Investigating protein structural plasticity by surveying the consequence of an amino acid deletion from TEM-1 β-lactamase

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Abstract While the deletion of an amino acid is a common mutation observed in nature, it is generally thought to be disruptive to protein structure. Using a directed evolution approach, we find that the enzyme TEM-1 β-lactamase was broadly tolerant to the deletion mutations sampled. Circum 73% of the variants analysed retained activity towards ampicillin, with deletion mutations observed in helices and strands as well as regions important for structure and function. Several deletion variants had enhanced activity towards ceftazidime compared to the wild-type TEM-1 demonstrating that removal of an amino acid can have a beneficial outcome.

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1. Introduction

Central to the evolutionary process is the diversification of protein structure and function through changes to the amino acid sequence [1,2]. Together with substitutions, the insertion or deletion (known as indels) of amino acids constitute major mutational mechanisms observed during divergent evolution [3,4]. This is highlighted by length variations that are routinely observed in comparisons of homologous proteins. Even though indels occur at a much lower frequency than substitutions, they can exert considerable influence on the properties of a protein. For example, amino acid deletions enhance the sequence and conformational diversity of immunoglobulin variable domains [5] and a single amino acid deletion in HIV reverse transcriptase has contributed to viral drug resistance [6]. However, the most common form of cystic fibrosis is the result of an amino acid deletion in the transmembrane conductance regulator, causing the protein to misfold [7].

The deletion of a single amino acid is one of the most commonly observed indel mutations in nature [4]. Nonetheless, mutations that remove an amino acid from the polypeptide chain are generally expected to be detrimental to proteins due to the anticipated major local and global structural changes, including register shifts in regular secondary structure elements, required to accommodate the removal of a residue. Therefore, long flexible loops are considered the places most likely to tolerate a deletion [3]. However, analysis of several specific deletion mutants of T4 lysozyme [8], the B1 domain of protein G [9] and Ricin A-chain [10] suggests this is not always the case. The difficulty in predicting the consequence of a deletion mutation means that most protein engineering and design efforts aimed at investigating or altering a protein’s properties tend to ignore them [11]. Thus our understanding of the consequence of deleting an amino acid remains limited. This is in spite of the fact that deletion mutations sample sequence space and conformational rearrangements not accessible by other mutations.

To provide further insight into the tolerance of a protein to deletion mutations, we have used a recently developed directed evolution method [12] to remove single amino acids at random positions in the antibiotic hydrolysing enzyme, TEM-1 β-lactamase [13]. We show that the majority of deletion mutations sampled were tolerated by TEM-1, including those in helices and strands, and that they had varying effects on the activity of the enzyme, including improvement of the in vitro activity towards a normally poor substrate.

2. Materials and methods

2.1. Construction of TEM-1 deletion variants

The library of deletion variants was constructed as described previously, using an engineered transposon (MuDel) containing a chloramphenicol resistance gene and the plasmid pNOM as the target DNA (Supplementary Fig. 1 and [12]). The selection of clones with MuDel inserted within the bla gene of pNOM was performed using a positive selection for chloramphenicol (Cam) resistance followed by a screen for ampicillin (Amp) sensitivity, as described previously [12]. Cells that grew on Cam but not Amp LB agar plates were picked and constituted the BLA<sup>®</sup> library. Each of the colonies was transferred to 96 deep-well culture plates containing LB and 25 μg/ml Cam and grown overnight at 37 °C. The diversity of transposon insertion positions within the bla gene of pNOM was analysed using restriction endonuclease digestion with XhoI and/or MlyI (see Fig. 1 for rational). A fraction (50 μl) of each culture was pooled and plasmid DNA isolated using the Qiagen HiSpeed plasmid midi kit. The cell density of each individual culture was not determined. Plasmid DNA was then digested and the reactions were analysed by agarose gel electrophoresis.

The individual deletion variants were constructed by removing MuDel from pNOM-MuDel DNA isolated from 76 separate cultures by digestion with MlyI. The linearised pNOM DNA was separated by agarose gel electrophoresis and purified. Intramolecular ligation was performed using the Quick Ligation™ kit (NE Biolabs) and ca. 10 ng DNA was added to 2 μg linearised pNOM DNA and ligated overnight at 16 °C in a solution containing 200 μl of 1X Quick Ligation™ reaction buffer, 10 μl Quick Ligation™ enzyme mix and 0.5 μl 100 μM ATP. The ligation reaction was then diluted 1:100 in 5X Quick Ligation™ reaction buffer and 100 ng DNA was added to each culture. After 18 h at 16 °C, cultures were diluted 1:10 in 2X Quick Select™ reaction buffer, 10 μl Quick Select™ enzyme mix and 0.5 μl 100 μM ATP and plated on LB plates containing 25 μg/ml Cam.

Abbreviations: Δ, amino acid deleted; Amp, ampicillin; Cam, chloramphenicol; CAZ, ceftazidime; Indel, insertion-deletion; MIC, minimum inhibitory concentration
of linear pNOM. The ligation mixture was used to transform *E. coli* DH5α and the cells plated on LB agar containing 15 μg/ml Amp (untransformed cells grew at [Amp] \( \leq 10 \) μg/ml). Ligation products that yielded no viable cells after repeated ligations and transformations were deemed to represent inactive TEM-1 deletion mutants. Ligation products that yielded viable *E. coli* cells were deemed active.

2.2. Characterisation of TEM-1 deletion variants

The *bla* gene of each of the 76 analysed variants was sequenced to determine the position and nature of the deletion mutation. In variants with a substitution adjacent to the deletion, the residue with two nucleotides deleted from its corresponding codon was designated as the deleted residue (see Fig. 1c for an example). The in vivo activity of each variant was estimated by measuring the minimum inhibitory concentration (MIC) of Amp or ceftazidime (CAZ) required to prevent *E. coli* growth. Each colony was transferred to LB agar containing 50, 100, 200, 500, 1000, 2000, 4000, 8000 and 16000 μg/ml Amp or 0.03, 0.06, 0.125, 0.250, 0.5, 1, 2, 4, 8 and 16 μg/ml CAZ and the plates incubated at 37 °C for 16 h. Two colonies expressing the wild-type TEM-1 were used as controls.

3. Results and discussion

3.1. Construction and sequence diversity of TEM-1 deletion variants

Directed evolution is a powerful approach for engineering proteins [14–16], generally by sampling amino acid substitutions. We recently described a new transposon-based directed evolution method used to sample single amino acid deletions from the polypeptide backbone by triplet nucleotide removal at random positions in a target DNA sequence (Supplementary Fig. 1 and [12]). The pNOM derived *bla* gene that encodes the clinically important TEM-1 β-lactamase was the target for mutagenesis using the engineered transposon MuDel that contained a chloramphenicol resistance gene for selection purposes. From a total of 816 colonies that were resistant to Cam, 391 were also sensitive to Amp and were deemed to have the MuDel inserted within *bla* so disrupting correct TEM-1 expression. The proportion of Amp sensitive cells was consistent with the proportion of pNOM comprising the *bla* gene. The 391 Amp sensitive clones were selected to constitute the BLA \( \text{D} \) 391 library. Restriction analysis using the endonucleases XhoI and MlyI was used to determine the diversity of transposon insertion in the BLA \( \text{D} \) 391 library (Fig. 1a). The band observed at ca. 1300 bp corresponds to MuDel (plus an additional 8 bp from the *bla* gene). The other digestion products formed a smear, as would be expected if MuDel insertion within *bla* was essentially random (Fig. 1b).

The pNOM-MuDel plasmid DNA was isolated from 76 individual clones and the deletion mutation introduced by the excision of MuDel and subsequent religation of pNOM. Sequence analysis of the variants revealed that there were 68 unique deletion mutations in TEM-1, with ca. 90% of the variants being unique at the DNA level (Fig. 2 and Supplementary Table 1). The R86D mutation was generated in two different ways at the genetic level due to transposon insertion positions that differed by 1 bp. The mutations were spread throughout the protein and sampled all elements of secondary structure (Fig. 2). The wild-type *bla* gene was not observed. An adjacent substitution accompanied the deletion mutation in 15 cases. This arose due to transposon insertion positions that result from the removal of 3 bp spanning two codons (Fig. 1c). The redundancy of the genetic code means that many triplet nucleotide deletions that span two codons do not cause an
amino acid substitution. This mirrors natural events whereby single amino acid deletions are introduced into a gene [4,17].

3.2. Influence of a deletion mutation on TEM-1 activity towards Ampicillin

It is generally thought that removal of an amino acid will be detrimental to the structural integrity of a protein. The anticipated drastic local and subsequent global structural rearrangements on removal of an amino acid should limit the acceptance of a deletion mutation to loops away from regions considered important for function. Removal of a residue from regular secondary structure would be considered unfavourable due to changes in the register of helices or strands.

Of the 68 unique TEM-1 variants, 18 could not confer Amp resistance on *E. coli* even at the very lowest Amp concentration of 15 μg/ml tested and therefore the enzyme was considered inactive (Fig. 2 and Supplementary Table 1). The majority of the debilitating mutations (13 of the 18) were found in helices, with one found in a short strand. The remaining four were within loops or turns linking regular secondary structure. This suggests that deletion mutations that severely disrupt the structure and function of a protein are likely to occur in regular secondary structure elements. Deletion of residues that contribute to the hydrophobic core would also be considered as highly destabilising. Seven of the inactive variants contained mutations that removed buried residues (e.g. L75Δ, L81Δ and L152Δ; Supplementary Table 1). Deletion of residues that are highly conserved in class A β-lactamases and/or considered essential for the structure and activity of TEM-1 [18] would also be expected to be deleterious. This is indeed the case for many of the inactive variants (e.g. L81Δ and S124Δ; Supplementary Table 1).

However, TEM-1 appears to have a broad tolerance to the deletion of an amino acid as 50 of the variants still retained TEM-1 activity. Mutations were found throughout the protein (Fig. 2 and Supplementary Table 1), with 22 found in loops or turns and 3 in the signal sequence. As expected, this suggests that deletion mutations in these regions, consisting of less organised structure, will generally be tolerated. However, over half of the deletion mutations found in helices (18 out of 31) and the majority found in strands (7 out of 8) produced TEM-1 variants that retained activity in vivo (Fig. 2 and Supplementary Table 1). The mutations were not always restricted to the termini of a helix or sheet (allowing adaptation by helix or strand shortening) but were also found towards the middle of these structures. For example, the core helix (H2) of TEM-1 that houses the catalytically important residues S70 and K73 contained 5 different deletion mutations, of which 3 (G78Δ, A79Δ and R83Δ) retained TEM-1 activity. This suggests that mutations in regular secondary structure elements are not, as predicted, always totally destructive.

Active variants also contained deletion mutations in other catalytically and structurally sensitive positions [19], such as the Ω-loop (e.g. P174Δ and D176Δ), strand S3 (e.g. G238Δ) and at the sub-domain interface (e.g. D214A and V262Δ). For example, both S130 and E166 form part of an intricate hydrogen bond network in the active site critical to the catalytic mechanism of TEM-1 [13]. Deletion of residues in their vicinity should potentially disrupt this hydrogen bond network and therefore catalysis. However, TEM-1 that contained the M129Δ or E168Δ mutations retained β-lactamase activity in vivo with the later having enhanced activity towards CAZ (see below). Several active TEM-1 variants contained deletion mutations at positions highly conserved and/or considered essential for activity of the enzyme. For example, A125 and
G242 are two such residues yet their deletion resulted in only a twofold drop of in vivo activity towards Amp compared to the wild-type TEM-1 (Fig. 2 and Supplementary Table 1). Deletion of residues whose side chain is buried within the hydrophobic core did not always abolish TEM-1 activity (e.g. L194Δ, W210Δ and L221Δ). Therefore, TEM-1 has a broad tolerance to the deletion of an amino acid. The ability of the enzyme to adapt its structure to accommodate the mutations without total loss of function provides a good demonstration of the inherent plasticity of proteins.

Only 4 variants conferred the same Amp MIC (16000 μg/ml) on E. coli as the wild-type TEM-1, with three containing single deletion mutations positioned in loops (A42Δ, E63Δ and G196Δ) and the other in the signal sequence (Q4Δ). In the vast majority of cases, the removal of an amino acid lowered the in vivo activity of TEM-1, with nearly half of the variants (23 of 50) conferring an Amp MIC on E. coli over 10-fold lower (Amp MIC ≤ 1000 μg/ml) than the wild-type enzyme. This suggests that the removal of an amino acid is having a detrimental effect on the protein so hindering activity towards Amp. Whether this is due to decreased stability of the variant making the protein more susceptible to proteolysis or altered arrangement of the substrate binding and catalytic sites is not known but evidence that the latter is occurring in some cases is discussed below.

The influence a deletion mutation has on TEM-1 depends on the position of the residue removed. For example, removal of Ala42 has no effect on activity towards Amp and improves activity towards CAZ (Table 1) but deletion of R43 or V44 lowers activity towards Amp (Fig. 1) but had little influence on the activity of TEM-1 towards CAZ. Therefore, deletion mutations in a localised section of primary and tertiary structure do not have generic effects but each has a distinct influence on the protein.

The triplet nucleotides deleted can span two codons in some cases cause substitution of residues immediately adjacent to the deleted residue (Figs. 1c and 2). In the present study, four cases were observed whereby the deletion mutation was present alone or in combination with an adjacent substitution. The effect of the accompanying substitution mutation varied. For example, the T114Δ mutation alone or in conjunction with D115N did not affect TEM-1 in vivo activity towards Amp. However, the variant containing L102Δ-V103F (or also read as L102F-V103Δ) mutations was inactive while TEM-1 containing the V103Δ mutation was still functional (Fig. 2). The combined effect of a deletion and adjacent substitution compared to a deletion alone will therefore be dependent on the nature and structural context of the substitution.

Table 1
Deletion variants of TEM-1 with enhanced in vivo activity towards ceftazidime

<table>
<thead>
<tr>
<th>Deletion mutation</th>
<th>Fold change in Amp MIC</th>
<th>Fold change in CAZ MIC</th>
<th>Secondary structure</th>
<th>Conservation of residue in class A β-lactamases</th>
</tr>
</thead>
<tbody>
<tr>
<td>A42Δ</td>
<td>0</td>
<td>16</td>
<td>Loop (H1-S1)</td>
<td>V</td>
</tr>
<tr>
<td>A86Δ</td>
<td>0.5</td>
<td>32</td>
<td>Turn (H2-SB1)</td>
<td>V</td>
</tr>
<tr>
<td>A125Δ</td>
<td>0.5</td>
<td>32</td>
<td>H4</td>
<td>HC</td>
</tr>
<tr>
<td>E168Δ</td>
<td>0.125</td>
<td>16</td>
<td>H7 (Ω loop)</td>
<td>V</td>
</tr>
<tr>
<td>P174Δ</td>
<td>0.125</td>
<td>64</td>
<td>Ω loop</td>
<td>V</td>
</tr>
<tr>
<td>D254Δ</td>
<td>0.031</td>
<td>16</td>
<td>Turn (S4-S5)</td>
<td>V</td>
</tr>
<tr>
<td>V262Δ</td>
<td>0.006</td>
<td>16</td>
<td>S5</td>
<td>V</td>
</tr>
</tbody>
</table>

* Fold change is in comparison to wild-type TEM-1 (Amp MIC = 16000 μg/ml; CAZ MIC = 0.125 μg/ml).
* Secondary structure elements as defined by Jelsch et al. [19]. H = Helix; S = strand.
* Based on sequence variation in Class A β-lactamases observed by Haung et al. [18]. HC = Highly conserved, V = variable.

3.3. Influence of deletion mutations on TEM-1 activity towards ceftazidime

Third generation cephalosporins such as ceftazidime (CAZ) are normally poor substrates for TEM-1 [13]. The removal of amino acids at certain positions was shown to have a beneficial effect on the activity of TEM-1 towards CAZ. Of the 50 deletion variants that retained TEM-1 activity towards Amp, 7 displayed a significantly improved activity towards CAZ (Table 1). The variant containing the P174Δ mutation exhibited the largest increase, conferring a CAZ MIC on E. coli 4 fold higher than wild-type TEM-1. It is the removal of P174 that appears critical for improved activity towards CAZ as deletion of nearby N170, D176 or E177 had a much lower influence (Supplementary Table 1). Apart from A42Δ, all the mutants that had a higher activity towards CAZ displayed lower activity towards Amp than wild-type TEM-1. This suggests that for these variants, the deletion of particular residues is influencing the conformation of substrate binding and catalytic regions of TEM-1. All but one of the mutations occurred at positions that are highly variable amongst the class A β-lactamase, the exception being the deletion of the highly conserved A125.

The deletion mutations that enhanced the activity of TEM-1 towards CAZ were found in all secondary structure elements.
The position of the mutations with respect to the tertiary structure of TEM-1 is shown in Fig. 3. Most of the mutations were close to residues involved in the catalytic process (e.g. A125A, E168A and V262A) and substrate binding (P174A and A42A). It is likely that these mutations result in a structural rearrangement of the active site leading to improved binding and/or hydrolysis of CAZ. Both the A86Δ and D254Δ mutations were relatively distant from these regions and their influence on TEM-1 activity towards CAZ is less obvious. The most likely explanation is that they impart long-range structural effects that propagate to the active site of TEM-1. For example, A86 lies at the bottom of the core helix H2 that houses S70. Deletion of A86 could lead to a minor shift in this helix that makes the active site more accessible to CAZ.

4. Conclusion

We have successfully applied a new directed evolution method to probe the consequence of an amino acid deletion from a protein. The deletion of an amino acid from TEM-1 was tolerated at the majority of positions sampled in this study, including helices and strands, and regions important for structure and catalysis. The ability of TEM-1 to compensate structurally and functionally to the removal of an amino acid provides an apt demonstration of the plasticity of proteins. The effect of a single amino acid deletion appears to be residue specific, and as with other mutations should therefore be considered within the context of the local primary and tertiary structure and not automatically assumed to be detrimental. This is exemplified by several deletion mutations occupying different positions in the tertiary structure enhancing the activity of TEM-1 towards CAZ. Deletion mutations provide nature with an added source of protein sequence and conformational diversity not sampled by substitutions. Therefore, deletions should not be ignored when designing or artificially evolving proteins with novel properties, especially with the advent of new directed evolution methods with the potential of sampling such mutations [12,20,21].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.018.

References


