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UDP-*N*-Acetylglucosamine enolpyruvyl transferase (MurA) of *Acinetobacter baumannii* (AbMurA): Structural and functional properties

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ABSTRACT

Peptidoglycan (PG) is the key component of the bacterial cell wall. The enzyme UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase (MurA) catalyzes the transfer of enolpyruvate from phosphoenolpyruvate (PEP) to uridinediphospho-*N*-acetylglucosamine (UNAG), which is the first committed step of PG biosynthesis. Here, we present the biochemical and structural features of the MurA enzyme of the opportunistic pathogen *Acinetobacter baumannii* (AbMurA). The recombinant AbMurA exists as a monomer in solution and shows optimal activity at pH 7.5 and 37 °C. The *K*m for UDP-*N*-acetylglucosamine was 1.062 ± 0.09 mM and for PEP was 1.806 ± 0.23 mM. The relative enzymatic activity was inhibited ~3 fold in the presence of 50 mM fosfomycin (FFQ). Superimposition of the AbMurA model with *E. coli* demonstrated key structural similarity in the FFQ-binding site. AbMurA also has a surface loop that contains the active site Cys116 that interact with FFQ. Sequence analysis indicates the presence of the five conserved amino acids, i.e., K22, C116, D306, D370 and L371, required for the functional activity like other MurA enzymes from different bacteria. MurA enzymes are indispensable for cell integrity and their lack of counterparts in eukaryotes suggests them to be a promising drug target.

1. Introduction

Gram-negative coccobacilli like *Acinetobacter baumannii* are important opportunistic bacterial pathogens that are responsible for 2–10% of all Gram-negative hospital infections [1]. They are classified as one of the six most important multidrug-resistant (MDR) microorganisms in hospitals worldwide by the Infectious Diseases Society of America [2]. It has been reported that *A. baumannii* causes a broad range of severe nosocomial infections, including skin and soft tissue infections, wound infections, urinary tract infections, and secondary meningitis [3–5]. Colistin is the most effective drug against MDR *A. baumannii*; however, its use has been associated with a number of side effects and is considered unsuitable for treating all types of infections [6]. There are also reports of colistin resistance worldwide [7], resulting in the emergence of strains that are resistant to all known antibiotics in certain geographical areas [8]. The emergence of MDR microbes and isolation of new pathogens has created an urgent need for novel antibiotics that could control the growth of such organisms.

One of the most validated targets for antibacterial therapy are the enzymes of peptidoglycan (PG) biosynthesis. PG, a unique structure present only in prokaryotes, is an essential component of the cell wall that provides structural integrity to bacteria against internal osmotic pressure and is responsible for maintaining cell shape [9]. The enzymes linked to PG synthesis remain conserved among the bacterial species throughout evolution. In the absence of a mammalian counterpart, they represent an attractive drug target. PG is made up of linear glycan chains composed of repeating units of two sugars: *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). With a few exceptions, each NAM residue of glycan chain carries a short stem made up of tetrapeptide having the following consensus sequence: L-Ala, D-Glu, meso-DAP/L-Lysine, and L-Ala. The adjoining tetra peptide sequences are linked directly (most

Abbreviations: AbMurA, *Acinetobacter baumannii* MurA; EcMurA, *Escherichia coli* MurA; PEP, phosphoenol pyruvate; FFQ, fosfomycin; UNAG, UDP-*N*-acetylglucosamine; UNAM, UDP-*N*-acetylmuramate; UNAGEP, UDP-*N*-acetylglucosamineenolpyruvate; SEC, size exclusion chromatography; RMSD, root-mean-square deviation; RMSF, root mean square fluctuation; PCA, principal component analysis.

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Gram-negative bacteria) or indirectly through an interpeptide bridge (most Gram-positive bacteria). The key precursor of PG is UDP-*N*-acetylmuramate (UNAM) that is synthesized in a two-step process by two cytoplasmic enzymes, UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) and UDP-*N*-acetyl enolpyruvyl glucosamine reductase (MurB). MurA catalyzes the transfer of an enolpyruvyl group from phosphoenolpyruvate (PEP) to UDP-*N*-acetylglucosamine (UNAG) to form UDP-*N*-acetylglucosamine enolpyruvate (UNAGEP). In the subsequent step, MurB reduces UNAGEP using NADPH to form UNAM [10].

Several structures of unbound MurA enzyme and its complexes with inhibitors/substrates are available in PDB [11–14]. The monomeric MurA enzyme consists of two domains connected by a double-stranded linker. The active site is present at the interphase of these domains. All reported MurA structures have a surface loop (Pro111–Pro121, *E. coli* numbering) containing the catalytic Cys115 residue. The surface loop is very flexible and can adopt different conformations depending on the presence or absence of ligands. The active site of MurA is created by induced fit mechanism. The binding of the UNAG facilitates a conformational change in the surface loop along with domain movement that results in the transition from the open to the closed form of the enzyme. This closed form is crucial for the catalysis as it brings active site Cys115 residue and PEP close enough to make contact so that a reaction takes place.

In the present manuscript, we report the biochemical, inhibition, and structural properties of AbMurA. Our results present interesting and important information on AbMurA and may help in the drug development against these opportunistic bacterial parasites.

2. Material and methods

2.1. Materials

The molecular biology kits and Ni-NTA agarose were purchased from Qiagen, CA, USA. All other reagents and chemicals were of the highest purity available and were purchased either from Sigma-Aldrich Chemical Company, St. Louis, MO, USA or Sisco Research Laboratories, Mumbai, India. Bacterial culture media was purchased from Himedia Laboratories, Mumbai, India.

2.2. Overexpression and purification of AbMurA

The *AbmurA* clone was a kind gift from Lt Dr. Vinod Bhakuni (CDRI, Lucknow). For the expression of AbMurA, the plasmid was introduced into C41(DE3) cells and grown in Luria-Bertani media containing 100 μg/mL ampicillin at 37 °C until an $A_{600}\sim$ 0.6 is achieved and then induced at 20 °C with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Induced cultures were grown further for 8 h with continuous shaking at 160 rpm and 20 °C. The cleared cell lysate was loaded onto a Ni-NTA column pre-equilibrated with 50 mM Tris-Cl, pH 8.0 containing 300 mM NaCl and washed with the same buffer followed by washing with 20 mM and 40 mM imidazole buffer. The protein was eluted with a linear gradient of 50–300 mM imidazole buffer and the desired peaks were dialyzed against 20 mM Tris-Cl buffer, pH 8.0 containing 100 mM NaCl. The purity and the molecular weight of the recombinant protein were determined using SDS-PAGE.

Size exclusion chromatography (SEC) experiment was carried out on a Superdex^{TM} 200, 10/300GL on an AKTA FPLC (GE Health care Biosciences) pre-calibrated with standard molecular weight markers. The column was pre-equilibrated and run with 20 mM Tris-Cl buffer, pH 7.5 containing 100 mM NaCl at 25 °C at a flow rate of 0.3 mL/min with detection at 280 nm.

2.3. Fluorescence and circular dichroism (CD) spectroscopy

Tryptophan fluorescence spectra were recorded with a Perkin Elmer Life Sciences LS55 fluorescence spectrophotometer in a 5 mm path length quartz cell at 25 °C. Excitation wavelength of 280 nm was used and the spectra were recorded between 300 and 400 nm [15]. The protein concentration of 2 μM was used for the studies. CD measurements were made on JASCO J810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate with a 2 mm path length cell at 25 °C. The values obtained were normalized by subtracting the baseline recorded for the buffer under similar conditions.

2.4. Activity assays

The MurA activity was assessed by quantitating the release of inorganic phosphate (Pi) from PEP as described earlier [16–18]. Reaction was performed in a 96 well plate at 37 °C for 10 min in the final volume of 50 μL reaction mixture containing 20 mM Tris-HCl pH 7.5, 10 mM KCl, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.6 mM UDPAG, and 3 μM of pre-dialyzed AbMurA. 1 mM PEP was added to initiate the reaction. The reaction was monitored by adding malachite green with the correction of the background reading in the absence of UDPAG or PEP at 620 nm. For control reactions, the conditions remained same as mentioned above; however, AbMurA was not added to the reaction mixture. The pH and temperature conditions for optimal enzymatic reaction were assessed at different pH (4–10) and temperature (10–80 °C) ranges.

2.5. Determination of kinetic constants

The steady-state kinetic parameters *K*m value and *V*max were determined under variable concentrations of one substrate with the other one fixed (UDPAG and PEP). Varying concentrations of UDPAG (0–5 mM) in the presence of 1 mM PEP or varying concentration of PEP (0–5 mM) in the presence of 0.5 mM UDPAG were used. *K*m and *V*max values were estimated by fitting the curve through non-linear regression by plotting Michaelis-Menton graph.

2.6. Inhibition of AbMurA by fosfomycin (FFQ)

Fosfomycin inhibits MurA enzyme by making a covalent adduct with the active site Cys residue. To determine the inhibitory effects of FFQ on enzymatic activity, different concentrations (1–50 mM) of FFQ were pre-incubated with the assay mixture for 15 min, and the enzymatic reactions were initiated by the addition of 5 mM of PEP. Since FFQ acts as a PEP analog, another assay was performed in which the reaction was initiated with different concentrations (1–50 mM) of PEP and a fixed concentration (30 mM) of FFQ so as to examine the competitive profile with the PEP. The concentrations of all other substrates were kept the same as in the activity assay reaction mixture.

2.7. Phylogenetic analysis

Phylogenetic analysis of AbMurA was carried out by ClustalW [19], Jalview [20] and Mega6.0 [21] tools. AbMurA sequence was submitted to BLAST search to predict the homologs. From NCBI, homologous sequences were selected and aligned by ClustalW algorithm. Color-coded alignment was generated using Jalview. Phylogenetic tree was constructed by distance-based NJ method using MEGA6.0 software [21].