Archives of Biochemistry and Biophysics 486 (2009) 19–26

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Steady-state kinetics of indole-3-glycerol phosphate synthase from *Mycobacterium tuberculosis*

Clarissa M. Czekster [a,b], Brenno A.D. Neto [c], Alexandre A.M. Lapis [c], Jairton Dupont [c], Diogenes S. Santos [a,*], Luiz A. Basso [a,*]

[a] *Centro de Pesquisas em Biologia Molecular e Funcional, Instituto de Ciência e Tecnologia em Tuberculose, Pontifícia Universidade Católica do Rio Grande do Sul, 6681/92A Ipiranga Av., 90619-900 Porto Alegre, RS, Brazil*

[b] *Programa de Pós-graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Porto Alegre, RS, Brazil*

[c] *Laboratory of Molecular Catalysis – Institute of Chemistry, UFRGS, Porto Alegre, RS, Brazil*

ARTICLE INFO

Article history:
Received 7 January 2009
and in revised form 13 March 2009
Available online 11 April 2009

Keywords:
Tuberculosis
Drug target
Indole-3-glycerol phosphate synthase
1-(*o*-Carboxyphenylamino)-1-deoxyribulose 5-phosphate
IGPS
Steady-state kinetics

ABSTRACT

Indole-3-glycerol phosphate synthase (IGPS) catalyzes the irreversible ring closure of 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CdRP), through decarboxylation and dehydration steps, releasing indole-3-glycerol phosphate (IGP), the fourth step in the biosynthesis of tryptophan. This pathway is essential for *Mycobacterium tuberculosis* virulence. Here we describe the cloning, expression, purification, and kinetic characterization of IGPS from *M. tuberculosis*. To perform kinetic studies, CdRP was chemically synthesized, purified, and spectroscopically and spectrometrically characterized. CdRP fluorescence was pH-dependent, probably owing to excited-state intramolecular proton transfer. The activation energy was calculated, and solvent isotope effects and proton inventory studies were performed. pH-rate profiles were carried out to probe for acid/base catalysis, showing that a deprotonated residue is necessary for CdRP binding and conversion to IGP. A model to describe a steady-state kinetic sequence for *Mt*IGPS-catalized chemical reaction is proposed.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Tuberculosis (TB)[1] is major public health concern, with nine million new cases and two million deaths per year (http://tballiance.org). The first-line treatment regimen for active TB prescribed today is the same since the 1980s [1], it is complex and has very low patient adherence due to the severe side effects. The causative agent of TB, *Mycobacterium tuberculosis*, is an easily transmitted airborne pathogen that infects primarily alveolar macrophages, beginning an infection in the lungs [2].

The emergence of multi and extensive drug resistant strains (MDR-TB, XDR-TB) and the lengthy treatment have led to efforts in identifying new targets for TB drug development. Smith and co-workers identified knockouts in the leucine, proline, and tryptophan biosynthetic pathways [3] that are unable to replicate in cultured murine macrophages and show attenuation in their ability to infect mice, indicating that these amino acids are unavailable or insufficient inside the macrophage. The most marked attenuation of virulence occurred in the tryptophan auxotrophic strain, which is essentially avirulent, even in immunodeficient mice. Additionally, tryptophan is not synthesized by mammals, making enzymes from the tryptophan biosynthetic pathway viable targets for the development of new anti-TB drugs. Even thought this is a very attractive pathway for drug design and virulence understanding, very few works were conducted concerning the TB enzymes [4–8].

The enzyme indole-3-glycerol phosphate synthase (IGPS) catalyzes the fourth step towards tryptophan biosynthesis, the irreversible ring closure of 1-(*o*-carboxyphenylamino)-deoxyribulose 5-phosphate (CdRP), through decarboxylation and dehydration steps, generating indole-3-glycerol phosphate (IGP), which contains the indolic ring of tryptophan (Fig. 1) [9]. The mechanistic studies data available for this catalyzed reaction comprise the crystallographic structures of the IGPS from *Sulfolobus solfataricus*, *Thermotoga maritima*, and *Escherichia coli*, along with pre-steady-state studies carried out with the *E. coli* enzyme [10–15]. Recently, a novel inhibitor targeting this TB enzyme has been proposed and the structure of *Mt*IGPS was modeled [7]. We have recently reported electrospray-ionization mass spectrometry (ESI-MS) data showing that two intermediates are part of *M. tuberculosis* IGPS chemical mechanism [8]. However, a steady-state kinetic charac-

* Corresponding authors. Fax: +55 51 33203629.
E-mail addresses: diogenes@pucrs.br (D.S. Santos), luiz.basso@pucrs.br (L.A. Basso).

[1] *Abbreviations used:* TB, tuberculosis; MDR-TB, multi-drug resistant tuberculosis; *Mt*IGPS, indole-3-glycerol phosphate synthase from *Mycobacterium tuberculosis* H37Rv; CdRP, 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate; IGP, indole-3-glycerol phosphate; AA, anthranilic acid; R5P, Ribose-5-phosphate; *Ec*IGPS, *E. coli* IGPS; *Ss*IGPS, *Sulfolobus solfataricus* IGPS; ESI-MS, electronspray-ionization mass spectrometry; ESIPT, excited-state intramolecular proton transfer; NMR, nuclear magnetic resonance.

0003-9861/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2009.04.001

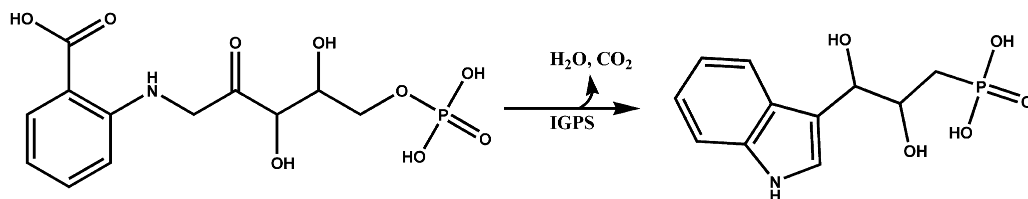

Fig. 1. *Mtb*IGPS-catalyzed reaction.

terization of this enzymatic reaction is absent in all organisms studied until the present date. In addition, there is a need for both a more thorough characterization of the substrate of IGPS-catalyzed chemical reaction, CdRP, and its behavior in solution.

Here, we describe cloning, expression, and purification of *trpC*-encoded *Mt*IGPS. In addition, we describe a modified protocol for the chemical synthesis and purification of CdRP, and its characterization by ESI-MS, IR, ^{1}H NMR, UV–vis, and fluorescence spectroscopy. The *Mt*IGPS-catalyzed reaction was characterized in steady-state conditions by initial velocity experiments, pH-rate profiles, temperature effects, solvent kinetic isotope effects, and proton inventory. A kinetic sequence for this reaction is proposed. It is hoped that the data presented here will contribute to improving our understanding on the mode of action of this attractive target for new anti-TB drug development.

Materials and methods

Materials

All chemicals were of analytical or reagent grade and were used without further purification. Deuterium oxide (99.9 atom% D) was purchased from Cambridge Isotope Laboratories. *Pfu* DNA Polymerase was from Stratagene, and restriction enzymes and T4 DNA ligase were from Invitrogen.

Cloning, expression, and purification of MtIGPS

The forward primer sequence for amplification of the *trpC* coding region was 5′-CA**CATATG**AGTCCGGCAACCGTGCTCGACTC-3′, having an NdeI restriction site (bold) at the N-terminal end. The reverse primer sequence was 5′-T**GGATCC**TCAGCGAGCCGGTTTC GGACAG-3′, which introduced a BamHI restriction site (bold) at the C-terminal end. All PCR reactions were carried out using *pfu* DNA polymerase (Stratagene), using conditions recommended by the manufacturer, and were supplemented by the addition of 10% (v/v) DMSO. The resultant PCR product, corresponding to 819 bp, was ligated into the pCR®-Blunt (Invitrogen) vector, and transformed into *E. coli* DH10B. The recombinant plasmid purified from these cells was cleaved with NdeI and BamHI restriction enzymes, and the resulting fragment subcloned into the pET-23a(+) vector (Novagen) previously digested with the same restriction enzymes. The plasmid was then transformed by electroporation into *E. coli* BL21(DE3) STAR host cells (Novagen). Cells were grown in 4YT containing 50 μg mL^{-1} carbenicillin, at 37 °C and after the OD_{600} reached 0.4–0.6, cells were allowed to grow for additional 9 h and harvested by centrifugation at 20,800*g* for 30 min. All purification procedures were carried out at 4 °C. Cells were resuspended in 50 mM Tris–HCl pH 7.3 containing 200 mM NaCl, 1 mM EDTA, and 0.1 mM DTT (buffer A), incubated with 0.2 mg mL^{-1} lysozyme, disrupted by sonication, and centrifuged at 48,000*g* for 30 min to remove cell debris. The supernatant was incubated with 20% (v/v) of streptomycin sulphate for 30 min and centrifuged at 48,000*g* for 30 min. After dialysis against buffer A, a buffer containing 50 mM Tris–HCl pH 7.3, 200 mM NaCl, 1 mM EDTA, 0.1 mM DTT, and 2 M $(NH_4)_2SO_4$ (buffer B) was used to bring the $(NH_4)_2SO_4$ concentration to 1 M, required for the first chromatographic purification step. The resulting preparation was loaded on a Phenyl Sepharose High Sub FF column (GE Healthcare) previously equilibrated with 50 mM Tris–HCl pH 7.3 containing 200 mM NaCl, 1 mM EDTA, 0.1 mM DTT, and 1 M of $(NH_4)_2SO_4$ (buffer C). The column was washed with 10 column volumes of buffer C, and the adsorbed material was removed with a linear gradient of 0–100% of buffer A. The fractions containing *Mt*IGPS were pooled and concentrated in an Amicon ultrafiltration membrane [molecular weight cutoff (MWCO) of 10,000 Da], and loaded on a Sephacryl S-100 column (GE Healthcare) pre-equilibrated with buffer A. The fractions containing *Mt*IGPS were pooled and precipitated with buffer B (50% v/v). The pellet (homogeneous *Mt*IGPS) was removed by centrifugation at 48,000*g* for 30 min and resuspended in buffer A. To remove any residual traces of $(NH_4)_2SO_4$, homogeneous *Mt*IGPS was dialyzed against buffer A prior to storage at −20 °C. Protein concentrations were determined by the method of Bradford et al. [16] using the Bio-Rad protein assay kit and bovine serum albumin as standard.

Oligomeric state determination

The molecular mass of purified *Mt*IGPS was estimated by gel-filtration chromatography employing a Superdex 200 HR column (1.0 cm × 30 cm) (GE Healthcare). All runs were at 0.4 mL min^{-1} in buffer A, and the eluate was monitored at 215, 254, and 280 nm. Molecular mass standards (GE Healthcare) were used for a calibration curve [ribonuclease A (13,700 Da) from bovine pancreas, chymotrypsinogen (25,000 Da) from bovine pancreas, ovalbumin (43,000 Da) from hen egg, albumin (67,000 Da) from bovine serum, aldolase (158,000 Da) from rabbit muscle, catalase (232,000 Da) from bovine liver, and ferritin (440,000 Da) from equine spleen]. Blue Dextran 2000 was used to calculate the void volume (V_0).

Chemical synthesis, purification, spectrometric, and spectrophotometric characterization of CdRP

Modifications to previously reported chemical synthesis protocols [17–20] were made, and the protocol employed for CdRP synthesis is as follows: 2.11 mmol of anthranilic acid (AA) (Sigma) were dissolved in 264 μL isopropanol (Merck) and mixed with 1.06 mmol of ribose-5-phosphate (R5P) (Acros Organics) dissolved in 1.32 mL of water plus 2.64 mL of isopropanol. The reaction mixture was kept in the dark, at room temperature overnight. Reaction was then cooled (4 °C) for 10 min, after which two layers were formed. The aqueous phase, containing unreacted AA and R5P, was removed, and the remnant dark yellow oil washed with isopropanol and ethyl acetate. After the addition of 4 mmol of barium acetate (from a 2 M solution), CdRP precipitated as a barium salt, being isolated by centrifugation. This precipitate was washed with water, isopropanol and ethyl acetate. After desiccation, CdRP was kept at −20 °C, protected from light. The stability of the compound was estimated by UV–vis spectra measured at different times after

Download English Version:

https://daneshyari.com/en/article/1926416

Download Persian Version:

https://daneshyari.com/article/1926416

Daneshyari.com