Amino acid type selective isotope labelling of the multidrug ABC transporter LmrA for solid-state NMR studies

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Abstract The ABC transporter LmrA in *Lactococcus lactis* confers resistance to a wide range of antibiotics and cytotoxic drugs and is a functional homologue of P-glycoprotein. Recently, solid-state NMR methods have shown potential for structural- and non-perturbing, site directed functional studies. These experiments require isotopic labelling of selected sites. We have developed a strategy to produce large quantities of selectively labelled LmrA reconstituted at a high density in lipid membranes. This makes the 64 kDa integral membrane protein LmrA and therefore the ABC transporter superfamily accessible to NMR analysis.

Keywords: Solid-state NMR; Multidrug resistance; Membrane protein; ^{13}C labelling

1. Introduction

The phenomenon of multidrug resistance, by which cells become resistant to structurally and functionally diverse pharmaceuticals, is often caused by active drug extrusion from the cell by primary-active or secondary-active transporters. Primary-active transporters such as P-glycoprotein, BCRP and MRP1 use ATP hydrolysis and are ubiquitous in prokaryotic and eukaryotic organisms. One member of this ATP-binding cassette (ABC) family is the *Lactococcus lactis* multidrug resistance protein LmrA, which can functionally substitute for P-glycoprotein in lung fibroblast cells [1]. LmrA is a 590 amino acid polypeptide with six transmembrane spannning segments in the N-terminal hydrophobic domain and a C-terminal hydrophilic nucleotide-binding domain (NBD). The protein is thought to function as a homodimer as suggested by the negative dominance of an inactive mutant over an active wildtype LmrA protein in a co-reconstituted liposomal system. Furthermore, covalent fusion of two wildtype LmrA monomers yields a functional transporter [2]. LmrA provides a potentially accessible system for advanced biophysical studies, since amplified expression at high levels has been previously demonstrated [3]. The first structural and dynamic studies on LmrA were carried out by ATR-FTIR and $^{1}H/^{2}H$ exchange experiments providing evidence for transmembrane oriented α-helices [4] as well as for long-range conformational changes during the transport cycle [5]. Indication of the existence of an aqueous chamber formed by the transmembrane segments open to the intracellular space in the non-energized state was found by cysteine scanning accessibility studies [6].

So far, no three-dimensional (3D) structure has been reported for full-length LmrA. However, a number of high resolution structures for other members of the ABC family have been obtained recently. The structure of *Escherichia coli* BtuCD [7], a vitamin B_{12} transporter, has been determined at 3.2 Å resolution. A 4.5 Å resolution structure has been reported for *E. coli* MsbA [8], a lipid A transporter [9] that has overlapping substrate specificity with LmrA [10]. The BtuCD structure has been proposed to be a framework for ABC transporter architecture [7] but interestingly has a somewhat different subunit arrangement compared to MsbA. BtuCD has the two membrane spanning subunits and the two NBDs in close contact with each other allowing ATP binding at the NBD:NBD interface. The structure of dimeric MsbA reveals that the NBD dimer occurs away from the dimer interface. The low resolution structure of YvcC, a homologue of MsbA and LmrA from *B. subtilis* [11] and *L. lactis*, is in agreement with the MsbA crystal structure and supports the existence of a central open chamber between the two subunits forming the homodimer. The differences between the structures of these ABC transporters may imply that alternative mechanisms of ATP dependent drug transport exist within the ABC transporter superfamily.

In addition to structures of whole transporters, crystal structures of NBDs crystallised in isolation from the transmembrane domains of the transporter may also shed light on potential transport and energy coupling mechanisms. For example, the crystal structure of MalK, a dimer of NBDs that form part of the *E. coli* maltose transporter when in complex with the two membrane spanning subunits MalG and MalF, has been determined. Its ATP bound and ATP free form shows a closed and an open structure [12] which, in complex with MalG and MalF, could represent a ''tweezer-like'' mechanism for transfer of ATPase activity. This mechanism would be difficult to reconcile with the MsbA and YvcC structures if,

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indeed, a common mechanism exists in this class of proteins. Recent electron diffraction structures of P-glycoprotein in both the ground and ATP bound states have provided hints about the way transmembrane domains alter conformation upon nucleotide binding and about the possibilities that hydrophobic drugs could enter the protein from the lipid bilayer [13]. Unfortunately, the NBDs are poorly resolved and do not offer a conclusive model for the nature of the subunit assembly and for the coupling of ATP binding in one domain to conformational changes in another.

Structural studies on ABC transporters rely on the availability of well diffracting 2D or 3D crystals. Meanwhile, NMR and EPR approaches can provide site-specific information on substrate–protein interactions, conformational changes and dynamics. Furthermore, solid-state NMR, which does not rely on fast molecular tumbling, allows studies in a native-like environment and has been increasingly applied to membrane proteins [14–16].

NMR on macroscopically aligned samples, as demonstrated by PISEMA experiments [17], can provide structural information via orientation constraints and resonance assignments. Similar methods were also used to determine the orientation of myristoylated recoverin, a soluble protein that attaches to lipid bilayers in the presence of high Ca^{2+} concentration [18]. The prospects of applying this approach to larger membrane proteins have been theoretically analysed [19] and also practically demonstrated with specifically ^{15}N-labelled purple membrane [20]. A more universal approach to larger membrane proteins, which does not require macroscopically ordered samples, is magic angle sample spinning (MAS) NMR in combination with residue-specific labelling. For example, a number of NMR studies have used residue-specific ^{13}C labelling in the protein backbone which revealed information on conformation and dynamics that are directly related to the protein function in the membrane [21]. Careful application of such labelling schemes can yield structural information from proteins as large as 60 kDa [22]. Recently, uniform $^{13}C/^{15}N$ labelling schemes for MAS-NMR have been demonstrated to provide a number of structural constraints in microcrystalline samples of SH3, which offers a potential approach for smaller membrane proteins [23]. MAS-NMR can also be used to study ligands bound to membrane proteins as demonstrated in the structure determination of neurotensin bound to the neurotensin receptor [24]. Magic angle oriented sample spinning (MAOSS) (a combination of alignment techniques and MAS-NMR) on specifically ^{15}N-labelled bacteriorhodopsin has been used to measure the orientations of trans-membrane helical residues [25].

These examples demonstrate that a variety of tools are available to study membrane embedded proteins, such as ABC transporters, by solid-state NMR. However, no applications to ABC transporters have been reported so far and all applications to secondary transporters were restricted to the observation of bound substrate by either using naturally abundant ^{31}P [26] or synthetically $^{13}C/^{2}H$ enriched [27–31] ligands.

A number of bottlenecks have to be addressed before solid-state NMR experiments on ABC transporters like LmrA can be performed successfully. Established expression systems have to be optimised so that large quantities (mg) of isotope labelled protein can be obtained. Efficient solubilisation and purification procedures must be established. Particular attention needs to be paid to reconstitution of purified transporter back into membranes at concentrations high enough to make an NMR experiment feasible (lipids/proteins < 1000 mol/mol). Furthermore, a functional assay should be used to monitor the activity of the reconstituted protein.

Examples of multi-domain membrane proteins that can be heterologously expressed at levels suitable for structural studies are rare and examples where such proteins have been labelled for NMR purposes are even more limited [32,33]. Cell free synthesis has recently been applied successfully to membrane proteins [34] but may not be capable of producing such large systems in a correctly folded and functional state. For this reason, we have sought to take advantage of the ability to produce large amounts of functional LmrA in *L. lactis*. Amplified expression in *L. lactis* offers a number of advantages. The cells grow rapidly to a high cell density and over-expressed membrane proteins are found exclusively within the cytoplasmic membrane and not in inclusion bodies. Proteins can then be solubilised directly from the membrane by mild detergents, while purification is simplified by the small genome size.

Here, we demonstrate the use of this homologous expression system to produce quantities of selectively [^{13}C]glycine-labelled LmrA (ca. 1 mg LmrA/l culture), purified and reconstituted into lipid vesicles in an active form and in sufficient quantities for solid-state NMR experiments. Successfully reconstituted protein in proteoliposomes was separated from protein free liposomes and protein aggregates, and the first solid-state NMR spectra were recorded. Our data demonstrate the suitability of this expression system for further studies of LmrA structure and function by NMR.

2. Materials and methods

2.1. Materials

Difco Laboratories' M-17 medium was obtained from BD, Sparks, MD; *n*-dodecyl-β-D-maltoside (DDM) from Glycon Biochemicals GmbH, Luckenwalde, Germany; Ni^{2+} nitriloacetic acid (NI–NTA) resin from Qiagen, Inc.; Bio-beads, SM-2 from Bio-Rad Laboratories Inc; *E. coli* lipids from Avanti Polar lipids, Alabaster, AL. [$^{13}C_1$]glycine was obtained from Campro Scientific GmbH, distributors for Isotec, Miamisburg, OH. All other reagents were of analytical grade or better.

2.2. Bacterial strains and growth conditions

Lactococcus lactis strain NZ9000 was used as a host for the expression vector pNHLmrA [35], whilst NZ9700 was used as a nisin producing strain. Cells were cultured overnight at 30 °C in M-17 medium or defined medium, both supplemented with 0.5% (w/v) glucose and 5 μg/ml chloramphenicol, and used to start large scale cultures (2 l) grown at 30° without shaking and in the dark. LmrA expression was induced at an OD_{660} of 1.0 (1 cm path length) through the addition of 1 ml (per litre culture) of the supernatant acquired during harvesting of NZ9700 cells by centrifugation. The defined medium was adapted from a previously published recipe [36] and contained, per litre double distilled water; L-alanine 0.3 g, L-arginine HCl 0.36 g, L-asparagine 0.48 g, L-aspartic acid 0.04 g, L-cysteine HCl 0.12 g, L-glutamic acid 0.48 g, L-glutamine 0.48 g, glycine 0.12 g, L-histidine 0.12 g, L-isoleucine 0.36 g, L-lysine HCl 0.72 g, L-methionine 0.20 g, L-phenylalanine 0.36 g, L-proline 0.60 g, L-serine 0.36 g, L-threonine 0.36 g, L-tryptophan 0.09 g, L-tyrosine 0.24 g, L-valine 0.60 g, NH_4Cl 3.75 g, KH_2PO_4 2.5 g, K_2HPO_4 3.25 g, $MgSO_4.7H_2O$ 0.87 g, NaCl 0.53 g, thiamine HCl 0.1 mg, riboflavin 1 mg, niacin 1 mg, pyridoxal-HCl 2.3 mg, *p*-aminobenzoic acid 0.1 mg, folic acid 1 mg, D-biotin 0.1 mg and trace amounts of adenine, guanine and uracil. The final pH was between 6 and 6.5.

2.3. Sample preparation

Inside out vesicles (ISOV) were prepared and histidine tagged LmrA was purified as described previously [35] with the following

modifications. 0.1 M Tris–HCl and 0.1 M NaCl buffer, pH 7, were used in place of potassium phosphate buffer throughout. During detergent solubilisation of LmrA containing ISOVs, the final concentration of DDM was 3% (w/v). Solubilised protein was incubated with 10 g of Ni–NTA resin and loaded on a column (Pharmacia) attached to an ÄKTA prime FPLC. Unbound protein was removed from the column using previously described buffers [35] and, when no further protein was detected in the flow (OD monitored at 280 nm), LmrA was eluted with buffer at pH 7.0 containing 250 mM imidazole. Washed biobeads were pre-incubated in a suspension of *E. coli* lipids to reduce removal of lipids from solution during the reconstitution process [37]. Lipids at a starting molar ratio of 200 lipids per protein were suspended in 50 mM Tris, 25 mM KCl, and 10 mM EDTA buffer at pH 7. The lipid suspension was sonicated briefly in a bath sonicator and then extruded (Northern Lipid Extruder) 11 times through a 400 nm filter (Whatman, Maidstone, UK). The liposome suspension was then titrated with a 0.02 M DDM solution as described [35] and mixed with detergent solubilised LmrA that had been concentrated (Centricon YM-50, average RCF 655g, 20 min; 4 °C). The detergent destabilised liposomes and purified protein were incubated for 30 min at room temperature before biobeads (final concentration of 80 mg/ml) were added. The mixture was incubated for a further 30 min at room temperature and then overnight at 4 °C. After removal of biobeads, the (proteo)liposomes were pelleted by centrifugation (average RCF $190\,000 \times g$; 30 min; 4 °C). Reconstituted protein in proteoliposomes was separated from liposomes and protein aggregates by sucrose density centrifugation. The reconstituted complexes were resuspended and layered over a discontinuous sucrose gradient (10–20–30%) (average RCF $83\,000 \times g$; 18 h; 4 °C). Bands were collected from the sucrose gradient and washed with 50 mM Tris, 25 mM KCl, and 10 mM EDTA buffer at pH 7 before being pelleted as before and loaded into 4 mm MAS rotors (Bruker GmbH, Kahlsruhe, Germany). Protein concentrations were measured using the Bio-Rad *DC* protein assay kit as adapted from Lowry et al. [38].

2.4. Enzyme assay

The ATPase assay of reconstituted LmrA was based on a colorimetric ascorbic acid/ammonium molybdate assay [39] to measure the liberation of P_i from ATP. 50 mM ATP and 10 mM Na orthovanadate stock solutions were freshly prepared. Reconstituted LmrA was washed twice with 20 mM K–HEPES (pH 7.0), 5 mM $MgSO_4$ buffer and resuspended at a final concentration of 2.5 mg/ml. ATPase assays were performed at 30 °C in a reaction volume of 20 μl. Following incubation of reconstituted LmrA and ATP (final concentration 5 mM) for 10 min, the ATPase reactions were terminated by addition of 40 μl of a freshly prepared acidic solution consisting of 0.48% (w/w) ammonium heptamolybdate tetrahydrate, 6.6% (v/v) concentrated sulfuric acid, 0.01% (w/w) potassium antimonyl tartrate and 0.42% (w/w) ascorbic acid. Following the addition of 140 μl H_2O and 30 min of incubation at 30 °C in the dark, the absorbance was measured at 690 nm. ATPase activity measurements in the presence of 1 mM orthovanadate were obtained in parallel and subtracted from the readings.

2.5. Electron microscopy

For freeze-fracturing reconstituted membrane vesicles in suspension placed between two small copper blades were frozen by plunging into ethane cooled to −180 °C by liquid nitrogen. Samples were fractured in a freeze-fracture unit (400T from Balzers, Lichtenstein) and shadowed with platinum/carbon at an angle of 45°. Replicas reinforced by pure carbon shadowing at an angle of 90° were cleaned from organic material in chromo-sulfuric acid and later-on analysed by electron microscopy (EM208S, Philips, Eindhoven, The Netherlands).

2.6. Solid-state NMR

All experiments were performed on a Bruker Avance 600 equipped with a 4 mm MAS DVT probe. Cross polarisation (CP) experiments were performed at 150.9 MHz for ^{13}C employing a 80–100% ramped proton pulse of 1.5 ms to satisfy the Hartmann–Hahn condition. Two pulse phase modulated (TPPM15) heteronuclear ^{1}H decoupling at a field of 62.5 kHz and a 49 ms acquisition time with a recycle delay of 2 s was used. Spectra were zero filled to 16k points and 50 Hz (^{13}C) exponential line broadening was applied during processing. Spectra were referenced externally to the carbonyl resonance of glycine at 176.03 ppm. All experiments were carried out at temperatures of 233 and 253 K.

3. Results and discussion

Growth of *L. lactis* on the chemically defined medium is comparable with that on M17 medium in terms of growth rate but with a reduced final cell density of 50%. This reduced growth is reflected in the final yield of purified LmrA. Here, we have typically purified 2 mg of LmrA per litre of M17 medium compared with 1 mg LmrA per litre of defined medium. The total protein yield in ISOVs was approximately 3 mg/l defined medium.

The incorporation of ^{13}CO-glycine demonstrates that *L. lactis* is able to grow and express LmrA sufficiently well, so that the high levels of protein required for solid-state NMR experiments can be obtained. Although LmrA is expressed at up to 30% of total membrane protein, spectra directly obtained from ISOVs prepared from *L. lactis* are not suitable for further NMR studies since signal from labelled LmrA cannot be discerned from other membrane constituents.

Therefore, we have sought to solubilise labelled LmrA, purify using established techniques and then reconstitute into a lipid environment at a high protein density. Detergent solubilised LmrA, eluted from the Ni–NTA column, was used immediately for reconstitution. As reported previously [35], a protein purity of 97% as judged by densitometric analysis of SDS–PAGE gels was achieved.

Existing protocols [35] combine LmrA and lipids at a ratio of 1/100 (w/w), which would allow approximately 0.1 mg of this 64 kDa protein to be fitted into the 20–80 μl internal volume of the standard MAS rotors with 4 mm diameter used for NMR experiments. Current solid-state NMR techniques are insufficiently sensitive to this amount of labelled protein and good quality spectra cannot be obtained. Therefore, we reconstituted LmrA in lipids at a ratio of between 1/1 and 1/10 (w/w) providing between approximately 100 and 1000 lipids per LmrA monomer and allow between 0.5 and 5 mg of labelled LmrA to be introduced into a MAS rotor per NMR experiment. The resulting protein–lipid vesicles were collected by centrifugation. The presence of LmrA associated with the vesicles was confirmed by SDS–PAGE gel analysis and by ^{13}C CPMAS NMR of ^{13}CO glycine labelled LmrA (data not shown).

A functional assay detecting ATP turnover confirmed the activity of the LmrA NBD for reconstituted LmrA using our method (Fig. 1). Reconstitution by co-dissolving protein and lipids in detergent solution after which the detergent was removed by incubation in the presence of biobeads has been tested as well but produced reconstituted complexes for LmrA with very poor ATPase activity (data not shown). The vanadate sensitive ATPase activity of the sample reconstituted by the detergent destabilisation method was determined to be 47 nmol/min/mg protein (Fig. 1), which can be compared with reported activity of about 150 nmol/min/mg for LmrA in ISOVs [2] and confirms the suitability of this reconstitution approach. The ATPase activity in our reconstituted system may be an underestimation of the total activity, since many ATP-binding sites may be located in the vesicle lumen and are not accessible to ATP.

Analysis of proteoliposome suspension directly after reconstitution by freeze fracture electron microscopy revealed the presence of proteoliposomes, both protein free liposomes and LmrA aggregates. Initially, the protein aggregates could not be separated from the successfully reconstituted protein using

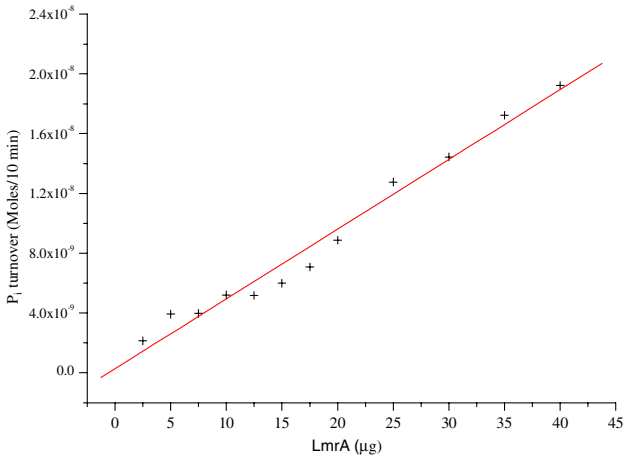

Fig. 1. The colorimetric ATPase assay performed on functionally reconstituted LmrA (in proteoliposomes) compares inorganic phosphate turnover with increasing amounts of LmrA over 10 min.

sucrose density centrifugation. However, addition of EDTA to the reconstitution buffer led to three well resolved bands on the gradient. The three bands were collected and analysed by freeze fracture electron microscopy and ^{13}C CPMAS NMR (Fig. 2). The lowest density band (9% sucrose) contained only protein free liposomes. The spectrum of this band was characterised by natural abundance signals from lipids and a lack of any intense signal in the carbonyl region from ^{13}CO glycine labelled LmrA (Fig. 2(a)). In the spectrum of the second band

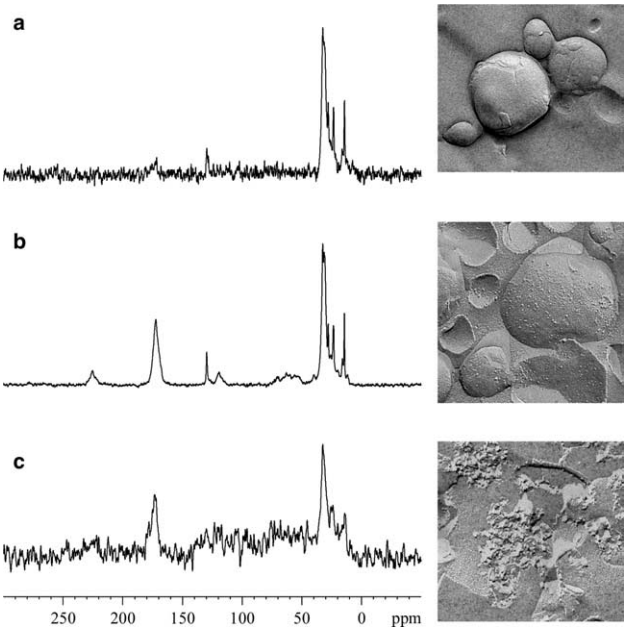

Fig. 2. ^{13}C CPMAS spectra of three fractions of a sucrose density centrifugation of LmrA functionally reconstituted in proteoliposomes prepared using *E. coli* lipids: (a) 9% sucrose: protein free liposomes, (b) 17% sucrose: proteoliposomes containing ^{13}CO glycine labelled LmrA, (c) 28% sucrose: protein aggregates. Spectra recorded at 8 kHz MAS spinning frequency and 253 K. 62k (b) and 56k (a, c) transients were acquired for each spectrum and 62.5 kHz TPPM heteronuclear ^{1}H decoupling was applied during acquisition. The corresponding freeze fracture electron micrographs are shown at 10k, 16k and 20k magnification for (a), (b) and (c), respectively.

(17% sucrose) (Fig. 2(b)), an increased peak intensity at 172.4 ppm indicated the presence of ^{13}CO glycine labelled LmrA. The corresponding electron micrographs for the second band showed well-formed lipid vesicles with protein incorporated and no evidence of aggregated protein (Fig. 2(b)). The aggregated protein was found to accumulate almost exclusively in the densest band (28% sucrose) (Fig. 2(c)). The ^{13}C CPMAS spectrum of this sample was characterised by low signal intensity and was composed largely of signal from aggregated protein (175.8 ppm) with some associated lipids.

The final lipid/protein ratio of our successfully reconstituted samples was estimated from the CP-MAS-NMR intensities of lipid and protein resonances and yielded approximately 1:3 (w/w) corresponding to ca. 250 lipids per protein monomer.

In the sucrose density centrifugation technique, the successfully reconstituted LmrA was separated from liposomes and protein aggregates to assess their individual contributions to the CPMAS spectrum of reconstituted LmrA. Unfortunately, the colorimetric ATPase assay showed negligible ATPase activity after this final purification stage (data not shown). This is probably due to the long time taken for the sucrose gradient and subsequent washing steps. However, the ratio of aggregated to lipid-reconstituted LmrA is estimated to be less than 1:9 as judged from the intensity of the observed NMR protein resonances, indicating that aggregated protein did not contribute dramatically to the observed signal. Therefore, the final sucrose density centrifugation stage can be omitted in future experiments in order to study LmrA in a functional form. Although LmrA is stable at 4 °C after reconstitution, all further steps such as storage or NMR experiments were carried out at low temperatures (233–253 K) to ensure long term stability. In our optimised protocol, the final reconstituted sample contained up to 3 mg labelled LmrA, which is compatible with a standard MAS-NMR rotor, and provided sufficient sensitivity to produce good signal to noise spectra within a realistic time frame.

Application of the preparatory techniques described here together with alternative ^{13}C labelling schemes using less abundant residues with larger chemical shift dispersion will give access to a range of solid-state NMR experiments to probe the environment of theses residues. Information about backbone conformation and dynamics of LmrA are now accessible in a non-perturbing, site-directed manner and will advance NMR analysis on this member of the ABC transporter superfamily.

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