Biochemical and Biophysical Research Communications 407 (2011) 552–556

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Isolation and characterization of functional *Leishmania major* virulence factor UDP-galactopyranose mutase

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ARTICLE INFO

Article history:
Received 2 March 2011
Available online 16 March 2011

Keywords:
UDP-galactopyranose mutase
Galactofuranose
Leishmaniasis
Flavoenzymes
Non-redox reaction

ABSTRACT

Human parasitic pathogens of the genus *Leishmania* are the causative agents of cutaneous, mucocutaneous, and visceral leishmaniasis. Currently, there are millions of people infected with these diseases and over 50,000 deaths occur annually. Recently, it was shown that the flavin-dependent enzyme UDP-galactopyranose mutase (UGM) is a virulence factor in *Leishmania major*. UGM catalyzes the conversion of UDP-galactopyranose to UDP-galactofuranose. The product, UDP-galactofuranose, is the only source of galactofuranose which is present on the cell surface of this parasite and has been implicated to be important for host-parasite interactions. The recombinant form of this enzyme was obtained in a soluble and active form. The enzyme was shown to be active only in the reduced state. A k_{cat} value of 5 ± 0.2 s^{-1} and a K_M value of 87 ± 11 μM were determined with UDP-galactofuranose as the substrate. Different from the dimeric bacterial and tetrameric fungal UGMs, this parasitic enzyme functions as a monomer.

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1. Introduction

Leishmaniasis constitutes a group of diseases caused by infection of parasites from the genus *Leishmania,* which are present in more than 80 countries worldwide [1,2]. There are three major forms of leishmaniasis: cutaneous, mucocutaneous, and visceral [1]. Symptoms of these diseases range from self-healing sores, as observed in cutaneous leishmaniasis, to infection of the liver, spleen, and lymph nodes, as observed in visceral leishmaniasis [3,4]. If untreated, leishmaniasis can lead to severe scarring, disfigurement, and in severe cases it leads to death [3,4]. The World Health Organization estimates that >2 million people are infected with these parasites, resulting in ~50,000 annual deaths [2]. Despite the significant health threat posed by these diseases, there are no efficacious drug treatments or vaccinations to prevent infection by *Leishmania* spp. [5].

The cell surface of *Leishmania* spp. has been shown to play a role in host-pathogen interactions and in the ability of the pathogen to evade the host immune system. Previous research has shown that galactofuranose (Gal*f*) found on the cell surface of *L. major* plays a role in pathogenesis and in pathogen-host interactions [6]. Gal*f* is a unique sugar; it is only found in parasites and other human pathogens and is present in cell surface lipids and proteins. Specifically, in *Leishmania* spp., Gal*f* is found in lipophosphoglycan (LPG) and glycoinositolphospholipids (GIPLs) [7]. LPGs are essential for adhesion of the parasite to the midgut of the insect and, therefore, are important for transmission of the parasite to the human host [8,9]. Deletion of LPG in *L. major* suggests that these glycosylated structures are involved in resistance to oxidative stress and the human immune system [10,11]. While GIPLs containing Gal*f* (specifically GIPL-1 from *L. major*) have been shown to aid in establishing the infection [6,12]. These results suggest that Gal*f* plays an important role in host specific cell recognition, parasitic growth, and pathogenesis. Since Gal*f* is not present in humans, the Gal*f* biosynthetic pathway is an attractive target for the development of novel anti-parasitic drugs [13,14]. In this pathway, UDP-galactopyranose mutase (UGM) catalyzes the conversion of UDP-galactopyranose (UDP-Gal*p*) to form UDP-Gal*f*, which serves as the precursor for all the Gal*f* found on the cell surface [15]. Deletion of the UGM gene in *L. major* (LmUGM) leads to attenuated virulence, suggesting that UGM is a virulence factor and a potential drug target [16,17]. We present the functional expression and characterization of recombinant LmUGM, which will allow further mechanistic and structural studies that might lead to the identification of specific inhibitors of this enzyme.

2. Materials and methods

2.1. Materials

UDP and UDP-Gal*p* were purchased from Sigma (St. Louis, MO). Accuprime polymerase, TOP-10, and BL21T1^R chemical competent

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0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2011.03.057

cells were obtained from Invitrogen (Carlsbad, CA). Restriction endonucleases *Sgf*I and *Pme*I, plasmid pFN18 K, chemical competent cells BL21(DE3), and HaloLink Resin were obtained from Promega (Madison, WI). The plasmid miniprep and PCR purification kits were from Qiagen (Valencia, CA). All other buffers and chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Expression plasmids, pVP55A and pVP56 K, were obtained from the Center for Eukaryotic Structural Genomics, University of Wisconsin, Madison [18].

2.2. Cloning

The LmUGM gene was provided by Dr. Françoise Routier (Medizinische Hochschule, Hannover, Germany) cloned into the pET22 plasmid. In this plasmid, the gene product appears to be toxic to BL21(DE3) cells. Therefore, the LmUGM gene was amplified by PCR using the forward primer ′5-ggttgcgatcgccatgagcgctgacaaggtggtc-3′ (*Sgf*I site underlined) and the reverse primer 5′-ggtcgtcgacggcctcgtaggtttaaactttt-3′ (*Pme*I site underlined). After running the resulting PCR product on a 0.8% agarose gel electrophoresis, the DNA was excised and purified using a Qiagen PCR clean up kit. This was followed by digestion with the restriction enzymes *Sgf*I and *Pme*I for 40 min at 37 °C, then heat inactivated for 25 min at 65 °C. The digested PCR product was then ligated into the plasmids pVP55A, pVP56K, and pFN18K, which were previously treated with *Sgf*I and *Pme*I. Cloning was designed for the expression of an N-terminus tagged fusion protein. In pVP55A, the gene was expressed with an 8×-His tag, in pVP56 K, the gene was expressed with an 8×-His tagged maltose binding protein (MBP), and in pFN18K, the gene was expressed with a HaloTag [19,20]. In the vectors pVP55A and pVP56K, the inserted gene was under the control of the T5 promoter and in pFN18K, the inserted gene was under control of the T7 promoter. Both promoters can be induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG). pVP55A provides resistance to ampicillin and both pVP56K and pFN18K provide resistance to kanamycin.

2.3. Protein expression

Cell growth was done in the presence of either 25 μg/mL kanamycin or 100 μg/mL ampillicin depending on whether the cells contained pVP56K, pFN18K, or pVP55A. BL21T1^{R} cells were transformed with pVP55A and pVP56K containing the LmUGM gene, while BL21(DE3) cells were transformed with the plasmid pFN18K containing the LmUGM gene. The transformed cells were plated onto Luria–Bertani (LB) agar plates with the appropriate antibiotic. A single colony was used to inoculate a 50 mL LB culture containing the appropriate antibiotic and incubated at 37 °C with agitation at 250 rpm overnight. The next morning, 15 mL of the overnight culture was used to inoculate each of 3 flasks containing 1 L of LB. The flasks were incubated at 37 °C with 250 rpm agitation until the optical density at 600 nm (OD_{600}) reached a value of 0.6, at which point 1 mM IPTG was added to each culture to induce the expression of the recombinant LmUGM enzyme. The temperature was reduced to 15 °C to increase solubility of the LmUGM enzyme. Four hours after induction, the cells were harvested by centrifugation at 5000*g* for 15 min and the resulting cell pellets (∼4.5 g) were stored at −80 °C until purification. LmUGM expressed in pVP55A and pVP56K was found to be insoluble. Expression of LmUGM in pVP56K was also performed using auto-induction medium to test if protein solubility was increased [21]. Using auto-induction medium, the yield of cell paste was ∼60 g from 6 L of medium.

2.4. Protein purification by metal affinity chromatography

Cell pellets from auto-induction (∼60 g) were resuspended in 150 mL 25 mM HEPES, 300 mM NaCl, 5 mM Imidazole, pH 7.5 containing 25 μg/mL each of lysozyme, DNase, and RNase. The resuspended cells were mixed for 30 min at 4 °C and cells were disrupted by homogenization (Nano DeBEE, BEE International) at 16,000 psi. The resulting lysate was then centrifuged at 30,000*g* for 30 min at 4 °C to remove the unlysed cells and insoluble proteins. The supernatant was collected and loaded onto a 5 mL nickel immobilized metal affinity chromatography (IMAC) column (GE Healthcare) previously equilibrated with buffer A (25 mM HEPES, 300 mM NaCl, 5 mM Imidazole, pH 7.5). The column was washed with three column volumes of buffer A and LmUGM was eluted with buffer A containing 300 mM imidazole. The 8×-His tag and MBP were removed by treating the fusion protein with tobacco etch virus (6×-His-Tev) protease (1:20 ratio LmUGM:Tev) overnight with slow stirring at 4 °C. The resulting sample was centrifuged at 30,000*g* for 20 min to pellet small amounts of denatured proteins. The supernatant was diluted 4-fold and loaded onto an IMAC. The 8×-His-MBP and 6×-His-Tev remained bound to the column, and the flow-through containing LmUGM was collected. This sample was concentrated and diluted by addition of 25 mM HEPES, pH 7.5, to decrease the NaCl concentration to less than 30 mM, and loaded onto a diethyl amino ethyl (DEAE) ion exchange chromatography column (GE Healthcare) equilibrated with 25 mM HEPES, pH 7.5. A gradient from 0 to 400 mM NaCl was used to elute the bound proteins; LmUGM eluted later in the gradient (∼250 mM NaCl). Fractions containing LmUGM were pooled, concentrated, and stored at −80 °C.

2.5. Protein purification with HaloTag

Cell pellets (∼4.5 g) were resuspended in 20 ml of 25 mM HEPES, 125 mM NaCl, pH 7.5, containing 25 μg/mL each of lysozyme, DNase, and RNase. The resuspended cells were lysed and a total volume of 75 mL of supernatant was obtained as indicated above. Approximately 30 mL of supernatant were mixed with 5 mL HaloLink resin for 1 h at room temperature. The resin was then precipitated by centrifuging at 1,000*g* for 5 min at 4 °C and supernatant was removed. The resin was then washed three times with 10 mL of 50 mM HEPES, 150 mM NaCl, pH 7.5. The resulting resin was resuspended with 2.5 mL 50 mM HEPES, 150 mM NaCl, pH 7.5 and LmUGM was cleaved from the HaloTag by addition of 5 mg 6×-His-Tev protease. After incubation for 2 h at room temperature, the resin was precipitated by centrifugation at 2,500*g* for 5 min and the resulting supernatant containing free LmUGM was saved. The resin was washed with 1 mL 50 mM HEPES, 150 mM NaCl, pH 7.5 and the supernatant was also saved. 6×-His-TEV was removed by passing the supernatant on a 1 mL nickel IMAC (GE Healthcare) column. Since LmUGM does not contain a His-tag it is collected in the flow through and TEV remains bound to the column. Analysis of the final product by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) indicated that the LmUGM sample was homogeneous. The sample was concentrated and stored at −80°C.

2.6. UV–visible spectrophotometry

The spectrum of recombinant LmUGM was recorded using an Agilent 8453 UV–visible spectrophotometer. The spectrum shows similar features to other UGM proteins with a bound flavin cofactor, with peaks at 276, 377, and 448 nm with a shoulder at 474 nm [22].

2.7. Activity Assay

The activity of recombinant LmUGM was tested with UDP-Gal*f* as the substrate following procedures previously described [22].

The protein concentration was calculated using the LmUGM flavin extinction coefficient at 450 nm (ε_{450} = 10 mM^{-1} cm^{-1}).

2.8. Molecular weight determination

The solution molecular weight was determined using size-exclusion chromatography. Purified LmUGM (10 μg) was loaded onto a Superdex 200 column (GE Healthcare) equilibrated with 50 mM phosphate buffer pH 7.2 with 150 mM NaCl. Using a set of protein standards (aproprotein (6.5 kDa), ribonuclease (13 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), canolbumin (75 kDa), aldolase (158 kDa), and ferritin (440 kDa)), a standard curve was obtained by plotting the log of molecular weight versus K_{av} for the standards [23].

3. Results and discussion

3.1. Expression and Purification of LmUGM

LmUGM was expressed using three plasmids: pVP55A, pVP56K, and pFN18K. It was found that expression of LmUGM in pVP55A with only an 8×-His tag led to completely insoluble protein whether expressed in LB or auto-induction media. However, when expressed by auto-induction as an N-terminal fusion to MBP in pVP56K it was partially soluble. The protein was purified first using nickel IMAC as affinity chromatography for the 8×-His Tag on the N-terminus of the MBP. The resulting protein was treated with 6×-His-Tev to remove the 8×-His-MBP tag. The protein was loaded back over a nickel IMAC to remove both the 8×-His-MBP and 6×-His-TEV. LmUGM eluted in the flow through and was further purified by passing over a DEAE column. The resulting protein was only ~70–80% pure, and the amount of protein recovered from 6 L auto-induction media (60 g cell paste) was only 3–4 mg.

To increase protein solubility, recovery, and purity, LmUGM was expressed as a Halo-tagged protein in the pFN18K plasmid. This system expresses the protein of interest as a fusion to a catalytically inactive derivative of *Rhodococcus* haloalkane dehalogenase (DhaA) [20]. This protein has been modified to form covalent bonds to chloroalkane-conjugated resins [20]. In addition, the 35-kDa DhaA protein was engineered to enhance the solubility of the fused protein [20]. LmUGM was found to be partially soluble when expressed as an N-terminal fusion to HaloTag in LB media. Halo-LmUGM was isolated using the HaloTag affinity resin. Since the fusion protein becomes covalently linked to the resin, this permits numerous washes to remove other contaminants. LmUGM can then be removed from the HaloTag and resin using Tev. LmUGM was isolated from 6×-His-Tev and other contaminants by loading the solution onto a nickel IMAC to remove the 6×-His-Tev yielding 3 mg of highly pure protein (~95%) (Fig. 1). In summary, 4.5 g of cell paste yielded 3 mg of homogeneous LmUGM using the HaloTag system. This is a ~20-fold increase in yield as compared to the expression of LmUGM as a fusion to MBP using auto-induction media (4 mg/60 g cell paste). The purified LmUGM contained a noncovalently bound flavin cofactor (50% incorporation), which was identified by mass spec analysis as FAD (data not shown). The UV/Vis spectrum displayed similar spectral characteristics to other flavoproteins with peaks at 377 nm and 448 nm and a shoulder at 474 nm (Fig. 2).

3.2. Molecular weight determination

The molecular weight for LmUGM of 55 kDa calculated by SDS–PAGE analysis is consistent with the predicted value of 54,960 Da based on amino acid composition, suggesting that the protein was not subjected to major modifications by *E. coli* proteases during purification. The solution molecular weight of 57,400 ± 500 Da was determined by size exclusion chromatography (Fig. 3). This value closely matches the predicted mass of a monomer of LmUGM. Interestingly, the oligomeric state of bacterial enzymes has been shown to be dimeric, while the eukaryotic UGM from *Aspergillus fumigatus* functions as a tetramer [22].

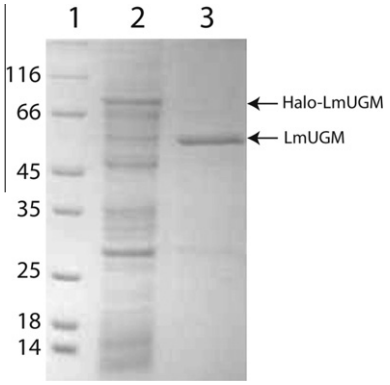

Fig. 1. SDS–PAGE gel of LmUGM protein purified by HaloTag using HaloTag resin. Lane 1. Molecular weight marker, Lane 2. Complete lysate supernatant, Lane 3. Supernatant after cleavage with Tev protease and after nickel IMAC to remove the Tev protease.

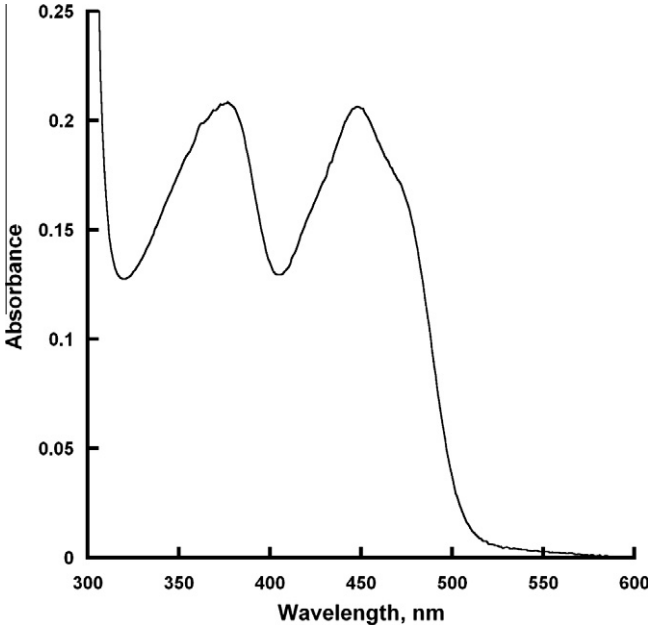

Fig. 2. UV–Visible spectrum of the purified LmUGM.

3.3. Activity

We tested the activity of recombinant LmUGM with UDP-Gal*p* as substrate in the presence or absence of dithionite as a source of reducing equivalents. Consistent to published work on bacterial UGM and more recently on a eukaryotic UGM from the fungus *A. fumigatus*, the enzyme was active only when the flavin cofactor is in the reduced form [22,24]. Since the equilibrium of the reaction favors the formation of UDP-Gal*p*, it is necessary to measure the activity in the reverse direction. Thus, the activity of LmUGM was measured as a function of UDP-Gal*f* concentration. As shown in Fig. 4, the activity of LmUGM follows typical saturation kinetics that can be analyzed using the Michaelis–Menten equation. The kinetic

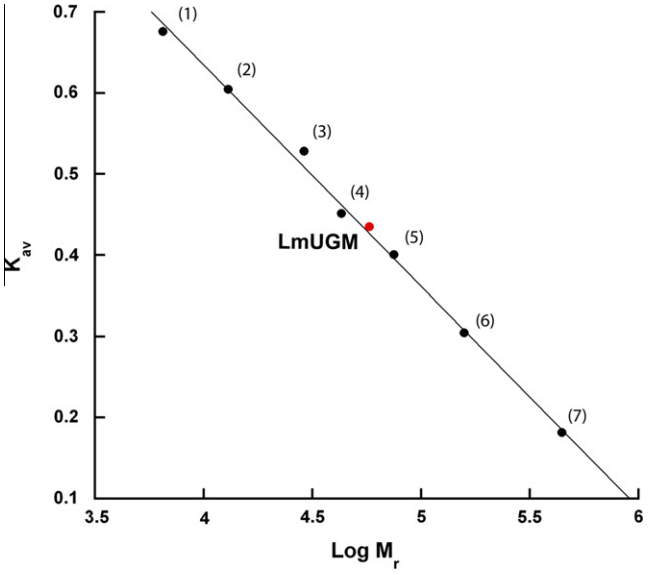

Fig. 3. Solution molecular weight determination of LmUGM using size exclusion chromatography. The elution volumes for aprotinin (1), ribonuclease (2), carbonic anhydrase (3), ovalbumin (4), conalbumin (5), aldolase (6), and ferritin (7) were used to calculate the K_{av} values ($K_{av} = (V_e - V_o)/(V_t\ V_o)$, where V_o is the void volume of the column, V_t is the total volume of the column, and V_e is the elution volume of the protein. The K_{av} value for LmUGM is also plotted).

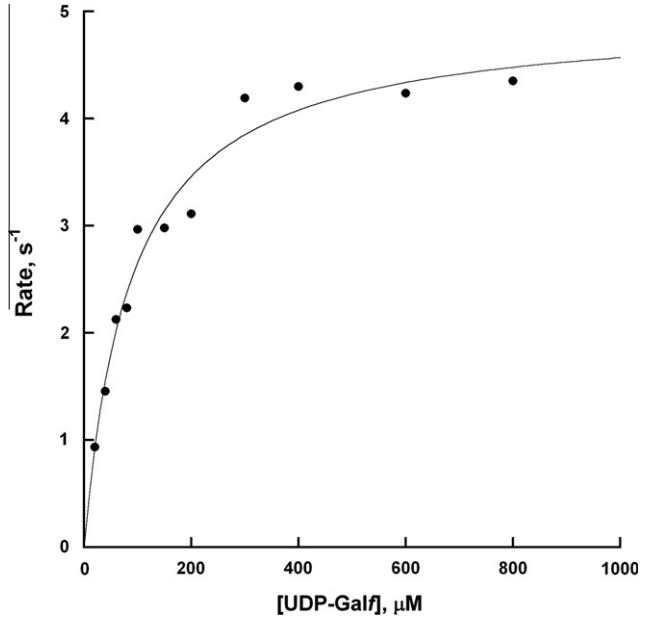

Fig. 4. Activity of LmUGM as a function of UDP-Gal*f*. The line is a fit to the Michaelis–Menten equation.

values obtained from the fit are summarized on Table 1. The k_{cat} values for some UGMs are more than 10-fold higher; however, it is clear that these enzymes have similar K_M values. The differences in the k_{cat} values might originate from the primary and quaternary structure variability between the members of this family of enzymes.

3.4. Concluding Remarks

In mycobacteria, UGM has been validated as a drug target, because this enzyme is essential for mycobacterial survival [25]. Similarly, the role of UGM in the virulence of eukaryotic pathogens has also been shown. In *A. fumigatus* and *L. major*, deletion of the UGM gene leads to attenuated virulence [16,26]. Thus, inhibition of UGM might lead to the identification of novel anti-fungal and anti-leishmanial drugs. Here, we report the functional expression of *L. major* UGM. The enzyme was expressed in a soluble form and the isolated enzyme contained the flavin cofactor. The enzyme was shown to be active in the reduced form and functions as a monomeric enzyme, which is different from the *A. fumigatus* and bacterial enzymes. Although, inhibitors of bacterial UGM have been identified, some are not active towards eukaryotic UGM [27], (Qi and Sobrado, unpublished results). This might be due the fact that bacterial and eukaryotic UGMs share less than 18% identity. Our results provide the opportunity for the screening of inhibitors specific to parasitic UGMs.

Table 1
Kinetic parameters of UDP-galactopyranose mutases[a].

Species	k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $\mu M^{-1}\ s^{-1}$	Ref.
L. major	5 ± 0.2	87 ± 11	0.057 ± 0.006	This work
A. fumigatus	72 ± 4	110 ± 15	0.65 ± 0.09	[22]
E. coli	27	22	1.22	[28]
K. pneumoniae	5.5 ± 0.66	43 ± 6	0.12 ± 0.02	[29]
D. radiodurans	66 ± 2.4	55 ± 7	1.18	[30]

[a] All the kinetic parameters are with UDP-Gal*f* as substrate in the presence of 5–20 mM dithionite.

Acknowledgments

We would like to thank Prof. Françoise Routier for providing us with the pET22 plasmid containing the LmUGM gene. This work was supported by NIH grant R01 GM094468 (P. Sobrado, PI). M.O. was supported by a fellowship from the American Heart Association. A.L.V was supported by a fellowship from the Ministry of Science and Technology of Costa Rica.

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