Quaternary Structure of the Lactose Transport Protein of
Streptococcus thermophilus in the Detergent-solubilized and
Membrane-reconstituted State*

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The quaternary structure of LacS, the lactose transporter of Streptococcus thermophilus, has been determined for the detergent-solubilized and the membrane-reconstituted state of the protein. The quaternary structure of the n-dodecyl-β-D-maltoside-solubilized state was studied using a combination of sedimentation velocity and equilibrium centrifugation analysis. From these measurements it followed that the detergent-solubilized LacS undergoes reversible self-association with a monomer to dimer mode of association. The association constants were 5.4 ± 3.6 and 4.4 ± 1.0 ml mg⁻¹ as determined from the velocity and equilibrium sedimentation measurements, respectively. The experiments did not indicate significant changes in the shape of the protein-detergent complex or the amount of detergent bound in going from the monomeric to dimeric state of LacS. Importantly, a single Cys mutant of LacS is labeled by 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid in a substrate-dependent manner, indicating that the detergent-solubilized protein exhibits ligand binding activity. The quaternary structure of membrane-reconstituted LacS was determined by freeze-fracture electron microscopy analysis. Recent developments in the analysis of freeze-fracture images (Eskandari, S. P., Wright, E. M., Freman, M., Starace, D. M., and Zampighi, G. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11235–11240) allowed us to directly correlate the cross-sectional area of the transmembrane segment to a dimeric state of the functionally membrane-reconstituted LacS protein. The cross-sectional area of the LacS protein was calibrated using the membrane-reconstituted transmembrane domain of the mannitol transporter enzyme II, an intramembrane particle for which the cross-sectional area was obtained from maps of two-dimensional crystals. The consequences of the determined quaternary structure for the transport function and regulation of LacS are discussed.

The lactose transport protein (LacS) of Streptococcus thermophilus is composed of a 51-kDa integral membrane domain and a 19-kDa regulatory domain that is located at the cytoplasmic surface of the membrane. The 51-kDa carrier domain is necessary and sufficient for translocation catalysis. The LacS protein is a member of the galactose-pentose-hexuronide family and studies on the membrane topology and structure prediction algorithms are most consistent with a carrier domain that is composed of a bundle of 12 transmembrane segments in α-helical configuration. Such an organization is typical for many membrane transport proteins (1). Besides the primary and secondary structure, very little structural information is available for membrane transport proteins. In this paper, we set out to study the quaternary structure of LacS, a system that is paradigmatic for proteins that catalyze the uptake of solutes in symport with a cation.

Based on genetic and biochemical studies of several secondary transporters, it has been proposed that there is a single structural principle for all these systems (2). For transporters containing 10 to 12 putative transmembrane α-helices, such as the LacY (3, 4), UhpT (2), and GLUT1 (5), the functional species appears to be monomeric. Whereas, secondary carriers with 6 to 7 putative transmembrane α-helices, such as the ADP/ATP carrier and the phosphate carriers of the inner mitochondrial membrane (6, 7), appear to function as a dimer. If this structural principle is indeed characteristic of all secondary transporters, it would predict LacS to be monomeric. Many integral membrane proteins, however, are oligomeric (8), which results in a large excluded volume effect (high protein concentrations in the membrane) and a significant restriction in translational mobility. Also, their orientation is fixed relative to the membrane plane, resulting in the loss of fewer degrees of freedom upon oligomerization than when a soluble protein oligomerizes.

Isolation of integral membrane proteins with nonionic detergents is often considered mild, and the proteins are usually un-denatured and intact (9). The quaternary structure of these detergent-solubilized proteins can then be derived from molecular weight measurements. The quaternary structure of membrane proteins in detergent has most often been studied by gel filtration chromatography. This method, however, suffers from a poor resolution due to the large amount of detergent binding to the protein and requires that the dissociation rate of the oligomeric species is slow compared with the time required to perform the gel filtration experiment. Molecular weight measurements can be made rigorously by employing analytical ultracentrifugation. The effect of the detergent on the hydrodynamic properties of the integral membrane protein can be corrected for using appropriate methods without the intrinsic assumptions described above (10, 11). The molecular organization in the membrane environment may be different from that in the detergent-solubilized form, that is when the protein

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requires particular alkyl chains, head group heads, or the strain of the membrane for its quaternary structure. Therefore, the quaternary structure of LacS was also studied for the membrane-embedded state of the protein. Freeze-fracture electron microscopy images of proteoliposomes allowed for the quantification of the cross-sectional surface area of the transmembrane domain, which correlates with the number of α-helices and, consequently, with the quaternary structure of LacS in the membrane environment (12).

Here, we report on the quaternary structure of the LacS protein in the detergent-solubilized and membrane-reconstituted state using analytical ultracentrifugation and freeze-fracture electron microscopy techniques.

**EXPERIMENTAL PROCEDURES**

**Materials**—[14C]-Dodecyl-β-D-maltoside (2.11 TBq/mol) was obtained from the Radiochemical Center Amersham, United Kingdom. [14C]-Dodecyl-β-D-maltoside (2 GBq/mmol) was a generous gift from M. Le Maire (Paris, France). Ni-NTA resin was from Qiagen, Inc. and Bio-Beads SM-2 from Bio-Rad, Inc. Triton X-100 was from Roche Molecular Biochemicals; n-dodecyl-β-D-maltoside from Anatrace. D_2O (99.9%) was purchased from Fluka. Total Escherichia coli lipids and α-phosphatidylcholine from egg yolk were from Avanti Polar Lipids and Sigma, respectively. All other materials were reagent grade and from commercial sources.

**Bacterial Strain and Growth Conditions**—S. thermophilus ST11(lacS) carrying plasmid pGKH was grown semi-aerobically at 42 °C in Elliker broth supplemented with 0.5% (v/v) beef extract, 20 mM lactose plus 5 μg/ml erythromycin (13).

**Construction of Single Cysteine Mutant C320A**—Using a polymerase chain reaction approach (13), Lys-373 was replaced for cysteine using the lacS(C320A) gene in pGKH(C320A) as parent. The oligonucleotide used for the mutagenesis was 5'-AATGGTGTCAGGTT-CACC (mutations are underlined); in addition to the AAX(Lys) to TGT (Cys) change, a BstEII site was created 3 base pairs downstream of the TGT codon. The fragment containing the mutant sequence was sequenced by nucleotide sequencing, and the resulting plasmid was named pGKH(C320A/K373C).

**Puriﬁcation of LacS-His**—All purification steps were performed at 4 °C. The protein was purified essentially as described (13) with the following modifications. Membranes of S. thermophilus were isolated and subsequently solubilized in 15 mM imidazole, pH 8.0, 10% (v/v) glycerol, 100 mM NaCl plus 0.5% (w/v) DDM. After 10–20 min of incubation, insoluble material was removed by centrifugation (280,000 × g, 15 min). The solubilized membrane proteins were mixed and incubated for 30 min with Ni-NTA resin (~4 mg of LacS/ml of resin) that was equilibrated with buffer A (15 mM imidazole, pH 8.0, 10% (v/v) glycerol, 100 mM NaCl plus 0.05% DDM). The column material was poured into a Bio-Spin column (Bio-Rad) and washed with 10 column volumes of buffer A containing 25 mM imidazole. The protein was eluted with 200 mM imidazole, pH 7.0, 10% (v/v) glycerol, plus 0.05% DDM. After 10-fold dilution with buffer B (5 mM Hepes, pH 7.2, plus 0.05% DDM). The diluted protein sample was applied to a Bio-Spin column containing Q-Sepharose fast flow resin (10 mg of LacS/ml of resin; Amersham Pharmacia Biotech) that was equilibrated with buffer B. After washing with 10 column volumes of buffer B plus 25 mM NaCl, the protein was step-eluted with buffer B plus 100 mM NaCl. Peak fractions were collected and purified by gel filtration. The purity of the protein was determined with Microcon-100 filters with a cut-off of 100 kDa (Amicon); the filters were centrifuged at 3,000 × g until the desired concentration was achieved without concentrating the DDM that is not associated with the protein.

**Spectral Characterization of LacS**—To determine the protein concentration and the degree of aggregation, absorption spectra were taken with an Aminco DW2000 spectrophotometer. Spectra were collected from 240–340 nm with wavelength increments of 1 nm. LacS concentrations were calculated from the absorbance at 280 nm (A_280) using an extinction coefficient (ε) of 76,320 M⁻¹ cm⁻¹ (14), following the appropriate corrections for light scattering (15). The LacS concentration as determined from the A_280 yielded values that were 15–20% lower than those estimated from the Lowry assay in the presence of 0.5% SDS using bovine serum albumin as standard (16). The Lowry assay was used for the LacS protein in Triton X-100 and the protein concentrations were normalized to the A_280 measurements of DDM-purified LacS. Circular dichroism spectra were collected using an Aviv 60 DS spectropolarimeter. The CD spectra were the average of five scans from 190 to 260 nm with wavelength increments of 1.0 nm, and the signal was acquired for 1 s at each wavelength. Experiments were performed at 25 °C with 0.14 mg/ml protein and a 0.1-cm path length cell. Prior to the measurements, the protein samples were extensively dia lyzed at 4 °C against 50 mM potassium phosphate, pH 6.0, 100 mM NaCl, 10% (v/v) glycerol plus the appropriate detergent as indicated in the legend of Fig. 1. Spectra were corrected by subtracting baseline spectra obtained with the buffer solution.

**Mal-ANS Labeling of LacS Protein**—The LacS(C320A), LacS(C320A/K373C), and other single cysteine mutants were purified as described above, using either Triton X-100 or DDM as detergent. Mal-ANS labeling was carried out in 2.0 ml of 50 mM potassium phosphate, pH 7.5, 100 mM NaCl plus 0.05% (v/v) detergent, and at a temperature of 30 °C. LacS protein was added to a final concentration of 10–15 μg/ml (i.e., 142–213 nmol), and after 3 min of equilibration, the reaction was started by adding 5 μM Mal-ANS (1 mM stock solution in Me2SO). The rates of labeling in the presence of galactose were estimated from the increase in fluorescence over a time period of 20 s, following the addition of ligand (at time is 5 min). Fluorescence changes were measured at excitation and emission wavelengths of 328 and 380 nm, respectively, using slit widths of 5 nm. The extent of LacS labeling by Mal-ANS was estimated from the protein and probe absorbances at 280 and 313 nm, respectively, using extinction coefficients of 76,320 for LacS and 14,000 M⁻¹ cm⁻¹ for ANS.

**DDM Binding to LacS**—The number of DDM molecules bound to purified LacS protein was determined with radiolabeled detergent using equilibrium desorption chromatography (17). The protein bound to Q-Sepharose (0.4 ml) was equilibrated with buffer B containing 0.05% (w/v) [14C]DDM. Following washing of the column with 4 ml of buffer B plus 25 mM NaCl, the protein was eluted with buffer B plus 100 mM NaCl. LacS concentration was determined spectrophotometrically and detergent concentrations in the various fractions were determined by liquid scintillation counting.

**Membrane Reconstitution of LacS**—Liposomes prepared from ace tone-ether-washed E. coli lipids and egg yolk α-phosphatidylcholine in a ratio of 3:1 (w/w) were made by dissolving lipids (20 mg/ml) in 50 mM potassium phosphate, pH 7.0, followed by three freeze/thaw cycles and extrusion through polycarbonate filters of 400-nm pore size (13), unless indicated otherwise. The liposomes (4 mg of phospholipid/ml) were then incubated with the appropriate amounts of detergent. The suspension was mixed with purified detergent-solubilized LacS and incubated for 30 min at 20 °C under gentle agitation. The final protein concentration was 10 μg/ml, resulting in a lipid to protein ratio of 400 (w/w). To remove the detergents, polystyrene beads were added at a weight ratio of 10,000 mg/ml and the sample was incubated for another 2 h at room temperature. Fresh Bio-Beads SM-2 were added twice and the samples were incubated at 4 °C for 3 h and overnight, respectively. The proteoliposomes were washed with 50 mM potassium phosphate, pH 7.0, and stored in liquid nitrogen.

**Analytical Ultracentrifugation**—Analytical ultracentrifugation experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge using an AN-50 Ti 8-place rotor with 2-channel charcoal-washed centerpieces at 20 °C. Prior to the measurements, the protein samples were extensively diluted at 4 °C against buffer C (100 mM potassium phosphate, pH 7.0, 2 mM K-EDTA plus 0.05% DDM (w/v)). The sedimentation velocity experiments were carried out at 38,000 rpm, and data were collected at 280 or 230 nm in a continuous mode with a radial step size of 0.003 cm, at 12-min intervals. Observed sedimentation coefficients were determined from the centroids of the sedimentation boundaries analogous to the method of Arikasa and Van Holde (22). Sedimentation equilibrium experiments were carried out on sample volumes of 100 μl at rotor speeds of 8,000, 10,000, and 12,000 rpm, and loading LacS-DDM concentrations of 0.020, 0.050, and 0.100 mg of protein/ml. Absorbance optics were used to collect data of the LacS-DDM samples at 280 nm, and data were acquired every 0.001 cm with 10 replicates. Data analysis was performed with the XL-I data analysis software. The partial specific volume was estimated from the amino acid composition using reported values (18). The solvent densities were determined with an Anton Paar densimeter, model DM 48.

**Data Treatment According to Multicomponent Systems**—The molecular weight of LacS was determined by treating the experimental data with equations appropriate for multicomponent systems (19, 10). At
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sedimentation equilibrium the experimentally determined protein concentration ($c$) as a function of the radial position ($r$) is given by,

$$(2RT/\omega)ln(c/\epsilon(r)) = M(1 - \phi \rho)$$  \hspace{1cm} (Eq. 1)

where $R$ is the gas constant, $T$ is the absolute temperature, $\omega$ is the angular velocity, $\rho$ is the density of the solvent, and $\phi$ is the effective partial specific volume of the protein moiety, which includes the contributions of bound detergent. The molecular weight ($M$) is that of the protein moiety of the particle, excluding bound detergent or solvent. This approach allows for the calculation of the buoyant density factor by replacing the factor $M(1 - \phi \rho)$ of Equation 1 by,

$$M(1 - \phi \rho) = M[(1 - \bar{\varphi}_p \rho) + \delta \rho(1 - \bar{\varphi}_D)]$$  \hspace{1cm} (Eq. 2)

where $\rho$ is the partial specific volume of LacS, which was calculated to be 0.7514 at 20 °C (18), and $\bar{\varphi}$ is the partial specific volume of DDM bound to LacS. In Equation 2, $\delta \rho$ is the DDM bound by LacS (g/g), which was determined experimentally using equilibrium desorption chromatography; $\bar{\varphi}$ is the partial specific volume of DDM which was determined experimentally by sedimentation equilibrium analysis at different solvent densities.

**Data Treatment According to Density Matching**—The behavior of a single, thermodynamically ideal solute in a centrifugal field can be described from the Lam equation (20),

$$(\ln c_r)R/T = M(1 - \bar{\varphi}_p \rho)S^2/2RT$$  \hspace{1cm} (Eq. 3)

where $c_r$ is the solute concentration as a function of the radial distance, $R$ is the gas constant, $T$ is the absolute temperature, $\omega$ is the angular velocity, $\rho$ is the density of the solvent, and $S$ is the partial specific volume of the protein moiety. Since the partial specific volume of the LacS-DM complex is unknown, the molar mass of the protein moiety cannot directly be derived from the effective molar mass of the complex, $M_{\text{st}} = M \times (1 - \bar{\varphi}_D)$. However, by determining the $M_{\text{st}}$ as a function of solvent densities by varying the ratio of H$_2$O and D$_2$O, the molar mass of the protein moiety can be evaluated by extrapolation to the density of DDM.

**Determination of DDM Density**—The density of DDM is an important parameter in the equations used to determine the molar mass of the protein moiety. The density of $1/n_{\text{DDM}}$ can be determined experimentally employing equilibrium sedimentation at different solvent densities by varying the ratio of H$_2$O and D$_2$O. Under conditions at which the solvent density equals $1/n_{\text{DDM}}$, the concentration distribution is no longer observed. However, the density of DDM is higher than the maximum obtainable solvent density using D$_2$O. Therefore, the density of DDM can only be determined by extrapolation to solvent densities that are $M_{\text{st}}$ becomes zero. Interference optics was used to collect data of the DDM samples to determine its partial specific volume.

**Freeze-Fracture Electron Microscopy**—Freeze-fracture replicas were prepared from pellets of proteoliposomes that were flash-frozen in liquid nitrogen and thawed at 4 °C in order to increase the vesicle size. Next, the proteoliposomes were washed with 15% glycerol in 50 mM K$_2$PO$_4$, pH 7.0, and placed on gold holders followed by ultra-rapid cooling by plunging into liquid propane (−190 °C) that was cooled with liquid nitrogen. Frozen samples were fractured in a Balzers BA501 instrument at 10 Torr. Tantalium/carbon was deposited at 45° elevation. Freeze-fracture replicas were picked up on 400-mesh uncoated grids for examination in a Philips CM10 electron microscope. Images enlarged at a final magnification of ×250,000 were digitized and particle diameters were measured perpendicular to the shadow direction using software from ScionImage. The results were plotted as frequency histograms at the center of the bin (0.5 nm) and fitted to a (multiple) Gaussian function,

$$f_s = \sum_i A_i \exp(-0.5(x - \mu_i/n)^2)$$  \hspace{1cm} (Eq. 4)

where $f_s$ is the frequency of occurrence of a particle of size $x$, $s$ is the measured diameter of a given particle (nm), $A$ is an approximation of the area below the curve of a given particle population, $\mu$ is the mean diameter of a given population (nm), $s$ is the standard deviation of the population diameter, and $i$ is an integer from 1 to the total number of particle populations. For sample sizes, $N$ refers to the number of diameter measurements, and $n$ refers to the number of particles counted for particle density determinations.

For particle density measurements fracture faces were enlarged to a final magnification of ×75,000, and intramembrane particles (IMP) from both the convex and concave proteoliposomes were counted from known areas of the membrane. No correction was made for the curvature of the vesicle surfaces, resulting in a slight overestimation of the particle densities.

**RESULTS**

**Spectroscopic Characterization of Detergent-solubilized and Purified LacS**—Using the His-tagged protein, LacS was purified in two steps (Ni affinity and anion exchange chromatography) yielding a single band after staining with silver or Coomassie Blue on a SDS-polyacrylamide electrophoresis gel. Previous purification trials often led to higher order aggregates and significant loss of activity, but the modified protocol yielded a protein that was highly pure, and stable in the Triton X-100 or DDM-solubilized state(s) for at least 3 days at 20 °C. The absorption spectrum of LacS purified with DDM exhibited a flat baseline in the 310–340 nm region (Fig. 1, inset), which indicates that higher order aggregates are not significantly present under these conditions. In contrast, LacS purified in OG was highly aggregated, as indicated by the absorbance in the 310–340 nm region, already in fractions eluting from the Ni-NTA column (Fig. 1, inset).

The secondary structure of LacS in micelles of DDM or Triton X-100 was monitored by circular dichroism (CD) spectroscopy (Fig. 1). Although the CD spectra of LacS purified in Triton X-100 exhibited more noise than those in DDM due to the absorption of Triton X-100 in the UV region, the spectra were otherwise superimposable. CD spectra of LacS purified in DDM were used to quantify the secondary structure elements by the method of Contin (23). The estimates of secondary structure amounted to 60% α-helix, 13% β-sheet, 15% β-turn, and 12% remainder. The addition of 10 mM lactose had no effect on the CD spectra (data not shown).

**Galactose Binding to Detergent-solubilized LacS**—To assess the functional integrity of detergent-solubilized and purified LacS protein, we determined the dissociation constant for galactose binding in a fluorometric assay. Mutants were constructed that lack the native cysteine (Cys-320) but carry a cysteine in interhelix loop 10–11, i.e. near the putative substrate-binding site (32). These mutants were tested for their ability to react in a ligand-dependent manner with Mal-ANS, a maleimide derivative that is non-fluorescent until it reacts with thiols. Moreover, its fluorescence quantum yield is highly
dependent on the polarity of the environment. Of different mutants tested, LacS(C320A/K373C) proved to be the best target as the rate of labeling was affected only in the presence of carrier substrates (Fig. 2A). Labeling of LacS(C320A/K373C) by Mal-ANS was quantitative as no further thiol-specific reaction was observed upon unfolding of the protein with SDS (not shown). The extent of SDS-treated LacS(C320A/K373C) was calibrated by titration with known amounts of reduced glutathione. Importantly, upon membrane reconstitution, the unlabeled and double mutant as well as the single mutant (C320A) displayed counterflow activities identical to the wild-type protein (data not shown). In contrast to LacS(C320A/K373C), detergent-solubilized and purified LacS(C320A) was not significantly labeled by Mal-ANS. The rate of LacS(C320A/K373C) labeling was enhanced in the presence of sugars (galactosides) that are known to be substrates of LacS (33), whereas other sugars such as glucose and sucrose had no effect (Fig. 2A). This property was used to estimate the dissociation constant ($K_d$) for equilibrium galactose binding by determining the rate of labeling by Mal-ANS at varying galactose concentrations. The kinetic analysis of galactose binding to LacS(C320A/K373C) as reported by the difference in the rate of labeling with and without ligand is shown in Fig. 2B. The data for the Triton X-100- and DDM-solubilized protein were very similar, yielding $K_d$ values for galactose binding of approximately 2 mM. Thus, both the spectroscopic and ligand binding measurements do not reveal significant differences between LacS in the Triton X-100 and DDM-solubilized state, and indicate that the detergent-solubilized LacS has retained its native conformation.

DDM Binding to LacS—Equilibrium desorption chromatography was employed to determine the molar ratio of DDM to LacS protein. Upon equilibration of LacS bound to the column matrix with buffer B containing $^{14}$C-DM (C320A/K373C) labeling was enhanced in the presence of sugars (galactosides) that are known to be substrates of LacS (33), whereas other sugars such as glucose and sucrose had no effect (Fig. 2A). This property was used to estimate the dissociation constant ($K_d$) for equilibrium galactose binding by determining the rate of labeling with and without ligand is shown in Fig. 2B. The data for the Triton X-100- and DDM-solubilized protein were very similar, yielding $K_d$ values for galactose binding of approximately 2 mM. Thus, both the spectroscopic and ligand binding measurements do not reveal significant differences between LacS in the Triton X-100 and DDM-solubilized state, and indicate that the detergent-solubilized LacS has retained its native conformation.

**FIG. 2.** Mal-ANS labeling of the purified LacS protein. The protein labeling reactions were performed as described under "Experimental Procedures." A, initial rate of Mal-ANS labeling in the absence and presence of 10 mM substrates (melibiose, galactose, and lactose) and non-substrates (glucose and sucrose). B, kinetics of Mal-ANS labeling of LacS purified in Triton X-100 (0.05%, □) or DDM (0.05%, ○). The solid lines represent hyperbola that fit the experimental data yielding $K_d$ values of 2.13 ± 0.29 and 1.74 ± 0.43 mM for galactose binding to the DDM and Triton X-100-solubilized LacS, respectively.

**FIG. 3.** DDM binding to LacS. Concentrations of LacS (bars) and $^{14}$C-DM (□) in the fractions eluting from the Q-Sepharose column; the buffers contained 0.05% (w/v) $^{14}$C-DM (−1 mM). As a negative control, the amount of $^{14}$C-DM was also determined in column fractions from an experiment without the addition of LacS protein (not shown), these values were on average 1.0 ± 0.04 mM. The amount of protein-associated DDM plotted against the protein concentration is shown in the inset. The line is a linear regression of the experimental data, from which a molar ratio of DDM to LacS of 197 ± 5 was calculated.
Hydrodynamic Properties of the LacS-DDM Complex—To determine the hydrodynamic properties of DDM-solubilized LacS, a combination of sedimentation equilibrium and velocity analytical ultracentrifugation was employed. Weight-average sedimentation coefficients of DDM-solubilized LacS were determined within the protein concentration range of 0.02–1.50 mg ml$^{-1}$ at 20 °C in buffer C. The weight-average sedimentation coefficient is a function of the protein concentration and converges to a value greater than 8 S at higher protein concentrations as depicted in Fig. 4, indicating that LacS-DDM undergoes reversible self-association with a defined stoichiometry. The experimental data were analyzed using non-linear least-squares curve-fitting procedures, assuming a monomer to dimer mode of association (Fig. 4, solid line). The observed sedimentation coefficients, $s_{obs}$, of the putative monomer and dimer are 5.6 ± 0.3 S and 8.9 ± 0.4 S, respectively, and the association constant of the dimerization is 5.4 ± 3.6 ml mg$^{-1}$. If indeed the experimental data can be described by a dimerization reaction then the sedimentation coefficients of the putative monomer and the dimer should relate as in Equation 5, assuming spherical symmetry for all species (9, 24).

$$s_2 = s_1(2)^{2/3}$$

(Eq 5)

Where $s_1$ and $s_2$ are the sedimentation coefficients of the monomer and dimer, respectively. Since the equilibrium desorption chromatography experiment (Fig. 3) showed that LacS binds similar amounts of DDM at protein concentrations that correspond to both the predominantly monomeric and dimeric form, the application of Equation 5 for this detergent protein system is justified. The sedimentation coefficient of the smallest associating species as determined from the least-squares fit of the data, 5.6 S, would, according to Equation 5, result in sedimentation coefficients of 8.9 and 14.2 S for the dimer and tetramer, respectively. The observed sedimentation coefficient of the highest associating species is thus in excellent agreement with that expected for reversible monomer to dimer self-association. Sedimentation equilibrium analytical ultracentrifugation was performed to confirm the monomer to dimer mode of association and monitor the shape contribution to the sedimentation velocity data.

After attainment of equilibrium, concentration distributions of three different LacS loading concentrations, at rotor speeds of 8,000, 10,000, and 12,000 rpm, respectively, were collected and used for further analysis (Fig. 5; only the data collected at 8,000 rpm are shown). The natural logarithm of the protein concentration exhibited a non-linear dependence on the square of the radius for each data set, which is indicative of heterogeneity. The data were then analyzed according to the method of multicomponent systems as described under “Experimental Procedures,” using 1.5 for $\delta$ as derived from the equilibrium desorption chromatography experiments (Fig. 3), and 0.814 for the partial specific volume ($\bar{\nu}$) of DDM (Ref. 11, and this study). To show that the LacS-DDM complex behaves as a single thermodynamic component, it needs to be demonstrated that the self-association reaction is reversible, that is, one should be able to describe the concentration distributions collected at different loading concentrations and speeds by a global equilibrium constant (25, 26). All nine data sets were analyzed simultaneously by global non-linear least-squares analysis assuming a single thermodynamic component. The results of this fit are shown in Fig. 5D (and Table I), represented by the solid lines through the data points, and the residuals as shown in Fig. 5, A–C. Fitting to models of increasing complexity or including a non-ideality factor did not result in significant improvement of the residuals. Therefore, the randomness of the residuals in the global analysis indicate that the LacS-DDM complex behaves as a single thermodynamic component with an apparent molecular mass for the protein moiety of 71.3 ± 2.3 kDa and a monomer-dimer equilibrium association constant of 4.4 ± 1.0 ml mg$^{-1}$.

Determination of the association state of LacS employing the method of multicomponent systems is based on two assump-
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<th>Method</th>
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<th>Association constant</th>
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<tr>
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*Within 95% confidence intervals.*

- Analyzed according to the method of multicomponent systems, see “Experimental Procedures.”
- Calculation based on the radius of a sphere with a volume obtained from the molar masses of LacS and associated DDM with their respective densities.
- Analyzed according to the method of density matching, see “Experimental Procedures.”
- ND, not determined.
- Deduced from the nucleotide sequence (15).
- Determined by a non-linear least squares fitting procedure of the experimental data, assuming a monomer to dimer mode of association.
- Approximations of the Stokes radii, using the Svedberg and Stokes equations, based on monomer molar masses and fitted sedimentation coefficients at 0.06 and 0.6 mg mL$^{-1}$.

**TABLE I**

Hydrodynamic properties of DDM-solubilized LacS

**FIG. 6.** The buoyant molar mass of DDM and the LacS-DDM complex as a function of the solvent density by varying the H$_2$O/D$_2$O ratio. DDM at 0.2 mg/ml in buffer C, uncorrected (▲) and corrected (△) for H-D exchange. Extrapolation of the open triangles (dotted line) and the intersection with the abscissa yields a density of 1.23 for DDM, LacS-DDM complex at an initial concentration of 0.050 mg/ml in buffer C, uncorrected (■) and corrected (□) for H-D exchange. From extrapolation of the open squares (dotted line) to the density of DDM (see arrow) a molar mass of 67.3 ± 35.0 kDa for LacS could be determined.

**Freeze-Fracture Electron Microscopy**—Having established that DDM-solubilized LacS undergoes self-association with a stoichiometry of monomer to dimer, freeze-fracture electron microscopy was employed to analyze the quaternary structure of LacS in the membrane-reconstituted state. A typical freeze-fracture image at low magnification of membrane reconstituted LacS is shown in Fig. 7A. This image shows several unilamellar concave and convex proteoliposomes with a similar morphology and diameter as monitored with cryo-transmission electron microscopy (27). In contrast to liposomes without LacS (data not shown), both the concave and convex proteoliposomes exhibit IMP's. The absence of complimentary pits in both the concave and convex proteoliposomes has been explained by the sublimation of frozen water into the apolar region where the protein has been removed upon fractionation (34). As a result of the curvature of the proteoliposomes, the angle of the tantalum shadowing varied significantly for IMP's within one pro-
teoliposome. This variation could result in a large error in both the cross-sectional area and the particle density determinations. To reduce this error, proteoliposomes were subjected to two cycles of rapid freezing in liquid nitrogen and slow thawing to allow proteoliposomes to fuse, resulting in about a 10-fold increase in proteoliposome diameter as shown in Fig. 7B. Images of proteoliposomes with a diameter greater than 2000 nm were used for further analysis. For particle density analysis, 1427 IMP's were counted in 15 different proteoliposomes, resulting in a particle density of $9.56 \pm 0.08$ particles/$\mu$m$^2$.

To determine the effect of a membraneous lipid environment on the molecular organization of LacS, freeze-fracture IMP's were further characterized. The diameter, perpendicular to the direction of shadowing, of 393 LacS IMP's were determined, plotted in a frequency histogram and fitted to a single Gaussian function (Equation 4) as depicted in Fig. 8A. The IMP's of LacS fell into a single size population with a mean diameter of $8.45 \pm 0.08$ nm ($n = 393$).

The IMP diameter were used to calculate the cross-sectional area, which corresponds to the number of transmembrane $\alpha$-helices and consequently to the quaternary structure. However, tantalium shadowing results in a deposition of metal onto the fractured face. As a consequence of this metal film, the experimentally determined IMP diameter will be larger than the diameter of the LacS transmembrane region. To determine the film thickness under these experimental conditions, the transmembrane domain of the reconstituted mannitol transport enzyme II (IICmtl) was used for calibration. The projection structure of IICmtl, as determined by cryo-electron crystallography, showed that the IICmtl consists of an asymmetric dimer with axis of about 40 and 90 Å (28). Based on these experimental dimensions the mean diameter corresponds to the length of the short axis (4.0 nm), plus half of the difference between the short and the long axis ((9.0 nm to 4.0 nm)/2), resulting in a mean diameter of 6.5 nm. Freeze-fracture images of 772 membrane reconstituted IICmtl molecules were obtained and analyzed under the same conditions as the LacS samples (Fig. 8B).

The IMP's of IIC fell into a single size population with a mean diameter of $8.52 \pm 0.08$ nm. Based on the difference of the mean diameters derived from the projection structure and that of the particle analysis of freeze-fracture images, it can be concluded that the metal film deposited on the fracture face is $2.02 \pm 0.08$ nm under these conditions. The mean diameter determined for the LacS IMP's can now be corrected for the metal film thickness, resulting in $6.43 \pm 0.08$ nm for the transmembrane region of LacS. From this mean diameter, the cross-sectional area can be calculated and with that the putative number of $\alpha$-helices (Table II). By using an average area of 1.40 nm$^2$/$\alpha$-helix, the LacS protein is predicted to have $23 \pm 1$ $\alpha$-helices which corresponds to a dimeric state for membrane-reconstituted LacS.
The sparsity of structural information of membrane transport proteins hampers the elucidation of their molecular mechanism(s). In the absence of high resolution structures, information on the quaternary structure contributes significantly toward defining the molecular organization of the functional unit. To obtain this information, LacS was solubilized using several non-ionic detergents and purified from the other membrane components. Spectral analysis, ligand binding assays, and hydrodynamic characterization of the highly purified LacS indicate that the protein is stable and functional in the detergent-solubilized state, irrespective of whether the protein is purified in DDM or Triton X-100. When OG is used to purify LacS, the protein aggregates and hardly any activity is recovered following membrane reconstitution.

The secondary structure of the carrier domain of LacS can be deduced from the CD spectra when the contributions from the IIA domain are accounted for. The x-ray structures of proteins homologous to the IIA domain of LacS are known (29), and these proteins consist of 10.5% α-helix, 34% β-sheet, and 55.5% remainder. If one uses these numbers together with the spectral analysis of the entire LacS protein to estimate the secondary structure of the carrier domain of LacS, then the fraction of α-helix increases to 75%. This is consistent with a protein domain that traverses the membrane 12 times in α-helical configuration and fits well with the predictions made on the basis of the hydropathy profile and the proposed membrane topology of the homologous MelB protein of E. coli (1, 30).

Since changes in protein fluorescence were not observed upon addition of lactose, galactose, or other substrates to detergent-solubilized LacS, an alternative method was devised to show that the detergent-solubilized protein was able to bind ligand. Previous work had shown that Cys-373 is in inter helix loop X-XI within 15 Å of the ligand-binding site, whereas this residue could be labeled without effecting transport (32). Here we show that labeling of Cys-373 with Mal-ANS can be used to probe ligand induced conformational changes. The C320A/K373C mutant is readily labeled by Mal-ANS, whereas C320A is not. The labeling of C320A/K373C is accelerated by the presence of substrate, and the concentration dependence of the acceleration was used to estimate the \( K_d \) of galactose binding of Triton X-100 and DDM-solubilized LacS. Besides demonstrating that the detergent-solubilized protein is in a functional state, the method offers an important tool to study, for instance, LacS mutants that are defective in transport but capable of binding ligands. Moreover, one does not require high affinity ligands to follow the binding kinetics. A similar strategy has been used for the LacY protein of E. coli, to probe ligand-induced conformational changes (35).

The spectral analysis and functional assays showed that the detergent-solubilized LacS is stable, isotropic, and exhibits ligand binding properties similar to those determined in the membrane (31). Therefore, the detergent-solubilized LacS can confidently be considered undenatured, and structural information obtained from these samples should report on the molecular organization of the protein in its native environment.

### DISCUSSION

Hydrodynamic characterization of the DDM-solubilized LacS showed that the sample behaves as a single thermodynamic species and undergoes reversible self-association with a monomer to dimer stoichiometry. The association constant as determined with sedimentation velocity and equilibrium centrifugation experiments are similar, that is 5.4 ± 3.6 and 4.4 ± 1.0 ml mg⁻¹, respectively. This consistency indicates that the dependence of the sedimentation coefficient on the protein concentration (Fig. 4) is predominantly affected by the reversible self-association reaction and not by factors such as shape and/or detergent binding (\( \delta \)) of the respective monomer and dimer. This result is consistent with the similar experimentally determined \( R_s / R_{s,min} \) values (see Table I for details), and DDM binding characteristics of the LacS-DDM monomer and dimer. The \( R_s / R_{s,min} \) values provide, in principle, information on the symmetry and hydration of the LacS-DDM complex and are similar to those reported for Ca⁺⁺-ATPase, mammalian rhodopsin, and photosynthetic reaction centers, all solubilized with Triton X-100, which have \( R_s / R_{s,min} \) values of 1.40, 1.54, and 1.32, respectively (36). The physical reality of the experimentally determined Stokes radius of the LacS-DDM monomer can be checked on the assumption that the 12-transmembrane α-helices form a compact transmembrane segment with a diameter of approximately 40 Å, and DDM detergent molecules that extend 30 Å perpendicular from the transmembrane segment of LacS (31, 17), resulting in a radius of approximately 50 Å. An estimate of the height of LacS, perpendicular to the normal of the membrane, includes approximate values for the membrane thickness, 40 Å, twice the hydrophilic extensions of the transmembrane helices, approximately 20 Å, and the diameter of the IIA domain based on the crystal structure of a homologous protein, 30 Å (29) resulting in a radius of 55 Å. This approximation of the molecular dimension of the monomeric LacS-DDM is consistent with the experimentally determined Stokes radius of 52 Å.

The quaternary structure of membrane-reconstituted LacS could be derived from the estimation of the transmembrane domain surface area of LacS particles observed in freeze-fracture images (12). The correlation between the transmembrane domain surface area and the quaternary structure is validated only if the transmembrane domain of LacS is constituted predominantly of α-helices (12), which is confirmed by the CD measurements (Fig. 1) and hydropathy analysis (1). The mean diameter of the LacS IMP’s corresponds to a dimeric LacS. These results are consistent with a recent study of the oligomeric structure of LacS employing saturation-transfer electron spin resonance spectroscopy (31). The results indicated that LacS reconstituted into lipid bilayers at lipid-to-protein ratio’s between 26 and 400 (w/w) is larger than a monomer.

Equilibrium sedimentation analysis indicates that 50% of LacS is monomeric at a protein concentration of 0.22 mg/ml. Freeze-fracture particle analysis, on the other hand, indicated that most of the LacS is dimeric. The concentration of LacS in the membrane, at lipid-to-protein ratio of 400 (w/w) is much higher than 0.22 mg/ml when calculated on the basis of the volume (“two-dimensional solution”) of the bilayer. For a pro-

### TABLE II

Freeze-fracture particle analysis of LacS

<table>
<thead>
<tr>
<th>Density (IMP/μm²⁻²)</th>
<th>Diameterᵃ</th>
<th>Areaᵇ</th>
<th>Number of α-helicesᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacS, L/P = 400</td>
<td>95 ± 8 (n = 1427)</td>
<td>6.40 ± 0.08 (N = 393)</td>
<td>32 ± 1</td>
</tr>
</tbody>
</table>

ᵃ Diameters were corrected for the thickness of the tantalium-carbon film by subtracting 2.02 ± 0.08 nm from the mean diameters obtained from the frequency histograms.

ᵇ Cross-sectional area was obtained by assuming a circular geometry for the LacS particles.

ᶜ Predicted number of helices was calculated by using 1.40 ± 0.03 nm²/helix (12). Values are mean ± S.E.
teoliposome with a diameter of 200 nm and a lipid to protein ratio of 400 (w/w), the “concentration in the membrane” is estimated to be 5–10 mg/ml. Moreover, comparison of self-association reactions of integral membrane proteins in the detergent-solubilized and the membrane-reconstituted state need to take into account the net effect of localization, orientational restriction, and volume exclusion, which enhance the self-association of proteins in membranes (8). At the protein to lipid ratios used here, the effect of volume exclusion is minimal. The largest contribution to the enhancement of self-association of proteins in membranes originates from the restriction in translational mobility, which manifests itself as an increased local concentration (8). Based on the restriction in translational mobility in a model system, an enhancement factor of 4300 times was calculated for a dimerization reaction in pelleted membranes, relative to the same dimerization reaction and the same average concentration in solution (8). Given these considerations, membrane-reconstituted LacS is expected to be dimeric even at lipid to protein ratios as high as 400 (w/w).

In this study we showed that LacS undergoes reversible self-association with a stoichiometry of monomer to dimer. The relation between this structural feature and the activity of LacS can be described by two models. In the first one, the active and inactive states of the protein are represented by the dimer and the monomer, respectively. In this model, transport activity can be regulated by conditions favoring LacS self-association, for instance through ligand and/or effector binding or increasing the LacS concentration. This model also allows for a LacS dimer in which the translocation pathway is constituted by both monomers, and, consequently, association of both monomers is a prerequisite for transport activity. In the second model, LacS can reversibly equilibrate between between a monomeric and a dimeric state which both are active. The fluorimetric assay showed that the detergent-solubilized LacS is able to bind ligand at LacS concentrations under which LacS is predominantly monomeric. This observation favors model two, assuming ligand binding is correlated directly to transport activity. More experiments are needed to quantitate the thermodynamic linkages between LacS dimerization, ligand, and/or effector binding and transport activity.

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