FEBS Letters 581 (2007) 3904–3908

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

Alan M. Simm[1], Amy J. Baldwin, Kathy Busse, D. Dafydd Jones*

School of Biosciences, Biomedical Sciences Building, Museum Avenue, Cardiff University, Cardiff CF10 3US, UK

Received 9 June 2007; revised 28 June 2007; accepted 9 July 2007

Available online 23 July 2007

Edited by Gianni Cesareni

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. *Circa* 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.**

Keywords: Protein plasticity; Directed evolution; Deletion mutations; Indels; Antibiotic resistance

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 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 vivo* 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 BLAΔ^{391} 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.

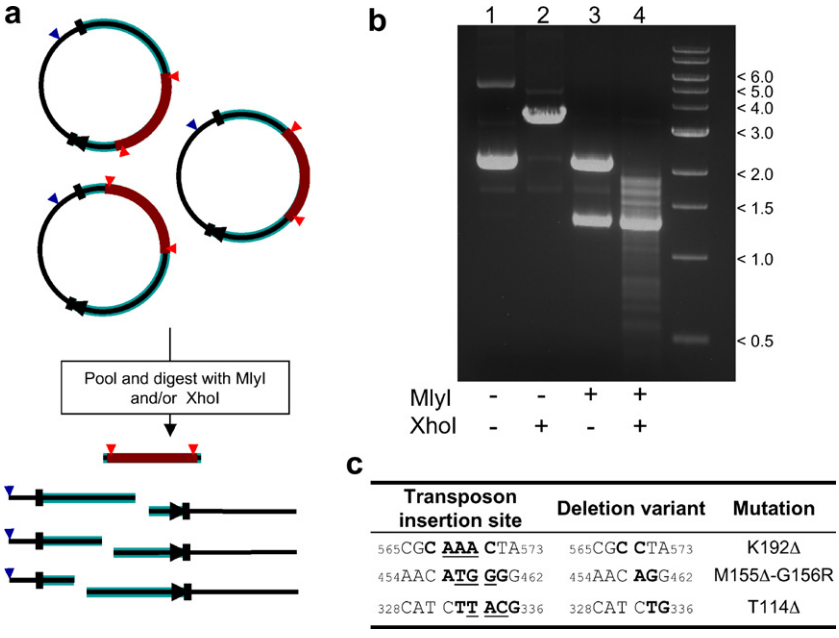
The individual deletion variants were constructed by removing MuDel from pNOM-MuDel DNA isolated from 76 separate cultures by digestion with MlyI. The linearised pNOM DNA was separated by agarose gel electrophoresis and purified. Intramolecular ligation was performed using the Quick Ligation™ kit (NE Biolabs) and ca. 10 ng

*Corresponding author.
E-mail address: jonesdd@cf.ac.uk (D.D. Jones).

[1]Present address: School of Engineering and Electronics, University of Edinburgh, King's Buildings, Edinburgh EH9 3JL, UK.

Abbreviations: Δ, amino acid deleted; Amp, ampicillin; Cam, chloramphenicol; CAZ, ceftazidime; Indel, insertion-deletion; MIC, minimum inhibitory concentration

0014-5793/$32.00
doi:10.1016/j.febslet.2007.07.018



c

Transposon insertion site	Deletion variant	Mutation
565CGC AAA CTA573	565CGC CTA573	K192Δ
454AAC ATG GGG462	454AAC AGG462	M155Δ-G156R
328CAT CTT ACG336	328CAT CTG336	T114Δ

Fig. 1. Analysis of the BLAΔ^{391} library. (a) Rational for determining MuDel (brown) insertion diversity within the *bla* gene (green with black stripe) of pNOM. Digestion by XhoI (recognition site depicted as blue triangle) of the pooled pNOM plasmid with MuDel inserted within the *bla* gene generates one major product of 3422 bp in size. Digestion by MlyI (recognition sequence depicted as red triangle) generates two major products of 1310 bp (MuDel plus 8 bp from *bla* gene) and 2112 bp in size. Digestion by both XhoI and MlyI generates the 1310 bp size MuDel fragment together with many different size fragments depending on the insertion position of the transposon. (b) Restriction analysis of the BLAΔ^{391} library with XhoI and/or MlyI. (c) The potential outcomes with respect to the insertion of MuDel at the three different positions within a codon by the mechanism described by Jones [12]. Nucleotides shown in bold correspond to the 5 bp sequence duplicated on transposon insertion and those additionally underlined represent the deleted triplet sequence. Each group of three nucleotides represents a single codon.

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] ⩽ 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 BLAΔ^{391} library. Restriction analysis using the endonucleases XhoI and MlyI was used to determine the diversity of transposon insertion in the BLAΔ^{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 R86Δ 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