Protein Expression and Purification 70 (2010) 81–87

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Purification and characterization of mammalian glucose transporters expressed in *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 3 August 2009
and in revised form 20 October 2009
Available online 31 October 2009

Keywords:
Glucose transporter
GLUT1
GLUT4
Membrane protein purification

ABSTRACT

The major bottleneck to the application of high-resolution techniques such as crystallographic X-ray diffraction and spectroscopic analyses to resolve the structure of mammalian membrane proteins has been the ectopic expression and purification of sufficient quantities of non-denatured proteins. This has been especially problematic for members of the major facilitator superfamily, which includes the family of mammalian glucose transporters. A simple and rapid method is described for the purification of milligram quantities of recombinant GLUT1 and GLUT4, two of the most intensively studied GLUT isoforms, after ectopic expression in *Pichia pastoris*. The proteins obtained were >95% pure and exhibited functional transport and ligand-binding activities.

Introduction

Membrane proteins are functionally diverse and include receptors for systemic or local messengers as well as transporters and channels that mediate the ability of various hydrophilic substrates to traverse lipid bilayer membranes. Many membrane receptors, channels, and transporters are the targets of pharmacological disease interventions and are thus of intense clinical interest. The determination of the structure of membrane proteins at high resolution can lead to the rational design and development of new drugs with greater efficacy and specificity and that lack undesirable iatrogenic effects.

The major facilitator superfamily (MFS)[1] is the largest superfamily of membrane transport proteins, containing over 5000 members identified in all three taxonomic kingdoms [1]. MFS proteins mediate the membrane transport of a highly diverse set of substrates via both active and passive mechanisms. Most MFS proteins appear to share a common membrane topology with 12 transmembrane alpha helices and are believed to exhibit highly flexible structures [1,2]. The latter property probably accounts for the extreme difficulty that has been encountered in attempts to obtain three-dimensional crystals of these proteins [3]. Only three members of the MFS have been crystallized to date (lactose permease [4,5], glycerol-3-P antiporter [6], EmrD multidrug transporter [7]) and none of these transporters is from a eukaryotic source. The principal roadblock to the crystallization of eukaryotic MFS proteins has been the inability to purify large quantities of these proteins in a non-denatured, non-glycosylated form.

The facilitative transport of glucose across the membranes of mammalian cells is mediated by members of the glucose transporter (GLUT) protein family that represents a subset of the MFS [8]. Reflecting their fundamental physiological importance, GLUT proteins are either directly or indirectly involved in a number of human diseases, including type 2 diabetes [9], tumor progression [10], GLUT1 Deficiency syndrome [11], Fanconi–Bickel syndrome [12], and metabolic syndrome associated with highly active antiretroviral therapy [13]. GLUT1 and GLUT4 are perhaps the most intensively studied of the 14 GLUT isoforms expressed in the human. The structure and function of GLUT1 has been studied for several decades using a variety of biochemical approaches including detailed kinetic studies, site-directed and glycosylation-scanning mutagenesis, chemical cross-linking, and many other techniques [14–20]. GLUT1 is expressed at high levels in the human erythrocyte and remains the only GLUT protein that has been purified from its native cell type [21–23]. Unfortunately, attempts to crystallize the purified native protein were unsuccessful, probably due in part to the presence of a heterogeneous N-linked oligosaccharide and to the flexible and insoluble nature of the protein in detergent solutions [22]. GLUT4 is the primary glucose transporter expressed in adipose and muscle tissues and has been the subject of intense investigation [24] because of its rate-limiting role in insulin-stimulated whole-body glucose disposal, a process that is defective in type 2 diabetes [9]. GLUT4 is also a direct cellular

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[1] *Abbreviations used:* MFS, major facilitator superfamily; GLUT, glucose transporter; AOX1, alcohol oxidase I; PI, protease inhibitor; DM, decylmaltoside; DDM, dodecylmaltoside.

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doi:10.1016/j.pep.2009.10.011

target of HIV protease inhibitors, which appears to contribute to the development of metabolic syndrome in patients treated with highly active antiretroviral therapy [25].

Herein we describe a rapid and simple procedure for the purification of milligram quantities of recombinant mammalian glucose transporter proteins that are suitable for functional studies and for crystallization assays.

Materials and methods

GLUT protein expression in Pichia pastoris

Human GLUT1 cDNA isolated from a HepG2 library [26] and rat GLUT4 cDNA isolated from an adipocyte library [27] were cloned into the expression vector pPICZ B (Invitrogen, Carlsbad, CA). The production of the GLUT1 construct is described here in detail, but similar procedures were used for all four mutant GLUT proteins using appropriate oligonucleotides. PCR was conducted using the pXOV GLUT1 [16] vector as a template in three different steps. In all of the reactions, the forward primer was 5′-GATACAGCGGCCG CATGGAGCCCAGCAGCAA containing a NotI restriction site. For the first reaction the reverse primer was 5′-CCGGTGGTGGTGATGATGGT GGTGGTGTCGACCTTCAATCACTTG. The purified DNA product was used as the template for a second amplification using the same forward primer and with the reverse primer 5′-CCGGTGGTGGTGAT-GATGGTGGTGGTGTCGACCTTCAATCACTTG for 15 cycles, and then a 10-fold molar excess of the primer 5′-TTCTAGATCAGTCCTTG TCCCGGTGGTGGTGATGATG was added for 25 additional cycles. These latter two primers inserted a Factor Xa cleavage site, a tag of eight histidines, and a stop codon at the C-terminus of the protein followed by a XbaI restriction site. The site of N-linked glycosylation was removed by mutation of Asp45 to a Thr residue. The NotI/XbaI restriction fragment was cloned into version B of the pPICZ vector. *P. pastoris* electrocompetent cells (strain X-33, Invitrogen, Carlsbad, CA), were prepared using a standard protocol and transformed by electroporation (Gene Pulser Xcell, BioRad, Hercules, CA) with 10 μg of plasmid linearized by BstX I digestion. Multiple copies of the expression cassette (P_{AOX1} promoter–gene–Zeocin) were integrated into the yeast genome by homologous recombination in a region upstream or downstream of the alcohol oxidase I (AOX1) gene. Transformed cells were selected in YPDS (Yeast extract Peptone Dextrose Sorbitol) with 100 μg/ml of Zeocin as the selection medium. Clones with multiple copies of the expression cassette were selected using 500–2000 μg/ml of Zeocin in YPDS, and then protein expression was evaluated by Western blot analysis using antibodies against the C-terminal region of GLUT1 [28]. Clones expressing the highest levels of the mutants were grown in a BioFlo 110 Fermentor & Bioreactor with gas mix controller (New Brunswick Scientific, Edison, NJ). Four liters of medium with 3% of glycerol were inoculated with 250 ml of an overnight culture of cells grown in BMGY (Buffered Glycerol-complex Medium). After the glycerol was completely exhausted from the culture medium (about 80–110 g cells/l), the cells were fed for 24–30 h with methanol on demand by monitoring oxygen levels in order to induce GLUT protein expression. Cells were then harvested and washed twice with PBS plus 1 mM EDTA and stored frozen at −80 °C.

GLUT protein solubilization

Forty-four grams of yeast cells were thawed and suspended at 30% w/v slurry to 135 ml (final volume) in breaking buffer (25 mM Na_2HPO_4/NaH_2PO_4, pH 7.40), protease inhibitor (PI) mix (0.01 U/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml benzamidine, 1 μg/ml antipain, 5 μg/ml trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, 1 mM PMSF), and 20 U/ml of DNAase I-type II (Sigma, St. Louis, MO). Cells were broken by 7 passes through a M-110S Microfluidizer (Microfluidics Co., Newton, MA) equipped with an interaction chamber of 85 μm at 23,000 psi. Before the last pass 2 M DTT and 0.1 M EDTA (4 mM and 2 mM final concentrations, respectively) were added and the homogenate was spun down at 4500*g* for 15 min. The supernate was used for the preparation of total stripped membranes. The pH of the supernate was adjusted to 10.5 using KOH, incubated for 15 min on ice, and then centrifuged at 200,000*g* for 1 h at 4 °C. The pellet was suspended in 50 ml of 25 mM Hepes, 2 mM EDTA, 2 mM DTT. Detergents were tested for GLUT solubilization using the total homogenate and the stripped membrane preparation. Triton X-100, CHAPS, decylmaltoside (DM) and dodecylmaltoside (DDM) were used at a concentration of 1% or 2% in 40 mM imidazole pH 6.80, 50 mM KCl, 5% glycerol, 1 mM TECP, 0.2 mM EDTA, containing the PI mix. The preparation was incubated for 45 min at 4 °C in a bath sonicator VWR M75D (VWR Int., Batavia, IL) containing ice and then spun down at 200,000*g* at 4 °C for 30 min. The supernates were analyzed by Western blotting using either polyclonal or monoclonal GLUT antibody [29] or poly-histidine antibody (Qiagen, Valencia, CA) and IRDye 680 donkey anti-rabbit or anti-mouse (LI-COR Biosciences, Lincoln, NE) IgG as secondary antibodies. Band intensities were measured using an Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE).

GLUT purification

For a standard large-scale preparation, 50 ml of yeast total membrane suspension was adjusted to 1% DDM, 40 mM imidazole pH 6.80, 50 mM KCl, 5% glycerol, 1 mM TECP, 0.2 mM EDTA, PI mix. After the 200,000*g* centrifugation step the pH was adjusted to 7.40 and the solubilized membrane protein was loaded onto a HisTrap HP 5 ml column (GE Healthcare, Uppsala, Sweden) in an AKTA FPLC (GE Healthcare, Uppsala, Sweden). The column was washed with four column volumes of 100 mM imidazole in loading buffer and the His-tagged GLUT proteins were eluted in 600 mM imidazole, pH 7.20, 50 mM KCl, 10% glycerol, 0.2% DDM, 3 mM TECP, 0.2 mM EDTA. The eluted protein in a total volume of 6 ml was concentrated to 1.5 ml using a Vivaspin 6 30K (Sartorius, Gottingen, Germany) tube and desalted on a 5 ml HiTrap de-salting column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 25 mM Hepes pH 6.80, 50 mM KCl, 10% glycerol, 0.2% DDM, 1 mM TECP, 0.2 mM EDTA. After de-salting, the protein solution was concentrated to a volume of 0.5 ml and then loaded onto a 24 ml 10 × 30 cm Superdex 200GL column (GE Healthcare, Uppsala, Sweden) equilibrated in the same buffer. The purity of the recombinant proteins was estimated by analysis of aliquots on 10% SDS–polyacrylamide gels stained with SimplyBlue Safe Stain (Invitrogen, Carlsbad, CA). The identity of the GLUT1 band was verified by Western blot analysis and mass spectrometry.

Photo-affinity labeling of aglyco-GLUT1 and aglyco-GLUT4

Fifteen micrograms of purified GLUT protein in 100 μl 25 mM Hepes pH 6.80, 50 mM KCl, 10% glycerol, 0.2% DM, 1 mM TECP, 0.2 mM EDTA were incubated with 5 μM of a biotin derivative of a photo-affinity labeling reagent for glucose transporters [30] *N*-[2-[2-[2-[(*N*-biotinyl-caproylamino)-ethoxy)ethoxyl]-4-[2-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoyl]-1,3-bis(mannopyranosyl-4-yloxy)-2-propylamine (PEG-biotincap-ATB-BMPA) (TRC Inc., Ontario, Canada) and UV irradiated for 1 min at 18 °C in a Rayonet RPR100 (The Southern New England UV Company, Branford, CT) and, in some of the samples, 1 μM cytochalasin B was included to test for inhibition. After photolabeling the protein was separated from the free reagent using a 0.5 ml G-25 Sepharose (GE Healthcare, Uppsala, Sweden) spin column and then subjected to Western