Analytical Biochemistry 498 (2016) 53–58

Contents lists available at ScienceDirect

Analytical Biochemistry

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

A 96-well microtiter plate assay for high-throughput screening of *Mycobacterium tuberculosis* dTDP-D-glucose 4,6-dehydratase inhibitors

Xiaoxia Shi [a, 1], Shanshan Sha [a, 1], Likun Liu [b], Xin Li [a], Yufang Ma [a, *]

[a] Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian 116044, China
[b] Department of Biotechnology, Dalian Medical University, Dalian 116044, China

ARTICLE INFO

Article history:
Received 26 October 2015
Received in revised form
5 January 2016
Accepted 6 January 2016
Available online 15 January 2016

Keywords:
Mycobacterium tuberculosis
RmlB
Microtiter plate assay
Kinetic properties
Inhibitor
High-throughput screening

ABSTRACT

Mycobacterium tuberculosis dTDP-D-glucose 4,6-dehydratase (RmlB) is the second enzyme for the biosynthesis of dTDP-L-rhamnose, which is a sugar donor to the synthesis of the cell wall linker, D-*N*-acetylglucosamine-L-rhamnose. RmlB is essential to mycobacterial growth and is not found in humans; therefore, it is a potential target for developing new anti-tuberculosis drugs. So far, there has been no suitable method for high-throughput screening of RmlB inhibitors. Here, the recombinant *M. tuberculosis* RmlB was purified and an absorbance-based microtiter plate assay was developed for RmlB activity. It could be used for high-throughput screening of RmlB inhibitors. The kinetic properties of *M. tuberculosis* RmlB, including optimal pH, optimal temperature, the effect of metal ions, and the kinetic parameters, were determined with this assay. The inhibitory effects of dTTP and dTDP on *M. tuberculosis* RmlB were also studied with the assay.

Tuberculosis (TB) remains a leading infectious cause of morbidity. In 2014, there were 9.6 million new TB cases and 1.5 million people died from the disease globally [1]. The emergence of resistant strains, particularly the multidrug-resistant (MDR) *Mycobacterium tuberculosis* (Mtb) strains, has further worsened the disease [1,2]. New drugs against novel targets are urgently needed for controlling the disease [3,4].

Mycobacteria have a unique cell wall that is required for bacterial survival in the hostile environment, and mycolic acid, peptidoglycan (PG), and arabinogalactan (AG) are the core structures of the cell wall. During recent years, a lot of research projects for developing anti-TB drugs have been carried out targeting the biosynthesis of the cell wall. Ethambutol and isoniazid are typical frontline drugs against TB that inhibit the biosynthesis of the cell wall of mycobacteria. Ethambutol inhibits the synthesis of arabinogalactan [5], and isoniazid, the effective therapeutic, targets the biosynthesis of mycolic acid [6]. Thus, the cell wall core is a crucial target for developing new anti-TB drugs.

The D-*N*-acetylglucosamine-L-rhamnose disaccharide links AG and PG, which is critical for the structural integrity of the cell wall [7,8]. L-Rhamnosyl residue of the disaccharide is from a sugar donor, dTDP-L-rhamnose. Four enzymes—D-glucose-1-phosphate thymidylyltransferase (RmlA), dTDP-D-glucose 4,6-dehydratase (RmlB), dTDP-4-keto-6-deoxy-glucose-3,5-epimerase (RmlC), and dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase (RmlD)—are involved in the biosynthesis of dTDP-L-rhamnose (Fig. 1). In previous studies, we confirmed that *rmlA*, *-B*, *-C*, and *-D* genes were essential for mycobacterial growth [9–11], which supported that the biosynthetic pathway of dTDP-L-rhamnose was a potential drug target. We also reported a microtiter plate-based assay containing RmlB, RmlC, and RmlD for screening inhibitors against these three enzymes [12] as well as a colorimetric assay for screening RmlA inhibitors [13]. However, there has not been a suitable assay that is solely for screening RmlB inhibitors by now. RmlB catalyzes the dehydration of dTDP-D-glucose to form dTDP-6-deoxy-D-*xylo*-4-hexulose. Traditionally, the dTDP-D-glucose 4,6-dehydratase activity of RmlB

Abbreviations: TB, tuberculosis; MDR, multidrug-resistant; Mtb, *Mycobacterium tuberculosis*; PG, peptidoglycan; AG, arabinogalactan; RmlA, D-glucose-1-phosphate thymidylyltransferase; RmlB, dTDP-D-glucose 4,6-dehydratase; RmlC, dTDP-4-keto-6-deoxy-glucose-3,5-epimerase; RmlD, dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase; OD, optical density; IPTG, isopropyl-D-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HPLC, high-performance liquid chromatography; IA, inhibitory activity.

* Corresponding author.
E-mail address: yufang_ma@hotmail.com (Y. Ma).

[1] The first two authors (Xiaoxia Shi and Shanshan Sha) contributed equally to this work.

http://dx.doi.org/10.1016/j.ab.2016.01.004
0003-2697/

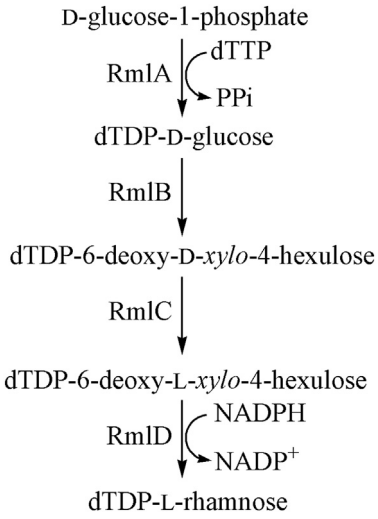

Fig.1. Biosynthetic pathway of dTDP-L-rhamnose.

was detected by measuring the production of dTDP-6-deoxy-D-*xylo*-4-hexulose at 320 nm in a 1-ml RmlB catalytic reaction [14–16]; however, it was unsuitable for high-throughput screening of RmlB inhibitors.

In this study, a rapid and simple microtiter plate assay for RmlB enzyme activity was developed. The kinetic properties of Mtb RmlB, including the optimal catalytic conditions (pH, temperature, and concentration of metal ions) and the kinetic parameters (K_m, V_{max}, and k_{cat} values), were determined. Besides, the inhibitory activities of dTTP and dTDP to Mtb RmlB, including the IC_{50} values and the inhibitory kinetics, were also studied using this assay.

Materials and methods

Chemicals and biochemicals

All growth media were purchased from Oxoid, and the Ni^{2+}-NTA agarose was purchased from Qiagen. The PageRuler prestained protein ladder was supplied by Fermentas. The substrate dTDP-D-glucose was purchased from Carbosynth. Berberine was obtained from Aladdin. Nova-Par C18 column was the product of Waters. The (anti)-His monoclonal antibody and the alkaline phosphatase-conjugated anti-mouse IgG antibody were obtained from Sigma. All other reagents and chemicals, including NAD^+, glycerol, dTTP, and dTDP, were also obtained from Sigma.

RmlB protein expression, purification, and identification

The pET29b–Mtb *rmlB* expression plasmid constructed previously [10] was transformed into *Escherichia coli* BL21(DE3). For Mtb RmlB protein expression, *E. coli* BL21(DE3) harboring pET29b–Mtb *rmlB* plasmid was grown in 200 ml of LB broth containing kanamycin at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.5 and then was induced with 0.5 mM isopropyl-D-thio-galactopyranoside (IPTG) at 16 °C for 12 h. The induced cells were harvested by centrifugation at 3000 ×g for 10 min, resuspended in 6 ml of lysis buffer (50 mM Tris–HCl [pH 8.0], 100 mM NaCl, 20% glycerol, 1 mM ethylenediaminetetraacetic acid, and 1 mM phenylmethyl sulfonylfluoride), and sonicated on ice. The lysate was centrifuged at 16,000 ×g for 25 min at 4 °C, and the supernatant was collected.

For RmlB purification, a 1.0-ml column volume of Ni^{2+}-NTA SuperFlow was prepared according to the manufacturer's instructions, and the supernatant was applied to the column. The column was then washed twice with 10 volumes of washing buffer (lysis buffer with 45 mM imidazole). Then, RmlB protein was eluted by 8 volumes of eluting buffer (lysis buffer with 200 mM imidazole).

The eluted RmlB protein was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot. For SDS–PAGE analysis, the gel was stained with Coomassie blue R-250. For Western blot analysis, the proteins in SDS–PAGE gel were electrotransferred to nitrocellulose membrane. Then, the membrane was incubated with (anti)-His monoclonal antibody, followed by alkaline phosphatase-conjugated anti-mouse IgG antibody, and finally visualized in NBT/BCIP solution. The purified RmlB protein was also identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS), which was performed by the Beijing Genomics Institution.

Furthermore, the activity of dTDP-D-glucose 4,6-dehydratase of the purified RmlB protein was confirmed by high-performance liquid chromatography (HPLC). The reaction of 100 µl contained 50 mM Hepes (pH 7.5), 10% glycerol, 0.1 mM dTDP-D-glucose, 0.01 mM NAD^+, 0.1 mM Mg^{2+}, and 12 µg of RmlB protein. After incubation at 30 °C for 35 min, 20 µl of the reaction mixture was injected into a Nova-Par C18 column (3.9 × 150 mm). KH_2PO_4 (500 mM) was used as mobile phase with a flow rate of 0.8 ml/min at 25 °C. The substrate dTDP-D-glucose was monitored at 254 nm. The negative control was performed with all reactants except RmlB protein.

Microtiter plate assay

An absorbance-based microtiter plate assay of RmlB was developed with the purified RmlB protein. The enzymatic reaction of 100 µl containing 50 mM Hepes (pH 7.5), 10% glycerol, 0.1 mM dTDP-D-glucose, 0.01 mM NAD^+, 0.1 mM Mg^{2+}, and 1.8 µg of purified RmlB protein was carried out in a 96-well microtiter plate (UV plate, cat. no. 3635, Corning, Corning, NY, USA). The reaction was initiated by adding the purified RmlB protein. After incubation at 30 °C for 15 min, the reaction was terminated by adding 10 µl of NaOH (1 M) at 30 °C for another 10 min. The OD_{320} was monitored by a microplate spectrophotometer (Multiskan GO, Thermo Scientific). The negative control was performed with all reactants except RmlB protein.

The mean values and standard deviations of "positive controls" and "negative controls," as well as the Z' value of this microtiter plate assay, were calculated according to a previous report [17]. The reactions with enzyme were positive controls, and the reactions without enzyme were negative controls.

Product quantification of microtiter plate assay

In the established microtiter plate assay, the product dTDP-6-deoxy-D-*xylo*-4-hexulose of RmlB reaction could not be quantified because the standard of dTDP-6-deoxy-D-*xylo*-4-hexulose was unavailable. Therefore, we quantified the substrate consumption of RmlB reaction through HPLC. The amount of substrate consumption represented the quantities of product formation. The reactions were carried out in a microtiter plate at different concentrations (0.022, 0.037, 0.075, 0.120, and 0.240 mM) of dTDP-D-glucose. After incubation at 30 °C for 35 min, each reaction (RmlB reaction and negative control) was monitored at 320 nm by the microplate reader, and then the reaction was quantified by HPLC. A standard curve of RmlB product concentration–OD_{320} was obtained ultimately.