Metabolic Engineering 39 (2017) 60–70

Contents lists available at ScienceDirect

Metabolic Engineering

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

Original Research Article

Overcoming the membrane barrier: Recruitment of γ-glutamyl transferase for intracellular release of metabolic cargo from peptide vectors

Tilmann Kuenzl[a], Magdalena Sroka[a], Puneet Srivastava[b], Piet Herdewijn[b], Philippe Marlière[c], Sven Panke[a,*]

[a] Bioprocess Laboratory, Department of Biosystems Science and Engineering, ETH Zurich, Mattenstrasse 26, 4058 Basel, Switzerland
[b] Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium
[c] Institute of Systems & Synthetic Biology, Génopole, 5 rue Henri Desbrueres, 91030 Evry Cedex, France

ARTICLE INFO

Keywords:
Retrobiosynthesis
Portage transport
Gamma-glutamyl transferase
Peptide transport
Xenobiotic import

ABSTRACT

Semipermeable membranes of cells frequently pose an obstacle in metabolic engineering by limiting uptake of substrates, intermediates, or xenobiotics. Previous attempts to overcome this barrier relied on the promiscuous nature of peptide transport systems, but often suffered from low versatility or chemical instability. Here, we present an alternative strategy to transport cargo molecules across the inner membrane of *Escherichia coli* based on chemical synthesis of a stable cargo-peptide vector construct, transport through the peptide import system, and efficient intracellular release of the cargo by the promiscuous enzyme γ-glutamyl transferase (GGT). Retaining the otherwise periplasmic GGT in the cytoplasm was critical for the functionality of the system, as was fine-tuning its expression in order to minimize toxic effects associated to cytoplasmic GGT expression. Given the established protocols of peptide synthesis and the flexibility of peptide transport and GGT, the system is expected to be suitable for a broad range of cargoes.

1. Introduction

Bacteria developed several ways to control the uptake of low molecular weight compounds from their surroundings. Many compounds reach the cell with active or passive assistance by transport proteins that evolved for a specific group of molecules. Others simply diffuse into the cell across membranes if the physicochemical properties allow that. However, most compounds do not reach the cytoplasm as they are neither recognized by existing carrier proteins nor do they diffuse across membranes due to their polar or charged nature. This can include intermediates of new artificial biochemical pathways, which limits pathway implementation to step-by-step development starting from a branching point in canonical metabolism rather than from both ends in parallel (Birmingham et al., 2014). Also, the uptake of many unnatural amino acids or nucleic acid analogs for xenobiology approaches remains difficult, unless specific transporters can be identified (Malyshev et al., 2014).

An alternative strategy for the transport of non-membrane going compounds into the cytoplasm of for example the model bacterium *Escherichia coli* is based on the promiscuous nature of peptide import. For this approach, also known as portage transport (Boehm et al., 1983), a compound of interest is attached to short peptides and "smuggled" into the cell via peptide transporters, where it is released. The two best-studied peptide transporters in *E. coli* are the dipeptide and oligopeptide permease systems DppABCDF and OppABCDF, which are both known to display broad substrate specificity (Guyer et al., 1986; Smith et al., 1999). Both transport systems have spacious hydrated binding pockets in their periplasmic binding proteins DppA and OppA that allow coping with a broad diversity of peptide substrates (Dunten and Mowbray, 1995; Nickitenko et al., 1995; Sleigh et al., 1999; Tame et al., 1995). Upon entering of a peptide into the binding pocket, water molecules are displaced while the remaining water molecules stabilize the peptide in the binding pocket and prevent strong interactions with the protein. Due to this high degree of flexibility, binding of peptides containing different side-chains or side-chain modifications is possible (Payne et al., 1984; Perry and Gilvarg, 1984).

In earlier studies, it was shown that several impermeable amino acid analogs and phosphorylated metabolic intermediates like histidinol phosphate and *o*-phosphohomoserine are transported into the cell by the oligopeptide permease (Opp) system as the C-terminal residue of a tripeptide (Ames et al., 1973; Fickel and Gilvarg, 1973). However, it is known that the permease's periplasmic peptide binding protein OppA forms several salt bridges with the backbone and C-terminus of

* Corresponding author.
E-mail address: sven.panke@bsse.ethz.ch (S. Panke).

http://dx.doi.org/10.1016/j.ymben.2016.10.016
Received 15 June 2016; Received in revised form 21 September 2016; Accepted 25 October 2016
Available online 27 October 2016

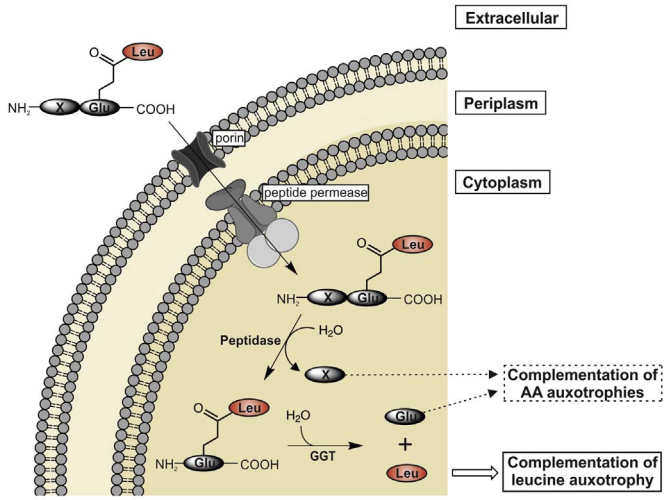

Fig. 1. Scheme of the proposed synthetic transport system and the proof-of-concept. A peptide loaded with leucine is transported into the cytoplasm of *E. coli* through porin channels and peptide permease systems. Inside the cell, the N-terminal amino acid is cleaved off by a peptidase and leucine is released by the activity of 6xHis_*Pn*GGT ΔN24.

the peptide substrate, suggesting that this approach is applicable mainly to amino acid analogs or closely related structures that do not significantly alter the conformation of the peptide backbone (Klepsch et al., 2011; Tame et al., 1995). This limits substantially the scope of the compounds that can be portaged. In a different approach, nucleophilic compounds were attached to the α-carbon atom of a glycine residue in a tripeptide and transported into the cell via the Opp system. The α-substituted glycine is stable only if it is part of a peptide but will decompose rapidly once it is liberated by cytoplasmic peptidases, releasing the leaving group (Hong and Park, 1993; Hwang et al., 1989; Kingsbury et al., 1984). The drawback of this method is that those peptides which contain a glycine residue with substituents that can function as good leaving groups are rather instable and have a half-life of only several minutes, making the system difficult to use in practical terms (Kingsbury et al., 1984).

We aimed at developing a new strategy that would retain the advantages of promiscuous peptide transport but would allow circumventing the mentioned disadvantages (Fig. 1). In general, it is chemically easy to attach a compound of interest to the γ-carboxyl side chain of a glutamate residue in a small oligopeptide via a stable amide linkage. Such a strategy would be suitable for a wide range of amine-containing cargo molecules, the resulting substrate would not display a significantly altered peptide backbone, and the substrate would be chemically stable. Once arrived in the cytoplasm, the oligopeptide would be hydrolyzed by one of *E. coli's* intracellular peptidases and the γ-substituted glutamate released. Crucially, such γ-glutamyl compounds could in principle be hydrolyzed by the enzyme γ-glutamyl transferase (GGT; EC 2.3.2.2) to unload the cargo molecule within the cytoplasm.

GGT is mainly responsible for the degradation of glutathione in the periplasmic space, but in addition, it is known to catalyze the hydrolysis of a broad range of other γ-substituted glutamyl compounds (Imaoka et al., 2010; Minami et al., 2003; Suzuki et al., 1986). Binding of a γ-glutamyl compound to the active site of the enzyme leads to the formation of a γ-glutamyl-enzyme intermediate and results in release of an amine cargo. The intermediate is then attacked by either water (hydrolase activity) or by an amino acid or peptide (transpeptidase activity) as a nucleophile (Fig. 2a) (Okada et al., 2006). Importantly, the enzyme displays a broad substrate specificity, which can be easily rationalized from the crystal structure of for example the *E. coli* GGT (*Ec*GGT): Only the γ-glutamyl moiety of the substrate is tightly bound within the substrate binding pocket while the substituent remains solvent exposed and does not or hardly interact with the active site (Okada et al., 2006). The binding of the γ-glutamyl moiety also implies that it has to be released from the oligopeptide first.

Even though the substrate specificity of *Ec*GGT seems to be favorable for the proposed approach, the cellular location of GGTs requires further attention. GGT is usually secreted from the cytoplasm of bacteria or anchored in the eukaryotic membrane with the catalytic activity in the extracellular domain (Finidori et al., 1984; Hanigan, 2014; Suzuki et al., 1986). In *E. coli,* GGT is expressed as an inactive proenzyme and secreted to the periplasmic space where it undergoes maturation into a (previously N-terminal) large and a (previously C-terminal) small subunit of approximately 40 and 20 kDa in size, respectively. The maturation process is essential for enzyme activity, as the threonine residue at the newly formed N-terminus of the small subunit is the catalytic residue of the enzyme (Suzuki and Kumagai, 2002). However, in the approach proposed here, we need to make sure that GGT is retained in the cytoplasm.

The aim of this study is to provide a proof of concept, making use of the broad substrate ranges of *E. coli's* peptide permeases and GGT and combining them in a synthetic and potentially universal transport system. Primarily, this required changing the cellular localization of GGT from the periplasmic space to the cytoplasm and fine-tuning its expression to overcome potential toxic effects of cytoplasmic GGT expression. With these modifications, we were able to demonstrate portage of different cargo molecules and unloading of these cargoes exclusively in the presence of cytoplasmic GGT.

2. Materials and methods

2.1. Strains and media

A list of all bacterial strains used in this study is provided in Table 1. For cloning purposes *E. coli* strains Top10 or DH5α λpir were used. Growth experiments in selective medium were carried out in the leucine auxotrophic strains TK054 or TK054 Δ*pcnB* and the methionine auxotrophic strain TK014. For the construction of TK054, the genes *ggt*, *leuB* and *brnQ* were replaced with disrupted versions by P1 phage transduction using respective donor strains from the KEIO collection (Baba et al., 2006; Thomason et al., 2007). The *livFGHMK* operon was inactivated by λred recombination with a PCR fragment containing a kanamycin resistance gene amplified from pKD13 with the primers TK140 and TK141 (Datsenko and Wanner, 2000). To subsequently remove the kanamycin resistance gene, plasmid pCP20 was used (Cherepanov and Wackernagel, 1995). The gene *pcnB* was deleted by plasmid-based gene replacement (Martinez-Garcia and de Lorenzo, 2011, 2012). For this, 500 bp fragments upstream and downstream of *pcnB* (TS1 and TS2) were amplified, combined by PCR and cloned into plasmid pEMG via *Eco*RI and *Bam*HI restriction sites. For the construction of TK014, the genes *metA* and *metE* were replaced with disrupted versions by P1 phage transduction using respective donor strains from the KEIO collection. To integrate 6xHis_*Pn*GGT ΔN24 into the chromosome, the N-terminally tagged 6xHis_*Pn*GGT ΔN24 gene together with its promoter and the chloramphenicol resistance gene was amplified with the primers TK417 and TK418 using the plasmid as template. The PCR product was recombined with the *E. coli* chromosome in the intergenic region between *yrhB* and *yhhA* assisted by the λred genes expressed from pKD46 (Datsenko and Wanner, 2000).

LB Miller broth (Becton Dickinson, Sparks, MD, USA) was used as standard growth medium for bacterial cultures (Sambrook, 2001). For the preparation of competent cells, SOB medium was used (Hanahan, 1983). Unless stated otherwise, growth experiments in selective medium were carried out in M9 minimal medium (Sambrook, 2001) supplemented with 0.5% glucose, 1 μg mL^{-1} thiamine, 0.5 mM IPTG and varying peptide concentrations. Alanyl-γ-glutamyl-leucine (> 95% purity) was custom synthesized by Pepscan (Lelystad, Netherlands)