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An internal hydrophobic helical domain of *Bacillus subtilis* enolase is essential but not sufficient as a non-cleavable signal for its secretion

Chun-Kai Yang [1], Xiao-Zhou Zhang [1,2], Chung-Dar Lu, Phang C. Tai *

Department of Biology, and Center of Biotechnology and Drug Design, Georgia State University, Atlanta, GA 30303, United States

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ABSTRACT

Many cytoplasmic proteins without a cleavable signal peptide, including enolase, are secreted during the stationary phase in *Bacillus subtilis* but the molecular mechanism is not yet clear. We previously identified a highly conserved embedded membrane domain in an internal hydrophobic α-helix of enolase that plays an important role in its secretion. In this study, we examined the role of the helix in more detail for the secretion of enolase. Altering this helix by mutations showed that many mutated forms in this domain were not secreted, some of which were not stable as a soluble form in the cytoplasm. On the other hand, mutations on the flanking regions of the helix or the conserved basic residues showed no deleterious effect. *Bacillus* enolase with the proper hydrophobic helical domain was also exported extracellularly in *Escherichia coli*, indicating that the requirement of the helix for the secretion of enolase is conserved in these species. GFP fusions with enolase regions showed that the hydrophobic helix domain itself was not sufficient to serve as a functional secretion signal; a minimal length of N-terminus 140 amino acids was required to mediate the secretion of the fused reporter GFP. We conclude that the internal hydrophobic helix of enolase is essential but is not sufficient as a signal for secretion; the intact long N-terminus including the hydrophobic helix domain is required to serve as a non-cleavable signal for the secretion of *Bacillus* enolase.

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1. Introduction

Many cytoplasmic proteins without any cleavable classical N-terminal signal peptides have been found to be secreted via 'non-classical or non-conventional secretion' [1–4]. Such proteins including enolase have been termed "moonlighting" proteins which display multiple unrelated functions in different subcellular locations, [3–6]. It has been proposed that the release outside of cells could be attributed to cell lysis [7,8]. Enolases (EC 4.2.1.11) are essential cytoplasmic enzymes that catalyze the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate. Although enolases lack a classical signal sequence and typical motifs for membrane or cell wall anchoring, many studies showed that various enolases can be exported to the cell surface or released to the culture medium in eukaryotic and prokaryotic organisms [5,9,10]. It has been long speculated that there might be an unknown signal for enolase export conserved over a long evolutionary period [11]. Extensive studies from different groups support the presence of an alternative secretion mechanism other than the classical Sec-pathway to drive enolases through membranes to the cell surface or into the extracellular medium [2,11]. *Escherichia coli* enolase was found to be exported into the medium and the export was dependent on covalent binding of its substrate, 2-phosphoglycerate [12]. *Bacillus subtilis* enolase has also been found in the extracellular compartment [8,13], though the mechanism of how the enolase is secreted remains uncertain. We have previously reported that enolase and other cytoplasmic proteins without a cleavable signal sequence can be secreted from *B. subtilis* cells into the medium in the absence of cell lysis [1].

Using the crystal structure of *Enterococcus hirae* enolase [14] as the template, a predicted molecular structure of enolase was modeled by Swiss-Model [15]. *Bacillus* enolase (SI Fig. 1) is composed of one large C-terminal domain (S_{139}-K_{430}) and one smaller N-terminal domain (P_2-N_{138}). According to Swiss-Plot database (Entry no. **37869**), the C-terminal barrel domain contains four phosphorylation sites, substrate and Mg^{2+} binding sites and two catalytic motifs.

Abbreviations: EnoBs, enolase of *Bacillus subtilis*; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria–Bertani; EM domain, embedded membrane domain; HH domain, hydrophobic α-helix domain; GFP, Green Fluorescent Protein.

* Corresponding author. Address: Department of Biology, Georgia State University, Room 592, Petit Science Center, 161 Jesse Hill Jr. Drive, Atlanta, GA 30303, United States. Fax: +1 404 413 5301.
E-mail address: biopct@gsu.edu (P.C. Tai).
[1] These authors contributed equally to this work.
[2] Present address: Gate Fuels, Inc., 2200 Kraft Drive, Suite 1200B, Blacksburg, VA 24061, United States.

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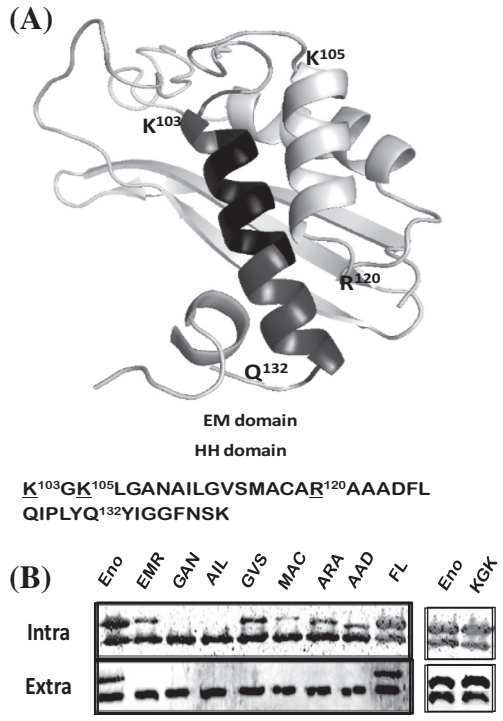

Fig. 1. Predicted ribbon structures of N-terminal domain of *Bacillus* enolase and the importance of HH domain on enolase secretion. (A). Top panel: predicted structures of the 140 aa N-terminal domains *Bacillus* enolase using the Swiss-Plot [15]. The HH α-helix (#106–126) and EM (#110–118) domains are highlighted. Three basic residues (K_{103}, K_{105}, R_{120}), and a small loop replacement (Q132) around HH domain are indicated. (B). Mutations in the HH domain affected the secretion of enolase. Different glycine-substituted mutants were expressed in *B. subtilis* WB600BHM. Equal volumes of soluble whole-cell lysate (Intra) and supernatant (Sup) fractions were examined by immunoblots. EnoBs antibodies detected both chromosomal wild-type (which were used as internal controls) and plasmid FLAG-tagged enolase (higher band, confirmed with FLAG antibodies). Eno: wild-type enolase; EMR: EM domain replaced enolase [1]; all other mutations are glycine replacements as described in Section 2.

In the N-terminal motif, a long unbent hydrophobic α-helix (HH) domain (A_{108}-L_{126}) of enolase resides (Fig. 1A). Within this HH domain, a core region (A_{110}-C_{118}) is predicted as a membrane-embedded (EM) domain [1] by the PSSM_SVM scheme [16] that may be involved in interacting with membranes. Mutation analyses by deletion of EM domain or replacement (EMR) of the enolase showed that this EM domain indeed is important for its secretion [1].

The EM domain is part of the larger HH domain that has been predicted as a signal peptide or a transmembrane domain (signalP 3.0 server; [17]). In this work we further examined the nature of this EM domain and the surrounding HH domain for the secretion of enolase in *B. subtilis* (EnoBs). We constructed a series of mutations in the domain of the cloned EnoBs to determine their effects on its secretion in *B. subtilis*. We found that the EnoBs with native hydrophobic helix, but not mutated, domain can also be exported in *E. coli* as in *B. subtilis*, indicating that the importance of the HH domain in the secretion of EnoBs. Moreover, we have identified an N-terminal region including the HH domain of EnoBs that facilitates the secretion of the reporter protein GFP. Taken all data together, we conclude that this highly conserved HH domain is important, but not sufficient in its secretion.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture conditions and growth

Bacterial strains and plasmids are listed in SI Table 1. *B. subtilis* WB600BHM and *E. coli* strain DH5α were grown in Luria–Bertani (LB) broth or agar plates containing 0.2% glucose at 37 °C. The following antibiotics were used as required: ampicillin (100 μg/mL), chloramphenicol (25 μg/mL for *E. coli* and 5 μg/mL for *Bacillus*), and kanamycin (10 μg/mL).

2.2. Cloning and expression of B. subtilis enolase mutants

Constructions of the wild-type *B. subtilis eno* gene and its mutations on shuttle vector pDG148 using overlapping PCR were described previously [1]. To construct FLAG-tagged enolase and mutants, the wild-type and mutated *eno* genes were PCR amplified and cloned into the *Sac*I/*Pst*I sites of pGTN-FLAG [1].

To express the EnoBs in *B. subtilis* WB600BHM, the transformants containing a plasmid with cloned *eno* gene were grown in LB medium with 0.2% glucose at 37 °C. When the cell cultures reached O.D. 0.2, IPTG (1 mM) was added to induce the cloned enolase expression as described [1]. After 10 h of cultivation for *B. subtilis,* 10 mL of cells were collected by centrifugation, whole cell lysate and medium supernatant were prepared as described [1]. The cells were re-suspended in 5 mL of 10 mM Tris–HCl (pH 8.0) buffer containing 1 mM EDTA (TE buffer), followed by French Press to break the cells. The whole cell lysates were centrifuged at 12,000×*g* for 10 min to remove cell debris and insoluble materials, and the supernatants were collected as soluble whole cell lysates. The samples were adjusted for protein amounts where necessary, using chromosomal enolase as internal control, and were mixed with the equal amount of 2× sample buffer for immunoblot analysis [1]. *B. subtilis* enolase antibodies were used under condition in which *E. coli* enolase was not detectable.

2.3. Construction of Green Fluorescent Protein fusions with N-terminus of enolase

Green Fluorescent Protein (GFP) was amplified from pBAD-gfpuv (Clontech, Mountain View, CA) and the restriction sites *Xba*I and *Sph*I were introduced at 5′ and 3′ sites of GFP, respectively. The PCR product was digested by *Xba*I/*Sph*I and subcloned onto the corresponding sites of *E. coli*/*B. subtilis* shuttle vector pDG148 in which the cloned gene was placed under the control of P_{spac} promoter, resulting in the plasmid pDGGFP. To construct the different lengths of N-terminus of enolase fusion with GFP, PCR-amplified N-terminus fragments were inserted into pDGGFP and resulted in nine different plasmids (SI Table 1). The sequences of the entire inserts in the pDGGFP-derived plasmids were confirmed by DNA sequencing.

2.4. Secretion of EnoBs and isolation of outer membrane vesicles in E. coli

The overnight cultures of *E. coli* DH5α containing cloned EnoBs gene were inoculated into fresh LB medium with 0.2% glucose at 37 °C with shaking. When cell cultures reached O.D. 0.2, IPTG (1 mM) was added to induce the cloned enolase expression as described [1]. After 8 h cells were collected by centrifugation and the supernatant was treated as with *B. subtilis* above. The outer membrane vesicles (OMV) were pelleted by ultracentrifugation (100,000 rpm, 30 min, 4 °C) in a TLA-100.3 rotor (Beckman Max-XP), and washed with 0.5 M NaCl to remove loosely bound enolase with membranes. The extracellular proteins in the spent medium were precipitated using DOC–TCA method [18].

2.5. Immunoblots

Protein samples were analyzed by immunoblots as described [1]. The rabbit primary antibodies for *Bacillus* enolase, *E. coli* OmpA, SecA and SecY were from the laboratory stock and for GFP and

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