Biochimie 94 (2012) 829–838

Contents lists available at SciVerse ScienceDirect

Biochimie

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

Research paper

Genetic and biochemical characterization of *Corynebacterium glutamicum* ATP phosphoribosyltransferase and its three mutants resistant to feedback inhibition by histidine

Yun Zhang [a], Xiuling Shang [a,b], Aihua Deng [a], Xin Chai [a,b], Shujuan Lai [a,b], Guoqiang Zhang [a,b], Tingyi Wen [a,*]

[a] Department of Industrial Microbiology and Biotechnology, Institute of Microbiology, Chinese Academy of Sciences, 100101 Beijing, China
[b] Graduate University of Chinese Academy of Sciences, 100049 Beijing, China

ARTICLE INFO

Article history:
Received 22 September 2011
Accepted 30 November 2011
Available online 8 December 2011

Keywords:
ATP phosphoribosyltransferase
Corynebacterium glutamicum
Feedback inhibition
Histidine
Mutagenesis

ABSTRACT

ATP phosphoribosyltransferase (ATP-PRT) catalyzes the condensation of ATP and PRPP at the first step of histidine biosynthesis and is regulated by a feedback inhibition from product histidine. Here, we report the genetic and biochemical characterization of such an enzyme, $HisG_{Cg}$, from *Corynebacterium glutamicum*, including site-directed mutagenesis of the histidine-binding site for the first time. Gene disruption and complementation experiments showed that $HisG_{Cg}$ is essential for histidine biosynthesis. $HisG_{Cg}$ activity was noncompetitively inhibited by histidine and the α-amino group of histidine were found to play an important role for its binding to $HisG_{Cg}$. Homology-based modeling predicted that four residues (N215, L231, T235 and A270) in the C-terminal domain of $HisG_{Cg}$ may affect the histidine inhibition. Mutating these residues in $HisG_{Cg}$ did not cause significant change in the specific activities of the enzyme but resulted in the generation of mutant ones resistant to histidine inhibition. Our data identified that the mutant N215K/L231F/T235A resists to histidine inhibition the most with 37-fold increase in K_i value. As expected, overexpressing a $hisG_{Cg}$ gene containing N215K/L231F/T235A mutations *in vivo* promoted histidine accumulation to a final concentration of 0.15 ± 0.01 mM. Our results demonstrated that the polarity change of electrostatic potential of mutant protein surface prevents histidine from binding to the C-terminal domain of $HisG_{Cg}$, resulting in the release of allosteric inhibition. Considering that these residues were highly conserved in ATP-PRTs from different genera of Gram-positive bacteria the mechanism by histidine inhibition as exhibited in *Corynebacterium glutamicum* probably represents a ubiquitously inhibitory mechanism of ATP-PRTs by histidine.

1. Introduction

Gram-positive actinomycete bacterium *Corynebacterium glutamicum* is applied as an industrial workhorse for production of amino acids and organic acids due to its metabolic and excreting capabilities [1–3]. As its genome sequence became available and many metabolic regulatory mechanisms have been extensively elucidated, *C. glutamicum* now serves as a model bacterium for construction of engineered strains on the base of a rational scheme including a series of genetic modifications [4–6]. Given that carbon flux through the pentose phosphate pathway (PPP) is higher than that through the TCA cycle [7,8], *C. glutamicum* is, therefore, an appropriate candidate for metabolic engineering of histidine biosynthesis with its precursor supplied through PPP pathway. To date, *de novo* engineering of histidine biosynthesis in *C. glutamicum* is not reported yet due to lack of knowledge about the functional role of its key enzyme and the regulation mechanism of histidine biosynthesis *in vivo*.

ATP phosphoribosyltransferase (ATP-PRT, EC 2.4.2.17) is the first enzyme in histidine biosynthesis pathway and plays a critical regulatory role in controlling carbon flux towards histidine biosynthesis [9]. In the presence of divalent magnesium ion, ATP-PRT catalyzes the condensation of 5-phosphoribosyl 1-pyrophosphate (PRPP) and ATP to form N1-(5-phosphoribosyl)-ATP (PR-ATP).

Abbreviations: $HisG_{Cg}$, ATP phosphoribosyltransferase from *Corynebacterium glutamicum*; ATP-PRT, ATP phosphoribosyltransferase; CD, circular dichroism; DTT, dithiothreitol; kDa, kilo Dalton; bp, base pairs; SDS-PAGE, soduim dodecyl sulphate polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; PCR, polymerase chain reaction; PDB, protein data bank; PPP, pentose phosphate pathway; PRPP, 5-phosphoribosyl 1-pyrophosphate; PR-ATP, N1-(5-phosphoribosyl)-ATP; N (or Asn), asparagine; K (or Lys), lysine; L (or Leu), leucine; F (or Phe), phenylalanine; T (or Thr), threonine; A (or Ala), alanine.

* Corresponding author. Tel.: +86 10 62526173; fax: +86 10 62522397.
E-mail address: wenty@im.ac.cn (T. Wen).

0300-9084/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2011.11.015

The best studied ATP-PRT is that from Gram-negative *Escherichia coli* and *Salmonella typhimurium* [9–12]. The reaction catalyzed by ATP-PRT is reversible and both directions are consistent with a Bi-Bi kinetic mechanism [10]. AMP and ADP are competitive inhibitors with respect to both substrates and become positively cooperative inhibitors in the presence of histidine [11,12]. ATP-PRT activity is subjected to feedback inhibition by the level of intracellular histidine to achieve strict regulation of histidine biosynthesis [9].

ATP-PRT is a member of a fourth (type IV) phosphoribosyltransferase (PRT) subfamily of enzymes, which are essentially involved in many pathways for the biosynthesis of cofactors, amino acids and nucleotides as well as the salvage of nucleotides [13]. As encoded by the *hisG* gene, ATP-PRTs have been found in archaea, bacteria, fungi and plants and share low sequence identity among various species [9,14,15]. These enzymes comprise two structurally distinct subfamilies referred as $HisG_L$ or long form and $HisG_S$ or short form, respectively. $HisG_L$ is composed of about 280–310 amino acids and found in lower eukaryotes and bacteria such as *E. coli*, *S. typhimurium* and *Mycobacterium tuberculosis*. In contrast, $HisG_S$ is about 80 residues shorter than $HisG_L$ at its C terminus and has no catalytic activity unless they bind the regulatory subunit HisZ as in *Lactococcus lactis* and *Thermotoga maritima* [16]. Crystal structures of ATP-PRTs have provided clues to understand the catalytic and regulatory mechanism of two subfamily enzymes. Enzymes from the short form ATP-PRT subfamily are hetero-octamers and composed of four catalytic $HisG_S$ subunits and four HisZ subunits. The latter acts as a regulatory domain to compensate for the absence of a C-terminal regulatory domain found in $HisG_L$. Therefore, $HisG_S$ exhibits the same catalytic mechanism as $HisG_L$ but with a different regulatory pattern [17–19]. To date, two different structures of hetero-octameric ATP-PRT have been identified to exist as a histidine-inhibited complex in *T. maritima* [18] and ATP-activated and PRPP-bound forms in *L. lactis* [17,19]. Enzymes from the $HisG_L$ subfamily are homo-hexamers which are the inactive, histidine-binding forms of the enzyme. The active form of $HisG_L$ is a homo-dimer and each subunit is composed of three distinct domains [20]. There are two different $HisG_L$ structures. In *E. coli*, the PRPP and parts of the ATP-binding sites locate in the first two domains in ATP-PRT structure, which clearly identifies that AMP can bind in both PRPP- and ATP-binding sites [21]. In *M. tuberculosis*, a comparison between the apo and AMP:His forms of ATP-PRT structure has revealed that the C-terminal domain directly interacts with histidine. This binding triggers the hexamer conformation change and thus leads to the steric hindrance in the active site [22]. Both of $HisG_L$ structures exhibit similar allosteric inhibition by histidine despite the fact that large differences exist in their amino acid sequences. However, there is no further study performed to identify or analyze the histidine binding sites in $HisG_L$.

The *hisG*$_L$ gene (GenBank accession no. AF050166) had been cloned previously from a genome library of *C. glutamicum* strain ASO19 by complementation of *E. coli* histidine auxotroph [23]. Genome sequencing of *C. glutamicum* strain ATCC13032 showed that this strain contains a gene called *hisG*$_{Cg}$ (NCgl1447) that encodes a protein annotated as an ATP-phosphoribosyltransferase [24,25]. In this study, we identified that *hisG*$_{Cg}$ gene encodes a functional ATP-PRT essential for histidine biosynthesis in *C. glutamicum* and showed detailed biochemical characterization performed on a long form ATP-PRT from a Gram-positive bacterium. According to sequence alignment and homology-based modeling using the crystal structure of ATP-PRT of *M. tuberculosis* [22], four residues in the C-terminal domain of $HisG_{Cg}$ were predicted to be involved in histidine binding. Functional analysis of $HisG_{Cg}$ mutants confirmed that three of the four residues were crucial to release the feedback inhibition by histidine. The findings about binding between histidine and $HisG_{Cg}$ as well as the electrostatic potential of protein surface account for the resistance of $HisG_{Cg}$ to histidine, shedding light on the molecular basis of how long-form ATP-PRTs are inhibited by histidine feedback.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

E. coli DH5α (F^- *supE44 Φ80 δlacZ ΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17* (r_k^-, m_k^+) *deoR thi-1* λ^- *gyrA96 relA1*) and BL21(DE3) (*hsdS gal* (*λcIts*857 *ind-l Sam7 nin-5 lacUV5-T7 gene 1)*) were used in this work as host strains for the gene cloning and expression of the recombinant enzymes, respectively. Plasmid pET28a (Novagen) was used for protein expression. *E. coli* strains were cultured aerobically on a rotary shaker at 180 rpm in Luria-Bertani (LB) medium supplemented with 50 μg/mL of kanamycin when needed. The *C. glutamicum* RES167 (restriction-deficient mutant of ATCC13032, *ΔcglIMΔcglIRΔcglIIR*) was used for genetic disruption and complementation using plasmids pK18*mobsacB* and pXMJ19 [26,27]. *C. glutamicum* strains were aerobically grown at 30 °C on a rotary shaker at 200 rpm in rich medium CGIII [28] or in the minimal medium CGX [29]. For the generation of mutant and maintenance of *C. glutamicum* brain heart broth with 0.5 M sorbitol medium was used [30].

2.2. Genetic disruption and complementation in C. glutamicum

Disruption of *hisG*$_{Cg}$ was performed by replacing its 3′-end sequence (corresponding to nucleotide 583–846) with the chloramphenicol resistance cassette which was amplified using pXMJ19 as template [27]. Three pairs of primers listed in Supplemental Table 1 were used to amplify the *hisG*$_{Cg}$ gene (163–582 bp), chloramphenicol resistance gene (861 bp) and downstream sequence of *hisG*$_{Cg}$ (408 bp). The products were fused by the splice overlap extension (SOE) PCR and ligated into pK18*mobsacB* to construct plasmid pK18*mobsacBΔhisG*$_{Cg}$. The resulting mutant RES167ΔNCgl1447 with disruption of *hisG*$_{Cg}$ gene in *C. glutamicum* RES167 was constructed by integration and excision of the plasmid pK18*mobsacBΔhisG*$_{Cg}$ according to the method of Schäfer *et al* [26].

For complementation, the plasmid pXMJ19-*hisG*$_{Cg}$ containing an intact *hisG*$_{Cg}$ gene was introduced into the *C. glutamicum* RES167ΔNCgl1447 and the expression of *hisG*$_{Cg}$ was induced by isopropylthio-β-D-galactopyranoside (IPTG).

2.3. Expression and purification of wild type $HisG_{Cg}$ and its mutants

The mutated *hisG*$_{Cg}$ genes were constructed by SOE PCR using a series of mutagenic primers as shown in Supplemental Table 1. Mutant and wild type genes were cloned into the vector pET28a and expressed in the BL21 (DE3) strains. The preparation of plasmid, digestion with restriction endonucleases, and electroporation were carried out as described by Sambrook *et al.* [31]. Cells were induced with 1 mM IPTG for 8 h at 16 °C when the OD_{600} reached 0.6, and then harvested by centrifugation and resuspended in the standard buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA and 1 mM DTT). The suspended cells were disrupted by sonication in an ice-water bath and the supernatants were collected by centrifugation at 12,000 × *g* for 20 min at 4 °C. The N-terminal 6 × His-tagged $HisG_{Cg}$ proteins were purified by His-Bind column chromatography according to the manufacturer's protocol (Novagen). The eluted protein solution was desalted in HiTrap columns (GE Healthcare, USA) and concentrated by ultrafiltration (10-kDa MW cut-off membrane, Millipore, USA). Subsequently, the $HisG_{Cg}$ was applied to a Superdex 200 10/300 GL column (10 × 300 nm, GE Healthcare, USA) equilibrated with the standard buffer using the ÄKTA purifier system (GE Healthcare, USA) and eluted at a flow rate of 0.5 mL/min.