**Pseudomonas aeruginosa** MurE amide ligase: enzyme kinetics and peptide inhibitor

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The enzyme kinetics of the amide ligase MurE, a cell wall biosynthesis enzyme, from *Pseudomonas aeruginosa* were determined using the synthesized nucleotide substrate UDP-MurNAc-Ala-Glu (uridine 5'-diphosphoryl N-acetylmuramoyl-L-alanyl-D-glutamate). When coupled to a competitive bio-panning technique using a M13 phage display library encoding ~2.7 × 10⁶ random peptide permutations and the specific substrates meso-A2pm (meso-diaminopimelic acid) and ATP, a peptide inhibitor of MurE was identified. The MurEp1 dodecamer selected and synthesized inhibited MurE ATPase activity with an IC₅₀ value of 500 μM. The inhibition was shown to be time-dependent and was reversed by the addition of meso-A2pm or UDP-MurNAc-Ala-Glu during the pre-incubation step. Kinetic analysis defined MurEp1 as a mixed inhibitor against both substrates with Kᵢ values of 160 and 80 μM respectively. MurEp1 was found to interfere in meso-A2pm and UDP-MurNAc-Ala-Glu binding necessary for amide bond formation. Modelling of *Ps. aeruginosa* MurE and docking of MurEp1 on the *Ps. aeruginosa* MurE surface indicated that MurEp1 binds at the juxtaposition of both meso-A2pm- and UDP-MurNAc-Ala-Glu-binding sites in the closed conformational state of the enzyme. Identification of the MurEp1 residues involved in MurE binding and inhibition will allow the development of a novel class of inhibitors having a novel mode of action against MurE.

Key words: meso-diaminopimelic-adding enzyme, peptide inhibitor, phage display, UDP- N-acetylmuramoyl-L-alanyl-D-glutamate:meso-diaminopimelate ligase.

**INTRODUCTION**

The bacterial cell wall biosynthesis pathway, for peptidoglycan synthesis and assembly, constitutes one of the best targets for the design of new antibacterial agents against the growing problem of antibiotic resistance. The intracellular enzymes MurA–MurF achieve the first step of the pathway by catalysing the synthesis of the precursor UDP-N-acetylmuramyl pentapeptide. These enzymes are essential for bacterial survival; inhibition of one of these enzymes gives a lethal phenotype. Mur enzymes are expressed during infection of the host, are highly conserved among bacteria and have no counterparts in eukaryotes [1,2]. Although many known antibiotics interfere with enzymes involved in the later steps of peptidoglycan production, few target the enzymes that catalyse the cytoplasmic step. The unavailability of suitable intermediates in *vitro* has severely hampered kinetic studies and development of inhibitors against these enzymes [2,3].

We have selected MurE as a key target enzyme for developing peptide inhibitors. MurE is part of the four ATP-dependent amide ligases that perform the stepwise addition of amino acids (L-Ala, D-Glu, a di-amino acid and D-Ala-D-Ala) to form the cell wall peptide moiety [4]. The non-ribosomal peptide bond formation involves a carboxy group activation of the nucleotide substrate to an acylphosphate intermediate by ATP, followed by nucleophilic attack by the amino group of the condensing amino acid and elimination of the phosphate group [2]. MurE is the only Mur ligase for which amino acid substrate specificity differs among bacterial species. Typically, Gram-negative bacteria and bacilli contain a meso-A2pm (meso-diaminopimelic acid) residue at the third position of the cell wall peptide moiety, whereas Gram-positive bacteria contain L-lysine at this position [5,6]. Addition of the di-amino acid residue at the third position of the cell wall peptide moiety is critical for the formation of the peptide side-chain cross-linkages by the periplasmic transpeptidases, establishing cell shape and integrity [6,7]. Furthermore, the flexibility of the meso-A2pm–meso-A2pm cross-bridge enables bacteria to adapt the peptidoglycan cross-linkage to growth conditions and environmental changes [7].

As a model target system, we studied the MurE UDP-N-acetyl- muramoyl-L-alanyl-D-glutamate:meso-diaminopimelate ligase from the Gram-negative bacterium *Pseudomonas aeruginosa*, an opportunistic pathogen highly resistant to most classes of antibiotics [8]. We first synthesized and purified the UDP-MurNAc-Ala-Glu (uridine 5'-diphosphoryl N-acetylmuramoyl-L-alanyl-D-glutamate) substrate to study the kinetics of the MurE amide ligase. We then developed a competitive bio-panning phage display screening approach using the substrates meso-A2pm and ATP to identify a peptide inhibitor of MurE. Phage display is a powerful tool for the selection of short peptides that interact specifically with target proteins by using a large pool of random peptide permutations fused to the phage M13 pIII minor coat protein [9]. This approach has been useful for the identification of various peptide inhibitors of enzymes, including MurC and MurD [10,11].

Abbreviations used: meso-A2pm: meso-diaminopimelic acid; PFU, plaque-forming unit(s); RMSD, root mean square deviation; UDP-GlcNAc, uridine 5'-diphosphoryl N-acetylglucosamine; UDP-MurNAc-Ala-Glu, uridine 5'-diphosphoryl N-acetylmuramoyl-L-alanyl-D-glutamate.

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EXPERIMENTAL

Purification of biologically active MurE enzyme

The MurE protein from *Ps. aeruginosa* PAO1 was overexpressed, purified and sequenced as described previously [12]. All reagents were purchased from Sigma–Aldrich unless indicated otherwise. The phosphate present in the MurE conservation buffer was removed by dialysing 3 ml three times against 3 litres of buffer A (25 mM Bis-Tris, pH 8, 1 mM 2-mercaptoethanol and 2.5 mM MgCl₂) at 4°C. Purified MurE protein was visualized by SDS/PAGE using SYPRO® Orange staining (Bio-Rad), and the protein concentration was determined using the Bradford method (Bio-Rad) with BSA as standard. Glycerol was added to the phosphate-free MurE solution to a concentration of 10% (v/v) and aliquots of 100 μl were conserved at −20°C. The biological activity of MurE was assayed by *in vitro* reconstruction of the biosynthetic pathway of peptidoglycan cytoplasmic precursor in the presence of purified MurA, MurB, MurC, MurD and MurF as described previously [12].

Synthesis and purification of UDP-MurNAc-Ala-Glu

Synthesis of UDP-MurNAc-Ala-Glu was performed according to the method described previously [13], except that the synthesis from UDP-GlcNAc (5′-diphosphoryl N-acetylglucosamine) was carried out in a single reaction using the combined activities of *Ps. aeruginosa* MurA, MurB, MurC and MurD enzymes. The Mur enzymes were removed by ultrafiltration and UDP-MurNAc-Ala-Glu was purified by loading the ultrafiltrate into a column packed with DEAE-Sephacel pre-equilibrated in 10 mM phosphate buffer at pH 7.5 and eluting at room temperature (20°C) with a 10–800 mM ammonium acetate gradient. A pyruvate kinase/lactate dehydrogenase-coupled assay for meso-A2pm-dependent ADP generation by MurE was used to identify fractions containing the cell wall intermediate. Positive fractions were freeze-dried three times to remove ammonium acetate. UDP-MurNAc-Ala-Glu purity was assessed by analytical anion-exchange FPLC on MonoQ™ using a 0–0.5 M ammonium acetate gradient. Structure of the purified UDP-MurNAc-Ala-Glu was confirmed using negative-ion electrospray MS, and the product was stored as frozen aqueous solution at 4.34 mM.

Kinetic characterization of MurE activity

The ATPase activity of the *Ps. aeruginosa* MurE protein was assessed by a spectrophotometric assay that quantifies released inorganic phosphate using the Lanzetta reagent [14]. The reaction was performed in 100 μl of reaction buffer B (50 mM Bis-Tris, pH 8.0, 1 mM dithiothreitol and 5 mM MgCl₂) containing 8.7 μM units of purified MurE (from a fresh aliquot for each assay), 1 mM ATP, 200 μM UDP-MurNAc-Ala-Glu and 10 mM meso-A2pm. The MurE enzyme was allowed to react for 15 min at room temperature before the addition of 800 μl of the Lanzetta reagent. The mixture was incubated for 5 min to allow proper colour development, 100 μl of a 34% (w/v) sodium citrate solution was added and the absorbance was immediately measured at 660 nm with a Cary spectrophotometer (Varian) [14]. The amount of inorganic phosphate was determined by comparison with the linear portion of a phosphate standard curve with a minimum R² value of 0.99. The phosphate content of each component was determined and subtracted from the total phosphate. Negative controls were performed without enzyme or substrate and assays were carried out in triplicate. Saturation curves were obtained for each substrate individually using fixed and optimal concentrations of the remaining substrates. The following parameters were determined for MurE with respect to each substrate: the kₘ, the maximal specific hydrolysis activity, the catalytic-centre activity, the Kₐ and the enzyme efficiency. Kinetic parameters were determined by non-linear regression analysis by means of a square matrix of enzyme velocities based on the Michaelis–Menten equations using the Enzyme Kinetics Module version 1 of SigmaPlot 8.

Affinity selection of phage-displayed peptides against MurE

Phage display screening was performed using the PH.D.-12 M13 phage library containing ~2.7 × 10⁹ peptide permutations (New England Biolabs) as described previously [10,15]. The screening specificity and stringency were optimized by increasing the Tween concentration during washing and by decreasing the time of contact between the phages and the targeted MurE protein during the three rounds of bio-panning. See Table 3 for the phage titres used as input. Phages with encoded peptides were eluted at the third round of bio-panning by 100 μl of 0.2 M glycine/HCl (pH 2.2), 100 μl of 1 mM meso-A2pm or 100 μl of 1 mM ATP for 30 min. The DNA of ten phages was sequenced for each elution condition with a ~96 gIII primer (New England Biolabs). The deduced peptide sequences were aligned and consensus amino acids were identified.

Peptide synthesis

The dodecameric peptide encoded by the strongest consensus sequence selected by the phage display screening (NHNM-HRTTQWPL) was synthesized and purified as described previously [10]. Called MurEp1, its purity (>95%) was analysed by HPLC and its molecular mass (1534.74 Da) was confirmed by MALDI–TOF–MS (matrix-assisted laser-desorption ionization–time-of-flight MS). The peptide was dissolved in buffer C (200 mM Tris/HCl, pH 8) at a concentration of 100 mM and the pH was adjusted to 7.0.

Bioinformatics analysis

The peptide consensus sequence was characterized using the Compute pi/Mw and ProtParam algorithms [16] as well as the PSlpred and SSpro secondary-structure prediction tools of the ExPasy proteomics server [17–19]. The sequence was also analysed for homologues in the UniProt database using the fasts34 program [11].

Evaluation of MurEp1 as an inhibitor of MurE ATPase activity

Inhibition of MurE ATPase activity by MurEp1 was observed at room temperature in reaction buffer B using the spectrometry-based method described above. Substrates were added after pre-incubation of the enzyme with the inhibitor. A test was carried out with 750 μM MurEp1 for 0, 10, 30, 60 or 120 min of pre-incubation to observe possible time-dependence of inhibition. Assays were also performed in which one substrate was added for 30 min of pre-incubation with or without 750 μM MurEp1. MurE specific activities were determined in the presence of 75, 150 or 600 μM MurEp1 with pre-incubation periods of 10 and 30 min and were compared with the reference activity without inhibitor. MurEp1 Kₐ was determined for meso-A2pm and UDP-MurNAc-Ala-Glu using reaction velocity data obtained with 75, 150 and 600 μM MurEp1 (with a 30 min pre-incubation) and either five concentrations of meso-A2pm (0.075, 0.25, 0.75, 3 and 10 mM) or five concentrations of UDP-MurNAc-Ala-Glu (12.5, 25, 50, 100 and 200 μM) with fixed optimal concentrations of the other
subicates. \(K\) values were determined regardless of the inhibition type, based on the following equation:

\[
v = V_{\text{max}} \times \left[ \frac{[1 + \beta \times I / (\alpha \times K_m)] \times [1 + (K_m / S)]}{[1 + (1 / K_m)] \times [1 + (1 / (\alpha \times K_m))] \times (1 + I / K_m) \times (1 + (1 / (\alpha \times K_m))}
\]

where \(\alpha\) is the \(K_m\) factor change when MurEp1 is bound to the enzyme–substrate complex, \(\beta\) is the \(K_m\) factor change when MurEp1 is bound to the enzyme–substrate complex, \(I\) is the inhibitor concentration and \(S\) is the substrate concentration.

All experiments were carried out in triplicate. Kinetic data were fitted to the appropriate model equations using the Enzyme Kinetics Module of SigmaPlot. Steady-state inhibition kinetic parameters were determined by non-linear regression analysis using the least-squares method as a quality control to ensure accurate dataset fitting and to generate error estimates. The expected \(K\) values for the inhibited MurE reactions were calculated from an increase in the uninhibited \(K\) value by a factor of \(1 + [\text{MurEp1}] / K\) for meso-A2pm and \(1 + [\text{MurEp1}] / 3K\) for UDP-MurNAc-Ala-Glu [20,21]. As controls, non-specific peptides issued from phage display were tested at different concentrations for inhibition of MurE activity [22].

Computer-assisted modelling of MurE and docking of MurEp1

Modelling of MurEp1 was performed using BetaTPred2 [23]; the peptide contains a \(\beta\)-turn as do many antibacterial peptides. The model of MurEp1 was built from the NMR structure (PDB:1WR3) using the first WW (Trp-Trp) domain of ubiquitin-protein ligase Nedd4-2 [24] with the nest modelling software from the Jackal package (available at http://wiki.c2b2.columbia.edu/honiglab_public/index.php/Software:Jackal); the interactions between MurEp1 and MurE were extracted using PIC software [25,26]. Alignment of both peptide sequences was done using the water algorithm from EMBOSS [27]. The secondary structure for the three-dimensional model was extracted using Stride [28], and the MurEp1 structure was refined using the minst software from the Jackal package [25,26]. The MurEp1 docking in MurE was carried out using Patchdock [29], and the structure was refined with FireDock [30]. Parameters used were: clustering RMSD (root mean square deviation) of 4.0 Å (1Å = 0.1 nm), complex type of enzyme inhibitors with 50 RBO (rigid body optimization) cycles and an atomic radius scale of 0.8. The quality of the MurE model was confirmed using procheck [31], and the localization of amino acids in the model was confirmed using Verify-3D [32].

RESULTS

Purification of biologically active MurE

The purified MurE protein was visualized as a single 55 kDa molecular mass band on SDS/PAGE (results not shown). Sequencing of the first 15 N-terminal residues confirmed its identity as MurE from Ps. aeruginosa (identical with the published sequence). Its biological activity was confirmed by reconstruction of the cell wall precursor UDP-N-acetylmuramyl pentapeptide in the presence of MurA, MurB, MurC, MurD and MurF [12].

Synthesis and purification of the UDP-MurNAc-Ala-Glu substrate

Analysis by anion-exchange chromatography indicated that the UDP-MurNAc-Ala-Glu product was 99.5% pure (Figure 1A). Characterization of the purified product by MS identified four different species with observed \(m/z\) values identical with the expected values (Table 1). Results obtained confirmed the UDP-MurNAc-Ala-Glu structure as presented in Figure 1(B).

MurE amide ligase activity

A series of preliminary experiments defined optimal conditions for the MurE assay as 15 min at room temperature in buffer B. Addition of NaCl did not improve MurE activity (results not shown). As depicted in Figure 2(A), the optimal concentration of ATP was 1 mM, whereas a concentration of 2 mM decreased MurE specific activity. The maximal specific activity was reached at 10 mM meso-A2pm (Figure 2B). The optimal concentration of UDP-MurNAc-Ala-Glu was 200 \(\mu\)M and concentrations of 300 \(\mu\)M or greater decreased enzyme activity (Figure 2C). On the basis of \(K\) values, the affinity of MurE for UDP-MurNAc-Ala-Glu was approx. 4.5-fold higher than for ATP (Table 2). The catalytic centre activity values indicated that each MurE active site hydrolyses 2.3 ATP molecules/s to ADP and inorganic phosphate and thus performed 2.3 amide ligase reactions/s between meso-A2pm and UDP-MurNAc-Ala-Glu according to the demonstrated stoichiometric relationship [33]. The enzyme efficiency indicated that MurE was more efficient with respect to UDP-MurNAc-Ala-Glu. The kinetic parameters were measured with reasonable precision, having S.D. values of less than 30%.

Selection of specific MurE-binding peptides

As shown in Table 3, each round of bio-panning enriched for MurE-specific-binding peptides, phage titres decreasing from approx. 10\(^{11}\) to 10\(^{6}\) PFU (plaque-forming units). Each round of bio-panning yielded a lower phage recovery in comparison with
Table 2 Kinetic parameters of MurE amide ligase activities associated with each of the three substrates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ATP</th>
<th>meso-A2pm</th>
<th>UDP-MurNAc-Ala-Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ ($s^{-1}$)</td>
<td>22.2 ± 2.6</td>
<td>22.2 ± 0.43</td>
<td>24.8 ± 0.86</td>
</tr>
<tr>
<td>Specific hydrolysis activity</td>
<td>2.6 ± 0.3</td>
<td>2.6 ± 0.05</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>Catalytic-centre activity</td>
<td>2.3 ± 0.42</td>
<td>2.3 ± 0.33</td>
<td>2.6 ± 0.42</td>
</tr>
<tr>
<td>$K_M$ ($\mu$M)</td>
<td>225 ± 90</td>
<td>140 ± 15</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>Enzyme efficiency ($s^{-1} \cdot \mu$M$^{-1}$)</td>
<td>0.01 ± 0.003</td>
<td>0.016 ± 0.003</td>
<td>0.087 ± 0.017</td>
</tr>
</tbody>
</table>

Note substrate inhibition by ATP and UDP-MurNAc-Ala-Glu.

Figure 2 Saturation profiles for (A) ATP, (B) meso-A2pm and (C) UDP-MurNAc-Ala-Glu in the MurE reaction

Table 3 Phage bio-panning using MurE and the dodecamer phage peptide library

<table>
<thead>
<tr>
<th>Round</th>
<th>Phage input</th>
<th>Eluted phages</th>
<th>Elution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gly-HCl</td>
<td>$4 \times 10^3$</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>2</td>
<td>Gly-HCl</td>
<td>$1 \times 10^4$</td>
<td>$1.5 \times 10^3$</td>
</tr>
<tr>
<td>3</td>
<td>Gly-HCl</td>
<td>$2 \times 10^3$</td>
<td>$8.7 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>$6.3 \times 10^4$</td>
<td>$3.2 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>Meso-A2pm</td>
<td>$5.3 \times 10^4$</td>
<td>$2.7 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

Peptide analysis

The theoretical pI of the consensus peptide sequence was established at 9.8, indicating that the peptide is positively charged at neutral pH. The consensus sequence does not contain any negatively charged residues and contains one positively charged residue. ProtParam confirmed the high proportion of asparagine, histidine and threonine residues in the consensus sequence and estimated the half-life of the peptide at more than 10 h in *Escherichia coli* and at 1.4 h in mammalian reticulocytes *in vitro*. The secondary-structure prediction indicated a putative random coil structure. Fasta34 did not identify peptide homologues having an E value <5 in the UniProt database.

Kinetics of MurE inhibition by MurEp1

As shown in Figure 4(A), the residual activity of MurE decreased exponentially when the pre-incubation time with MurEp1 increased, indicating that the inhibition is time-dependent. The $IC_{50}$ values for MurEp1 determined with pre-incubation periods of 10 and 30 min were 800 and 500 $\mu$M respectively (Table 4). The specific activity of MurE decreased gradually as a function of the MurEp1 concentration, giving an inverse linear dose–response with a varying slope (Figure 4B).
Figure 3  Peptide sequences obtained from the 12-mer phage display library analysed by sequencing of selected phages eluted after the third round of bio-panning against MurE

Consensus sequences and related conserved motifs are boxed in black and the superscript letters indicate peptide sequences recovered more than once.

Figure 4  Time-dependent inhibition of MurE ATPase activity and IC₅₀ values for peptide MurEp1

(A) Inhibition of the ATPase activity of MurE by 750 μM MurEp1 at pre-incubation times of 0, 10, 30, 60 and 120 min, revealing the time-dependence of the inhibition. (B) IC₅₀ determination for MurEp1 for MurE ATPase activity with a 30 min pre-incubation.

Table 4  Kinetics of Ps. aeruginosa MurE inhibition by MurEp1

The expected Kₘ values for the inhibited MurE reactions were calculated from increases in the uninhibited Kₘ value by a factor of 1 + [MurEp1]/Kᵢ for meso-A₂pm and 1 + [MurEp1]/3Kᵢ for UDP-MurNAc-Ala-Glu. Kinetic parameters α and β (Kᵢ, and Kᵢ factor changes when MurEp1 is bound to the enzyme–substrate complex) are shown with the corresponding competitive Kᵢ values with respect to meso-A₂pm and UDPMurNAc-Ala-Glu.

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Value</th>
<th>Expected Kᵢ value μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ for 10 min pre-incubation</td>
<td>800 ± 25 μM</td>
<td></td>
</tr>
<tr>
<td>IC₅₀ for 30 min pre-incubation</td>
<td>500 ± 15 μM</td>
<td></td>
</tr>
<tr>
<td>α meso-A₂pm</td>
<td>3 ± 1</td>
<td></td>
</tr>
<tr>
<td>β meso-A₂pm</td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Kᵢ meso-A₂pm</td>
<td>0.05 ± 0.02 μM</td>
<td></td>
</tr>
<tr>
<td>α UDP-MurNAc-Ala-Glu</td>
<td>6.5 ± 0.3 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>β UDP-MurNAc-Ala-Glu</td>
<td>60 ± 25 μM</td>
<td></td>
</tr>
<tr>
<td>Kᵢ UDP-MurNAc-Ala-Glu</td>
<td>80 ± 25 μM</td>
<td></td>
</tr>
</tbody>
</table>

Meso-A₂pm or UDP-MurNAc-Ala-Glu added during the 30 min pre-incubation period completely restored MurE activity, whereas adding ATP did not affect inhibition by MurEp1 (results not shown). MurE reaction velocity was identical with or without UDP-MurNAc-Ala-Glu in the pre-incubation step, but was slightly reduced in the presence of ATP or meso-A₂pm. This effect was taken into account when determining MurEp1 inhibition (results not shown). Since meso-A₂pm and UDP-MurNAc-Ala-Glu both competed against MurEp1 inhibition, the Kᵢ values for MurEp1 were determined for these substrates. The slopes and x-intercepts of fitted lines from both Lineweaver–Burk plots varied as a function of MurEp1 concentration, indicating a catalytic component in the inhibition (Figure 5). The y-intercepts
varied according to the different MurEp1 concentrations used for the meso-A2pm plot, indicating a specific component in the inhibition (Figure 5A). The UDP-MurNAc-Ala-Glu plot did not show significant y-intercept variation, with the common intersection point between the fitted lines standing closer to the y-axis (Figure 5B). The common intersection point between the fitted lines of both Lineweaver–Burk plots was at the left of the y-axis and above the x-axis, indicating that MurEp1 caused a reversible mixed type of inhibition against both substrates (Figure 5). This model of inhibition gave the best fit to the experimental data according to the Runs test, with $R^2$ values of 0.96 and 0.94, Akaike values of around 230 and 195 and S.D. of the residuals of 0.14 and 0.18 for meso-A2pm and UDP-MurNAc-Ala-Glu respectively. Dixon plots established the competitive $K_i$ values at 160 ± 45 μM for the meso-A2pm substrate and at 80 ± 25 μM for UDP-MurNAc-Ala-Glu (Table 4). Even if MurEp1 behaved as a time-dependent inhibitor, the tight binding inhibition model was excluded since the MurE concentration was not approximately equal to or higher than the apparent $K_i$ values. The $\sigma$ parameter indicated that binding of MurEp1 to the enzyme–substrate complex decreased MurE affinity for meso-A2pm by a factor of 3, and decreased the MurE affinity for UDP-MurNAc-Ala-Glu by a factor of 6 (Table 4). The $\beta$ parameters indicated that the dissociation of the enzyme–substrate complex to enzyme and product was slightly affected by MurEp1 in the case of the meso-A2pm complex, and strongly inhibited in the case of the UDP-MurNAc-Ala-Glu (Table 4).

The $V_{\text{max}}$ values for MurE decreased as a function of MurEp1 concentration with respect to meso-A2pm; the $V_{\text{max}}$ obtained with 600 μM of MurEp1 was half of the typical value. This effect was not observed for UDP-MurNAc-Ala-Glu (Table 4). The $K_m$ values for meso-A2pm increased as the MurEp1 concentration increased, by a factor of 1 + [MurEp1]/$K_a$, as normally observed for competitive inhibitors. Observed $K_m$ values for meso-A2pm were nearly identical with the expected values, except for the lower value obtained with 600 μM MurEp1 (Table 4). $K_m$ values for UDP-MurNAc-Ala-Glu also increased as a function of MurEp1 concentration. In this case, the factor 1 + [MurEp1]/3/$K_a$ was more accurate than 1 + [MurEp1]/$K_a$ in predicting $K_m$ values (Table 4). The kinetic parameters were measured with reasonable precision, the S.D. values being generally smaller than 30% of the reported values.

**Computer-assisted modelling of MurE and docking of MurEp1**

The structure of the *P. aeruginosa* MurE enzyme was modelled from the structural data of its *E. coli* counterpart. The majority of the amino acid residues forming the active site of MurE are conserved in *P. aeruginosa* and *E. coli*, with the exception of the loop at positions 42–47 in the *E. coli* MurE which interacts with pyrophosphate of the UDP-MurNAc-Ala-Glu-meso-A2pm product, and Asn 150, which is replaced by His 150, in the *P. aeruginosa* MurE (Figures 6A and 6B). The quality of the *P. aeruginosa* MurE model was confirmed using procheck [31], and the Ramachandran plot showed 99.2% of positions positioned correctly in the model. The localization of amino acid residues in the model was confirmed using Verify-3D [32], giving an average 3D-1D (three-dimensional and primary structure comparisons) of 91.14%. Alignment with *E. coli* MurE gave an RMSD of 0.2 Å for 457 amino acids.

The MurEp1 inhibitor was successfully docked into the structure of *P. aeruginosa* MurE. According to the model presented in Figure 6, MurEp1 binds to the active site of MurE that assembled at the common domain interfaces. The predicted MurEp1-binding site overlapped with the meso-A2pm and UDP-MurNAc-Ala-Glu substrate-binding sites in the closed conformational state of the enzyme (Figures 6A and 6B). The docking also suggested that MurEp1 was close enough to the shape and size of both meso-A2pm and UDP-MurNAc-Ala-Glu to compete for their binding sites on the enzyme surface (Figure 6B).

The specific residues involved in the *P. aeruginosa* MurE–MurEp1 molecular interactions are presented in Figure 6C and indicate that MurEp1 binds to MurE via three pairs of residues. The two N-terminal pairs are composed of the residues at positions one and two in the peptide sequence (Asp-His) and the residues at positions four and five (Asp-His). These four residues allow the binding of MurEp1 to the meso-A2pm-binding site of MurE in close proximity to the UDP-MurNAc-Ala-Glu-binding site. The third pair of residues, composed of the C-terminal tryptophan and proline residues at positions 10 and 11, allow the binding of MurEp1 to the UDP-MurNAc-Ala-Glu-binding site on MurE (Figure 6). Leu 31 of MurE forms a hydrophobic bond with the tryptophan and proline residues of MurEp1. Val 37 and Tyr 146 of MurE display hydrophobic interactions with the proline and methionine residues of MuEp1 respectively.
Kinetics of a novel MurE peptide inhibitor

Figure 6 Computer-assisted modelling of Ps. aeruginosa MurE and docking of MurEp1 into the Ps. aeruginosa MurE active site

(A) MurE from E. coli with UDP-MurNAC-Ala-Glu in orange and meso-A2pm in red. (B) MurE/MurEp1 Ps. aeruginosa complex. (C) Close-up view of MurEp1 with the side chains of key interacting residues of MurE shown as sticks. The atomic distances are: Lys112 –His5, 3.14 Å; Asp205 –Asn1, 2.60 Å; Glu457 –Met4, 3.64 Å; and Glu461 –His2, 2.81 Å.

Glu461 of MurE form ionic bonds with the histidine and arginine residues of MurEp1. Lys112, Asp205, Glu457 and Glu461 of MurE form hydrogen bonds with histidine, aspartate, methionine and histidine residues of the peptide respectively.

Glu457 of Ps. aeruginosa MurE is critical for the binding of MurEp1 as it interacts with the N-terminal arginine, histidine and methionine residues of the peptide. Glu468 of E. coli MurE is the functional equivalent of Ps. aeruginosa MurE Glu457, and it has been shown to interact with the meso-A2pm moiety of the UDP-MurNAC-Ala-Glu-meso-A2pm product [34]. This indicates that MurEp1 interacts with at least one residue of Ps. aeruginosa MurE directly involved in the binding of the meso-A2pm. His43, Glu44 and Ala45 of E. coli MurE interact with the UDP-MurNAC moiety of the UDP-MurNAC-Ala-Glu-meso-A2pm product, but they are not conserved in the Ps. aeruginosa MurE. According to the modelling of Ps. aeruginosa MurE (Figures 6A and 6B), Val157 of Ps. aeruginosa MurE may be involved in the interaction with the UDP-MurNAC moiety. This residue interacts with the C-terminal proline residue of MurEp1, suggesting that MurEp1 may bind to a residue directly involved in the binding of the nucleotide substrate. Modelling of Ps. aeruginosa MurE also indicates that Leu23 that interacts with the C-terminal Trp-Pro residues of MurEp1 may also be involved in the binding of the nucleotide substrate of MurE.

DISCUSSION

The lack of commercially available pathway intermediates suitable for kinetic analysis and studies of inhibitors have severely hampered the discovery and characterization of inhibitors of the MurE amide ligase, a potent antibacterial target. We synthesized the UDP-MurNAC-Ala-Glu substrate of the enzyme (Figure 1 and Table 1). Biologically active MurE from Ps. aeruginosa was purified and detailed kinetic analysis was carried out by monitoring the release of inorganic phosphate for MurE ATPase activity. The MurE ATPase activity has been shown to be absolutely dependent on the di-amino acid and UDP-MurNAC-Ala-Glu substrates [33]. The vast majority of MurE kinetic studies have measured enzyme activity using addition of radioactive di-amino acid substrate to UDP-MurNAC-Ala-Glu, and quantifying the radioactive UDP-MurNAC-tripeptide after separation by reverse-phase HPLC, HPLC cation-exchange chromatography, high-voltage electrophoresis or TLC [6,33,35–41]. Compared with our simple, rapid and sensitive spectrophotometric assay, these methods are time-consuming, costly and labour-intensive. A MurE ATPase assay based on the ADP- and NAD-coupled reaction with pyruvate kinase and lactate dehydrogenase has been reported [42]. Our assay measures ATP hydrolysis directly rather than through an NAD-dependent additional enzymatic reaction, thus offering better screening capacities to analyse specific MurE inhibitors. Furthermore, our assay could be easily adapted to HTS (high-throughput screening) in microtitre plates. Recently, Bratkovi et al. [43] reported a similar assay, quantifying the release of inorganic phosphate with the Biomol Green reagent.

A high concentration of ATP was detrimental to MurE activity (Figure 2A); this has been observed previously with the Staphylococcus aureus MurE enzyme [33]. MurE is highly sensitive to pH; a pH value of 8.5 has been found to be optimal and enzyme activity ceases below pH 7 [33,44,45]. A UDP-MurNAC-Ala-Glu concentration above 300 μM caused strong substrate inhibition of MurE activity (Figure 2C); this has also been observed with MurE from Thermotoga maritima, S. aureus and...
Bacillus cereus [33,44,45]. Similar inhibition by the nucleotide cell wall precursor has been noted for the cell wall biosynthesis enzymes MurA and MurF [46,47]. Adding NaCl did not suppress substrate inhibition for MurE as it does for the MurF enzyme [46].

Such substrate inhibition is presumably involved in regulation of the cell wall synthesis rate, allowing cells to respond to different growth and environmental conditions. The optimal substrate concentrations and $K_v$ values obtained for $Ps. aeruginosa$ MurE were similar to those obtained for other MurE enzymes. The $K_v$ values obtained for UDP-MurNAc-Ala-Glu and meso-A2pm (Table 2) were nearly identical and higher respectively than those reported for the E. coli enzyme: 35 μM for UDP-MurNAc-Ala-Glu and 36 μM for meso-A2pm [36]; 76 μM for UDP-MurNAc-Ala-Glu and 36 μM for meso-A2pm [48]; 55 μM for UDP-MurNAc-Ala-Glu [49]; and 40 μM and 11 μM for meso-A2pm [39,50]. The $Ps. aeruginosa$ MurE $K_v$ values were similar to those obtained for the B. cereus meso-A2pm-adding enzyme: 340, 320 and 130 μM for ATP, meso-A2pm and UDP-MurNAc-Ala-Glu respectively [44]. The T. maritima MurE enzyme showed higher $K_v$ values: 3.6 mM for ATP, 2.8 mM for L-lysine, 4.8 mM for meso-A2pm and 0.45 mM for UDP-MurNAc-Ala-Glu [45].

The synthesized MurEp1 peptide encoding the strongest selection factor for phage specifically binding to MurE than increasing the wash stringency during the second round. This was indicated by the lower percentages of phages obtained in the third round (Table 3). This selection pattern had also been observed in phage display screening of peptides against MurD [11]. In contrast, screening of MurC gave higher phage recovery titres for the second and third rounds [10]. Earlier selection of MurC-specific peptides during the phage display screening compared with MurE and MurD could explain this enrichment [10,11]. It should also be noted that several other factors affect phage recovery, such as phage infection and replication efficiency, protein translocation and folding bias as well as pH coat stability [51].

Limiting the time of contact between the phages and MurE during the third round of bio-panning presented a more stringent selection factor for phage specifically binding to MurE than increasing the wash stringency during the second round. This was indicated by the lower percentages of phages obtained in the third round (Table 3). This selection pattern had also been observed in phage display screening of peptides against MurD [11]. In contrast, screening of MurC gave higher phage recovery titres for the second and third rounds [10]. Earlier selection of MurC-specific peptides during the phage display screening compared with MurE and MurD could explain this enrichment [10,11]. It should also be noted that several other factors affect phage recovery, such as phage infection and replication efficiency, protein translocation and folding bias as well as pH coat stability [51].

The synthesized MurEp1 peptide encoding the strongest consensus sequence selected by the phage display screening represented a more stringent selection factor for phage specifically binding to MurE than increasing the wash stringency during the second round. This was indicated by the lower percentages of phages obtained in the third round (Table 3). This selection pattern had also been observed in phage display screening of peptides against MurD [11]. In contrast, screening of MurC gave higher phage recovery titres for the second and third rounds [10]. Earlier selection of MurC-specific peptides during the phage display screening compared with MurE and MurD could explain this enrichment [10,11]. It should also be noted that several other factors affect phage recovery, such as phage infection and replication efficiency, protein translocation and folding bias as well as pH coat stability [51].

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Few MurE inhibitors have been reported to date and none of them has antibacterial activity [2]. The most potent compound is a phosphinate inhibitor, which interferes with the tetrahedral intermediate of MurE and inhibited MurE with an IC₅₀ value of 1.1 μM [37]. Two analogous substrates close to the natural UDP-MurNAc-Ala-Glu substrate have a significant inhibitory effect on MurE at 1 mM, with the best inhibitor giving a MurE residual activity of 71% [49]. Recently, phosphinate transition-state inhibitors of MurD were shown to inhibit MurE, acting as UDP-MurNAc-Ala-Glu substrate analogues. The more promising compound gave a MurE residual activity of 12% at a concentration of 1 mM [41]. Several analogues of meso-A2pm have been tested as competitive inhibitors of MurE [2]. The LD₅₀ diastereoisomers of N-α-propionyl-dipeptides displayed moderate inhibitor effects [53]; (25,3R,6S)-3-fluro-A2pm and N-hydroxy-A2pm respectively had IC₅₀ values of 2.3 and 0.56 mM with an expected Kᵢ value of 0.4 mM for N-hydroxy-A2pm [40], while a monophosphonomonocarboxy analogue had an IC₅₀ value of 10 mM [38]. The MurEp1 peptidopeptide does not have structural homology with these meso-A2pm analogues, and was shown to be a more potent inhibitor (Table 4).

During the course of the present work, Bratkovič et al. [43] performed a phage display screening against MurD from E. coli and MurE from S. aureus. After three rounds of bio-panning, elution of MurE-specific peptides was performed with UDP-MurNAc-Ala-Glu, L-lysine and glycine. Ten phages were sequenced and no clear amino acid motif was identified, but four prevalent peptides were synthesized. The three dodecameric peptides were shown to be inactive, whereas the cyclic C7C peptide CQANLRSQC inhibited the ATPase activity of MurE with an IC₅₀ value of 1100 μM [43]. Peptides selected against MurD from E. coli have no similarity to those identified previously [11]. Peptides selected against MurE from S. aureus display few resemblances with the peptide sequences reported in Figure 3. For comparative purposes, it should be noted that MurE from Ps. aeruginosa and S. aureus recognize different di-amino acid substrates. The dodecamer ME-12_2 selected by Bratkovič et al. [43] begins with THLP (Thr-His-Lys-Pro), whereas two peptide sequences from the glycine elution and one from the ATP elution reported in the present study begin with TGLP (Thr-Gly-Lys-Pro). The dodecamer ME-12_3 begins with SYS (Ser-Tyr-Ser) and one peptide sequence selected with the ATP competitive elution begins with SFS (Ser-Phe-Ser) (Figure 3). In an attempt to achieve a conformational homogeneous population of target enzymes during the screening process, Bratkovič et al. [43] exposed MurD and MurE to the ATP substrate. ATP binding initiates the conformational change of the Mur ligases, but these enzymes assemble into an active closed tertiary structure only when the three substrates are bound [34,54–56]. The MurD and MurE enzymes used by Bratkovič et al. [43] were likely to be present as a transitional tertiary structure state.

We have identified and characterized the first dodecameric peptide inhibitor of the MurE cell wall biosynthesis enzyme. The peptide has a novel mode of action against the amide ligase, binding at the juxtaposition of both meso-A2pm- and UDP-MurNAc-Ala-Glu-binding sites in the closed conformation of the enzyme and preventing amide bond formation. Identification of the MurEp1 residues involved in MurE binding and inhibition will allow the development of optimized inhibitors by peptidomimetics. This is of particular interest because targeting MurE may also lead to perturbation in the regulation of antibiotic resistance. MurE has previously been shown to influence methicillin resistance in S. aureus by regulating the expression of two penicillin-binding proteins [57,58].

**AUTHOR CONTRIBUTION**

Catherine Paradis-Bleau performed most experiments, analysed the data and wrote the manuscript. Adrian Lloyd assisted in the design of MurE assays, François Sanschagrin designed phage screening, Halim Maaroufi assisted in the molecular modelling, Tom Clarke performed substrate analysis, Anne Blewett designed and purified substrates, Chris Dowson supervised substrate analysis, David Roper assisted in design and purification, Timothy Bugg designed MurE strategies and Roger Levesque designed the project, supervised experiments and revised manuscripts.

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