Protein Expression and Purification 89 (2013) 241–250

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

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

Optimized protocol for expression and purification of membrane-bound PglB, a bacterial oligosaccharyl transferase

Marcie B. Jaffee, Barbara Imperiali *

Department of Biology and Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

ARTICLE INFO

Article history:
Received 1 February 2013
and in revised form 3 April 2013
Available online 12 April 2013

Keywords:
Membrane protein
Purification protocol
Recombinant expression
Asparagine-linked glycosylation
Oligosaccharyl transferase
PglB

ABSTRACT

Asparagine-linked glycosylation (NLG) plays a significant role in a diverse range of cellular processes, including protein signaling and trafficking, the immunologic response, and immune system evasion by pathogens. A major impediment to NLG-related research is an incomplete understanding of the central enzyme in the biosynthetic pathway, the oligosaccharyl transferase (OTase). Characterization of the OTase is critical for developing ways to inhibit, engineer, and otherwise manipulate the enzyme for research and therapeutic purposes. The minimal understanding of this enzyme can be attributed to its complex, transmembrane structure, and the resulting instability and resistance to overexpression and purification. The following article describes an optimized procedure for recombinant expression and purification of PglB, a bacterial OTase, in a stably active form. The conditions screened at each step, the order of screening, and the method of comparing conditions are described. Ultimately, the following approach increased expression levels from tens of micrograms to several milligrams of active protein per liter of *Escherichia coli* culture, and increased stability from several hours to greater than six months post-purification. This represents the first detailed procedure for attaining a pure, active, and stable OTase in milligram quantities. In addition to presenting an optimized protocol for expression and purification of PglB, these results present a general guide for the systematic optimization of the expression, purification, and stability of a large, transmembrane protein.

Introduction

Biological researchers are invariably familiar with the importance of membrane proteins in therapeutic development and throughout cell biology. Several well-known statistics demonstrate this import; most notably, membrane proteins account for approximately one-third of the human proteome and comprise a majority of current drug targets [1–3]. However, standard overexpression and purification techniques are often unsuitable for this class of proteins, which presents a major obstacle to research progress. There exist promising developments for acquiring large quantities of membrane proteins, including cell-free translation systems, directed evolution of well-expressing bacteria, and the ever-increasing advances in the efficiency of current approaches [4,5]. However, expressing and purifying membrane proteins currently remains a largely empirical, time-consuming, and high-risk endeavor, leaving many important membrane-bound enzymes uncharacterized and presenting significant gaps in the understanding of cellular pathways.

The proteins in the eukaryotic N-linked glycosylation (NLG)[1] biosynthetic pathway provide an example of this phenomenon, as virtually all of the enzymes are membrane-bound. NLG plays a major role in many cell processes, including immune-system response, protein signaling and trafficking, and pathogenic invasion strategies [6–9]. In addition, NLG introduces several prospective tools in therapeutics; N-linked sugars are capable of functioning as indicators of cell state and type [10–12] and represent a novel chemical platform for developing new therapeutics and enhancing efficacy of current drugs [10,13]. Most current studies involving NLG, however, focus on determining the specific effects of the glycan modification on a target of interest, often aimed primarily at

* Corresponding author. Address: BI Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139. USA. Fax: +1 (617) 452 2419.
E-mail address: imper@mit.edu (B. Imperiali).

[1] *Abbreviations used:* NLG, asparagine-linked glycosylation; OTase, oligosaccharyl transferase; TM, transmembrane; CEF, cell envelope fraction; O.D., optical density; PCR, polymerase chain reaction; TMHMM, TM hidden Markov model; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LB, Luria–Bertani; Ni–NTA, nickel–nitrilotriacetic acid; IPTG, isopropyl-β-D-thiogalactoside; EDTA, ethylenediaminetetraacetic acid; DDM, *n*-dodecyl-β-D-maltopyranoside, OG, *n*-octyl-β-D-glucoside; MBP, maltose binding protein; GB1, G-protein B1 domain; SUMO, small ubiquitin-like modifier; Und-PP-Bac-[^{3}H]GalNAc, undecaprenyl diphosphate-linked-α-Bac- α-*N*-acetylgalactosamine; Bac, bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucose); *p*F, *para*-nitrophenylalanine; CMC, critical micelle concentration.

1046-5928/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2013.04.001

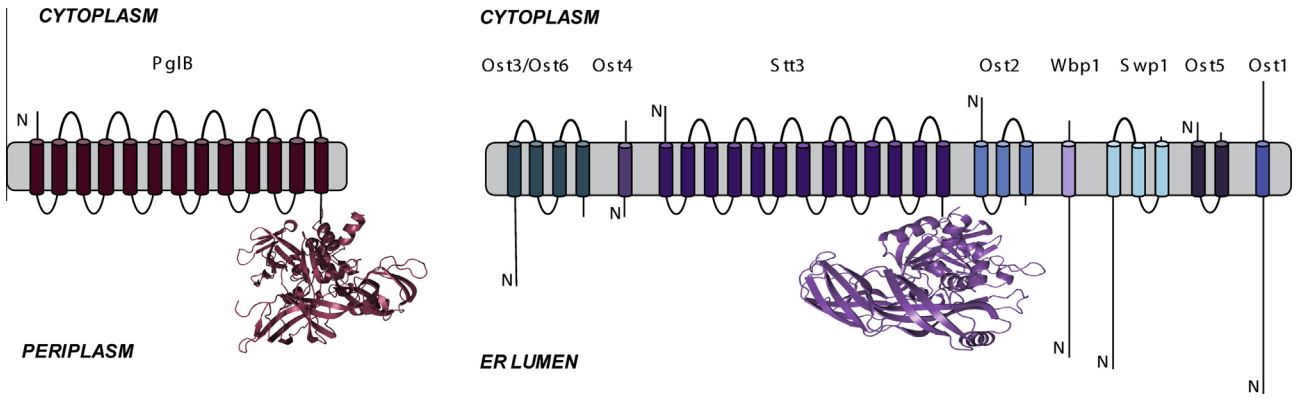

Fig. 1. Comparison of the OTases in *C. jejuni* (bacteria) and *S. cerevisiae* (eukaryotes). Images highlight the similarity between the *S. cerevisiae* catalytic subunit 'STT3' and PglB.

establishing the glycosylated sites within a protein and the effects of the modification on function [14–17]. Far less is known about the enzymes comprising the biosynthetic pathway of NLG due to the difficulty of expressing and purifying the involved membrane proteins in a stable and active state. Closing this knowledge gap would allow for enhanced ability to efficiently manipulate, study, and thus control and utilize NLG.

The system of N-linked glycosylation in the bacteria *Campylobacter jejuni* is now well recognized as an important and tractable model for studying biochemical principles of the pathway, both in bacteria and more broadly (Supplementary Information, Fig. 1) [18–20]. The central enzyme in the NLG pathway is the oligosaccharyl transferase (OTase), which catalyzes the transfer of a specific oligosaccharide to asparagine side chains. In yeast, the OTase is a complex composed of eight subunits, all of which have one or more transmembrane domains. In comparison, the OTase in *C. jejuni* (termed "PglB") is comprised of a single subunit, which is homologous to the catalytic subunit of the eukaryotic OTase (Fig. 1). Thus, PglB presents an exceptional opportunity for learning about the fundamental biochemistry involved in asparagine glycosylation, as well as studying the effects of NLG in bacteria and as a tool for protein engineering and high-level N-glycoprotein synthesis [21,22]. The recent structural and biochemical data published on PglB show the motifs responsible for catalysis are conserved throughout all kingdoms of life, solidifying its role as an important and general mechanistic model for N-linked glycosylation [19,20].

Although PglB is ostensibly a tractable target relative to the eukaryotic OTase, the enzyme represents a challenge in its own right. PglB has thirteen transmembrane domains and is fairly large (82 kDa), which accounts for poor recombinant expression and instability in *Escherichia coli*. Thus, characterization of PglB has lagged relative to other *C. jejuni* NLG enzymes, despite the potential of this OTase to reveal fundamental principles about the mechanism of OTases across the evolutionary spectrum. This manuscript describes the systematic approach used to optimize the expression, purification, and stability of active PglB. The conditions screened at each step, the order of screening, and the method of comparison for each condition are described. Specific activity values are used to determine the optimal conditions for balancing protein recovery with activity recovery. This information provides the first available protocol for expressing and purifying milligram quantities of a stable and active OTase. The method is intended to aid researchers interested in *C. jejuni* N-linked glycosylation, and also to illustrate an activity-guided approach to optimizing expression, purification, and stability of a specific membrane protein of interest.

Materials and methods

Vectors and cloning

The PglB gene was amplified by PCR from the *C. jejuni* genome NCTC 11168 [23,24]. Primers used in the PCR encoded a BamHI site at the N-terminus prior to the start codon and His10-UGA-XhoI on the C-terminus prior to the native stop codon. The PCR product was purified and digested with BamHI and XhoI (New England Biolabs, NEB) and ligated into the corresponding sites in the pET24a(+) vector (Invitrogen) using T4 DNA ligase (NEB) and standard molecular biology procedures. The resulting vector was sequenced and then transformed using manufacturer-supplied protocols into BL21 (DE3) RIL *E. coli* competent cells (Agilent) for expression. Additional vectors screened for expression of PglB with alternate fusion tags include pGEX with Glutathione-s-transferase (GST) (GE Healthcare), pMAL-c2X with MBP (NEB), pET SUMO with SUMO (Invitrogen), pET Trx with Thioredoxin (EMD Millipore), and pGBH with GB1 [25] (Supplementary Information, Table 1).

Protein expression

Pre-optimized expression was carried out according to the following procedure except when specifically noted: 5 mL solutions of LB at 25 g/L were at 37 °C, until reaching an O.D. = 0.6–0.8. At this point the temperature was turned to 16 °C and cultures were induced by adding IPTG to a final concentration of 1 mM. Cultures were left to shake overnight. The following day, cells were harvested and lysed according to the conditions described below. Competent cells screened included BL21 (DE3) RIL, BL21 (DE3) Gold, BL21 (DE3) RP, BL21 (DE3) pLys (all BL21 strains from Agilent), Rosetta 2 (DE3) (Novagen), Rosetta gami-2 (DE3) (Novagen), and C41 (Lucigen). In contrast, the optimized expression procedure involves using autoinduction media ZYM-5052, a high-density growth media [26]. For autoinduction expression, one liter of autoinduction media was made up in a six-liter flask to allow adequate aeration of the cultures. 10 g tryptone and 5 g yeast extract were combined with 960 mL of deionized water and autoclaved. Once the media had cooled, Kanamycin and Chloramphenicol were added for final concentrations of 100 μg/mL and 170 μg/mL, respectively. Just before inoculation, the following media components were added:

- 25 mL of 40X Salt Solution: 1 M Na_2HPO_4, 1 M KH_2PO_4, 2 M NH_4Cl, 0.2 M Na_2SO_4.
- 20 mL of 50X 5052: 25% glycerol (v/v), 2.5% glucose (w/v), 10% α-lactose monohydrate (w/v).