



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Rv2477c is an antibiotic-sensitive manganese-dependent ABC-F ATPase in *Mycobacterium tuberculosis*

Jaiyanth Daniel*, Liz Abraham, Amanda Martin, Xyryl Pablo, Shelby Reyes

Department of Biology, Indiana University-Purdue University Fort Wayne, Fort Wayne, IN 46805, United States of America

ARTICLE INFO

Article history:

Received 13 October 2017

Accepted 30 October 2017

Available online xxx

Keywords:

Mycobacterium tuberculosis

ATPase

ABC-F

ATP-binding cassette

Manganese

Antibiotic

ABSTRACT

The Rv2477c protein of *Mycobacterium tuberculosis* (*Mtb*) belongs to the ATP-binding cassette (ABC) subfamily F that contains proteins with tandem nucleotide-binding domains but lacking transmembrane domains. ABC-F subfamily proteins have been implicated in diverse cellular processes such as translation, antibiotic resistance, cell growth and nutrient sensing. In order to investigate the biochemical characteristics of Rv2477c, we expressed it in *Escherichia coli*, purified it and characterized its enzymatic functions. We show that Rv2477c displays strong ATPase activity ($V_{\max} = 45.5$ nmol/mg/min; $K_m = 90.5$ μ M) that is sensitive to orthovanadate. The ATPase activity was maximal in the presence of Mn^{2+} at pH 5.2. The Rv2477c protein was also able to hydrolyze GTP, TTP and CTP but at lower rates. Glutamate to glutamine substitutions at amino acid residues 185 and 468 in the two Walker B motifs of Rv2477c severely inhibited its ATPase activity. The antibiotics tetracycline and erythromycin, which target protein translation, were able to inhibit the ATPase activity of Rv2477c. We postulate that Rv2477c could be involved in mycobacterial protein translation and in resistance to tetracyclines and macrolides. This is the first report of the biochemical characterization of an ABC-F subfamily protein in *Mtb*.

© 2017 Published by Elsevier Inc.

1. Introduction

Mycobacterium tuberculosis (*Mtb*), causes more than 10 million new cases of tuberculosis and about 1.8 million deaths [1]. Latent tuberculosis infection occurs when the pathogen enters a dormant state and persists in the human body without causing active clinical disease [2]. ATP binding cassette (ABC) proteins play critical roles in all organisms [3]. The ABC-F subfamily of ABC proteins contain tandem nucleotide-binding domains (NBDs) but no transmembrane domains (TMDs). Unlike the membrane-localized ATP transporters, these soluble proteins are involved in cellular processes such as translational control and antibiotic resistance by ribosomal protection [4,5]. The *Mtb* genome encodes several ABC family proteins. Although several TMD-containing ABC transporters in *Mtb* have been shown to be involved in transport processes including antibiotic efflux, the soluble *Mtb* ABC proteins that contain tandem NBDs but lack TMDs have not been studied [6,7].

The Rv2477c protein encoded in the *Mtb* genome shows strong

sequence identity with EttA, the ABC-F protein in *Escherichia coli*, that associates with the ribosome to control translation [8]. The transcript levels of Rv2477c were upregulated when the pathogen was subjected to antibiotic stress [9]. Recently, it was reported that a multi-drug resistant *Mtb* strain isolated from a human patient harbored a single nucleotide polymorphism in the gene encoding Rv2477c that altered the pathogen's phenotype from antibiotic susceptible to resistant [10]. In spite of these indications of its potential importance, Rv2477c has not been studied. Therefore, we investigated the biochemical characteristics of Rv2477c in this study.

2. Materials and methods

Bacterial strains and growth conditions. *Escherichia coli* TOP10 and *E. coli* BL21Star (DE3) (ThermoFisher, Waltham, MA) were grown at 37 °C in Luria broth (LB) or LB-agar containing kanamycin (50 μ g/ml).

Bioinformatic analyses. The amino acid sequence of Rv2477c was obtained from TubercuList [11]. Other amino acid sequences were obtained from UniProt (<http://www.uniprot.org/>). ClustalOmega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to align the sequences. BoxShade (http://www.ch.embnet.org/software/BOX_

* Corresponding author. Department of Biology, Indiana University-Purdue University, SB 334, 2101 E Coliseum Blvd., Fort Wayne, IN 46805, United States of America.

E-mail address: danielj@ipfw.edu (J. Daniel).

form.html) was used to shade similar and identical amino acids in the aligned sequences. The information on the genetic organization of the locus around Rv2477c and its orthologs in mycobacteria was obtained from Genolist (<http://genolist.pasteur.fr>) and KEGG (<http://www.kegg.jp>).

Cloning of Rv2477c and Rv2477c-EQ₂ mutant proteins. Rv2477c was amplified from the genomic DNA of *Mycobacterium tuberculosis* H37Rv by polymerase chain reaction using the following primers (Forward: 5'-CACCATGGCTGAGTTTCATCTACACGATG-3'; Reverse: 5'-TTAGCCGCGCTCAGCTTGCG-3'). The amplified product was cloned into pET200 D-TOPO plasmid (ThermoFisher, Waltham, MA) and sequence integrity was verified by DNA sequencing. The Rv2477c-EQ₂ mutant protein containing glutamate to glutamine substitutions at amino acid positions 185 and 468 in the two Walker B motifs on the Rv2477c protein was generated using the Rv2477c-pET200 plasmid construct as template by site-directed mutagenesis using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA) following the manufacturer's protocols in two stages. The primers used in the first stage to generate the E to Q mutation at residue 185 were: SDM553F: 5'-GTTGCTCGACACGCC-GACCAA-3'; SDM553R: 5'-AACAGTTCGGGTTTGGAC-3'. The mutated plasmid construct was then used as template to generate the second mutation (corresponding to amino acid residue 468 on Rv2477c) using the following primers: SDM1402F: 5'-TCTGCTCGACCAACCGACGAAC-3'; SDM1402R: 5'-ATCAGTTGCCGCCCTGT-3'. The mutations were confirmed by DNA sequencing of the plasmid construct.

Expression and purification of Rv2477c and Rv2477c-EQ₂ proteins. The Rv2477c-pET200 or Rv2477c-EQ₂-pET200 plasmid construct was used to transform chemically competent *E. coli* BL21 Star (DE3). A 300 ml LB culture containing 0.4 M sucrose was grown at 37 °C until log-phase (OD₆₀₀ ~0.6). The culture was heat-shocked at 42 °C for 20 min (to enhance soluble protein production) and after cooling to 22 °C, expression of Rv2477c protein was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; IBI Scientific, Peosta, IA) for 16 h at 22 °C with shaking. Protein expression was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. Cells from the induced culture were lysed by ultrasonication on ice in the presence of 0.2 mM phenylmethylsulfonyl fluoride. The Rv2477c protein was purified from the 16,000 × g supernatant of the lysate by cobalt-affinity chromatography using the TALON cobalt-affinity resin (Takara Bio USA, Inc., Mountain View, CA). Following binding of the protein at 22 °C to 1 ml bed-volume of TALON resin equilibrated with equilibration/wash buffer (50 mM Tris-HCl pH 7.0, 500 mM NaCl), the column flow-through was collected. The resin was washed with 10 bed-volumes of equilibration/wash buffer containing 5 mM imidazole followed by 10 bed-volumes of equilibration/wash buffer containing 10 mM imidazole. Elution of bound Rv2477c protein from the resin was done using 5 bed-volumes equilibration/wash buffer containing 50 mM imidazole. The fractions were checked for purity by 12% SDS-PAGE followed by Coomassie staining and fractions containing Rv2477c were pooled, concentrated and buffer-exchanged into 50 mM Tris-HCl pH 7.0, 300 mM NaCl, 10% glycerol by six cycles of ultra-filtration using a 50,000 molecular weight cut-off (MWCO) centrifugal membrane filter (EMD Millipore, Billerica, MA). The purified protein was quantitated by Bradford protein assay (Bio-Rad, Hercules, CA) and purity of the protein was confirmed by SDS-PAGE and Coomassie staining.

The Rv2477c-EQ₂ protein was purified from 600 ml induced cultures following the same protocol described above. Elution of the mutant protein from the affinity resin was done using 200 mM imidazole. After concentration and buffer-exchange of protein as described above, the purity of the protein was confirmed as above.

ATP hydrolysis assays. The ATPase activity assays were carried out in a 30 µl reaction mix containing 2 µg (1 µM) of purified Rv2477c (or Rv2477c-EQ₂) protein, 500 µM ATP, 100 mM sodium acetate pH 5.2, 100 mM NaCl, 2.5 mM MnCl₂ and 10% glycerol. ATP (Calbiochem- Millipore, Burlington, MA) was dissolved in 100 mM Tris-HCl pH 7.0 to make a fresh 10 mM stock solution from which appropriate amounts were added to assay. ATP concentrations between 50 and 2000 µM were used to determine the kinetic parameters. A 2 mM excess concentration of divalent cation over ATP concentration was maintained throughout. The apparent maximal reaction velocity (V_{max}) and Michaelis-Menten constant (K_m) were determined using the Enzyme Kinetics Nonlinear Fit Results from Michaelis-Menten plots in SigmaPlot 13 (Systat Software, Inc., San Jose, CA). In pH-dependence assays, 100 mM sodium acetate pH 4.0 or 100 mM Tris-HCl pH 7.0/7.5/8.0/8.8 were substituted appropriately. In assays for divalent cation dependence, 2.5 mM MgCl₂ or CaCl₂ or CoCl₂ or ZnSO₄ were used instead of MnCl₂. GTP (MP Biomedicals, Solon, OH), CTP (Alfa Aesar, Ward Hill, MA) or TTP (Sigma Aldrich, St. Louis, MO) were substituted for ATP in appropriate assays.

The malachite green reagent (MGR) assay was used to determine the amount of phosphate released by the ATP hydrolase activity of purified Rv2477c following a previously reported procedure [12]. MGR is a 2:1:1:2 mixture of 0.0812% (w/v) malachite green, 2.32% (w/v) polyvinyl alcohol, 5.72% (w/v) ammonium molybdate in 6 M HCl and water, respectively. After incubation at 37 °C for 30 min, 150 µl MGR and 15 µl of 34% (w/v) sodium citrate were added. After 30 min at 22 °C, absorbance at 620 nm was measured using an accuSkan™ GO UV/Vis Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Absorbance readings of negative control reactions without Rv2477c protein were subtracted from respective readings in reactions containing the protein. The quantity of inorganic phosphate released was estimated using a standard curve that was linear up to 200 µM in the reaction buffer. The specific activity of the purified protein was determined from the rate of inorganic phosphate released from ATP. The effects of ATPγS (TOCRIS Bioscience, Bristol, UK) which is the non-hydrolysable substrate analog and known inhibitors of ATPases (sodium orthovanadate, sodium azide and KNO₃) on ATPase activity were investigated by pre-incubating the purified Rv2477c protein with the substrate analog or inhibitor for 15 min on ice prior to assay. Sodium orthovanadate (MP Biomedicals, Solon, OH) was prepared by following a previously described protocol [13]. To investigate the effects of antibiotics on the ATPase activity of Rv2477c, the purified protein was pre-incubated for 15 min at room temperature with antibiotic prior to addition of ATP. Antibiotic stock solutions were prepared in water with the exception of erythromycin which was prepared in ethanol. Ethanol solvent control was used for erythromycin in assays.

3. Results

The mycobacterial protein Rv2477c shows similarities with bacterial ABC-F subfamily proteins involved in ribosome function and antibiotic resistance. The protein encoded by the Rv2477c gene is an essential gene annotated in the mycobacterial genome as a "probable macrolide transport ATP-binding protein/ABC transporter" [11]. The Rv2477c protein does not contain any predicted transmembrane domains and displays homology to the ABC-F protein EttA in *E. coli* that controls ribosomal processes [8]. The ABC-F subfamily members are soluble proteins that lack transmembrane domains but contain tandem nucleotide-binding domains and are involved in diverse cellular processes such as translation, cell division and antibiotic resistance [5]. Several genes that encode lipid metabolizing enzymes are located in the genomic neighborhood of

Download English Version:

<https://daneshyari.com/en/article/8295284>

Download Persian Version:

<https://daneshyari.com/article/8295284>

[Daneshyari.com](https://daneshyari.com)