each of the samples sodium chloride, glycerol and/or water was added to yield the following final solutions: sample A: protein in 40 mM sodium phosphate, pH 7.5; sample B: protein in 40 mM sodium phosphate, pH 7.5, 200 mM NaCl; sample C: protein in 40 mM sodium phosphate, pH 7.5, 20% glycerol and sample D: protein in 40 mM sodium phosphate, pH 7.5, 200 mM NaCl and 20% glycerol. One aliquot of each sample was stored at 4 °C, a second aliquot at room temperature.

After 14 days of storage, the samples were analyzed on a Waters 600 HPLC system with a Waters 996 Photodiode Array detector using a Shodex KW-804 gel filtration column and buffer E as running buffer. Absorption spectra from 200 to 600 nm were measured at time points of 0.03 min. An aliquot of sample D was analyzed at the start of the experiment as a reference sample. Several samples were measured as duplicates or triplicates for error estimation, and the room temperature experiment for the cytochrome$_{b562}$ fusion was repeated with an independently purified protein sample for batch-to-batch comparison. All samples were filtered through a 0.22-μm spin filter before application onto the HPLC column. Ferritin, catalase, aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A were used as standards for calibrating the column. The void volume of the column was measured with dextran blue, which eluted at 6.7 ml.

The amount of unaggregated protein was quantified by integration of the protein peak at 280 nm. For the L6_cyt_N2C6 fusion, integration at the Soret absorption band (426 nm) gave identical results as integration at 280 nm. In the case of overlapping monomer and aggregate peaks, two Gaussian curves were fit to the total protein peak. The area of the vitamin B_{12} peak was integrated at 360.5 nm and used to correct the areas of the protein peaks to adjust for slight variations in the injected volumes. The corrected peak areas were then normalized to the corrected integrated peak area of the control sample that was measured at the start of the experiment. Curve fitting and integration were performed with the computer software Matlab (MathWorks, Natick, MA, USA). For the statistical analysis of the HPLC results, the commercial software package SAS/STAT (SAS Institute, Cary, NC, USA) was used. Fisher (F)-values and probabilities were calculated using the General Linear Model (GLM) procedure of this software and a Type III sum of squares and estimable function.

3. Results

3.1. Protein stability in different detergents

L6_cyt_N2C6 protein purified in DDM was mixed with an excess of test detergent and stored for 3 days at room temperature prior to analysis by SDS-PAGE (Fig. 1). Controls included protein diluted with detergent-free buffer sampled at the start of the experiment (lane C2) and at 3 days (lane C1). The C2 sample was frozen at −20 °C in SDS-sample buffer at the start of the experiment and thawed 3 days later. The appearance of this material on SDS-PAGE

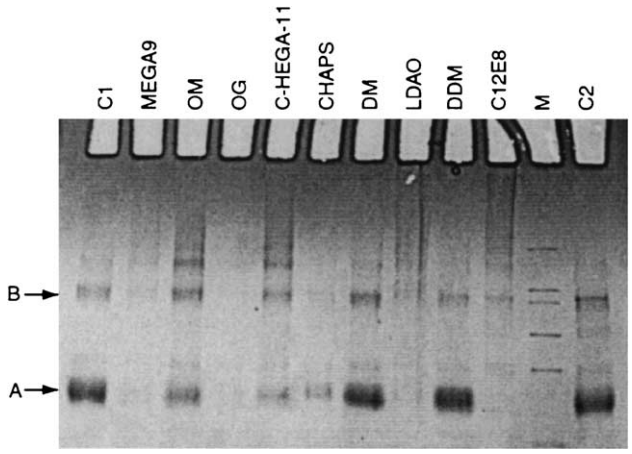

Fig. 1. Effect of the addition of various detergents on the stability of purified L6_cyt_N2C6 as assayed by SDS-PAGE (15% gel, no stacking gel, stained with Coomassie blue). Samples were stored for 3 days at room temperature after adding the test detergent, and the same amount of sample was added to each lane (equivalent to 0.6 μg unaggregated protein). The indicated test detergents are arranged by decreasing critical micelle concentration from left to right. "A" indicates the position of the monomeric protein, and "B" of the first order aggregate, presumed to be a dimer. Control samples with no added test detergent were run in lane C1 (3 days at room temperature) and in lane C2 (fresh-frozen).

was indistinguishable from a fresh, unfrozen sample (data not shown). A minor band migrating at the position of a dimer is visible, possibly indicating that some aggregation of the sample occurred prior to the start of the experiment. None of the samples was heated in SDS sample buffer prior to PAGE. As is commonly observed for highly hydrophobic membrane proteins, heating in the presence of SDS leads to further irreversible aggregation and would add an additional level of complexity in the interpretation of these results.

While the addition of excess DDM does not destabilize the protein, the protein sample with added decyl maltoside shows slightly more aggregation, and severe aggregation occurs in all of the other detergents that were tested. In particular, the addition of MEGA-9, OG, LDAO and C12E8 resulted in a complete loss of the monomeric product, indicating severe aggregation. The samples containing CHAPS, OM or C-HEGA-11® had intermediate levels of aggregation as evidenced by the ladder of higher order species and a concomitant reduction in the intensity of the monomer band. Although the final samples contained mixtures of DDM and test detergent prior to the addition of SDS sample buffer, the test detergent was present at 30–50-fold molar excess over DDM over the course of the incubation, and well above the test detergent cmc in all cases. Trials involving detergent exchange to remove the DDM were consistent with the behavior in the mixed detergent systems. Thus, severe aggregation was seen in pure OG solutions, while moderate aggregation was observed in CHAPS solutions (data not shown). It is remarkable that the relative ranking of the harshness of the tested detergents is consistent with a detergent survey carried out on diacyl glycerol kinase [17]. Similarities are also seen with human erythrocyte anion exchanger (AE1), with the exceptions that in this case, C12E8 is stabilizing and CHAPS is especially unfavorable [31].

3.2. Protein stability comparison by SDS-PAGE and SE-HPLC

The stability of the permease as measured by SDS-PAGE under denaturing conditions and SE-HPLC under nondenaturing conditions is shown in Fig. 2. L6_cyt_N2C6 in DDM

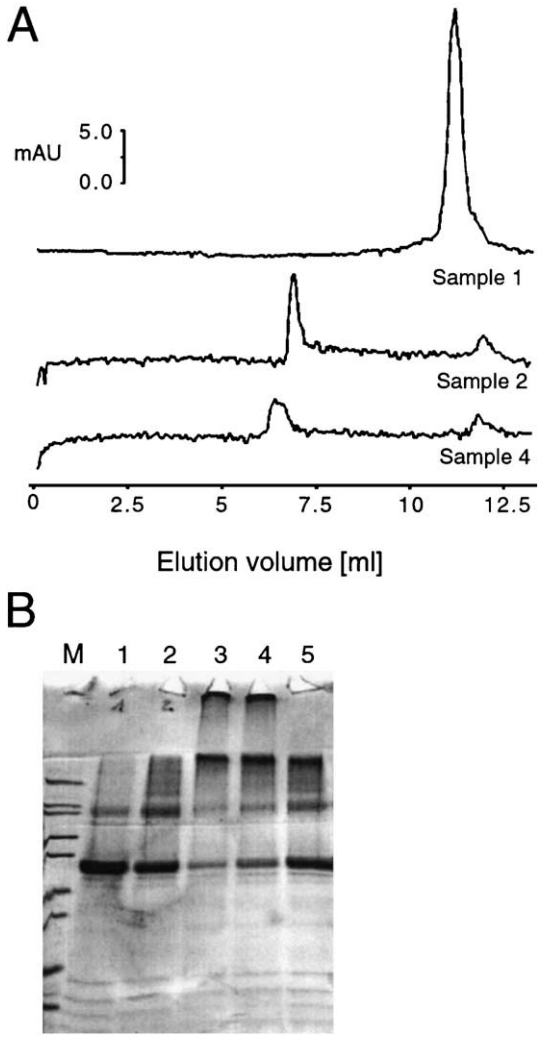

Fig. 2. Stability of purified L6_cyt_N2C6 in DDM as assayed by nondenaturing (SE-HPLC) and denaturing (SDS-PAGE) techniques. All samples were divided in two: one aliquot was investigated by SDS-PAGE, the second was filtered through a 0.22-μm spin filter and applied onto a Shodex KW-804 gel filtration column. Sample 1: protein stored for 4 h at room temperature; Sample 2: similar to sample 1, except that OG was added to 1.5% (w/v) prior to the 4-h incubation. Samples 3–5: similar to sample 1, but heated to 45 °C for 12 h, 6 h, and 45 min, respectively. All volumes were adjusted by the addition of protein buffer to yield the same final protein concentration at the start of the experiment. (A) Chromatograms of the SE-HPLC runs were recorded at 280 nm. (B) The SDS-PAGE gel contained a 4% stacking gel and a 15% running gel, and was silver-stained. Aggregates accumulated at the interface of the stacking gel and running gel, as well as at the top of the running gel.

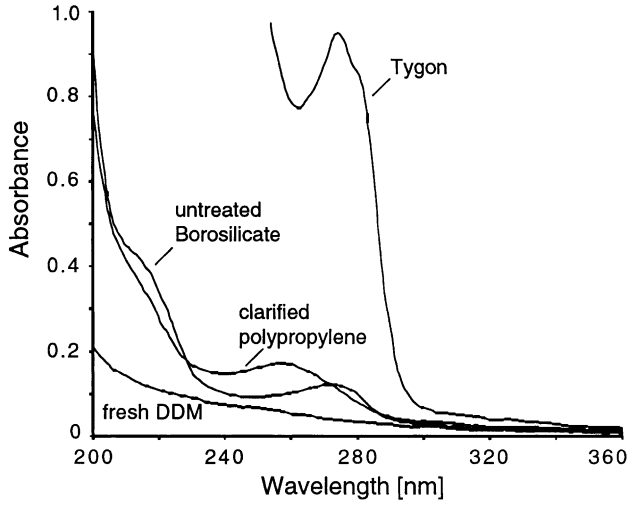

Fig. 3. Extraction of UV-absorbing materials from storage containers by DDM solutions. The spectra of 1.5% DDM solutions stored for 3 days with a sample of Tygon® R3603 laboratory tubing, in a clarified polypropylene microfuge tube and in an unwashed borosilicate sample vial, are shown in comparison to the spectrum of a fresh solution. The other containers that were tested (including an ethanol-rinsed borosilicate vial) did not affect the spectrum of the DDM solution.

solutions shows a strong monomer band and a weak dimer band on SDS-PAGE, and a single, approximately symmetrical peak by SE-HPLC. The addition of a 10-fold molar excess of OG over DDM leads to a moderate increase of the strength of the aggregate bands on the SDS gel, but to a complete transition from a monomer to a high molecular weight species in the HPLC chromatogram. These HPLC aggregates elute in the void volume of the column, with a Stokes radius (R_S) of greater than 10 nm, while the R_S of the monomeric protein is 4.55 nm. The aggregation induced by OG could not be prevented by DDM, although these aggregates were mostly reversible by SDS. Thus, the SDS-PAGE test presented in Figs. 1 and 2 underestimate the amount of aggregate in a sample. The protein sample heated to 45 °C for 6 h shows a total loss of the monomeric species, and a similar but reduced aggregate peak in the HPLC chromatogram. Severe aggregation is also seen on the SDS-gel, including species that remain at the top of the well in the stacking gel and at the boundary of the stacking and running gel, although, as with the OG-induced aggregation, some of the heat-aggregated protein could be dissociated into monomers by SDS. The reduced amount of total protein in the HPLC sample is most likely due to the removal of large aggregates by filtration of the sample prior to injection onto the HPLC column, and may be the same material that is not dissociated by SDS.

3.3. Extraction of UV light-absorbing substances

Initial attempts at monitoring the protein stability by HPLC with quantitation by UV absorption were hampered by an increasing level of signal at 280 nm upon protein storage. This was traced to the extraction of UV-absorbing substances from the storage containers by DDM solutions, as shown by experiments performed with simple detergent solutions containing no protein (Fig. 3). After 3 days of storage at room temperature, the detergent solutions stored in contact with Tygon® tubing, a 1.5-ml clarified polypro-

Table 1
Native SE-HPLC stability assay

Storage condition			Recovery of unaggregated protein					
NaCl [mM]	Glycerol [%]	Temperature [°C]	Exp.[a]	Wild-type	Exp.[a]	L6_cyt_N2C6	L6_fla_N5C5	L6_lyso_N1C1
0	0	4	1	0.7673	9	0.7160 (1)	0.4425	0.3059
								0.3119
200	0	4	2	0.7474	10	0.7161 (1)	0.5175	0.3293
0	20	4	3	0.8533	11	0.8815 (1)	0.6081	0.4007
								0.4116
200	20	4	4	0.8069	12	0.8769 (1)	0.6720	0.4765
				0.8852		0.8779 (1)	0.6794	0.4823
								0.4811
0	0	22	5	0.5383	13	0.4912 (1)	0.3515	0.1670
						0.5085 (2)		
200	0	22	6	0.5650	14	0.5449 (2)	0.4012	0.1728
						0.5200 (2)		
0	20	22	7	0.9030	15	0.8319 (1)	0.5101	0.3198
						0.8460 (2)		
200	20	22	8	0.8771	16	0.8076 (1)	0.6281	0.3615
				0.8875		0.8274 (1)	0.6147	0.3527
						0.8131 (2)		
						0.8217 (2)		

The recovery of unaggregated protein after 14 days of storage are given for eight conditions for the wild-type lac permease (experiments 1–8) and for each of the three fusion proteins (experiments 9–16). Multiple entries represent repeated measurements for error estimation, and "(1)" and "(2)" distinguish two separately purified L6_cyt_N2C6 fusion protein samples for batch-to-batch comparison.

[a] Experiment number.

pylene microcentrifuge tube or an unwashed borosilicate sample vial showed distinct absorption maxima that were not present in uncontaminated DDM solutions. By far, the strongest absorption peak was seen in the solution that was stored in the presence of Tygon® tubing. The absorption peak of the detergent solution stored in the borosilicate vial was not seen when the vial was first rinsed with ethanol, and thus in this case, the UV absorbing material was most likely due to apolar contaminants on the surface of the glass. In contrast, the spectra for the solutions that came in contact with Tygon® tubing or plastic microfuge tubes exhibited the same spectra even when first rinsed with ethanol, and most likely became contaminated with aromatic compounds extracted from the plastics. The extraction of plasticisers such as di(2-ethylhexyl)phthalate (DEHP) from polyvinyl chloride (PVC) plastics by lipophilic compounds is well documented (see, for example Ref. [32]). The Material Data Safety Sheet for Tygon® R-3603 states that the formulation of the tubing is a PVC polymer that contains DEHP. In the case of clarified polypropylene, derivatives of dibenzylidene sorbitol (DBS) are used to improve the optical characteristics of the plastic, and are likely to be the UV-absorbing compounds extracted by the detergent. Analysis of the UV spectra of HPLC chromatograms of lac permease samples stored in clarified polypropylene indicated that the extractable materials partitioned between the free detergent micelles and the PDCs. DDM solutions in contact with washed borosilicate vials, scintillation vials, polystyrene or polypropylene tubes did not show any change in the absorption spectrum upon storage.

3.4. Quantitative assessment of protein stability

The stability of the wild-type lac permease relative to each of three different fusion proteins was evaluated by SE-HPLC by full factorial 4-factor 2-level experiments. The factors and levels were: (1) sodium chloride concentration (0 or 200 mM), (2) glycerol concentration (0% or 20%), (3) storage temperature (4 °C or room temperature) and (4) fusion carrier (absent (wild-type lac permease) or present (carrier domain constructs)). There are $4^2 = 16$ measurements for each comparison of the wild-type to a given fusion protein: 8 storage conditions for the wild-type and 8 for the fusion protein. The data are reported as the amount of monomeric protein recovered after 14 days (Table 1). The reduction in the area of the monomer peak was attributed to protein aggregation. Large aggregates were removed by filtration or eluted in the void volume of the column. In some cases, "small aggregate" species were observed as a distinct leading shoulder or peak to the main monomer peak in the HPLC chromatogram (Fig. 4). In these cases, the recovery of monomeric protein was obtained by fitting two Gaussian curves to the total protein peak and separately integrating the two curves, as described in the Section 2. The R_S of the monomeric protein was 4.3 nm for the wild-type protein and approximately 4.5 nm for the three fusion

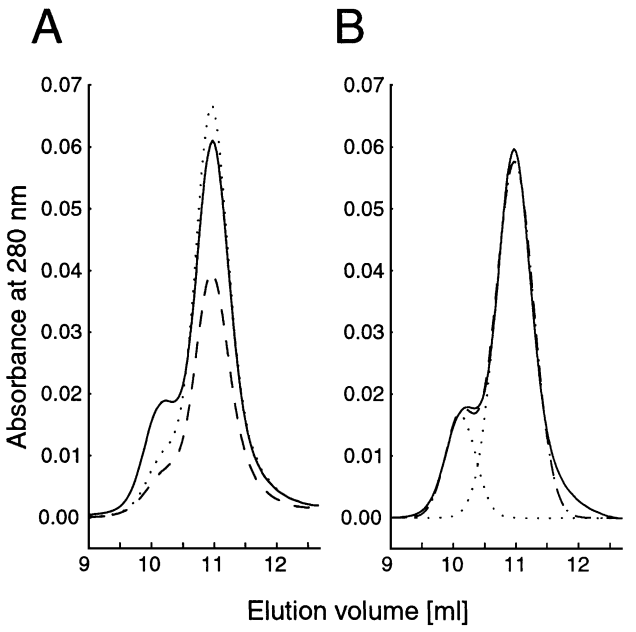

Fig. 4. (A) SE-HPLC elution profiles of wild-type lac permease. Dotted line: freshly purified sample. Solid Line: sample stored for 14 days in 20% glycerol, 200 mM NaCl at 4 °C (Table 1, experiment 4). Dashed line: sample stored for 14 days with no added glycerol or salt at 22 °C (Table 1, experiment 5). (B) Integration of the area under the monomer peak by line fitting. Solid line: measured profile (same curve as solid line in part A). Dashed line: calculated profile resulting from the sum of two ideal Gaussian curves centered at 10.19 ml for the "small aggregate" and at 10.98 ml for the monomer (dotted lines).

proteins, which is consistent with a particle containing a single lac permease chain and 150–200 bound DDM molecules. Similar levels of DDM binding have been reported for other proteins [33,34]. The small aggregate peak with an R_S of 6.4 nm is well modeled by a single Gaussian curve, suggesting that this is a relatively well-defined species. Because the amount of bound detergent in the small aggregate was not determined in these experiments, we make no attempt at estimating the number of permease chains in this complex. Multiple measurements were used for error estimation in the statistical analysis. For the cytochrome$_{b562}$ fusion protein, the experiment was repeated with independently purified samples to ensure batch to batch reproducibility. All peak areas were normalized relative to the internal vitamin B_{12} standard and reported as a ratio to the area of the monomer peak on the first day of the experiment.

Under the most favorable storage conditions (4 °C and in the presence of 20% glycerol), 80–90% of the wild-type protein remained in the unaggregated state after 14 days. The cytochrome$_{b562}$ fusion protein showed a comparable level of stability under all the tested conditions. However, the N5C5 flavodoxin and the N1C1 lysozyme fusion proteins were more prone to aggregation.

The full factorial experimental design enabled us to evaluate the influence of main factors and the interactions between these factors. The four main effects and 11 possible

	A	B	C	D	AB	AC	BC	AD	BD	CD	ABC	ABD	ACD	BCD	ABCD
Experiment	NaCl	Glyc	Temp	Fusion	NaCl* Glyc	NaCl* Temp	Glyc* Temp	NaCl* Fusion	Glyc* Fusion	Temp* Fusion	NaCl* Glyc* Temp	NaCl* Glyc* Fusion	NaCl* Temp* Fusion	Glyc* Temp* Fusion	NaCl* Glyc* Temp* Fusion
1	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+
2	+	-	-	-	-	-	+	-	+	+	+	+	+	-	-
3	-	+	-	-	-	+	-	+	-	+	+	+	-	+	-
4	+	+	-	-	+	-	-	-	-	+	-	-	+	+	+
5	-	-	+	-	+	-	-	+	+	-	+	-	+	+	-
6	+	-	+	-	-	+	-	-	+	-	-	+	-	+	+
7	-	+	+	-	-	-	+	+	-	-	-	+	+	-	+
8	+	+	+	-	+	+	+	-	-	-	+	-	-	-	-
9	-	-	-	+	+	+	+	-	-	-	-	+	+	+	-
10	+	-	-	+	-	-	+	+	-	-	+	-	-	+	+
11	-	+	-	+	-	+	-	-	+	-	+	-	+	-	+
12	+	+	-	+	+	-	-	+	+	-	-	+	-	-	-
13	-	-	+	+	+	-	-	-	-	+	+	+	-	-	+
14	+	-	+	+	-	+	-	+	-	+	-	-	+	-	-
15	-	+	+	+	-	-	+	-	+	+	-	-	-	+	-
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L6_cyt_N2C6	0.0	**568.4**	**116.0**	7.7	2.0	0.6	**106.8**	0.3	0.9	4.7	2.6	0.2	0.0	7.9	0.0
L6_fla_N5C5	7.1	**217.7**	**41.5**	**344.7**	0.0	0.2	**27.0**	9.3	2.4	0.1	0.0	0.7	0.0	17.3	1.5
L6_lyso_N1C1	2.3	**353.8**	**116.6**	**2130.0**	0.4	0.1	**57.8**	4.2	**13.3**	5.6	1.1	2.4	1.2	**28.0**	0.2

Fig. 5. Full factorial analysis of the HPLC stability data. The 15 possible effects involving the 4 factors tested are listed. A–D: main effects of sodium chloride concentration (NaCl), glycerol concentration (Glyc), temperature (Temp) and fusion (Fusion). AB–ABCD : interactions between the four factors. For the main effects, " – " indicates a low level for a particular factor (absence of salt or glycerol, 4 °C storage temperature or absence of fusion insert (i.e. wild-type)), and " + " indicates the high level for this factor (200 mM sodium chloride, 20% (w/v) glycerol, 22 °C storage temperature or presence of fusion insert). Experiment numbers correspond to those given in Table 1. Only one generic experimental matrix is given. The HPLC stability experiment is evaluated as separate comparisons of wild-type lac permease with each of the three fusion proteins, and the bottom three lines give the *F*-values for the three independent comparisons. The threshold for the *F*-values at a given significance probability level depend on the degrees of freedom of the error estimate and therefore on the number of measurements per experiment. The *F*-value threshold values at a significance probability level of 0.01 are 11.3 for L6_cyt_N2C6, 21.2 for L6_fla_N5C5, and 12.2 for L6_lyso_N1C1. *F*-values that exceed their threshold values are indicated in bold type.

interactions are shown in Fig. 5. Columns A through D represent the experimental matrix, where " – " indicates a low level for a particular factor and " + " a high level. For the interactions in columns AB through ABCD, the tabulated signs are the result of the multiplication of the signs from the contributing main effects. Finally, in each column, contributions of eight " + " and eight " – " terms are used to determine the significance of each main effect or interaction.

The statistical evaluation of this data by an analysis-of-variance procedure was performed using a general linear model (GLM) and a type III sum of squares and estimatable function as defined in the computer package SAS/STAT. Because there are no missing measurements in our full-matrix experiment, the Type III sum of squares coincides with the more familiar Yates' weighted squares-of-means technique. Instead of a classical ANOVA procedure, a GLM is preferable for unbalanced experimental designs with varying numbers of measurements for the different conditions. Due to limitations in the available material, not all measurements could be repeated an equal number of times and our experimental design is therefore unbalanced.

The statistical evaluation results in the calculation of *F*-values. Large *F*-values lead to rejection of the null hypothesis, which is that the main factors or the interactions between them have no significant effect on the stability of the protein and that the differences in the measurements are caused by random fluctuations. The probability of calculating an *F*-value larger than the *F*-value given that the null hypothesis is true is called the significance probability value

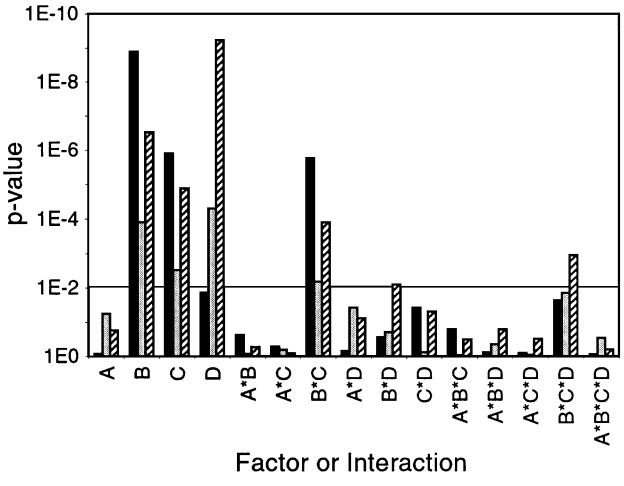

Fig. 6. Significance probability values for the full factorial comparisons of wild-type lac permease with the cytochrome$_{b562}$ fusion protein (solid black bars), with the N5C5 flavodoxin fusion protein (shaded bars) and with the N1C1 lysozyme fusion protein (striped bars). The horizontal line marks the threshold level of 0.01.

(*P*-value). A significance probability of 0.01 means that the null hypothesis is incorrectly rejected less than 1% of the time. If the null hypothesis is not rejected, then either the hypothesis was true (i.e. no effect), or there were not enough data to detect the differences to be tested.

The *F*-values and *P*-values for the comparisons between the wild-type lac permease and each of the three fusion proteins are presented in Figs. 5 and 6. *F*-values that surpass the significance probability of 0.01 are marked in bold (Fig. 5), and are taken to be significant. The glycerol concentration, the storage temperature and the interaction between the glycerol concentration and the storage temperature had a significant effect on the stability of the protein in all three experiments. In contrast, the NaCl concentration had no significant effect in any comparison. While the stability of L6_cyt_N2C6 is not significantly different than that of the wild-type protein, the introduction of carriers in the N5C5 flavodoxin and N1C1 lysozyme fusion proteins strongly influences the stability of the protein, resulting in protein that is more prone to aggregation.

4. Discussion

4.1. Stability of the lac permease in detergent solutions

The behavior of the lac permease in the solubilized state is strongly detergent-dependent, and DDM proved to be the most suitable detergent tested. The effectiveness in stabilization depends on both the nature of the head group and the alkyl chain length of the detergent, and there is not a simple correlation with the critical micelle concentration (cmc) of the detergent. Detergents with maltoside headgroups are favored, and in this series, longer alkyl chains improve the properties of the PDC, as expected. DDM is well known for its gentle properties [8], but has a very low cmc (approximately 0.008% w/v) and forms large micelles (aggregation number 110–140) [33,35,36]. Although this first property is advantageous, the latter two properties are problematic for purification and crystallization. This situation with the lac permease serves to underscore the overall need in this field for new detergents with improved properties [37–39].

The simple SDS-PAGE stability test is an easy way to rapidly screen a large number of trial detergents for their stabilizing properties, and the ranking that we observe is similar to that seen with diacyl glycerol kinase [17] and AE1 [31]. The final sample that is actually run on the gel is a complex mixture of the test detergent, the purification detergent (here, DDM) and SDS. We expect that the DDM may tend to reduce the aggregation behavior of the test detergent, although care should be taken in evaluating these results because mixed detergent systems do not necessarily have properties intermediate of their contributing pure components. Also, since high concentrations of a detergent can lead to protein aggregation [40,41], several levels of a test detergent can be tested by this method. In the data presented here, it is clear that DDM offers little protection to the protein when OG is added to the mixture, and this is consistent with the well-established observation that OG leads to the severe aggregation of the lac permease in pure detergent solutions. In this respect, it is interesting to note that an essential feature of the original protocol for the functional reconstitution of purified lac permease into defined proteoliposomes was the addition of *E. coli* lipids in the OG chromatography buffer [42].

4.2. Protein aggregation

The simple SDS-PAGE test assumes that aggregates formed in the presence of the test detergent are not dissociated by the addition of SDS. This is the case for severely aggregated lac permease, but the SE-HPLC measurements clearly demonstrate that some of the aggregates can be reversed by SDS. Thus, the SDS-PAGE method gives an underestimate of the degree of aggregation of a sample. There are intermediates in the aggregation pathway, and early "small aggregates" can be dissociated by SDS, while the "large aggregates" that form more slowly (possibly from the small aggregates) cannot be dissociated by SDS under the conditions tested.

Since the HPLC assay measures the protein concentration based on its absorbance at 280 nm, it was essential to eliminate all contact of our solutions with plastics in order to avoid the extraction of UV-active compounds. These materials become tightly associated in the PDC and are presumably present in most solutions of membrane proteins that have been in contact with these plastics. Impurities in detergents can affect the stability of the solubilized membrane proteins [43,44], but the effects of the compounds extracted here have not been studied.

4.3. Stability of lac permaese carrier fusion proteins

One of the main goals of this work was to quantitatively measure the stability of promising carrier fusion proteins relative to that of the wild-type lac permease. Unexpectedly, the introduction of a carrier domain had either no effect (in the case of cytochrome$_{b562}$) or had a negative effect (flavodoxin, lysozyme) on the aggregation of the protein in DDM solutions. The reduced stability of the flavodoxin and lysozyme fusion proteins were not due to internal strain caused by linkers that were too short, since in both cases, fusion proteins with short "N1C1" and longer "N5C5" linkers had identical properties [22], and residues from the central hydrophilic loop can be deleted without ill effect [22,45]. Thus, the simple addition of additional polar surface domains in these fusion proteins did not improve the properties of the solubilized lac permease. A similar result has been described in a head-to-tail fusion of bacteriorhodopsin and aspartyl transcarbamylase [46], although in this case, the poor properties of the resulting fusion protein was thought to be due to exposed hydrophobic surfaces of the

transcarbamylase domain. The carriers we describe here are monomeric, and the reasons for the diminished stability in some but not all of these fusion proteins are not clear.

4.4. Factorial search for optimal solution conditions

Common laboratory manipulations to reduce protein aggregation include the optimization of salt and pH, the use of reducing agents, low temperature, and the addition of co-solvents such as glycerol. Our factorial experiment clearly points to the importance of glycerol and the fact that there is a synergistic effect of glycerol and temperature. The presence of a strong interaction between these factors means that the stabilizing contributions of glycerol and temperature are not simply additive. The full factorial design makes it possible to detect such interactions, but because of the large size of the experimental matrix, it is impractical to test an extensive set of test conditions. Incomplete factorial designs are more efficient at testing a large number of primary effects or levels, but are generally unsuitable for detecting interactions between effects [25,26,47].

In all cases tested, aggregation was not affected by the two chosen levels of salt (0 and 200 mM), suggesting that electrostatic effects are not important in the aggregation process. Knowing that the lac permease and its fusion constructs are stable in low and high salt buffers is helpful for the design of crystallization screens, since the protein can be stored in a minimal sample buffer containing little or no salt prior to the addition of precipitant mixtures. This makes it possible to investigate a wide range of salt concentrations in a DDM/polyethylene glycol crystallization screen, for example, allowing a thorough investigation of the phase separation behavior [48] of this system. Similar conclusions for the effect of detergent, glycerol and temperature on the aggregation behavior of the human erythrocyte glucose transporter as measured by SEC-HPLC have been reached by Boulter and Wang [49].

In this quantitative study, glycerol was by far the most effective factor that was tested. Although the effectiveness of glycerol in stabilizing membrane proteins is generally appreciated, the magnitude of the effect has not been systematically studied, and the mechanisms of stabilization in these complicated systems are not understood. With soluble proteins, glycerol and other protecting osmolytes stabilize the folded states of proteins through preferential exclusion and/or osmotic pressure effects [50–52]. The net result is the reduction of the conformational heterogeneity of a protein population by favoring more rigid and compact structures [53–56] with decreased specific volumes and adiabatic compressibility [57,58].

The situation is more complicated with solubilized membrane proteins such as the lac permease because only the exposed surface loops interact with the aqueous phase, and in general, these make only minor contributions to the overall stability of the protein [7,20,59]. It seems likely that in these cases, glycerol stabilizes interhelical packing in the transmembrane segments of the protein through poorly understood detergent-mediated effects. Because integral membrane proteins in their native environment may be stabilized by the lateral pressure generated within lipid bilayers [60,61], the increased rate of aggregation of membrane proteins in detergent solutions relative to that of membrane-bound states may be due to the loss of the lateral bilayer pressure. This would allow the protein in the PDC to sample a wider distribution of conformational substrates, and intermolecular collisions between peptides with partially or fully unfolded conformations would lead to kinetically trapped aggregates. Glycerol (and possibly other co-solvents) may affect the structure of detergent assemblies to create a more tightly packed and less compressible hydrocarbon interior, resulting in a more native membrane-like environment in the belt region of a PDC. It is not known if glycerol affects the detergent/protein stoichiometry within the PDC, or the dynamics of detergent exchange. Because of these effects, glycerol/detergent mixtures may prove to be more effective surrogates for phospholipids bilayer environments than simple detergent systems.

The effect of temperature on the stability of the protein can be rationalized with similar arguments. Zhou et al. [18] have determined that irreversible thermal inactivation of diacyl glycerol kinase in OG and DM solutions is due to conformational effects. A reasonable model is that conditions that favor compact, low energy states of detergent-solubilized membrane proteins will favor protein stability and reduce aggregation. Conditions in which partially and fully unfolded intermediates are more populated will lead to increased rates of aggregation. Clearly, the design of crystallization screens should emphasize on conditions in which protein aggregation is minimized. Further investigations of the effect of glycerol and other co-solutes on these systems may lead to improved strategies for the handling of membrane proteins in detergent solutions, and provide further insight into the forces that stabilize the structures of membrane proteins.

Acknowledgements

Support for this work was provided in part by a grant from The Canadian Institutes for Health Research (MT-13335). C.K.E. was supported by Fellowships from the Deutsche Forschungsgemeinschaft and from the Canadian Institutes of Health Research. We thank Ron Kaback, David Trichler, Chris Richardson, Reinhart Reithmeier and Avi Chakrabartty for helpful discussions, and Peter Henderson for the protocol for purifying inner membranes on a preparative scale.

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