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Size and Shape of the *Escherichia coli* Lactose Permease Measured in Filamentous Arrays

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ABSTRACT: The *Escherichia coli* lactose permease has been purified on cation exchanger to contain a minimal amount of phospholipids, i.e., 4–5 mol/mol of permease, in the presence of the detergent dodecyl β-maltoside at its critical micelle concentration. This preparation is active in galactoside binding. When the detergent level is further reduced by dialysis, the lactose permease forms filaments one molecule wide and up to several micrometers long. The filaments tend to associate laterally to form sheets. Analysis of electron micrographs of negatively stained filamentous arrays indicates an average filament spacing of 51 Å and a subunit period of 26–30 Å along individual filaments. These values most probably correspond to the dimensions of the lactose permease molecule measured parallel to the membrane plane. In many filaments, the subunits show a stain-penetrated cleft. It suggests that the lactose permease molecule comprises two domains, which may be correlated with internal repeats between the N- and C-terminal halves of the polypeptide sequence.

The lactose permease is an integral protein of the *Escherichia coli* cytoplasmic membrane and has a molecular weight of 46 500. It catalyzes the cotransport of 1 mol of proton with 1 mol of galactoside across the membrane (Kaback, 1983; Overath & Wright, 1983). Therefore, at the expense of the electrochemical gradient of protons generated by the cell's metabolic activities, the cell can accumulate the sugar against a concentration gradient (Mitchell, 1968).

The *lac y* gene coding for the permease has been cloned into a multicopy plasmid (Teather et al., 1978), allowing the gene

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sequence and hence the polypeptide sequence to be determined (Büchel et al., 1980). Of 417 residues, 71% were found to consist of nonpolar amino acids, making the lactose permease one of the most hydrophobic proteins known. The plasmid-carrying strain T206 overproduces the permease, which accumulates in the cytoplasmic membrane to reach 15% of the total membrane protein (Teather et al., 1980). From such cells, the lactose permease has been solubilized in detergent, either octyl β-glucoside (Newman & Wilson, 1980) or dodecyl β-maltoside (Wright et al., 1982), and purified to homogeneity with respect to the protein (Newman et al., 1981; Wright & Overath, 1984). The solubilized lactose permease has been reincorporated into phospholipid vesicles and shown to catalyze proton–galactoside symport with similar kinetic constants to those in the native membrane [see reviews by Kaback (1983) and Overath and Wright (1983)].

Both the N- and C-termini of the polypeptide have been localized by specific antibodies to the cytoplasmic surface of the membrane (Seckler et al., 1983; K. Beyreuter, unpublished results), while two other sites recognized by distinct monoclonals were localized to the periplasmic surface (Carrasco et al., 1982; Herzlinger et al., 1984). Therefore, the lactose permease spans the membrane and traverses it an even number of times. Circular dichroic spectra of the lactose permease in octyl β-glucoside micelles and in proteoliposomes indicate that 85% of the amino acid residues are in the α-helical conformation (Foster et al., 1983). On the basis of this evidence and the hydropathy profile (Kyte & Doolittle, 1982) of the sequence, Foster et al. (1983) proposed a model of 12 transmembrane helices. Raman spectroscopy confirmed the high helical content, at about 70% (Vogel et al., 1985). This figure and considerations of the amphiphilic character of the sequence led Vogel et al. to propose a model comprised of 10 transmembrane helices and 4 other transmembrane segments which may or may not be helical. These attempts at determining the arrangement of the lactose permease in the membrane highlight the need to visualize the three-dimensional structure. However, direct observation of the lactose permease molecule is so far limited to the visualization of intramembrane particles of freeze-fractured proteoliposomes (Costello et al., 1984).

An impediment to structural examination of the lactose permease molecule is the presence, in the previously obtained preparations, of large amounts of phospholipids and detergents. They hinder any attempts to use X-ray diffraction or electron microscopic methods to observe the structure directly and prevent the specific interactions between protein molecules required to form ordered arrays which would be even more suitable for molecular structural determination by X-ray diffraction or electron microscopy. In this study, we introduced a positive purification step involving binding of the lactose permease to a cation exchanger in order to minimize the amount of associated lipid and detergent. From this state, the lactose permease molecules have been induced, by further reduction of the detergent level, to form arrays of filaments lying side by side. Examination of these arrays by electron microscopy determined the dimensions of the lactose permease molecule and revealed that this molecule comprises two domains separated by a cleft.

Experimental Procedures

Materials. The *E. coli* strain T206 which carries the *lac Y* gene on a multicopy plasmid (Teather et al., 1980) was a kind gift from Dr. Peter Overath. Crude *E. coli* lipids purchased from Avanti Biochemicals were washed with acetone–ether according to Newman and Wilson (1980) to obtain the phospholipids, which were stored at 100 mg/mL in benzene at −20 °C. Dodecyl β-maltoside (DM)[1] was purchased from Calbiochem, Ecteola-23 from Fluka, and CM-52 from Whatman. [^{3}H]NPG (8 μCi/μmol) was prepared by the procedure of Kennedy et al. (1974). [^{14}C]DMOAc (27.9 μCi/μmol) was prepared according to Gaylor and Delwiche (1966) using pyridine as the solvent during acetylation.

Initial Purification of Lactose Permease. The lactose permease was partially purified from cytoplasmic membranes of *E. coli* T206 cells by extraction into DM and chromatography on Ecteola using previously described procedures (Wright et al., 1983a; Wright & Overath, 1984) with minor modifications: during preextraction of the membranes with cholate and 5-sulfosalicylate (Wright & Overath, 1984) sonication was limited to 30 s to reduce protein loss; the Ecteola column buffer consists of 10 mM triethanolamine, 0.1 mM EDTA, 0.02% NaN_3, and 1 mg/mL DM. Column fractions containing lactose permease were identified by SDS–polyacrylamide gel electrophoresis.

CM-52 Chromatography. CM-52 was preequilibrated with 20 mM MES–K^+ buffer, pH 6.2, containing 0.1 mM EDTA and 0.02% NaN_3 (MEA6.2). A 1 × 12 cm column was prepared and equilibrated at a flow rate of 0.3–0.4 mL/min with 2 volumes of MEA6.2, followed by 2–5 volumes of MEA6.2 plus DM, which was at its critical micelle concentration of 0.1 mg/mL (De Grip & Bovee-Geurts, 1977) unless otherwise stated. The pooled lactose permease fractions from the Ecteola column were dialyzed overnight at 4 °C against 100 times the sample volume of MEA6.2, and brought to room temperature before being loaded onto the column. Up to 40 mL at a maximum OD_{280} of 0.4 was loaded for each run. Unbound protein was eluted with MEA6.2 plus DM until the OD_{280} returned to the base line, and bound protein was eluted by a step of 250 mM KCl, or by a gradient of 0–250 mM KCl in the MEA6.2 plus DM. The KCl concentration at which the bound proteins were eluted was estimated from the conductivity of the column fraction, calibrated by the conductivity of KCl solutions in MEA6.2.

Determination of Protein, Phospholipid, and Detergent Concentrations. Protein was determined according to Peterson (1977), using BSA as standard and applying a correction factor (Wright & Overath, 1984) of 1.3 mg of BSA/mg of lactose permease. On the basis of the protein assay, the absorption coefficient of the lactose permease sample eluted from the CM-52 column was evaluated to be 1.05 OD_{280} units per 1 mg/mL. Phospholipids were determined by the assay of Ames (1966). The DM concentration was calculated from the radioactivity of [^{14}C]DMOAc (Wright et al., 1983b; also see Materials) which was present as a marker for DM throughout the preparation at $^1/_{12000}$th mole fraction of the DM.

Reconstitution into Phospholipid Vesicles. Five milligrams of washed *E. coli* phospholipids (see Materials) was dried under N_2 to a thin film in a glass test tube and redispersed in 100 μL of a 50 mg/mL solution of DM in H_2O by stirring with a glass rod and sonication in a bath-type sonicator. One milligram of lactose permease eluted from the Ecteola column, or from the CM-52 column, was diluted to 0.2 mg/mL in the

[1] Abbreviations: BSA, bovine serum albumin; CMC, critical micelle concentration; DM, dodecyl β-maltoside; [^{14}C]DMOAc, dodecyl β-maltoside [1-^{14}C]acetate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; KP_i, potassium phosphate; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MEA6.2, 20 mM MES, 0.1 mM EDTA, and 0.02% NaN_3, pH 6.2; [^{3}H]NPG, 4-nitrophenyl-α-[6′-^{3}H]galactopyranose; pCMBS, *p*-(chloromercuri)benzenesulfonate; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography.

column buffer, and 1 mM DTT, 10 mM EDTA, and 12% (w/v) glycerol were added. The lactose permease was mixed into the phospholipids and detergent by stirring with the rod and sonication for 30 s to 5 min, until the mixture was clear. After another 30 min of incubation at room temperature, 1 g of washed BioBeads SM-2 (Holloway, 1973) was added and gently tumbled with the sample at 4 °C overnight. The beads were removed by filtration through a plug of glass wool in a syringe. From the turbid filtrate, vesicles were collected by centrifugation at 200000*g* for 90 min and resuspended in 0.5 M KP_i (pH 7.0) for the NPG binding assay by microequilibrium dialysis.

NPG Binding Assay in the Airfuge Pellet. The permease content of isolated cytoplasmic membrane fragments was evaluated by measuring the binding of [^{3}H]NPG (see Materials) as follows: 10 μL of a membrane suspension in the Ecteola column buffer at about 10 mg of protein/mL was incubated at room temperature with 150 μL of 6.4 μM [^{3}H]NPG dissolved in 0.5 M KP_i (pH 7.0). After 15 min, the membranes were pelleted in the Beckman airfuge operated at 35 psi for 20 min. Aliquots of the supernatant were counted to determine the concentration of the free ligand in equilibrium with the membranes, and the entire pellet was counted to determine the bound NPG per milligram of membrane protein. Nonspecific binding was subtracted by counting a control pellet of membranes incubated in the presence of 400 μM pCMBS, an inhibitor of the lactose permease. The number of available binding sites was calculated from the nanomoles of bound NPG per milligram of protein, using the experimentally determined dissociation constant K_s in the relationship

$$\text{nmol of sites/mg} = (\text{nmol bound/mg})(1 + K_s/[\text{NPG}]_{\text{free}})$$

Determination of the Dissociation Constant for NPG from Lactose Permease in the Cytoplasmic Membrane. NPG binding was measured in airfuge pellets obtained after incubating the membranes with NPG at six different concentrations between 2 and 34 μM. The dissociation constant was given by the reciprocal of the slope of a Scatchard plot of bound NPG per milligrams of protein divided by the free NPG concentration vs. free NPG concentration. This determination was repeated for the membrane-bound lactose permease under two different ionic conditions, namely, in 0.5 M KP_i (pH 7.0) and in the Ecteola column buffer.

NPG Binding Assay by Microequilibrium Dialysis. The NPG binding activity of the purified lactose permease was assayed before and after reconstitution into phospholipid vesicles by equilibrium dialysis. The microequilibrium dialysis cell was constructed by modifying the design of Englund et al. (1969) and consisted of a pair of 60-μL chambers separated by SepctraPor-2 membrane (12000–14000 molecular weight cutoff), with a bleed hole added to each chamber to facilitate filling. One chamber was filled with the lactose permease sample and the other with a solution of 6.4 μM [^{3}H]NPG in 0.5 M KP_i (pH 7.0), with or without the inhibitor pCMBS at 400 μM. A glass stirring bead was included in each chamber, and the sealed cell was mounted on a wheel revolving at 5 rpm. Equilibrium was attained in 3 h at 20 °C, as established from the equalization of counts between a control pair of chambers from which the lactose permease was omitted; 45-μL aliquots were withdrawn from each chamber to determine the concentrations of free and bound NPG. Nonspecific binding, measured in the presence of pCMBS, was at the background level and therefore ignored.

SDS–Polyacrylamide Gel Electrophoresis. Slab gels (9 cm × 9 cm × 0.4 mm) containing 12% acrylamide in the separating gel and 3% in the stacking gel (1 cm) were prepared by using the buffer system of Laemmli (1970). Samples for electrophoresis were mixed with an equal volume of 6% SDS, 200 mM DTT, 0.125 M Tris-HCl (pH 6.8), 10% glycerol, and 0.004% bromophenol blue. Solubilization was assisted by sonication in the bath sonicator for 30 s. After electrophoresis, the gel was stained with Coomassie blue, or with silver by the method of Wray et al. (1981), and dried between Bio-Rad cellophane membranes. Relative amounts of protein in the bands were estimated by densitometry of the silver-stained gel.

Preparation of Filaments. Either the pooled fractions of lactose permease eluted by KCl from the CM-52 column or selected fractions (see Results) were used to prepare the filaments. Detergent was slowly removed from the sample by dialysis at 4 °C across a SpectraPor-2 membrane against a gently stirred reservoir 200 times the sample volume. The reservoir pH was from 6 to 8, buffered by MES, triethanolamine, or Tris; 5–10% PEG 20000 may be used in the dialysis to concentrate the protein, before, during, or after detergent reduction. In addition, the reservoir contained 0.1 mM EDTA, 0.02% NaN_3, and 50 mM KCl or 100 mM NH_4Cl. Filament formation was discernible from the opalescence of the sample and was confirmed by electron microscopy.

Electron Microscopy and Image Analysis. Samples of filaments were adsorbed to glow-discharged carbon grids, negatively stained with 1% uranyl formate or acetate, or 2% phosphotungstate (pH 6.0), and examined in a Phillips 420 electron microscope operated at 80 kV. Images were taken at 60000× magnification. Areas of the micrographs were densitometered at 20-μm steps, corresponding to 3.3-Å sampling intervals at the specimen. The digitized image was displayed on an AED 676 graphics terminal, and selected areas of filaments were boxed off for Fourier transformation. In some instances, before Fourier transformation the filaments were straightened by spline fitting a curve to the long axis of a filament and reinterpolating the image area so as to straighten this curve (Egelman, 1986).

RESULTS

Purification by CM-52 Chromatography. The sample of lactose permease after elution from Ecteola typically contained protein at 0.16 mg/mL, phospholipids at 0.28 mM, and DM at 2.9 mg/mL. Using an average molecular weight of 653 for *E. coli* phospholipids (Ingraham et al., 1983), the protein:lipid:detergent mass ratio was 1:1.2:18 before CM-52 chromatography. The silver-stained SDS gel (Figure 1, lane 1) indicates that the lactose permease accounted for 70% of the protein. The maximum concentration of lactose permease was estimated to be 3.4 μM ignoring the contaminants. It follows that the phospholipids were present at about 80-fold molar excess relative to the lactose permease. Likewise, the sample contained DM at a higher concentration than that necessary to maintain the lactose permease in solution. That minimal DM concentration would include the 1.3 mg of bound DM/mg of lactose permease (Wright et al., 1983b) plus the free DM which must be above its CMC of 0.1 mg/mL (De Grip & Bovee-Guerts, 1977). For a protein concentration of 0.16 mg/mL, the minimal DM concentration can be estimated to be 0.3 mg/mL, which is a tenth of the DM concentration present.

Chromatography on the CM-52 removed one-third of the protein contaminants, as well as separating the lactose permease from the excess phospholipids and detergent. The silver-stained SDS gel of column fractions (Figure 1) shows clearly that over 85% of the lactose permease was bound to the cation exchanger in 20 mM MES buffer (pH 6.2) containing 0.1 mg/mL DM. The bound lactose permease can be

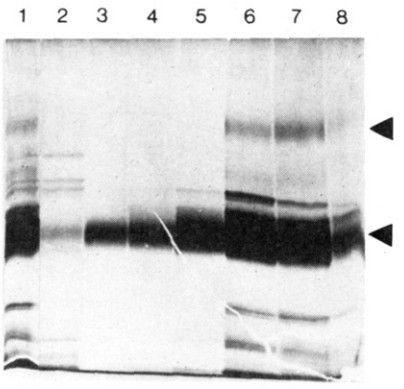

FIGURE 1: SDS gel electrophoresis of the lactose permease preparation before and after purification on CM-52. Electrophoresis and silver staining were carried out as described under Experimental Procedures. Fifteen microliters from each column fraction was loaded on the gel without prior concentration. Lane 1, the starting sample obtained from the Ecteola column; lane 2, the unbound proteins eluted from CM-52; lanes 3–8, the bound proteins, eluted by the addition of 50 mM KCl (lane 3) followed by 250 mM KCl (lanes 4–8). Arrows indicate the diffuse bands due to the monomer and dimer of the lactose permease. Note that certain contaminant bands in the starting sample were quantitatively eluted in the unbound fraction, while most of the lactose permease was obtained in the bound fractions.

eluted from the column by addition of KCl at 50–140 mM. The fractions eluted at 50–70 mM KCl (lane 3, Figure 1) contained lactose permease as the only protein component, which appears as a diffuse band of M_r 33 000 on the gel. Of the applied lactose permease, 2% was recovered in these fractions. Another 52% was recovered in the fractions eluted at the higher ionic strength. The latter fractions contained minor impurities that were also bound to the column. At the highest ionic strength required to elute the lactose permease, the gel showed additional diffuse bands at higher molecular weights, evidence of a small degree of aggregation (dimerization and higher polymers) of the lactose permease. Densitometry of the gel indicated that, when aggregation was detected in a fraction, the aggregated material did not exceed 6–11% of the lactose permease in that fraction, and the aggregated material from all these fractions amounted to less than 3% of the lactose permease applied.

Results of phosphate determination on the column fractions indicate that in 20 mM MES (pH 6.2) with 0.1 mg/mL DM, 84–89% of the phospholipids in the sample were removed with the unbound proteins. The concentration of phospholipids in the bound protein fractions amounted to 4–5 mol/mol of lactose permease.

Excess DM was also removed with the unbound protein. Figure 2 plots the DM concentration together with the OD_{280} of the column fractions. The DM can be seen to be eluted in two peaks, a large peak coinciding with the unbound proteins and a smaller one with the bound protein. Furthermore, the DM concentration in the bound protein peak can be accounted for as the sum of a protein-associated component, estimated at 1.3 times the concentration of eluted proteins, and a free component at the concentration of DM in the column buffer. This was found to be true in each case whether the column was equilibrated and run at 0.1 mg/mL DM, as illustrated in Figure 2, or at 0.2 or 1.0 mg/mL DM. Thus, by running the column at the CMC of DM, the detergent eluted with the lactose permease was reduced to the minimum level. The final protein:lipid:DM mass ratio was 1:0.1:1.8, giving approximately a 10-fold reduction in both lipid and detergent.

In experiments where the lactose permease was solubilized in DM containing a trace of [^{14}C]DMOAc, but chromatographed on the CM-52 column in nonradioactive detergents,

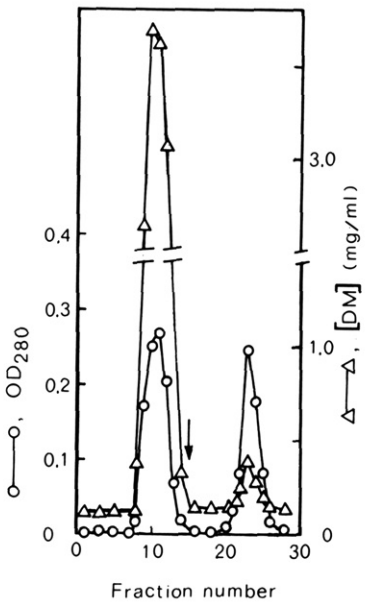

FIGURE 2: Removal of excess detergent from the lactose permease preparation by chromatography on a CM-52 column preequilibrated with the detergent DM at its CMC of 0.1 mg/mL in MEA6.2. Excess DM in the starting sample was eluted in a peak with the unbound proteins (fractions 8–14). The bound proteins, consisting mainly of lactose permease (see Figure 1), were eluted by the addition of 250 mM KCl (arrow) and so was the protein-bound detergent. The DM concentration in the bound protein peak (fractions 20–26) was equal to 1.3 mg/mg of the lactose permease present plus the 0.1 mg/mL contained in the column buffer.

all of the radioactivity in the sample was eluted in the unbound protein fractions (data not shown). This observation taken together with the kind of results shown in Figure 2 demonstrates that the DM in the micelles surrounding the column-bound lactose permease was exchanging with the free detergent in the column. This exchange allowed the column-bound lactose permease to remain in a solubilized state and be eluted fully solubilized when the free detergent level was reduced to the CMC.

Galactoside Binding Activity. The galactoside binding activity was measured with [^{3}H]NPG as the substrate. Two disparate dissociation constants for NPG (K_s) have been reported previously. Kennedy et al. (1974) found it to be about 7 μM from measurements in the membrane pellets obtained after incubation with the radioactive sugar, whereas Overath et al. (1979) found it to be 22 μM from flow dialysis assays. We reinvestigated the dissociation constant by measurements in the airfuge pellet. Our procedure is similar to that of Kennedy et al. (1974) except that nonspecific counts were corrected for by inhibiting the permease with pCMBS. We obtained from Scatchard plots a dissociation constant of 8.8 μM (correlation coefficient = 94.5%) in 0.5 M KP_i (pH 7.0) and 25 μM (correlation coefficient = 97.0%) in the Ecteola column buffer. The former result is in close agreement with Kennedy et al. (1974) and the latter with Overath et al. (1979). Hence, the difference in the diossociaton constants can be attributed to the differences in the ionic conditions during assay. Thereafter, our binding assays were carried out in 0.5 M KP_i (pH 7.0), and the number of binding sites was calculated by using K_s = 8.8 μM. According to the airfuge procedure, the T206 cytoplasmic membranes were found to contain 2–3 nmol of NPG binding sites/mg of protein, in agreement with the results of Teather et al. (1980).

The galactoside binding activity of purified lactose permease was measured by equilibrium dialysis assay of reconstituted proteoliposomes containing of protein and lipid at a weight

ratio of 1:5. When identical reconstitution procedures were used, proteoliposomes containing the lactose permease eluted from the Ecteola column showed 3.8 ± 0.2 nmol of NPG binding sites/mg of protein, and those containing the permease eluted from the CM-52 column showed 4.0 ± 0.5 nmol of NPG binding sites/mg of protein. Therefore, there was no loss of galactoside binding activity associated with the reduction, achieved by CM-52 chromatography, of phospholipid and detergent levels in the lactose permease preparation. The same level of NPG binding was also found in equilibrium dialysis assay of the solubilized lactose permease after the CM-52 step, but the value is indeterminate for the solubilized lactose permease before the CM-52 step, because it aggregated during dialysis in 0.5 M KP_i.

Compared to the number of NPG binding sites in the cytoplasmic membrane vesicles measured by the airfuge assay, the column-purified lactose permease in the reconstituted vesicles showed a 2-fold increase of binding activity when expressed per milligram of protein. This is inconsistent with the 5–8-fold enrichment of the lactose permease observed by gel electrophoresis, but the reason for this is not clear. It is not due to aggregation of the lactose permease, because the degree of initial aggregation detected by gel electrophoresis was insignificant, and the CM-52-purified samples can be stored for 1 month at 4 °C or for at least 10 days at room temperature with no aggregation detectable by electron microscopy.

Formation of Filaments. When the CM-52-purified lactose permease was dialyzed at 4 °C against the column buffer MEA6.2 without added detergent, an opalescence developed in 3–12 h depending on the protein concentration which was from 0.1 to 0.8 mg/mL. The light-scattering material was found by electron microscopy to be aggregates of filaments.

In further experiments, three conditions were found to be necessary for filament formation, namely, reduction of the free DM concentration to below the CMC, a protein concentration above 0.1 mg/mL, and low temperature (4 °C) during these operations. Low temperature was essential, as either concentrating the protein or removing the detergent at room temperature led to dense, amorphous precipitates suggestive of protein denaturation. However, the order and manner in which the first two conditions were attained were not critical. For example, the following procedures all led to filament formation. In the first, the sample was alternatively concentrated against solid PEG 20000 across a dialysis bag for periods up to 30 min at a time and dialyzed against the MEA6.2 for an accumulated dialysis time of 20 h. In the second, the sample was dialyzed for 3 h against MEA6.2 containing both 5–10% PEG 20000 and 0.1 mg/mL DM to increase the protein concentration and then dialyzed for 30 h against MEA6.2 to lower the DM concentration. In the third, the sample was dialyzed overnight against MEA6.2 to remove DM and then against MEA6.2 plus 10% PEG 20000 for 30 min to bring the protein concentration to the threshold. We have estimated from pilot experiments using [^{14}C]DMOAc as marker for DM that the $t_{1/2}$ for dialyzing out monomeric DM is about 10 h under the conditions used to prepare filaments. Therefore, the dialysis regime in these procedures can be expected to reduce the free DM level in the CM-52-purified lactose permease sample to below the CMC, and consequently to reduce the amount of bound DM per molecule of lactose permease.

When the necessary conditions for filament formation were satisfied, the addition of 50 mM KCl or 100 mM NH_4Cl in the dialysis reservoir resulted in filaments that were straighter and had a tendency to associate laterally into parallel arrays. Most strikingly, if instead of the pooled lactose permease fractions from the CM-52 column, only the fractions eluted at 50–70 mM KCl, which were of higher purity and contained lactose permease as the only protein component (see Figure 1, lane 3), were used to prepare filaments, then very long filaments, over 2 μM, were obtained. By contrast, the filaments prepared from the fractions eluted at the highest KCl concentration, which contained 30% impurities (see Figure 1, lane 8), were short and stubby, indicating an inhibition of filament elongation. Therefore, the filaments appear to arise from linear addition of lactose permease molecules.

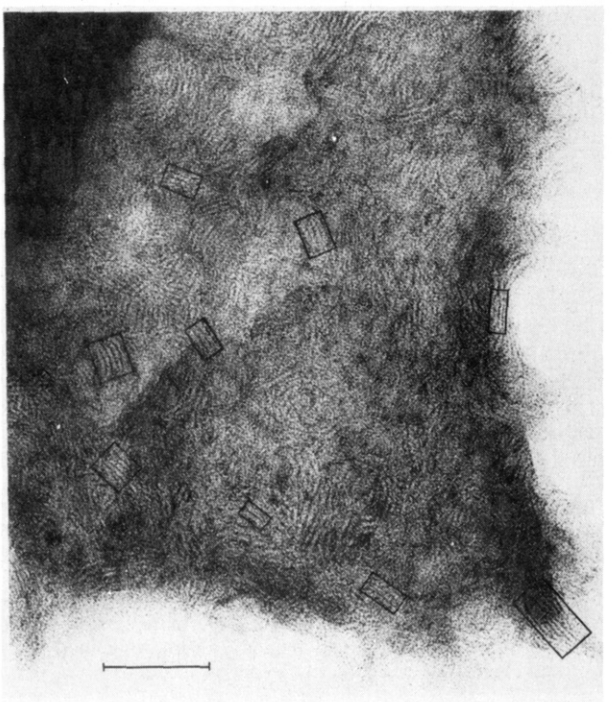

FIGURE 3: Electron micrograph of a sheet formed by the side-to-side association of lactose permease filaments. The filaments have been negatively stained with uranyl formate. Areas of the sheet showing two-dimensional order are indicated by boxes. Note that the subunits in many filaments display a stain-penetrated cleft oriented parallel to the filament axis. Scale bar: 1000 Å.

Electron Microscopy and Image Analysis. In electron micrographs (Figure 3), stain accumulation between the filaments gave areas of parallel-packed filaments a grooved appearance. To a lesser degree, the uranyl stains were also present between subunits within the filament, giving rise to a cross-striation. With both the uranyl stains and phosphotungstate, the subunits in many filaments showed a stain-penetrated cleft which was nearly parallel to the filament axis, so the subunits appeared bilobate and the filament double stranded. The staining of the cleft was more prominent than the cross-striation. As these filaments associated laterally forming sheets, two-dimensional order was present in small areas of the sheet, about 300 Å on a side. First-order optical diffraction spots have been observed from such filamentous arrays, at about 50-Å spacing on the equator due to the lateral spacing between filaments, and at about 30-Å spacing in the near-axial direction due to the row spacing between subunits in adjacent filaments.

Twenty-two image areas of various filament specimens (Figure 4) were digitized for Fourier transformation. Each computed transform showed 1 or 2 orders of equatorial spots, giving a total of 34 measurements of the interfilament spacing. These fell into 3 sets as follows: in 14 arrays of close-packed filaments found in the center of the sheets, the interfilament

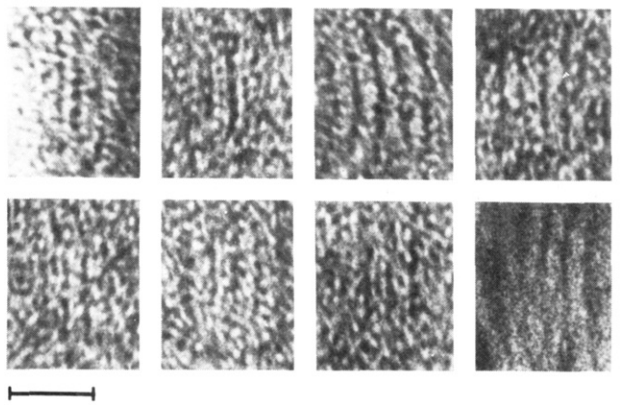

FIGURE 4: Selection of the small two-dimensional arrays of lactose permease filaments lying side by side. Fourier transforms were computed from 22 such image areas, in some cases after straightening the filaments. The transforms indicate an interfilament spacing of 50.7 ● 1.6 Å and an average subunit repeat along the filament axis of 28.4 ± 1.6 Å. In the images, the stain-penetrated cleft can be seen to bisect the subunit area. Scale bar: 200 Å.

spacing was 50.7 ● 1.6 Å (n = 23); in 5 arrays taken from the edge of the sheets, this spacing was 42.9 ± 1.9 Å (n = 6); and in 3 arrays of rather loosely packed filaments formed in the absence of added KCl or NH_4Cl, it was 59.5 ● 2.1 Å (n = 5). We take the spacing of close-packed filaments in the center of the sheets, 50.7 ± 1.6 Å, to be most characteristic of the width of the filament. The smaller spacing between filaments at the edge of the sheets may be due to the filaments being slightly turned on the side or distorted during drying on the specimen grid.

The computed transforms from 15 of the image areas showed a more or less axial spot that was clearly above the background. The relative weakness of this near-axial spot compared to the equatorial is expected because less stain accumulated between subunits within the filaments than between filaments. For different arrays, the angle between the direction of the near-axial spot and the equator varied in the range of 67–86°. Dividing the spacing of the near-axial spot by the sine of this angle, one obtains the repeat distance per subunit along the filament axis. The average of all these values was 29.0 ● 1.8 Å (n = 15). When data from filaments at the edge of sheets, which may have been distorted, were excluded, the average decreased very slightly, to 28.4 ± 1.6 Å (n = 12), and this was taken to indicate the subunit dimension in the axial direction.

In 17 out of the 22 image areas processed, the cleft was notable in every subunit, although the size of the cleft cannot be determined from our data which only extended to 25-Å resolution.

Discussion

The large amounts of lipids and detergent in the previously obtained preparations of the lactose permease came about because the preparation procedures (Newman et al., 1981; Wright & Overath, 1984) consisted of negative purification steps designed to yield the lactose permease in the unbound column fractions, into which the large amount of detergent required for dissolution of the membrane and the solubilized membrane lipids were also collected. For example, using the procedure of Wright and Overath (1984) up to the Ecteola step, we obtained the lactose permease with roughly a 10-fold excess of phospholipids and the detergent DM (see Results). Similarly, the procedure of Newman et al. (1981) calls for the addition of extra phospholipids with the octyl β-glucoside, to a protein:lipid:detergent mass ratio of 1:3:10.

Removal of the excess lipid and detergent is a prerequisite to structural examination of the lactose permease molecule, because they present a barrier to the formation of ordered arrays. We found that dialyzing out DM from the Ecteola-purified lactose permease sample resulted in an aggregation which was in fact lipid vesicle formation (data not shown). This is a common occurrence among lipid-rich preparations of membrane proteins (Furth, 1980). Moreover, the excess lipids contributed charge heterogeneity among the lactose permease–lipid–detergent mixed micelles. Hence, when the DM-solubilized lactose permease sample eluted from the Ecteola column was applied onto a DEAE-5PW (HPLC) column, it resolved into distinct peaks, among which four contained only lactose permease as the protein component, but showed increasing retention times on the column in direct correlation with their increasing phospholipid:protein ratios (J. Li and P. Tooth, unpublished results). This observation implies that on anion exchangers, the lactose permease–lipid–detergent micelles are adsorbed at least partly through negative charges of the phospholipids.

Reduction of lipids and detergent can be achieved by any positive purification method that retains the mixed micelles through the protein component. Noting that the lactose permease contains 24 basic residues compared to 16 acidic ones (Ehring et al., 1980), so that at neutral pH a net positive charge on the protein will bind the mixed micelles to a cation exchanger, we rechromatographed the Ecteola-purified material on CM-52. The lactose permease was indeed bound, whereas much of the phospholipids, negatively charged, and the neutral detergent were removed as unbound material. The result was a 10-fold reduction of lipids and detergent relative to the lactose permease, and a new preparation containing 4–5 mol of phospholipids/mol of permease, and only the protein-bound detergent in addition to free detergent at the CMC level. We showed that the galactoside binding activity was maintained after the excess lipid and detergent were removed.

Using the CM-52-purified lactose permease which contains a minimal amount of lipid and detergent, we have obtained small two-dimensional arrays of filaments lying side by side by further reduction of the free detegent level to below the CMC. This condition would reduce the bound detergent per protein molecule and favor protein associations that internalize the hydrophobic surfaces thus exposed. Therefore, subunit contacts in the filaments can be expected to involve the hydrophobic surfaces of the lactose permease molecule normally facing the membrane lipids. Filaments up to several micrometers in length have been obtained from pure lactose permease. In the presence of 50 mM KCl or 100 mM NH_4Cl, the filaments associate laterally into sheets. The bonding between subunits in the filament is apparently stronger than that between the filaments. This is suggested by the observation that the negative stains penetrated to a lesser degree into the intersubunit boundary in the filament than into the interfilament boundary. Furthermore, in the computed transforms, the angle of the near-axial spot from the equator varied between 67° and 86° from array to array, indicating a degree of freedom in the relative register of adjacent filaments.

In the majority of the filaments examined, the subunits display a stain-penetrated cleft, which is parallel to the filament axis. The common orientation of the clefts shows that the lactose permease filaments are not helical, like the bacteriorhodopsin filaments (Michel, 1982), but they are ribbons, like the dimer ribbons of the (Na^+,K^+)-ATPase dimer (Mohraz et al., 1985; Zampighi et al., 1986) and the Ca^{2+}-ATPase

dimer (Buhle et al., 1983; Taylor et al., 1984). The computed transforms of the lactose permease arrays showing a near-axial spot are handed. Hence, the subunits in the array all point the same way from the plane of the array, and not alternatively in and out of this plane. This is best accounted for by adjacent lactose permease molecules having the same orientation as they would in the natural membrane.

The area per subunit in the filamentous array of the lactose permease is 1440 Å^2, defined by the filament width of 50.7 ± 1.6 Å and by the repeat distance in the axial direction of 28.4 ± 1.6 Å. This value is within 10% of the estimated cross-sectional area of the molecule, which is 1322–1487 Å^2 on the basis of a molecular weight of 46 500, a partial specific volume of 0.77 cm^3/g for membrane proteins, and a membrane thickness of 40–45 Å. The ratio of the 1440 Å^2 area to the molecular weight also agrees well with the ratio for bacteriorhodopsin, another membrane protein of high α-helical content which has an experimentally determined cross-sectional area of about 800 Å^2 (Engelman et al., 1980; Henderson & Unwin, 1975) and a molecular weight of 26 000. Therefore, the subunit in the filamentous array is probably the lactose permease molecule seen in the face-on view as if it were held in the membrane. Thus, the lactose permease molecule has an elongated cross section in the membrane plane, of 51 Å by 28 Å. That the stronger bonding between subunits in the array occurs over the long edge of this cross section can be understood in terms of the internalization of a greater part of the hydrophobic lateral surface.

Previously, Costello et al. (1984, 1986) measured the diameter of the intramembrane particles due to the lactose permease and found it to be 51 ± 9 Å in the thinnest tantalum replicas. Dornmair et al. (1985) derived an elliptical molecular cross section of radii 22 and 18 Å from the rotational diffusion coefficient of lactose permease molecules in lipid bilayers. In comparison, our results, in addition to indicating a molecular size consistent with the molecular weight, revealed a greater elongation of the molecule (axial ratio = 1.8:1) in the membrane plane. This is probably because we have measured the molecule in ordered arrays and not in vesicles where the molecular orientation is random in the membrane plane.

The stain-penetrated cleft bisects the elongated subunit area into two roughly equal parts. This observation suggests strongly that the lactose permease molecule is comprised of two globular domains of similar size, separated by a solvent-accessible cleft. The galactoside binding site is very likely contained within this cleft. Such a location of the sugar binding site can be anticipated from earlier reports indicating that the monomer of lactose permease is active in galactoside binding (Wright et al., 1983b), that the sugar binding site is protected from proteolysis from the membrane surface (Goldkorn et al., 1983), and that the bound substrate is in a low-dielectric region within the folded polypeptide and yet the site is also connected by an aqueous channel to the membrane surface (Mitaku et al., 1984). Costello et al. (1984) have pointed out substructures in some of the intramembrane particles on the rotary-replicated fracture face of proteoliposomes incorporating the lactose permease, but the evidence was inconclusive.

Inspection of the lactose permease sequence revealed two pairs of homologous oligopeptide segments from the N and C halves of the molecule, each separated by about half of the 417-residue polypeptide length. These segments are ...(68)-DKLGLR(73)...(136)SNFE(139)... and ...(284)NRIGGK-(289)...(339)SQFE(342).... Sequences in the neighborhood of these segments can be aligned such that the corresponding residues from the N and C halves of the molecule are similar in hydrophobicity. It is tempting to infer from the limited internal homology that the N and C halves of the polypeptide each fold into one of the two globular domains and in such a way that the two domains are related in structure. An analogy may be drawn to several high-affinity binding proteins of the *E. coli* periplasm, including the arabinose binding protein (Gilliland et al., 1981), for which the molecular structures have been determined to atomic resolution. These proteins all show a bilobate domain organization with a buried substrate binding site in the cleft between the domains. Furthermore, in each case, the two domains are made up of similarly arranged secondary structural elements, and they can be related by a local 2-fold rotation, although significant sequence homology is lacking between the superimposable structural elements.

If indeed the two globular domains of the lactose permease molecule contain similarly arranged secondary structural elements, then given that both the N- and C-termini of the whole polypeptide are located on the cytoplasmic surface (Seckler et al., 1983; Beyreuther, unpublished results), the N- and C-termini of the partial sequence forming each of the two domains must both be located on the same membrane surface. This requires the lactose permease polypeptide to traverse the cytoplasmic membrane for a multiple of 4 times. Models of secondary structure arrangements that predict 12 transmembrane helices, such as proposed by Foster et al. (1983), are consistent with this implication, whereas models predicting 10 or 14 transmembrane segments would not be. With reference to the model of Foster et al. (1983; Kaback, 1986) depicting six helices in each half of the molecule, the two pairs of homologous oligopeptides cited above can be recognized in approximately corresponding places in the N and C halves of the model, namely, in the cytoplasmic link region between helices II and III vs. that between helices VIII and IX and in the cytoplasmic link region between helices IV and V vs. that between helices X and XI.

Our approach of minimizing the amount of lipid and detergent copurified with the lactose permease has facilitated the formation of small arrays, from which some structural measurements can be made. This approach is applicable to other hydrophobic membrane proteins.

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