Peptide inhibitors of the essential cell division protein FtsA

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The revolutionary era of antibiotics has been overwhelmed by the evolutionary capacity of microorganisms such as Pseudomonas aeruginosa to develop resistance to all classes of antibiotics. In the perspective of identifying new antimicrobials using novel strategies, we targeted the essential and highly conserved FtsA protein from the bacterial cell division machinery of P. aeruginosa. In a series of experiments we cloned, overproduced and purified the FtsA and FtsZ proteins. Expression of FtsA into Escherichia coli cells led to its accumulation in inclusion bodies. We developed a protocol permitting the purification and refolding of enzymatically active FtsA hydrolysing ATP. The purified enzyme was used to screen for peptide inhibitors of ATPase activity using phage display. Selective biopanning assays were done and phages were eluted using ATP, a non-hydrolysable ATP analogue and the protein FtsZ known to interact with FtsA in the divisome during the process of bacterial cell division. We identified two consensus peptide sequences interacting with FtsA and a competitive ELISA was used to identify peptides having high affinity for the target protein. Five of the six peptides synthesized showed specific inhibition of ATPase activity of FtsA with IC_{50} values between 0.7 and 35 mM. Discovery of peptides inhibiting the essential cell division machinery in bacteria is the first step for the future development of antimicrobial agents via peptidomimetism.

Keywords: FtsA/FtsZ/inhibitory peptides/phage display/ Pseudomonas aeruginosa

Introduction

The alarming increase and spread of resistance among emerging and re-emerging bacterial pathogens to all clinically useful antibiotics is one of the most serious public health problems of the last decade (Cohen, 2000; Fauci, 2001; Normark and Normark, 2002). This critical situation necessitates the design of novel classes of antibacterial agents having new modes of action against essential and novel bacterial targets.

Recent developments in genomics, proteomics and bio-informatics coupled to bacterial genetics facilitates the identification of novel essential genes and their products as antibacterial targets (Breithaupt, 1999; Projan, 2002). The perfect bacterial target should be essential for bacterial survival, highly conserved in bacterial evolution but absent in the animal kingdom, easily accessible for genetic manipulations and sufficiently expressed during the infection of the host. We selected the prokaryotic cell division machinery to identify attractive new targets because they encode essential proteins leading to a lethal phenotype when inhibited. These proteins are highly conserved in bacterial species but absent in eukaryotic cells. They are extremely sensitive to inhibition because the division process depends on recruitment of specific proteins in an absolutely essential cascade forming the divisome (Dai and Lutkenhaus, 1992; Projan, 2002; Errington et al., 2003). To our knowledge, the intrinsic cell division inhibitors MinC (Errington et al., 2003) and SulA (Cordell et al., 2003) which constitute active regulators of this physiological process do not select resistance. Among bacterial division proteins, we chose FtsA as a specific target. This highly conserved protein (van den Ent et al., 2001) presumably constitutes a key bacterial component because of its essential protein–protein interaction with FtsZ and its ATPase enzymatic activity (Errington et al., 2003) that can be exploited to screen and analyse inhibitory molecules.

The FtsZ protein is at the top of hierarchical recruitment in the divisome and its polymerization into the Z-ring allows the physical separation of daughter cells (Lutkenhaus and Addinall, 1997). The localization of FtsA follows the mid cell accumulation of FtsZ and is essential for protein recruitment in the divisome (Pichoff and Lutkenhaus, 2002) and for Z-ring constriction (Begg et al., 1998). The FtsZ–FtsA protein–protein interaction and the FtsZ:FtsA ratio are crucial for the progress of bacterial cell division (Dai and Lutkenhaus, 1992; Yan et al., 2000; Lowe et al., 2004). Accumulating evidence suggests that FtsA plays the role of a motor protein in providing energy for constriction by way of its ATPase activity (Nanninga, 1998; Feucht et al., 2001; Errington et al., 2003). Hence this cytoplasmic enzyme and its protein interactions remain to be fully characterized but can be an elegant tool with the aim of developing antibacterials with novel modes of action.

In order to identify specific inhibitors of FtsA, we used the phage-display technique, which represents a powerful tool for the selection of short peptide ligands having high binding affinities to proteins of interest among a large pool of random peptide permutations (Sidhu, 2000; Christensen et al., 2001). This approach has been useful for the detection of various enzyme inhibitors (Hyde-DeRuyscher et al., 2000), including the cell division protein FtsZ (Paradis-Bleau et al., 2004) and MurC implicated in the biosynthesis of the bacterial cell wall (El Zeeiby et al., 2003).

In this paper, we describe the purification and biochemical characterization of FtsA and FtsZ cell division proteins of Pseudomonas aeruginosa. We systematically exploit the use of two different phage display libraries and demonstrate how the design of a strategic biopanning approach can lead to the identification of specific peptide inhibitors. Three rounds of phage display screening led to the identification of clear consensus peptide sequences and high affinity binding peptides presumably active against FtsA. Of six peptides synthesized,
five showed a significant and specific inhibition of the ATPase activity of FtsA. In order to obtain promising lead compounds with appropriate pharmacological properties, inhibitory peptides can be chemically modified and their sequences will constitute the core for the synthesis of libraries of peptidomimetic molecules.

Materials and methods

Plasmid construction, bacterial strains, reagents and media
All reagents were purchased from Sigma Aldrich (Oakville, Ontario, Canada) unless indicated otherwise. Restriction endonucleases and T4 ligase were obtained from New England Biolabs (Mississauga, Ontario, Canada). Agarose gel electrophoresis and plasmid DNA preparations were performed according to published procedures (Sambrook et al., 1989). Recombinant plasmids containing P. aeruginosa PAO1 ftsZ and ftsA genes were maintained in Escherichia coli NovaBlue, endA1 hsdR17 (rK12 mK12 + ) supE44 thi-1 recA1 gyrA96 relA1 lac [F' proA’ B’ lacZ’ A’ M15::Tn10] prior to protein synthesis in E. coli BL21, F- ompT hsdSb (rB–mB–) gal dcm (ΔDE3) (Novagen, Madison, WI).

Cloning of P. aeruginosa ftsA, ftsZ and DNA sequencing
Polymerase chain reaction (PCR) cloning was used to obtain DNA fragments encoding FtsA and FtsZ proteins and a His-tag at their C-terminus. The ftsA insert was amplified from genomic DNA of P. aeruginosa PAO1 (70 ng) with primers FtsSA–NdeI 5’-GTA ATA CAT ATG GCA AGC GTG CAG A-3’ and FtsA–HindIII 5’-TTG AAG CTT AAT TGC CCT GGA CCC-3’ in frame with the His-tag changing the two last amino acids N–F for K–L. Amplification of the ftsZ insert was done with forward and reverse primers FtsZ–NdeI 5’-GGA GAG GGC ATA TGT TTG AAC TGT TTG AAC TGG-3’ and FtsZ–XhoI 5’-TTA CTT CAC TCG AGC TGA CGA GCG-3’ changing the two last amino acids A–D for L–E. PCRs were done with primers at a concentration of 0.1 μM each, dNTPs (Amersham Biosciences, Baie d’Urfe, Quebec, Canada) at 0.2 mM each and 2 mM MgCl2. DNA amplification was done using 30 cycles with 2.5 U of Hot Start TAQ polymerase (Qiagen, Mississauga, Ontario, Canada) and annealing at 56.3°C for ftsA or with 2.5 U of the Expand High Fidelity TAQ polymerase (Roche Diagnostics, Laval, Quebec, Canada) using annealing at 60°C for ftsZ. PCR products were digested with appropriate restriction enzymes and cloned into the corresponding sites of the expression vector pET30a for ftsA and pET24b (Novagen) for the ftsZ insert. The recombinant plasmids pMON2023 encoding ftsA and pMON2020 expressing ftsZ were electroporated into competent E. coli NovaBlue cells. For both plasmids, the insert was completely sequenced for both DNA strands using the universal T7 primers (Novagen).

Overexpression of FtsA
The pMON2023 plasmid was transformed into competent E. coli BL21 (ΔDE3) cells by CaCl2 transformation for expression of FtsA. A bacterial culture of 1.9 l of LB broth (Diffco Laboratories, Detroit, MI) supplied with 0.05 g/l of kanamycin was inoculated with an overnight culture of 100 ml of E. coli BL21 (ΔDE3) carrying pMON2023. Cells were incubated at 37°C with agitation at 260 r.p.m. until an optical density (O.D.) of 0.65 at 600 nm. Protein expression was carried out by adding 1 mM of IPTG and incubating for 6 h; cells were centrifuged and the pellets were immediately stored at −80°C.

Purification of inclusion bodies and refolding of FtsA
The bacterial pellet was resuspended in wash buffer from the Protein Refolding Kit (Novagen) as recommended by the manufacturer and treated with lysozyme at 100 μg/ml for 15 min at 30°C. Cells were lysed by sonication for 30 s/ml using a Virsonic Digital 475 ultrasonic cell disrupter (Virtis, Gardiner, NY) and a cocktail of protease inhibitors was immediately added as recommended by the manufacturer (Roche Diagnostics). Disrupted cells were centrifuged for 10 min at 5000 g; the pellets were resuspended in wash buffer (Novagen) and centrifuged for 10 min. The pellets were resuspended again, centrifuged for 10 min at 10 000 g and stored at −80°C. Solubilization of inclusion bodies was performed by gently resuspending the pellets in solubilization buffer (Novagen) with 0.3% N-laurylsarcosine and 0.1% dithiothreitol (DTT) to a final concentration of 5 mg/ml. The suspension was incubated for 30 min, centrifuged for 30 min at 25 000 g at room temperature and the supernatant was conserved at 4°C for a maximum of 2 months. Refolding of FtsA was carried by multistep dialysis of solubilized inclusion bodies, first in 75 volumes of dialysis buffer (20 mM Tris–HCl pH 8.5) with 0.1% (v/v) DTT for 4 h at 4°C and overnight at 4°C with fresh buffer. The suspension was then dialysed in 100 volumes of dialysis buffer with 10 mM MgCl2 overnight at 4°C and 5 h at 4°C with fresh buffer. Refolded FtsA protein was visualized by SDS–PAGE with Coomassie Brilliant Blue staining, the concentration was measured by the Bradford method (BioRad, Mississauga, Ontario, Canada) and it was stored at 4°C.

Overexpression and purification of FtsZ
The recombinant plasmid pMON2020 was introduced into competent E. coli BL21 (ΔDE3) cells for expression of FtsZ. Protein expression was carried as described for FtsA except that the culture was induced at an O.D. of 0.8 and incubated for 4 h. Cells were centrifuged and the pellet was resuspended in 3.33 times their weight of sonication buffer (50 mM Tris–HCl pH 8.6 and 2 mM EDTA), treated with lysozyme and lysed by sonication as for FtsA. Cellular debris were removed by centrifugation at 17 000 g for 30 min. Recombinant FtsZ protein was purified to homogeneity by affinity chromatography using a His-bind nickel resin (Novagen) with 150 mM imidazole during elution. Purified FtsZ was dialysed in conservation buffer (20 mM Tris–HCl pH 7.6, 10 mM NaCl and 1 mM EDTA) (Zhulanova and Mikulik, 1998) and conserved in 50% (v/v) glycerol at −80°C.

FtsA and FtsZ N-terminal sequencing
After separation by SDS–PAGE (Sambrook et al., 1989), 2 μg each of FtsA and FtsZ protein preparations were transferred on PVDF membrane using a transfer buffer without glycin [10 mM CAPS pH 11 and 10% (v/v) methanol]. The membrane was stained with Ponceau Red and FtsA and FtsZ protein bands were cut out and rinsed with water. N-terminal sequencing was done by the Edman degradation technique at the Biotechnology Research Institute (National Research of Council Canada, Montreal, Quebec, Canada).

Biochemical characterization of FtsA and FtsZ
The ATPase and GTPase enzymatic activities of FtsA and FtsZ were confirmed using a thin-layer chromatographic (TLC) assay with 32P-labelled nucleotides as substrates. The FtsA ATPase assay was done using 4 μM renatured FtsA in reaction
buffer A (50 mM Tris–HCl pH 7.2, 50 mM potassium acetate, 10 mM MgCl2 and 1 mM DTT) (Feucht et al., 2001) and 1 μl of 10 μCi/μl [32P]ATP (PerkinElmer, Woodbridge, Ontario, Canada) in a final volume of 20 μl. The FtsZ GTPase assay was performed using 12 μM FtsZ in reaction buffer Z (Paradis-Bleau et al., 2004) and 1 μl of 10 μCi/μl [32P]GTP (PerkinElmer) in a final volume of 20 μl. The mixtures were incubated for 2 and 1 h, respectively, at 37°C and 2 μl of each sample were deposited on a TLC plate along with negative controls (without enzyme). Hydrolysis of radioactive substrates was measured by autoradiography using a Phosphor imager (Fuji, Stanford, CA). A UV cross-link specific nucleotide-binding assay was performed with FtsA and FtsZ against the four radioactive nucleotides. Amounts of 3 μg of FtsA or FtsZ protein were mixed with each of the four [32P]-labelled nucleotides (PerkinElmer) and the UV cross-link assay was carried out as described (Feucht et al., 2001).

Briefly, the samples were incubated for 30 min at 0°C, eluted peaks.

The inhibitory capacities of the synthesized peptides were determined by preincubating FtsA in buffer A lacking DTT with various concentrations of peptide buffered solutions for 20 min at room temperature. [32P]-labelled ATP was then added and mixtures were immediately incubated for 2 h at 37°C. Each sample was separated by TLC along with positive and negative controls and the 50% inhibitory concentrations (IC50) of peptides were calculated (Paradis-Bleau et al., 2004). As controls, non-specific peptides were tested for inhibition of the ATPase activity of FtsA and a competitive assay was done with casein at 10 times the enzyme concentration. The non-specific peptides were a circular C-7-C mer that had previously been shown to inhibit L-1 β-lactamase and a 12 mer known to bind to the herpes virus capsid (Sanschagrin and Levesque, 2005). The reducing DTDT effect was analysed by evaluating the peptide inhibition with 10 mM DTT.

**Results**

**Purification of P. aeruginosa FtsA and FtsZ**

The expressed FtsA protein was detected solely in inclusion bodies and FtsZ was found in the soluble cytoplasmic fraction. The renaturation of purified inclusion bodies containing the FtsA peptide inhibitors

**Affinity ELISA**

This experiment was adapted from Carettoni et al. (2003). Briefly, 150 μl of FtsA (100 μg/ml) was adsorbed overnight at 4°C on Ni-NTA strips (Qiagen) along with BSA as a control. The strips were washed four times with TBS (50 mM Tris–HCl pH 7.5, 150 mM NaCl) and blocked with BSA for 1 h at room temperature. After a brief wash, 2×106 plaque-forming units (pfus) per well of each amplified phage samples were incubated in triplicate for 1 h in FtsA-coated wells. TBS was added in triplicate to BSA-coated wells to evaluate non-specific signal. The strips were washed six times with TBS 1% Triton, twice for 10 min and six times again. A 150 μl amount of biotin-labelled anti-fd rabbit polyclonal antibodies diluted 1:2500 in TBS 1% Triton was incubated for 1 h at room temperature. After four washes, 150 μl of 1 μg/ml HRP-labelled streptavidin (Roche Diagnostics) were added to the strips for 30 min at room temperature and the excess was removed with four washes. The strips were incubated for 20 min with 100 μl of ABTS (Roche Diagnostics) and the results were analysed at an O.D. of 405 nm. The results were an average from triplicates and the BSA non-specific signal was subtracted from all values. Specific affinity ratios for phages from the third round of biopanning were determined by dividing the value by the non-specific control phage value.

**Selection and synthesis of peptides**

Specific peptides were synthesized on an ABI 433A Peptide Synthesizer using FastMoc chemistry (El Zoeiby et al., 2003) from the consensus peptide sequences and high-affinity peptides were identified.

**FtsA inhibitory enzymatic assay**

The inhibitory capacities of the synthesized peptides were determined by preincubating FtsA in buffer A lacking DTT with various concentrations of peptide buffered solutions for 20 min at room temperature. [32P]-labelled ATP was then added and mixtures were immediately incubated for 2 h at 37°C. Each sample was separated by TLC along with positive and negative controls and the 50% inhibitory concentrations (IC50) of peptides were calculated (Paradis-Bleau et al., 2004). As controls, non-specific peptides were tested for inhibition of the ATPase activity of FtsA and a competitive assay was done with casein at 10 times the enzyme concentration. The non-specific peptides were a circular C-7-C mer that had previously been shown to inhibit L-1 β-lactamase and a 12 mer known to bind to the herpes virus capsid (Sanschagrin and Levesque, 2005). The reducing DTDT effect was analysed by evaluating the peptide inhibition with 10 mM DTT.

**Affinity selection of phage displayed peptides against FtsA**

Phage display screening was carried out with the PH.D.-12 and PH.D.-C-7-C phage libraries (New England Biolabs) containing ~2.7×106 12 mers and ~3.7×106 C-7-C mers random peptide sequences. Specificity during the three rounds of biopanning was obtained by increasing the stringency of the washes and decreasing the time of contact between the peptides and the target protein as described (El Zoeiby et al., 2003), but using the following modifications. The second- and third-round phage inputs were calculated as 2×1011 phages for each library. Phages adsorbed on FtsA at the third round of biopanning were eluted by non-specific disruption of binding interactions using glycine and by a competitive elution using 100 μl of 1 mM ATP, with 100 μl of 1 mM 5′-adenyllylimidodiphosphate (a non-hydrolysable analogue of ATP) and with 100 μl of 1 mM FtsZ.

**Phage DNA preparation and sequencing**

The M13 DNA purification and sequencing were done as described (El Zoeiby et al., 2003) except that 12 phages were sequenced for each of the two phage libraries eluted using four sets of elution conditions.

**Gel filtration of FtsA**

To improve the purity of the inclusion body purified FtsA, an aliquot was analysed by gel filtration chromatography. A refolded FtsA sample was first concentrated five times with a stirred ultrafiltration cell using an ultrafiltration membrane with a cut-off of 10 kDa (Millipore, Bedford, MA, USA). A 200 μl volume of concentrated FtsA was injected onto an analytical Tricorn Superdex 75 column (Amersham Biosciences) equilibrated with buffer A and elution was performed with buffer A at a flow rate of 0.8 ml/min. Collected fractions were analysed by SDS–PAGE with SYPRO Orange staining (BioRad) and tested for ATPase activity as described. The mixtures were analysed by SDS–PAGE with SYPRO Orange staining.

**Purification of P. aeruginosa FtsA and FtsZ**

The expressed FtsA protein was detected solely in inclusion bodies and FtsZ was found in the soluble cytoplasmic fraction. The renaturation of purified inclusion bodies containing the FtsA dimer of 85.2 kDa and trace peaks eluted after one column volume. SDS–PAGE analysis of the collected fractions showed that the
principal peak contains a single protein of 42.6 kDa and that the three minor peaks contain no protein detected by the sensitive SYPRO Orange staining (data not shown). The nickel affinity chromatography permitted the purification of FtsZ with a yield of 20 mg/l. The N-terminal sequencing of the first 15 amino acid residues confirmed the nature of both proteins (100% identity with published sequences in both cases).

**Characterization of FtsA and FtsZ ATPase and GTPase activities**

As depicted in Figure 2A, analysis by autoradiography showed that 4 μM FtsA hydrolysed 85% of ATP, giving as substrates 8% ADP, 3.4% AMP and 73.6% inorganic phosphate, after 2 h at 37°C. The ATPase assay was also performed on fractions obtained from gel filtration by FPLC. A single significant peak obtained corresponding to the expected FtsA size on SDS–PAGE had ATPase activity (data not shown, but available upon request). The FtsZ enzyme used at 12 μM hydrolysed 85% of GTP to GDP after 1 h at 37°C. These data confirmed that both enzymes were active and that FtsA was properly refolded. To determine the nucleotide substrate specificity for each enzyme, a UV cross-link binding assay was performed and autoradiography confirmed that FtsA binds preferentially ATP (Figure 2B) and that FtsZ binds preferentially GTP among the four radioactive nucleotides tested as substrates.

**Affinity selection of FtsA binding peptides**

After the third round of biopanning and depending on the type of elution strategy utilized, the DNA sequencing of randomly selected phages identified a variety of peptide sequences (Figure 1 in the Supplementary data available at PEDS Online). The analysis of the frequency of phage recovery revealed a consensus peptide sequence for each library that both contained a P-S-P motif (Table I). The 12 mer consensus sequence was the only peptide sequence recovered when using FtsZ as a biopanning competitor against FtsA (Figure 1 in the Supplementary material). A FASTA search was carried out with each peptide reported in Figure 1 in the Supplementary material but no homologous proteins were identified in databases.

**Affinity ELISA for binding specificity**

The relative affinity of peptides expressed from phages selected randomly was evaluated by ELISA to identify the best binding peptide sequences. The relative affinity ratios defined as the value of specific binding phage divided by the value of non-specific binding phage varied from 2.7 to 43.1 (Table I). For example, a 43.1 relative affinity ratio indicated that the specific phage had 43.1 times more affinity for the target FtsA protein compared with the binding of random peptide.
peptides. We noted that the frequency of peptide recovery did not correlate with the values of affinity ratios (Table I). The average ratio of the 12 mer phage expressed peptides was $25^\pm 6$ and was significantly higher than the C-7-C mer mean ratio of $11^\pm 6$. An interesting fact is that the two best binding peptides with relative affinity ratios of 43.1 and 42.4 have the same G-P-H conserved motif at their N-termini and ended with a P. The second and third strongest binding peptides with ratio values of 42.4 and 32 contained a G-M motif at their centre and ended with an R-P motif (Table I). We noted also that the two C-7-C mer peptide sequences with the highest ratio values 16.0 and 23.3 have a W-A motif (Table I). We noted that the 12 mer and C-7-C mer lowest binding peptide sequences were identified when using the non-hydrolysable analogue 5'-adenylylimidodiphosphate in the biopanning. Indeed, elution at the biopanning step with glycine, ATP and FtsZ gave phage expressing peptides with similar affinity ratio values; although the best binding peptides came from the glycine and ATP (Table I, and Figure 1 in the Supplementary data).

Selection and synthesis of peptides
To establish the inhibitory capacity of selected peptides against the ATPase activity of FtsA, peptide sequences were chosen on the basis of their frequency of recovery and their relative affinity ratios. Indeed, the two consensus peptides were synthesized along with the two peptides having the highest relative affinity values from each phage library (Table I).

Inhibition of FtsA ATPase activity
Of the six peptides synthesized, five inhibited the ATPase activity of FtsA and had $IC_{50}$ values between 0.7 and 35 mM (Table I). The C-7-C mer consensus peptide FtsAp1 gave an $IC_{50}$ value of 22 mM but the 12 mer consensus peptide FtsAp2 did not inhibit the enzymatic activity of FtsA. The 12 mer peptide with the highest relative affinity ratios, FtsAp3 and FtsAp4, gave $IC_{50}$ values of 0.7 (Figure 3) and 25 mM, respectively. The C-7-C mer peptides FtsAp5 and FtsAp6 had $IC_{50}$ values of 2.5 and 35 mM, respectively. Competitive rescue assays using BSA gave identical $IC_{50}$ values (data not shown).

**Table I. Frequency of recovery, ELISA relative affinity ratio and $IC_{50}$ values of selected peptide sequences for ATPase inhibition**

<table>
<thead>
<tr>
<th>12 mer sequence</th>
<th>Frequency of recovery</th>
<th>Ratio</th>
<th>$IC_{50}$ (mM)</th>
<th>C-7-C mer sequence</th>
<th>Frequency of recovery</th>
<th>Ratio</th>
<th>$IC_{50}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVSVGKPSPRP$^a$</td>
<td>21</td>
<td>32.0</td>
<td>N.D.</td>
<td>CLAPSPSK$^b$</td>
<td>14</td>
<td>3.2</td>
<td>22</td>
</tr>
<tr>
<td>FTTSNHTSRHGS</td>
<td>2</td>
<td>26.7</td>
<td></td>
<td>CSSATGKSC</td>
<td>3</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td>TPSLPPTMFRLLT</td>
<td>3</td>
<td>17.8</td>
<td></td>
<td>CGLQTKMRC</td>
<td>2</td>
<td>15.4</td>
<td></td>
</tr>
<tr>
<td>GPHYWYHRLRP$^c$</td>
<td>1</td>
<td>43.1</td>
<td>0.7</td>
<td>CGHRPYYC</td>
<td>3</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>QSPVNHYYHYH$^d$</td>
<td>1</td>
<td>7.7</td>
<td></td>
<td>CWAFPPLHHC$^e$</td>
<td>1</td>
<td>16.0</td>
<td>2.5</td>
</tr>
<tr>
<td>NMTTPMPHNNTV</td>
<td>1</td>
<td>5.1</td>
<td></td>
<td>CTLNSHSC</td>
<td>1</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>SLLPHSNHAKHY</td>
<td>1</td>
<td>31.4</td>
<td></td>
<td>CEISAKRTC</td>
<td>1</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>EFYFHPATFRSL</td>
<td>1</td>
<td>16.5</td>
<td></td>
<td>CHILHAQAC</td>
<td>1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>GPHLGMNQRRRP$^f$</td>
<td>1</td>
<td>42.4</td>
<td>25</td>
<td>CTRPPSLEC</td>
<td>1</td>
<td>4.9</td>
<td></td>
</tr>
</tbody>
</table>

$^b$C-7-C mer consensus peptide FtsAp1.
$^c$12 mer peptide that had the highest relative affinity ratio value FtsAp3.
$^d$C-7-C mer peptide that had the second highest relative affinity ratio value FtsAp5.
$^e$12 mer peptide that had the second highest relative affinity ratio value FtsAp4.
$^f$C-7-C mer peptide that had the highest relative affinity ratio value FtsAp6.
The reducing agent DTT had no effect on the inhibitory capacity of FtsAp3, FtsAp4 and FtsAp5. The C-7-C mer FtsAp1 lost 20% of its inhibitory capacity when DTT was added to the reaction and the C-7-C mer peptide FtsAp6 was affected by DTT as its inhibitory potential was raised by a factor of 1.5. The IC<sub>50</sub> value of FtsAp6 was 52 mM without DTT and 35 mM with DTT. Analysis of FtsA activity using random peptides at high concentrations showed that the reaction rate was not reduced by the peptides (data not shown), indicating that the ATPase activity of FtsA was not inhibited by non-specific C-7-C mer or 12 mer peptides.

**Discussion**

There has been growing interest in identifying new bacterial targets from essential mechanisms (Breithaupt, 1999) such as cell division (Wang et al., 2003; Jennings et al., 2004; Margalit et al., 2004; Paradis-Bleau et al., 2004). In this work, we selected specific peptide inhibitors of the highly conserved and essential cell division protein FtsA (Feucht et al., 2001).

The purification of biologically active FtsA was a challenge because this protein accumulates in inclusion bodies. We developed a multi-step solubilization and renaturation protocol coupled with gel filtration that may be useful for any insoluble protein when taking into account the presence of disulfide bonds and the cofactor or stabilizing molecule.

The ATPase activity of FtsA has been demonstrated (Feucht et al., 2001) but we are the first to show that FtsA hydrolyses ATP to ADP, AMP and inorganic phosphate (Figure 2A). To confirm that ATPase activity is not due to traces of a contaminating ATPase, the inclusion body containing FtsA was separated by gel filtration chromatography and ATPase activity was determined for eluted fractions. The unique FtsA peak obtained had ATPase activity.

Our results support the hypothetical role of FtsA as a motor protein in providing energy for the Z-ring constriction via ATP (Feucht et al., 2001) and that FtsZ utilizes GTP (Diaz et al., 2001). We showed that FtsA binds preferentially ATP amongst the four nucleotides and confirmed the native dimer nature of FtsA (Feucht et al., 2001).

We noted that the non-specific disruption of phage interactions with glycine, the allosteric competition by FtsZ and the active site competition by ATP and non-hydrolysable analogue gave different peptide sequences binding FtsA. This confirmed the validity of using different elution conditions to obtain a variety of peptide sequences interacting with diverse sites on the target protein. We hypothesize that the FtsA nucleotide binding site is functionally related to the FtsZ interaction site(s) as the ATP binding and/or hydrolysis may lead to a conformational change that could affect the FtsZ interaction site(s) or vice versa. Indeed, ATP elution could yield phage encoded peptides binding to the nucleotide binding site and to the FtsZ interaction site(s).

We did not find peptide homologues in databases but we assumed that peptide sequences have conformational homology with cell division proteins interacting with FtsA itself, FtsZ, PBP3, FtsQ or FtsN (Yim et al., 2000; Di Lallo et al., 2003). FtsA binds to the conserved consensus sequence LD<sub>P</sub>X<sub>L</sub>P<sub>X</sub>FOR/K (O, hydrophobic residue; X, any residue) at the C-terminus of FtsZ (Yan et al., 2000; Haney et al., 2001). We noted that the perfect 12 mer consensus sequence selected by the FtsZ elution contained a KPSPR motif and the C-7-C mer consensus sequence had a APSPSK motif sharing homology with the interaction site of FtsZ indicated in bold.

Anderluzzi’s group recently reported a phage display using E.coli FtsA against the PH.D.-7 library and a single glycine elution (Carettone et al., 2003). There is no redundancy between the two phage display screenings since different phage libraries were used. The inhibitory potential of some selected 7 mer peptides needs to be determined.

ELISA showed that the 12 mer peptides bind more tightly to the scattered and elongated structure of FtsA (van den Ent and Lowe, 2000) than the C-7-C mer peptides, requiring less space since C residues constrain the peptide in a disulfide loop. We noted that elution with non-hydrolysable ATP analogue gave peptides with the lowest binding affinities. Hence this artificial molecule presumably binds less tightly to FtsA. Conserved motifs identified in peptides having the highest affinity ratios must be important for binding to FtsA. We noted that the relative affinity values did not correlate with the frequency of phage recovery. Apart from the binding affinity, several factors such as phage infection and replication efficiency, protein translocation and folding bias, pH coat stability and retained phages will affect phage recovery (Carettone et al., 2003). Furthermore, phages with very high binding properties cannot be recovered with the mild elution conditions used to preserved phage particle integrity (Hoess, 2001).

The inhibitory capacity of peptide against the FtsA ATPase activity was evaluated for consensus peptides and the two peptides having the highest relative affinity ratios for each phage library (Table I). Five synthesized peptides were able to inhibit ATPase activity of FtsA. DTT had no effect on the inhibitory capacity of 12 mer peptides; the three C-7-C mer inhibitory peptides reacted differently to the reducing agent. FtsAp1 partially lost its inhibitory capacity, indicating that the disulfide loop was important for inhibition of FtsA. In contrast, the loop conformation of FtsAp5 was not essential for inhibition since DTT did not change the IC<sub>50</sub> value. FtsAp6 gave a higher inhibition value with DDT, suggesting that this peptide was more active in the linear monomeric form.

The perfect 12 mer consensus peptide eluted with FtsZ (Figure 1 in the Supplementary data) could bind into a FtsA allosteric site. The corresponding peptide FtsAp2 having the KPSPR motif homologous with the FtsA interaction site of FtsZ was the only one that did not inhibit ATPase activity. We assume that it could be an inhibitor of the essential FtsA–FtsZ interaction that represented a more sensitive and specific target than the ATPase activity of FtsA alone (Yan et al., 2000; Haney et al., 2001). The C-7-C mer FtsAp1 with a APSPSK motif had homology with the conserved C-terminal interaction site of FtsZ and showed inhibition of FtsA. FtsAp1 could inhibit ATPase activity of FtsA due to binding to an FtsZ interacting site. The FtsA allosteric site(s) binding FtsZ could regulate the enzymatic activity even if FtsA binds and hydrolyses ATP without FtsZ. It as already been hypothesized that FtsZ interaction may change the FtsA conformation and enhance ATP hydrolysis (van den Ent and Lowe, 2000). This may regulate the ATPase motor force required for the Z-ring constriction.

Peptide IC<sub>50</sub> values did not correlate with the frequency of phage recovery, with the relative affinity ratio values or with the phage library (Table I). The inhibitory capacity depends primarily on localization of the peptide interaction with the target protein and secondarily on the affinity strength.
Characterizing the specificity and inhibition mechanism of five peptides identified will be essential as kinetic analysis did not reveal a general trend in peptide inhibitory mechanisms (Hyde-DeRuyscher et al., 2000). To obtain promising lead compounds, inhibitory peptides will undergo chemical modifications; this will constitute the core for the synthesis of peptidomimetic molecules (Nefzi et al., 1998; Bursavich and Rich, 2002). Inhibition of FtsA in Gram-negative bacteria needs to be studied since ZipA absent in Gram-positive bacteria share overlapping functions with FtsA (Pichoff and Lutkenhaus, 2002; Geissler et al., 2003; Lowe et al., 2004).

Acknowledgements

We express our gratitude to Le Service de Série de Peptides de l’Est du Québec and Le Service d’Analyse et de Synthèse d’Acides Nucléiques de l’Université Laval. This work was funded by the Canadian Bacterial Diseases Network via the Canadian Centers of Excellence, an FCAR infrastructure team grant to R.C. Levesque and a CRSNG studentship to Catherine Paradis-Bleau.

References


FtsA peptide inhibitors


Received November 24, 2004; revised February 4, 2005; accepted February 8, 2005

Edited by Mirek Cygler