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Oligomeric State of Membrane Transport Proteins Analyzed with Blue Native Electrophoresis and Analytical Ultracentrifugation

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Blue native electrophoresis is used widely for the analysis of non-dissociated protein complexes with respect to composition, oligomeric state and molecular mass. However, the effects of detergent or dye binding on the mass and stability of the integral membrane proteins have not been studied. By comparison with analytical ultracentrifugation, we have evaluated whether the oligomeric state of membrane transport proteins is reflected reliably with blue native electrophoresis. For the analysis we have used two well-characterized transporters, that is, the major facilitator superfamily protein LacS and the phosphotransferase system EII^{Mtl}. For another member of the major facilitator superfamily, the xyloside transporter XylP from *Lactobacillus pentosus*, the complete analysis of the quaternary structure determined by analytical ultracentrifugation and freeze-fracture electron microscopy is presented.

Our experiments show that during blue native electrophoresis the detergent bound to the proteins is replaced by the amphipathic Coomassie brilliant blue (CBB) dye. The mass of the bound CBB dye was quantified. Provided this additional mass of bound CBB dye is accounted for and care is taken in the choice and concentration of the detergent used, the mass of LacS, XylP and EII^{Mtl} and four other membrane (transport) proteins could be deduced within 10 % error. Our data underscore the fact that the oligomeric state of many membrane transport proteins is dimeric.

Keywords: membrane transport proteins; quaternary structure; blue native electrophoresis; analytical ultracentrifugation; freeze fracture electron microscopy

Introduction

For soluble proteins, it is generally accepted that protein-protein interactions play an important role in their function and regulation, but the quaternary structure of membrane transport proteins is less well established due to lack of high or medium-resolution structural information. Until now, the tetracycline efflux protein (TetA) and the Na^+/H^+ antiporter (NhaA) from *Escherichia coli* and the oxalate/formate transporter (OxlT) from *Oxalobacter formigenes* are the only 12 α-helical secondary transporters for which medium-resolution structures are available.[1–3] In the two-dimensional crystals, NhaA and TetA are organized as dimers and trimers, respectively, whereas OxlT is monomeric. For a number of transporters, analytical ultracentrifugation, freeze-fracture electron microscopy (freeze-fracture EM), chromatography, rotational correlation spectroscopy, radiation inactivation, chemical cross-linking, reconstitution titration experiments, or complementation studies have been used to address their oligomeric state and function. Due to difficulties associated with the handling of membrane proteins, as well as data analysis, the results are not always unambiguous. There is some consensus, however, that the galac-

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Abbreviations used: BN-PAGE, blue-native-polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue G-250.

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toside transporter LacY from *E. coli* and the sodium-dependent glucose transporter SGLT1 from intestinal brush borders are monomeric,[4,5] although oligomeric species of these proteins have been observed. The sugar-phosphate antiporter UhpT from *E. coli* has been observed only as a monomer.[6] Secondary transporters for which an oligomeric subunit organization is proposed include the glucose transporter GLUT1 from erythrocytes,[7] the tetracycline transporter TetA and the Na^+/H^+ antiporter NhaA from *E. coli*,[1,8] the ion exchanger band 3 from erythrocytes,[9] the drug efflux system EmrE from *E. coli*,[10,11] several members of the neurotransmitter:sodium symporter family[12–14] and mitochondrial carrier proteins.[15,16] Functional analysis demonstrated that the multimeric state of these proteins is coupled to their function.

As most methods to study the oligomeric state of membrane proteins make high demands on the amount and the purity of the proteins, it would be of great advantage to have a reliable, simple method to assay quaternary structure of protein in the detergent-solubilized state. Blue native PAGE (BN-PAGE) is used widely for the isolation of non-dissociated membrane protein complexes and the analysis of their composition, oligomeric state and molecular mass. Using BN-PAGE, electrophoretic mobility of proteins is obtained through binding of negatively charged amphiphilic Coomassie brilliant blue (CBB) dye. However, in general, the technique is used without taking into account the fact that membrane proteins bind large amounts of detergent or CBB dye, which adds to the mass of the proteins, and without considering that membrane proteins may aggregate aspecifically if the detergent is replaced by the CBB dye.

To study the versatility of BN-PAGE, the technique was compared with other methods in the analysis of three well-characterized membrane transport proteins, the lactose transporter (LacS) from *Streptococcus thermophilus*, the xyloside transporter (XylP) from *Lactobacillus pentosus* and the mannitol-specific enzyme II (EII^{mtl}) from *E. coli*. Analytical ultracentrifugation, freeze-fracture EM,[17] rotation correlation spectroscopy,[18] and *in vitro* complementation studies[19] have shown that the LacS protein is organized as a dimer, in which each subunit comprises a full translocation pathway. The two subunits interact with each other cooperatively.[19] The monomer to dimer equilibrium of EII^{mtl} has been shown by size-exclusion chromatography,[20] cross-linking[21] and *in vitro* and *in vivo* complementation studies.[22,23] The association into dimers was also reflected in the 5 Å projection structure of the transmembrane domain.[24] The determination of the quaternary structure of XylP is described here.

Here, we show that BN-PAGE can be used to determine the molecular mass of transmembrane proteins, provided one takes into account the fact that bound detergent and CBB dye contribute to the observed mass. With the aid of a calibration curve that corrects for CBB binding, the oligomeric state of a number of other membrane transport proteins was determined.

Results

Hydrodynamic properties of the XylP-DDM complex

The hydrodynamic properties of *n*-dodecyl-β-D-maltoside (DDM)-solubilized XylP were analyzed using sedimentation velocity and sedimentation equilibrium experiments. Protein stability studies revealed that XylP was stable and monodisperse for some days at 4 °C. The weight-average sedimentation coefficient of the DDM-XylP complex did not depend on the protein concentration, in the range of 0.025 to 1 mg/ml. After correction for buffer density and temperature, the observed mean sedimentation coefficient at 20 °C in water ($s_{20,w}$) was 6.2(±0.3) S. Similar studies had shown that LacS undergoes reversible self-association with an association constant of 5.4(±3.6) ml/mg, and $s_{20,w}$ values of 5.7(±0.3) S and 9.1(±0.4) S for the monomer and dimer, respectively.[17] The $s_{20,w}$ of 6.2 S for XylP suggests a particle bigger than a monomer, because the protein lacks the 20 kDa regulatory IIA domain that is present in LacS (and assuming that these proteins have similar shapes). Sedimentation equilibrium centrifugation was performed to determine the mass of the DDM-XylP complex. After equilibrium was attained for three different XylP loading concentrations, the collected data were analyzed simultaneously by global, non-linear, least-squares analysis assuming a single thermodynamic component (Figure 1). The randomness of the residuals indicates that XylP behaves as a single thermodynamic component with an apparent mass of 91.5(±8.8) kDa. When these data were fitted and assuming the protein exists in a monomer to dimer equilibrium, the association constant is 62(±30) ml/mg. This indicates that the protein was present predominantly in its higher oligomeric state, which is in agreement with the independence of the sedimentation coefficient on the XylP concentration.

Calibration of the migration behavior of membrane proteins

The electrophoretic mobility on BN-PAGE of LacS, XylP, EII^{mtl} and soluble marker proteins was followed in time (Figure 2(a)). After one hour running time at 35V plus five hours at 200V, LacS, XylP, EII^{mtl}, thyroglobulin, ferritin, catalase and lactate dehydrogenase had reached their final position. Bovine serum albumin (67 kDa) is too small and was not trapped at the highest percentage polyacrylamide (17 %) used here. We conclude that the polyacrylamide gradient gel indeed works as a molecular sieve, in which the electrophoretic mobility decreases at areas of restrictive pore size, such

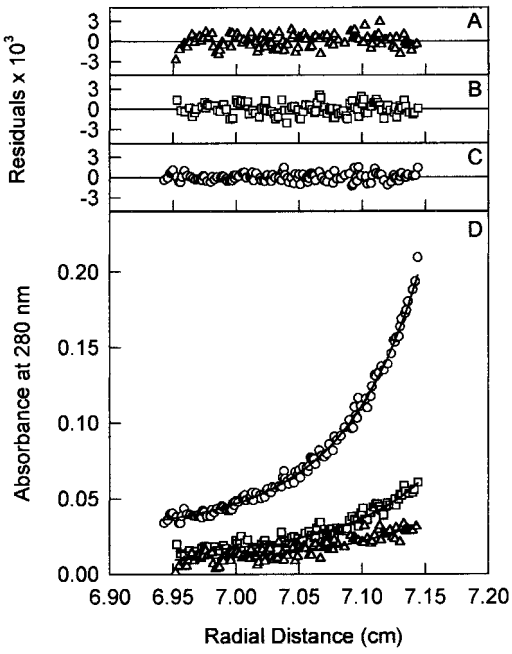

Figure 1. Sedimentation equilibrium profiles of XylP in 0.05% DDM. The equilibrium profiles of XylP-DDM at 10,000 rpm with initial protein concentrations of 0.005 (△), 0.01 (□) and 0.05 mg/ml (○). The continuous lines represent the best fits described by the global non linear least square analysis of all three data sets, and residuals of the fits are indicated in (a)-(c).

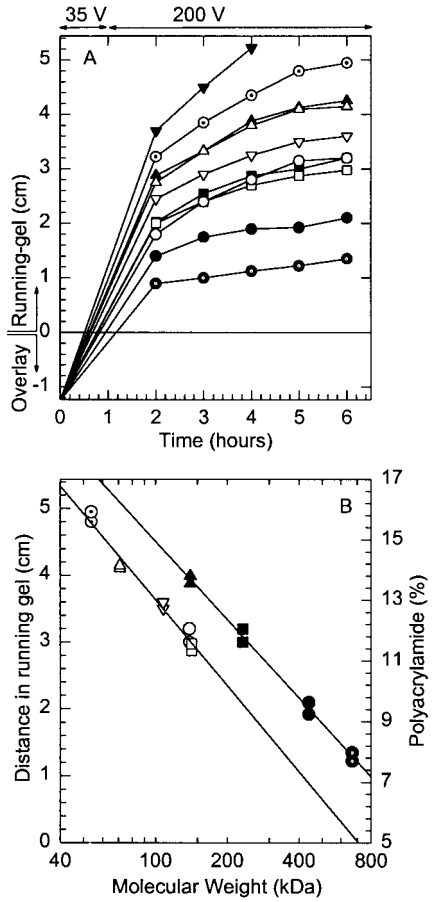

Figure 2. Time and molecular mass-dependent migration of proteins on BN-PAGE. (a) The migration distance of protein bands corresponding to LacS monomers and dimers (△ and □), XylP monomers and dimers (⊙ and ▽), EII^{mtl} dimers (○;), and of the soluble marker proteins bovine serum albumin (67 kDa, ▼), lactate dehydrogenase (140 kDa, ▲), catalase (232 kDa, ■), ferritin (440 kDa, ●) and thyroglobulin (669 kDa, ✪ was followed in time. Different gels were run for one hour at 35 V followed by one, two, three, four or five hours at 200V and stained. (b) The migration distances are plotted against the calculated molecular mass of the proteins and fitted with linear regression. The symbols are the same as in (a) and data are from independent experiments.

that, on the time-scale of hours, proteins keep their position.

When purified LacS was analyzed by BN-PAGE, we observed two protein bands at approximately the same migration distances as those occupied by lactate dehydrogenase (140 kDa) and catalase (232 kDa). These molecular masses are different from the predicted masses of the monomer and dimer of LacS on the basis of the amino acid composition, which are 71 kDa and 142 kDa, respectively. To establish that the protein band with highest mobility corresponds to monomeric LacS, BN-PAGE was carried out under conditions comparable to those of analytical ultracentrifugation at which virtually all LacS is monomeric; that is, with protein samples of 0.05 mg/ml (four times below the K_D of the monomer to dimer equilibrium of LacS) and in the presence of 0.05% (w/v) DDM in the gel as well as in the buffers. Gels run for various periods of time, including times at which the 67 kDa soluble marker protein was still observable, showed only one protein band at approximately the same distance as that occupied by lactate dehydrogenase of 140 kDa even after prolonged silver-staining. We thus conclude that this band corresponds to the monomer of LacS, whereas the band at approximately 232 kDa is the dimer.

The migration distances of LacS, XylP and EII^{mtl} and the soluble marker proteins were plotted as function of the molecular mass deduced from the amino acid sequence (Figure 2(b)); data from two independent experiments are shown. LacS, XylP and EII^{mtl} had a similar size-dependent migration behavior, which was different from the soluble marker proteins. This variation can be explained by differences in dye binding, as in aqueous solution, membrane proteins have a much higher capacity to bind amphipathic molecules like CBB than soluble proteins. For accurate determination of the molecular mass of a membrane protein, one needs to know the amount of detergent and/or CBB bound per molecule.

Detergent and CBB binding and CBB/detergent exchange

The amount of DDM bound to purified XylP (not shown) or LacS (Figure 3(a)) immobilized on a Ni-affinity column was determined. After equilibration with a buffer containing 1 mM [^{14}C]DDM, the proteins were eluted from the column in a buffer containing an equal concentration of [^{14}C]DDM. The amount of DDM bound to LacS was 197(±5) molecules per LacS monomer, yielding a molar mass of 170 kDa for the protein detergent complex. The molar ratio of DDM to XylP was 184 (± 7). This yields a molar mass of 148 kDa for the monomeric DDM-XylP complex.

The rate of detergent/CBB exchange was estimated from the [^{14}C]DDM elution profiles from a column with bound LacS upon washing with buffer without [^{14}C]DDM but with CBB at the same concentrations as used in the sample- (Figure 3(b)) or cathode buffer (Figure 3(c)). Immediately after switching to a buffer without [^{14}C]DDM but with 0.5 % CBB, a peak in the [^{14}C]DDM elution profile appeared. This peak corresponds to the amount of [^{14}C]DDM displaced from the protein, which results temporarily in [^{14}C]DDM concentrations above the equilibration level. The detergent/CBB exchange was slower when the column was washed with 0.02 % (w/v) CBB. However, in both cases after ten column volumes, virtually all of the [^{14}C]DDM was replaced. The total amount of DDM displaced from the protein upon washing with CBB-containing buffer without DDM could be calculated by subtracting the displacement from the empty control column (Figure 3(b), continuous line). This amount was approximately equal to the amount of DDM that co-eluted with LacS when [^{14}C]DDM was not displaced. The amount of CBB bound to LacS was quantified by measuring the absorbance at 580 nm and estimating the concentration of protein by SDS-PAGE, using calibrated amounts of LacS. The ratio of LacS-CBB to LacS was approximately 1.8 (w/w).

Detergent effects on the monomer to dimer equilibrium on BN-PAGE

Purified LacS and XylP solubilized with different concentrations of DDM or Triton X-100, detergents with a comparable CMC, were separated by BN-PAGE (Figure 4(a)). With both DDM and Triton X-100, the dimer to monomer equilibrium shifted towards the monomer with increasing detergent concentration, but at identical concentrations the dimer was more stable in Triton X-100 than in DDM. Comparison of LacS and XylP showed that the dimer of XylP was more stable, as at 0.9 % DDM a considerable fraction of XylP was still dimeric, whereas all LacS was monomeric. The detergent effects on the monomer to dimer equilibrium were similar when LacS-containing membranes were solubilized and the solubilisate was subjected to BN-PAGE; for these experiments, the proteins were visualized by immunodetection (Figure 4(b)). Correct analysis of the oligomeric state of membrane proteins in solubilized membrane extracts requires the use of sufficient amounts of detergent to prevent aberrant migration due to the presence of associated lipids (incomplete solubilization). LacS and XylP membranes solubilized at DDM concentrations above 0.5 % and 1 %, respectively, yielded protein bands migrating at the positions of the monomeric and dimeric species observed with purified protein. At

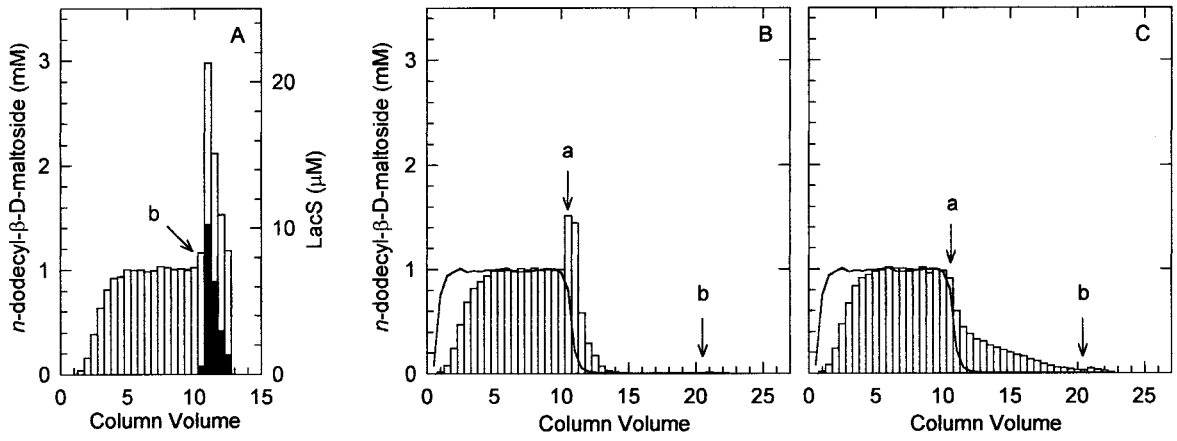

Figure 3. Detergent and CBB binding and exchange. LacS on a Ni-NTA column is equilibrated with 10 ml (20 column volumes) of 1 mM [^{14}C]DDM in buffer A plus 10 mM imidazole. (a) At the arrow, LacS is eluted from the column by switching to buffer A (pH 7.0), with 200 mM imidazole and 1 mM [^{14}C]DDM. Filled bars represent the LacS concentrations as determined from the absorbance at 280 nm. (b) and (c) [^{14}C]DDM is displaced upon washing with a buffer A without [^{14}C]DDM but with 0.5 % CBB ((b) arrow a) or 0.02 % CBB ((c), arrow a). Subsequently, LacS is eluted in buffer A at pH 7.0, containing 200 mM imidazole and 0.5 % CBB ((b) arrow b) or 0.02 % CBB ((c), arrow b). Continuous lines indicate the equilibration and displacement of [^{14}C]DDM from a column without protein bound.

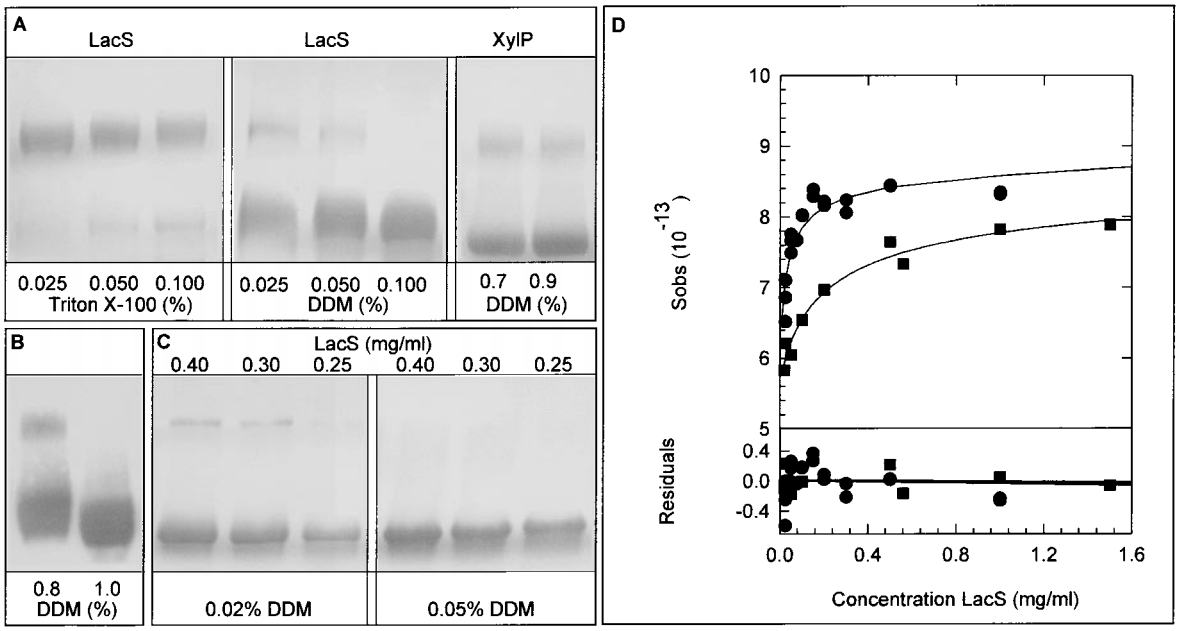

Figure 4. Detergent effects on the oligomeric state of LacS and XylP. (a) Purified LacS and XylP at 0.5 mg/ml in DDM or Triton X-100 and separated by BN-PAGE. (b) Western blot of LacS solubilized from membranes with 0.8 % or 1.0 % DDM and separated by BN-PAGE. (c) LacS (20 µl) purified in 0.02 % or 0.05 % DDM was separated by BN-PAGE. Gels and buffers contained the equivalent concentration of DDM; the LacS concentrations are indicated. (d) Mass average sedimentation coefficients of LacS solubilized in 0.05 % DDM (■) (data from Friesen *et al.*[17]) or 0.02 % DDM (●) as a function of protein concentration were determined by sedimentation velocity analytical ultracentrifugation. The lines are least-square fits assuming a monomer to dimer mode of association. Residuals are indicated.

lower detergent concentrations, the protein bands migrated less far or appeared as a smear (not shown).

The relevance of detergent concentration-dependent association-dissociation was confirmed by analytical ultracentrifugation. The association constant for dimerization of LacS in 0.05 % DDM was approximately 5 ml/mg. The sedimentation coefficients of the monomer and dimer were the same in 0.02 % DDM, but the association constant was ten times higher (Figure 4(d)). Identical protein samples were run on BN-PAGE gels that contained 0.02 % or 0.05 % DDM in the gels and the buffer system (Figure 4(c)). Samples of 20 µl were loaded in each lane so that each protein sample was diluted by the same factor upon pipetting into the sample chamber. In the presence of 0.02 % DDM and at a concentration of 0.25 mg/ml LacS or higher, the dimer band was visible, whereas in the presence of 0.05 % DDM dimers were absent. A difference between the data from analytical ultracentrifugation and from blue native electrophoresis is that the fraction dimeric LacS is less prominent in the electrophoresis experiments.

BN-PAGE reports the oligomeric state of membrane transport proteins

The migration of purified membrane transport proteins other than LacS, XylP and EII^{mtl} on a BN-PAGE gel was determined. The masses of the protein bands from purified samples of LacS, XylP and EII^{mtl}, were deduced from the soluble marker calibration line and plotted as function of their actual molecular mass based on amino acid sequence (Figure 5). The data could be fitted with linear regression, yielding a factor of 1.8 for the conversion of the molecular mass of membrane proteins on the basis of amino acid sequence (M^{AA}) and the molecular mass on BN-PAGE calibrated with soluble marker proteins (M^{BNP}); that is, $M^{BNP} = 1.8 \times M^{AA}$. The experimentally determined CBB binding to LacS is in agreement with this conversion factor of 1.8.

The migration of the protein bands from purified samples of the Na^+/citrate transporter CitS from *Klebsiella pneumonia*, the multidrug export protein LmrA and the drug/proton antiporter LmrP from *Lactococcus lactis* in 0.05 % DDM were calibrated using soluble marker proteins. Purified CitS migrated on BN-PAGE with an apparent mass of 160 kDa, yielding a mass of 89 kDa after conversion, close to what is expected for the dimer (98 kDa). LmrA, with a calculated molecular mass of 66 kDa, migrated on a BN-PAGE gel at 108 kDa and 221 kDa, which corresponds to the monomeric and dimeric species after correction for CBB binding. LmrP has a calculated molecular mass of 46 kDa and ran on BN-PAGE as a 87 kDa protein, yielding a mass of 48 kDa after correction. Also, the monomeric and dimeric species of the inner

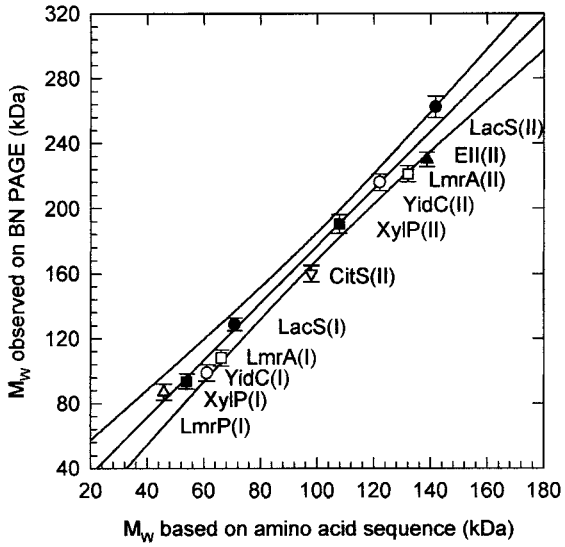

Figure 5. Oligomeric state of membrane transport proteins analyzed by BN-PAGE. The indicated membrane proteins were purified in the presence of 0.05% DDM using Ni-affinity chromatography and separated on BN-PAGE. The observed molecular masses of LacS (●), XylP (■), EII^{mtl} (▲) monomers (I) and dimers (II) as calibrated using soluble marker proteins are plotted as a function of their calculated molecular mass. The data are from at least four independent experiments and the error in the measurements is indicated. Linear regression and the 95% confidence interval are indicated. The observed molecular masses of purified CitS (▽), LmrA (□), LmrP (△), and YidC (○) in 0.05% DDM are plotted as function of their monomeric (I) or dimeric (II) masses.

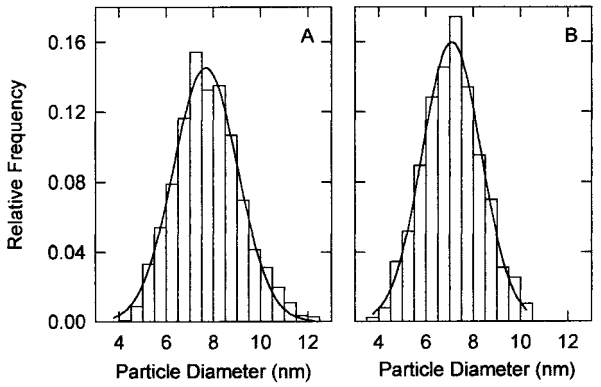

Figure 6. Size distribution of (a) XylP and (b) LacS IMPs in proteoliposomes. The continuous line indicates a Gaussian fit.

membrane protein YidC from *E. coli*[25] were obtained with the calibration procedure.

Oligomeric state of membrane-reconstituted XylP

Freeze-fracture EM was employed to analyze the quaternary structure of XylP in the membrane-reconstituted state. The diameter of 1478 XylP and 872 LacS IMPs was determined. Both the IMPs of XylP and LacS fell into a single size population with a mean diameter of 7.67(±0.04) nm and 7.09(±0.04) nm, respectively. Based on published data, where the mean diameter of LacS was calculated to be 6.43(±0.08) nm,[17] the film thickness was determined to be 0.66(±0.12) nm under these conditions. The mean diameter for the XylP IMPs corrected for this film thickness is 7.01(±0.16) nm. Using an average area of 1.4 nm^2/α-helix,[5] XylP is predicted to have 27.6(±1.3) α-helices, which approximates a dimeric species.

Discussion

The determination of the oligomeric state of membrane proteins has proven to be difficult because of their tendency to aggregate when isolated from the native membrane environment. Moreover, the binding of detergents to the hydrophobic surfaces adds greatly to the total protein mass. We have addressed the usefulness of BN-PAGE in the analysis of the quaternary structure of membrane proteins by comparison with analytical ultracentrifugation. Freeze-fracture EM was used to demonstrate that the oligomeric structures observed in detergent solution are related to the membrane-embedded states of the proteins. The oligomeric state of a protein can be deduced from BN-PAGE, provided the amount of CBB and/or detergent bound is known and the membrane proteins do not aggregate when detergent is displaced by the dye. Aggregation can be observed as a ladder of greater molecular mass species on BN-PAGE, the inability to reach equilibrium in sedimentation equilibrium centrifugation, and time-dependent increases in the absorbance between 320 nm and 340 nm due to light-scattering.

LacS and XylP, two sugar transport proteins from the GPH family of major facilitators, and EII^{mtl}, a member of the PTS superfamily, were used for the analysis. At a concentration below 0.05 mg/ml, LacS is predominantly monomeric, whereas EII^{mtl} and XylP are dimeric, indicating that the strength of the interactions between the monomers in the dimer is quite different when solubilized in DDM. Despite variations in the association constants, all three proteins are largely dimeric when embedded in the membrane,[17,18,23] which is expected when the restrictive volume of the two-dimensional space and the orientation of the proteins is taken into account.[26] The observation of dimers of XylP, a homologue of LacS lacking the regulatory IIA domain, suggests that dimerization of members of the GPH family is an intrinsic property of the membrane-embedded carrier domain.

The observation that [^{14}C]DDM is exchanged rapidly for CBB has as consequence that the majority of detergent will be replaced under gel

electrophoresis conditions. The estimated amount of bound CBB is consistent with the approximately 80 % greater mass observed on BN-PAGE when the migration of LacS, XylP and EII^{mtl} is calibrated with soluble marker proteins. Using the conversion factor of 1.8, the masses of monomers and dimers of LacS, XylP and EII^{mtl} could be determined within an error of 10 %. Although the fraction of membrane and soluble domains is different in all three proteins, the amount of CBB bound was comparable. The presence or absence of up to 0.05 % DDM and/or 0.02 % to 0.1 % CBB did not alter the mass calibration, seemingly because equivalent amounts of detergent and dye are bound.

BN-PAGE has been used to determine the oligomeric state of membrane proteins.[27–30] In order to calibrate the oligomeric state, the complexes were dissociated using harsh conditions, e.g. 8 M urea, 100 mM DTT plus high detergent concentrations. Such conditions affect the folding of the proteins and lead to an altered detergent/CBB to protein ratio. Moreover, according to our experiments, proteins smaller than 80 kDa are not trapped by gels with 15 % polyacrylamide. As in most previous reports, the 68 kDa marker protein is still observed on 15 % polyacrylamide gels, we conclude that electrophoresis was not carried out for long enough times. Finally, in the original studies by Schägger *et al.*, the additional mass associated with membrane proteins may not have been noted because the migration of proteins was analyzed over a wide range of sizes (12 kDa to 880 kDa), which decreases the accuracy.[31,32]

To rule out aggregation artifacts induced by the electrophoresis method, we correlated the data with those obtained by analytical ultracentrifugation. The association constant determined with sedimentation velocity centrifugation for dimerization of LacS was lowered about one order of magnitude when the detergent concentration was increased from 0.02 % to 0.05 % DDM. Also on BN-PAGE, the population of LacS monomers increased with increasing DDM concentrations. Moreover, the monomer to dimer equilibrium was dependent on the detergent concentration and on the type of detergent. Since LacS has a net negative charge at pH 7.0, the electrophoresis could be performed in the presence of detergent but in the absence of CBB. The absence or presence of up to 0.1 % CBB in the cathode buffer did not affect the observation of the equilibrium between monomers and dimers (not shown). The association constants for the monomer to dimer equilibrium of both LacS and XylP estimated from BN-PAGE are lower than those determined by analytical ultracentrifugation. This can be expected, as BN-PAGE is not an equilibrium method; that is, concentrations are changing when the species are migrating. Whether higher oligomeric species are observed depends on the kinetics of association and dissociation of the subunits. It is thus possible that higher oligomeric species are not observed at all or only as a small fraction, whereas they are present in the detergent solution.

We determined the oligomeric state of four other α-helical membrane transport proteins. Calibration of the protein bands from LmrA revealed monomeric and dimeric species. This is in accordance with the generally accepted view of subunit organization of ABC transporters. CitS appeared only as dimeric species. A strong interaction between the subunits, even in the detergent-solubilized state, is supported by the finding that non-biotinylated protein was co-purified with biotinylated protein using a streptavidin column.[33] LmrP showed a single monomeric species.

In conclusion, we show that BN-PAGE can be used to obtain reliable information on the oligomeric state of membrane (transport) proteins, provided care is taken in the choice and concentration of detergent used, the protein is stable in the detergent-solubilized state, and appropriate corrections are made for detergent/dye binding to the protein. The relative simplicity of the technique, e.g. compared to analytical ultracentrifugation, allows a more general use of this technique also for those proteins for which large amounts of stable detergent-solubilized purified protein are difficult to obtain. The method is particularly useful for the analysis of the effects of mutations on the association equilibrium or to screen for the effects of detergents/co-solvents on protein stability/aggregation. Moreover, proteins need not be purified, as membrane solubilisates can be analyzed directly, provided a suitable antibody is available to detect the protein. Finally, the analysis presented here underscores the suggestion that many secondary transport proteins actually have an oligomeric structure higher than monomeric.

Materials and Methods

Materials

Coomassie brilliant Blue G-250 (CBB) was purchased from Serva (Heidelberg, Germany). Acrylamide/*N*,*N*′-methylene-bisacrylamide (32.5:1, w/w) was from Fluka. [^{14}C]Dodecyl-β-D-maltoside (2GBq/mmol) was a generous gift from M. Le Maire (Paris, France). The high molecular mass marker set was from Pharmacia, Triton X-100 was from Boehringer Mannheim, and *n*-dodecyl-β-D-maltoside (DDM) was from Anatrace.

Strains, plasmids and membrane isolation

Strains and plasmids used are listed in Table 1. Membrane vesicles were isolated as described and stored in liquid nitrogen.[34,37,38]

Membrane solubilization and protein purification

Membranes from either *S. thermophilus*, *L. lactis* or *E. coli* were solubilized in 50 mM potassium phosphate (pH 7), 10 % (v/v) glycerol and various concentrations of DDM or Triton X-100. Purification and membrane reconstitution of LacS,[17,37] XylP[34] and EII^{Mtl} [38] were performed as described. The concentrations of LacS and XylP were

Table 1. Strains and plasmids used in this work

Protein	Strain	Plasmid	Reference
	L. lactis		
XylP	NZ9000	pNZ804xylP	[34]
LmrP	NZ9000	pHLP5	[35]
LmrA	NZ9000	pNHLmrA	[36]
	S. thermophilus		
LacS	ST11(ΔlacS)	pGKHis	[37]
	E. coli		
EII^{Mtl}	LGS322	pMahismtlAPr	Unpublished
CitS	ECOMUT2	pA1CitS	Unpublished
YidC	SF100	pEH1hisYidC	[25]

calculated from the absorbance at 280 nm, using extinction coefficients of 76,320 and 100,800 M^{-1} cm^{-1}, respectively. Purification of all other membrane proteins was performed as described for XylP.

BN-PAGE

BN-PAGE was performed as described.[31,32] Linear 5%-17% (w/v) polyacrylamide-gradient gels were formed with a 4% overlay. Gel dimensions were 70 mm × 80 mm × 1 mm. Protein samples were supplemented with a tenfold concentrated loading dye (5% CBB, 500 mM 6-amino-*n*-caproic acid and 100 mM bis-Tris, pH 7.0). Unless indicated otherwise, the electrophoresis was started at 35 V for one hour and continued for five hours at 200 V at room temperature. Gels were stained with Coomassie brilliant blue. Immunoblotting was performed as described[31] using anti-Histag antibodies. For the analysis of the molecular mass of proteins, the distances of the protein bands on the gels were measured from the middle of the band to the start of the 4% overlay.

DDM binding and detergent-CBB exchange

The number of DDM molecules bound to XylP and LacS, and the displacement of detergent by CBB, was measured with [^{14}C]DDM using equilibrium desorption chromatography. Proteins were solubilized and purified as described, except that after the wash steps the columns were equilibrated with 20 column volumes of buffer A (50 mM potassium phosphate (pH 8.0), 100 mM NaCl, 10% (v/v) glycerol) containing 10 mM imidazole plus 0.05% (w/v) [^{14}C]DDM; 0.05% DDM corresponds to 1 mM. To determine the amount of DDM bound, proteins were eluted in buffer A at pH 7.0 containing 200 mM imidazole plus 0.05% [^{14}C]DDM. For the determination of DDM-CBB exchange, the columns, equilibrated with 0.05% [^{14}C]DDM, were washed with 20 column volumes of buffer A containing 10 mM imidazole plus 0.02% or 0.5% CBB before protein elution in buffer A at pH 7.0 containing 200 mM imidazole and 0.02 or 0.5% CBB. Protein concentrations were determined spectroscopically from the absorbance at 280 nm and the detergent concentration in the various fractions was measured by liquid scintillation counting.

Analytical ultracentrifugation

Analytical ultracentrifugation experiments were performed in a Beckman Optima XL-I, using an AN-50 Ti rotor with two-channel charcoal-filled centerpieces at 4 °C or 20 °C. Prior to the measurements, LacS and XylP were dialyzed at 4 °C against buffer C (100 mM potassium phosphate (pH 7.0), 2 mM K-EDTA) plus 0.05% or 0.02% DDM and buffer D (50 mM potassium phosphate (pH 7.0), 10% glycerol, 2 mM K-EDTA) plus 0.05% DDM, respectively. Sedimentation velocity experiments were performed at 38,000 rpm and 20 °C for LacS and 4 °C for XylP. Data were collected at 280 nm and 230 nm in a continuous mode with a radial step size of 0.005 cm and 6 or 8.5 minute intervals. Sedimentation equilibrium experiments with XylP were carried out at 4 °C and a rotor speed of 10,000 rpm on a sample volume of 100 µl with loading concentrations ranging from 0.005 to 0.1 mg/ml. The absorbance optics system was used to collect data every 0.001 cm with ten replicates at 280 nm.

Data treatment according to multicomponent systems

Data analysis was performed with the XL-I data analysis software. The molecular mass of XylP was determined by analyzing the data using equations appropriate for multicomponent systems as described.[17,39,40] The partial specific volume of XylP was calculated to be 0.7446 ml/mg at 4 °C and for the partial specific volume of DDM a value of 0.814 ml/mg was used.[17] The DDM/XylP (w/w) ratio was 1.7. Solvent densities were determined using an Anton Paar DM48 densitometer.

Freeze-fracture EM

Freeze-fracture replicas of XylP proteoliposomes were prepared as described.[17] From images at a final magnification of 250,000×, particle diameters were measured perpendicular to the shadow direction using software from ScionImage. Data were analyzed according to Eskandari *et al.*[5] The results were plotted as frequency histograms at the center of the bin (0.5 nm) and fitted to a (multiple) Gaussian function.

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