Identification and characterization of UDP-\(N\)-acetylenolpyruvylglucosamine reductase (MurB) from the Gram-positive pathogen *Streptococcus pneumoniae*

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The UDP-\(N\)-acetylenolpyruvylglucosamine reductase (MurB) from a Gram-positive pathogen, *Streptococcus pneumoniae*, was identified and characterized. The enzyme from *S. pneumoniae* shows 31% identity with the MurB protein from *Escherichia coli*, and contains the catalytic residues, substrate-binding residues and FAD-binding motif identified previously in the *E. coli* protein. The gene was cloned into the pET28a + expression vector, and the 34.5 kDa protein that it encodes was overexpressed in *E. coli* strain BL21(DE3) to 30% of total cell protein. The majority of the protein was found to be insoluble. A variety of methods were used to increase the amount of soluble protein to 10%. This was then purified to near homogeneity in a two-step process. The absorption spectrum of the purified protein indicated it to be a flavoprotein, like its *E. coli* homologue, with a characteristic absorption at 463 nm. The enzyme was shown to be active, reducing UDP-\(N\)-acetylenolpyruvylglucosamine enolpyruvate with the concomitant oxidation of NADPH, and was characterized kinetically with respect to its two substrates. The enzyme showed properties similar to those of its *E. coli* counterpart, being activated by univalent cations and being subject to substrate inhibition. The characterization of an important cell wall biosynthesis enzyme from a Gram-positive pathogen provides a good starting point for the discovery of antibacterial agents against MurB.

Key words: bacterial cell wall, peptidoglycan biosynthesis, UDP-\(N\)-acetylmuramate.

**INTRODUCTION**

UDP-\(N\)-acetylmuramate (UNAM) is the sugar building block for peptidoglycan biosynthesis. It is synthesized in a two-step process by the cytoplasmic enzymes UDP-\(N\)-acetylgulosaminyl enolpyruvyl transferase (MurA) and UDP-\(N\)-acetylenolpyruvylglucosamine reductase (MurB). MurA transfers an enolpyruvyl group from phosphoenolpyruvate to UDP-\(N\)-acetylgulosaminyl to form UDP-\(N\)-acetylenolpyruvyluridine (UNAGEP). MurB then reduces UNAGEP using NADPH to form UNAM (Scheme 1). The pentapeptide chain is then added to this sugar by the amino acid ligases MurC, MurD, MurE and MurF at subsequent steps in the pathway [1]. Finally this intermediate is transported across the bacterial cell membrane and incorporated into the bacterial cell wall.

Cell wall biosynthesis is an important target of several antimicrobials, including fosfomycin, which acts on MurA [2]. However, there are no known antibiotics targeted against the second step in the formation of UNAM, catalysed by MurB. The murB gene has been shown previously to be essential in both *Escherichia coli* and *Bacillus subtilis* [3,4], indicating that the MurB enzyme is a suitable target for antibiotics. The enzyme from *E. coli* has been studied extensively. It has been cloned, overexpressed and purified by several groups in both the native [5,6] and tagged [7] forms, and has been thoroughly characterized both mechanistically and structurally [5,7,8]. *E. coli* MurB was shown to be a flavoprotein containing a bound FAD cofactor which mediates the two-electron transfer from NADPH to UNAGEP [5]. Kinetic studies on the enzyme have indicated a Ping Pong Bi-Bi double-competitive substrate inhibition mechanism [7].

The *E. coli* enzyme has been crystallized and the crystal structure solved, both with and without the substrate (UNAGEP) bound [8–10]. The protein has three structural domains and a novel binding motif for FAD. The residues responsible for substrate and FAD binding were identified, and a catalytic role for Ser-229 was proposed and later confirmed by kinetic experiments [11].

As an essential enzyme for peptidoglycan biosynthesis, MurB is of great interest as a target for novel antibacterial agents. Despite the wealth of information available on the *E. coli* enzyme, little work has been described on MurB from any Gram-positive organism. We have previously studied the MurA enzymes from the Gram-positive pathogen *Streptococcus pneumoniae* [12]. Here we report the identification, overexpression, purification and characterization of the MurB enzyme from the same organism. A fuller understanding of the cell wall biosynthesis
enzymes in Gram-positive organisms may aid the discovery of novel antibiotics against these targets.

MATERIALS AND METHODS

Materials

Biochemical reagents were from Sigma. UDP-N-acetylglucosamine was obtained as described in [5]. Enzymes and reagents used in cloning were from Gibco BRL (Gaithersburg, MD, U.S.A.). DNA preparations were performed with assorted kits from Qiagen (Valencia, CA, U.S.A.). Column chromatography was performed on an AKTA FPLC system (Pharmacia) with Pharmacia Hi-Load columns. SDS/PAGE analyses were performed on NOVEX (San Diego, CA, U.S.A.) 4–12 % (w/v) Bis-Tris gels. The N-terminal amino acid sequence of the purified enzyme was deduced by automated Edman degradation with a Hewlett Packard model G1000A sequencer.

Identification of MurB from S. pneumoniae

A S. pneumoniae homologue of the B. subtilis MurB protein sequence was identified in translations of library sequences of S. pneumoniae 0100993 (N. G. Wallis, unpublished work) using BLAST version 2.0.4 software [13]. The gene sequence was completed by PCR amplification and sequencing. Alignments of MurB protein sequences were carried out using the Jotun Hein software [13], shows greatest similarity to MurB from B. subtilis (37 % identity at the amino acid level [4]). The protein also shows identity with MurB proteins from many other bacteria, including E. coli, Salmonella typhimurium and Haemophilus influenzae. Its level of identity with the E. coli protein is 31 %; however, despite the relatively low sequence identity, the sequence still contains the characteristic motifs implicated in substrate and FAD cofactor binding in the E. coli MurB structure [8]. The catalytic residues are also conserved in the S. pneumoniae enzyme, including the active-site residue Ser-229 (E. coli numbering) [11] (Figure 1), suggesting that the active sites are very similar, despite the overall low sequence identity. Interestingly, low identity between murB genes from different species has already been noted [14]. The MurB enzyme from S. typhimurium is only 82 % identical with the E. coli enzyme, which is one of the lowest recorded identities between proteins from these two highly related organisms.

Expression of MurB protein

E. coli BL21(DE3) cells, transformed with plasmid pET28a + murB, were grown overnight at 37 °C in LB medium containing 50 μg/ml kanamycin and 1 % (w/v) glucose. A 30 ml portion of this culture was used to inoculate 3 litres of LB medium containing 50 μg/ml kanamycin and 1 % glucose. The cells were grown at 37 °C until the A₆₀₀ reached 0.55. At this point the cells were chilled to 18 °C, and isopropyl β-D-thiogalactoside (IPTG) was added to 40 μM in order to induce protein expression. Cell growth was continued for 20 h at 18 °C, and then cells were harvested. The cell pellet was resuspended for lysis in 20 ml of 100 mM Tris, pH 8.0, containing 5 mM dithiothreitol (DTT) and 500 mM NaCl.

Purification of MurB

Cells were lysed by sonication and lysozyme treatment, followed by three freeze/thaw cycles. Following centrifugation (6500 g for 10 min), (NH₄)₂SO₄ was added to the supernatant to 1.5 M and purification was carried out on a Hi-Load (16/10) phenyl-Sepharose column (Pharmacia), equilibrated with 100 mM Tris, pH 8.5, 5 mM DTT and 1.5 M (NH₄)₂SO₄. Proteins were eluted with an (NH₄)₂SO₄ gradient (1.5–0 M) over 200 ml. Fractions containing MurB were then pooled and dialysed against 100 mM Tris (pH 8.5)/5 mM DTT. MurB was purified further by loading on a Hi-Load (16/10) Q-Sepharose column (Pharmacia) equilibrated with 100 mM Tris (pH 8.5)/5 mM DTT. Protein was eluted with a 0–1 M KCl gradient over 200 ml. Fractions containing MurB were identified by SDS/PAGE and by MurB activity. These fractions were then pooled, and (NH₄)₂SO₄ was added to 70 % saturation to concentrate the protein. The resulting pellet was resuspended in 1 ml of 100 mM Tris (pH 8.0)/1 mM DTT and dialysed against the same buffer overnight.

Assay procedures

The activity of MurB was assayed by monitoring the change in absorbance at 340 nm upon oxidation of NADPH. Assays were carried out in Costar 9443 plates in a final assay volume of 100 μl on a SpectraMax plate-reader. Standard assay mixtures were composed of 50 mM Bis-Tris propane, pH 8.0, 200 μM UNAGEP, 200 μM NADPH, 0.2 μg/ml enzyme and the relevant cation. Reactions were carried out at 37 °C for 20–60 s. Enzyme activity was determined by measuring the decrease in NADPH absorbance at 340 nm. A molar absorption coefficient of 6220 cm⁻¹·M⁻¹ for NADPH absorbance at this wavelength was used for calculations of the specific activity of the enzyme. Kinetic constants were determined by means of a square matrix of enzyme velocities. Saturation curves were obtained at 10 concentrations of UNAGEP, with NADPH fixed at each of the same 10 concentrations. The concentrations used were 16.6, 25, 33, 50, 66, 100, 125, 175, 250 and 500 μM. Substrate saturation curves and mechanistic data were fitted to the equations described in [7]. Kinetic parameters were determined by non-linear regression analysis (SigmaPlot; Grafit).

RESULTS AND DISCUSSION

Identification of S. pneumoniae MurB

The murB gene from S. pneumoniae encodes a 316-amino-acid polypeptide which, when compared with all the proteins in the non-redundant protein database using BLAST version 2.0.4 software [13], shows greatest similarity to MurB from B. subtilis (37 % identity at the amino acid level [4]). The protein also shows identity with MurB proteins from many other bacteria, including E. coli, Salmonella typhimurium and Haemophilus influenzae. Its level of identity with the E. coli protein is 31 %; however, despite the relatively low sequence identity, the sequence still contains the characteristic motifs implicated in substrate and FAD cofactor binding in the E. coli MurB structure [8]. The catalytic residues are also conserved in the S. pneumoniae enzyme, including the active-site residue Ser-229 (E. coli numbering) [11] (Figure 1), suggesting that the active sites are very similar, despite the overall low sequence identity. Interestingly, low identity between murB genes from different species has already been noted [14]. The MurB enzyme from S. typhimurium is only 82 % identical with the E. coli enzyme, which is one of the lowest recorded identities between proteins from these two highly related organisms.

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Only one copy of the murB gene was identified, unlike the preceding enzyme in the pathway (MurA), which has two copies in Gram-positive organisms [12]. In B. subtilis the murB gene is found in the dcv cluster [4], but in other bacteria, including S. pneumoniae [15,16], the murB gene does not cluster with other genes from the cell wall biosynthesis pathway. S. pneumoniae murB is found downstream of yhfC and upstream of potA.

Expression and solubilization of MurB

In order to study the MurB protein from S. pneumoniae further, it was overexpressed and purified. Initially, cells containing the overexpressing plasmid were grown at 37 °C and induced with 1 mM IPTG. Expression levels were excellent (30% of total cell protein); however, all the MurB protein formed inclusion bodies. Alternative methods of induction were tried with various combinations, by varying the concentration of IPTG and incubation at 18 °C. Protein expression levels were greater than 30% of total cell protein under all these conditions. At 18 °C approx. 7% of the MurB was soluble when induced with 1 mM IPTG, while 10% of the MurB was soluble when induced with 40 µM IPTG. Increased solubility of the overexpressed protein appears to be correlated with conditions that slow down the production of protein.

Purification of MurB

MurB protein was purified using a combination of Hi-Load phenyl-Sepharose and Hi-Load Q-Sepharose anion-exchange columns, as described above. In both cases the MurB activity co-eluted with a yellow-coloured protein, indicating the presence of a bound flavin. The final purified protein migrated as a single band on 12.5% SDS-PAGE with a molecular mass of 35 kDa.

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Kinetic characterization of MurB

Enzyme activity was tested with a range of different pH values. The optimum was found to be pH 8, similar to that reported previously for the E. coli enzyme. Substrate inhibition of this enzyme was observed with both substrates. Substrate inhibition was strong at pH 6 for UNAGEP, but weaker at pH 8, whereas for NADPH little inhibition was seen at pH 6, but some was seen at pH 8, when working at low UNAGEP concentrations (Figure 4). Similar effects have been described previously for the E. coli enzyme [7].

The activation of both E. coli and Enterobacter cloacae MurB enzymes by univalent cations has been described previously [7,17]. We therefore investigated their effect on the S. pneumoniae enzyme. No activation was observed with Li⁺, but Na⁺, K⁺, NH₄⁺, Rb⁺ and Cs⁺ resulted in a range of activation (Table 1). When no univalent cations were present, activity was very low. The activation constants determined are rather higher than those reported for the E. coli enzyme, but for both enzymes K⁺ causes the greatest activation of the enzyme. The bivalent cations Cu²⁺, Fe²⁺, Hg²⁺, Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Ni²⁺ and Pb²⁺ did not activate the enzyme, with Hg²⁺ and Pb²⁺ actually causing inhibition.

Determination of the kinetic parameters of the enzyme was carried out at pH 8 and 100 mM KCl. Under these conditions the Kₗ values for UNAGEP and NADPH were 89 μM and 79 μM respectively, and the kₗ value was 18 s⁻¹. These Kₗ values are higher than those described in [7] for the E. coli enzyme (24 and 17 μM respectively). The kₗ value for the S. pneumoniae enzyme is rather lower than the value reported in [7] for the E. coli enzyme (62 s⁻¹), but in the same range as the value (1300 min⁻¹) reported in [5] for a recombinant MurB from E. coli.

The substrate specificity of the enzyme was also investigated, by determining the kinetic parameters of the enzyme for analogues of NADPH (Table 2). These data show that S. pneumoniae MurB can utilize different NADPH analogues in a similar way to the E. coli enzyme, but is unable to use NADH. NMR experiments on MurB have suggested previously that the NADP⁺-binding site may be relatively flexible [18,19]. This flexibility may explain the ability of MurB to accept these alternative NADPH analogues as substrates, with relatively small effects on the kinetic parameters.

Conclusions

Overall, S. pneumoniae MurB shows great similarity to its E. coli counterpart, despite the low level of sequence identity between

![Figure 4](image-url) Substrate inhibition by NADPH and UNAGEP at pH 6.0, 7.0 and 8.0

Table 1 Kinetic constants for activation by Na⁺, K⁺, NH₄⁺, Rb⁺ and Cs⁺ of S. pneumoniae MurB

<table>
<thead>
<tr>
<th>Cation</th>
<th>kₗ (s⁻¹)</th>
<th>Kₗ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>4 ± 2</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>K⁺</td>
<td>14 ± 3</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>13 ± 3</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>10 ± 2</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>ND</td>
<td>9 ± 5</td>
</tr>
</tbody>
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the two. The *S. pneumoniae* enzyme is activated by univalent cations in a similar manner to that reported for the *E. coli* and *Enterobacter cloacae* enzymes. The enzyme also shows the effects of substrate inhibition, which vary with pH, as described for the *E. coli* enzyme. Experiments showing the reduction of FAD by NADPH also indicate a Ping Pong mechanism similar to that proposed for the *E. coli* enzyme.

Its key position in bacterial cell wall biosynthesis and its essential nature in both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) organisms illustrate the importance of MurB as an antibacterial target. We have shown that, overall, the characteristics of the *E. coli* and *S. pneumoniae* enzymes are very similar. These data, and the fact that key residues in the active site identified in the *E. coli* MurB structure are conserved, suggest that, despite the low level of overall sequence identity, the active sites are very similar. These similarities in the active-site region suggest that it may be possible to find a broad-spectrum inhibitor that acts on both Gram-negative and Gram-positive enzymes. The characterization of this enzyme from *S. pneumoniae* should lead the way towards the discovery of inhibitors of this important antibacterial target.

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REFERENCES


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