Communication

Insertion Mutagenesis as a Tool in the Modification of Protein Function

EXTENDED SUBSTRATE SPECIFICITY CONFERRED BY PENTAPEPTIDE INSERTIONS IN THE Ω-LOOP OF TEM-1 β-LACTAMASE

(Received for publication, August 26, 1997, and in revised form, September 19, 1997)

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The TEM-1 β-lactamase enzyme efficiently hydrolyzes β-lactam antibiotics such as ampicillin but cleaves third generation cephalosporin antibiotics poorly. Variant β-lactamases that conferred elevated levels of resistance to the cephalosporin ceftazidime were identified in a set of β-lactamase derivatives previously generated by pentapeptide scanning mutagenesis in which a variable 5-amino acid cassette was introduced randomly in the target protein. This mutagenesis procedure was also modified to allow the direct selection of variant β-lactamases with pentapeptide insertions that conferred extended substrate specificities. All insertions associated with enhanced resistance to ceftazidime were targeted to the 19-amino acid Ω-loop region, which forms part of the catalytic pocket of the β-lactamase enzyme. However, pentapeptide insertions in the C- and N-terminal halves of this region had different effects on the ability of the enzyme to hydrolyze ampicillin in vivo. Larger insertions that increased the length of the Ω-loop by up to 2-fold also retained catalytic activity toward ampicillin and/or ceftazidime in vivo. In accord with previous substitution mutation studies, these results emphasize the extreme flexibility of the Ω-loop with regards the primary structure requirements for ceftazidime hydrolysis by β-lactamase. The potential of pentapeptide scanning mutagenesis in mimicking evolution events that result from the insertion and excision of transposons in nature is discussed.

Even prior to the clinical introduction of the first β-lactam antibiotic, penicillin, infectious bacteria resistant to the activity of this antibiotic were identified (1). This resistance subsequently proved to be due to the expression in these bacteria of one of four classes (A, B, C, and D) of β-lactamase enzyme that efficiently hydrolyzes the antibiotic substrate (2). Furthermore, the introduction of later generation β-lactamases was followed inexorably by the appearance of resistant strains expressing mutated β-lactamase enzymes with correspondingly altered substrate specificities (1, 3). To date over 50 naturally occurring class A TEM-1 β-lactamase variants have been isolated.¹

Among the variant β-lactamases that have emerged are derivatives that cleave third generation cephalosporin antibiotics, e.g. ceftazidime, which the wild-type protein recognizes poorly (3). Natural mutations that give rise to these variants are confined to amino acids at positions 104, 164, and 237–240, which are close to or which form part of the catalytic pocket of TEM-1 β-lactamase (5–7). However, artificial substitution mutations at many positions in the Ω-loop (residues 161–179) also alter the specificity of the enzyme (8–11). Ω-Loop substitutions appear to increase the conformational flexibility of the catalytic region, thereby allowing access of the bulkier side chain of third generation cephalosporin substrates (9–13).

Pentapeptide scanning mutagenesis is a method by which a variable five amino acid cassette is introduced at random into a target protein (14, 15). In this study, pentapeptide insertions were constructed in the active pocket Ω-loop of the TEM β-lactamase, which extended the activity spectrum of the enzyme to include the third generation cephalosporin, ceftazidime. The extreme tolerance of the Ω-loop to alteration was emphasized further by the construction of β-lactamase derivatives in which the length of this region was increased up to 2-fold but which retained catalytic activity toward ceftazidime and/or ampicillin in vivo.

EXPERIMENTAL PROCEDURES

Medium, Enzymes, and Materials—Escherichia coli cultures were grown in L medium at 37 °C unless otherwise stated. The following antibiotics were added to L medium when required (Sigma): 10–5500 μg/ml ampicillin, 0.075–1.5 μg/ml ceftazidime, 50 μg/ml kanamycin, 50 μg/ml streptomycin, and 5 μg/ml tetracycline. Enzymes were obtained from New England Biolabs or Life Technologies, Inc..

Transposons, Plasmids, and Bacterial Strains—Transposon Tn443015 is a derivative of Tn4300 that encodes kanamycin resistance (16). Plasmid pHT385 (16) was used as a source of Tn443015. The bla gene on plasmid pBB322 (17) was used as the target for Tn443015 mutagenesis. E. coli FH1046A, which was obtained by cotransformation of pHT385 and pBR322 into strain XL1-Blue (18), and the streptomycin-resistant strain DS941 (19) were used as donor and recipient, respectively, in conjugation trials. Strain DH5α (20) was used for plasmid propagation and in cloning experiments.

General Procedures—Plasmid DNA was prepared and manipulated essentially as described by Sambrook et al. (21). The maximum antibiotic concentrations allowing growth were determined by making 10⁴ dilutions of overnight cultures grown in L broth containing tetracycline at 30 °C and spotting 2-μl volumes of these dilutions on L plates containing the appropriate concentration of ampicillin or ceftazidime. Results were read after incubation at 30 °C for 48 h. Nucleotide sequencing of insertion sites in pBR322-15-bp² constructs was performed with a set of custom-made bla-specific primers using dye terminator chemistry after which samples were run on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Warrington, Cheshire, UK).

Pentapeptide Scanning Mutagenesis—Pentapeptide scanning mutagenesis is a technique whereby 5-amino acid insertions are introduced at random in a target protein (14). Briefly, a donor strain containing the target plasmid and pH385, a conjugative Tn443015 delivery vector, is mated with a plasmid-free recipient strain. By plating the mating mix simultaneously on antibiotics selecting for the

¹G. Jacoby and K. Bush, World Wide Web site, http://www.lahey.hitchcock.org/pages/lhc/studies/webt.htm.

²The abbreviation used is: bp, base pair(s).
resuspended in 300 µl of L broth, and 2.5-µl volumes of these cell suspensions were spread on an L plate. Following incubation for 3 h at 37 °C, the conjugation mix was resuspended in 300 µl of L broth, and 50-µl volumes were spread on L plates containing streptomycin, tetracycline, and kanamycin to select for transconjugants that appeared following overnight incubation at 37 °C.

**Immunoblot Analysis**—The levels of variant β-lactamase proteins present in exponentially growing cultures were assessed by immunoblot analysis. Lysates of cells grown at 30 °C with tetracycline selection were prepared as described by Palzkill et al. (10). Proteins in the cell lysate were resolved by SDS-polyacrylamide gel electrophoresis on a 12.5% (w/v) polyacrylamide gel at 16 V/cm for 75 min at room temperature. Proteins in the gel were transferred to polyvinylidene difluoride membrane by electroblotting, and β-lactamase protein was visualized by immunoblotting with β-lactamase antiserum, a secondary antibody conjugated to alkaline phosphatase, and AttoPhos (JBL Scientific, San Luis Obispo, CA). Immunoblots were scanned with a Fluorimag (Molecular Dynamics, Chesham, Buckinghamshire, UK) and band intensity was quantified using ImageQuant software (Molecular Dynamics).

**Nomenclature of Pentapeptide Insertions**—Pentapeptide insertions in the β-lactamase protein are named by the amino acid residue to the N-terminal side of the insertion followed by the sequence of the insertion itself. Amino acid numbering corresponds to that recommended elsewhere (22).

**RESULTS**

**Indirect Selection of Variant β-Lactamases with Extended Substrate Specificities**—Substitution mutations of certain residues extend the resistance spectrum of the TEM-1 β-lactamase enzyme to include third generation cephalosporins, which wild-type β-lactamase hydrolyzes poorly (10, 11, 13, 23). Using the pentapeptide scanning mutagenesis technique, a set of pentapeptide insertions was constructed previously in the β-lactamase protein encoded by the bla gene of pBR322 (14). By determining the maximum concentrations that allowed growth at 30 °C on the cephalosporin antibiotic ceftazidime, the effect of these insertions on β-lactamase substrate specificity was assessed. The wild-type β-lactamase protein expressed by pBR322 conferred resistance to 0.075 µg/ml of ceftazidime under the conditions used in this study. The 163GVPLD and 164WGTPR variants had elevated ceftazidime resistance levels of 0.5 and 1.25 µg/ml, respectively (Table I). Resistance levels provided by these mutated β-lactamase proteins were reduced at higher temperatures. Strains expressing β-lactamases with pentapeptide insertions at 21 other locations were as sensitive to ceftazidime at all temperatures tested as was a strain containing the wild-type protein. These 21 variants conferred different levels of ampicillin resistance (14). Significantly, the insertions that resulted in increased resistance to ceftazidime abolished detectable ampicillin resistance (Ref. 14 and Table I) and are located one amino acid apart in the active site Ω-loop (Fig. 1). This result demonstrates that, in addition to substitution mutations, insertion mutations in the Ω-loop can profoundly alter the resistance spectrum of the β-lactamase protein.

**Direct Selection of Variant β-Lactamases with Extended Substrate Specificities**—The utility of pentapeptide scanning mutagenesis in engineering β-lactamase derivatives with altered substrate profiles was demonstrated further by modifying the pentapeptide scanning mutagenesis procedure to facilitate the direct selection of variant enzymes that conferred elevated levels of resistance to ceftazidime. Because the number of positions at which mutations extend β-lactamase substrate specificity may be relatively few (3), a pool of plasmids containing a large number of independent 15-bp insertions in bla was generated as follows. Plasmid pBR322 was transformed into a strain containing the Tn443015 donor plasmid, pHT385. More than 300 colonies from this transformation were pooled into 1 ml of L broth, and dilutions of this cell suspension were used as donor material in mating trials with the streptomycin-resistant recipient, DS941. Transconjugants in which a plasmid-pBR322 cointegrate was transferred to DS941 were isolated by plating the mating mix on streptomycin, kanamycin, and tetracycline. Plates containing >1000 transconjugant colonies were selected, and plasmid DNA was isolated from a pool of these colonies. Because resolution of the pHT385-pBR322 cointegrate is efficient in vivo (14–16), this plasmid preparation consisted primarily of pBR322 derivatives containing Tn443015 insertions. As has been shown previously, these Tn443015 insertions are distrib-
uted randomly in the target plasmid (14, 15). The plasmid preparation was digested with KpnI and religated to generate a bank of pBR322 derivatives in which the bulk of Tn443030I5 was deleted but leaving 15-bp insertions. Derivatives in which 15-bp insertions in bla resulted in elevated levels of resistance to ceftazidime were isolated from this bank by transforming the ligation mixture into strain DH5α and selecting at 30 °C on tetracycline and also on ceftazidime at a concentration of 0.25 μg/ml. This level of ceftazidime is three times higher than the level of resistance conferred by the wild-type β-lactamase protein (Table I). Using this procedure involving pooled plasmid DNA generated from a large pool of donors, ceftazidime-resistant colonies were isolated in 9 of 30 independent experiments.

One ceftazidime-resistant candidate from each of the nine successful trials was characterized further (Table I). All of the pentapeptide insertions in these derivatives were targeted to the active site Ω-loop of the β-lactamase protein. Seven unique insertions distributed throughout the Ω-loop were identified among the nine variant β-lactamase proteins (Fig. 1). Insertions at different positions within the same codon resulted in β-lactamase derivatives with different pentapeptide insertions at both position 163 and position 170. The pair of ceftazidime-resistant mutations (163GVPLD and 164WGTPR) described in the preceding section that were isolated indirectly from the collection of pentapeptide insertions previously constructed in the β-lactamase protein (14) reoccurred in the direct selection trials.

The resistance levels conferred by the new variant β-lactamase enzymes were determined on both ampicillin and ceftazidime (Table I). Ceftazidime resistance levels among the pentapeptide-containing variants ranged from 7 to 17 times the level of resistance associated with the wild-type protein. These resistance levels are comparable with those conferred by substitution mutations in the Ω-loop (10, 11). Insertions in the N-terminal segment of the Ω-loop abolished activity toward ampicillin. In contrast, all four insertions in the C-terminal segment of this region retained different but significant levels of ampicillin resistance. These results indicate that insertions at multiple positions in the Ω-loop can extend the activity of the β-lactamase protein to include a cephalosporin substrate. In addition, the results also suggest that, whereas all insertions in the Ω-loop reduce the ability to hydrolyze ampicillin, insertions in the C-terminal segment of the loop have a less deleterious effect than insertions in the N-terminal segment.

**Properties of β-Lactamase Derivatives with Ω-Loop Insertions Larger than Five Amino Acids**—All 15-bp insertions generated by pentapeptide scanning mutagenesis include a KpnI site (14). The effect on the β-lactamase substrate profile of Ω-loop insertions larger than five amino acids was assessed by introducing a 42-bp in-frame double-stranded synthetic oligonucleotide into the KpnI site in the bla alleles specifying the 164WGTPR and 170GVPLN mutated proteins that have lost and retained activity toward ampicillin, respectively. This resulted in variant proteins containing a total of 19 amino acids introduced at positions 164 and 170. The cloned oligonucleotides included a series of contiguous unique hexanucleotide recognition sites for restriction endonucleases that generate blunt ends. By digesting plasmid DNA containing the 42-bp insertions with appropriate combinations of these restriction enzymes and religating the digested DNA, sets of shorter derivatives specifying β-lactamase proteins with a total of 11, 15, or 17 amino acids at positions 164 or 170 were produced. None of the >11-amino acid insertions at positions 164 and 170 significantly altered the levels of resistance toward ceftazidime and ampicillin in comparison with the parental derivatives containing pentapeptide insertions at these positions. These results indicate that β-lactamase derivatives in which the length of the Ω-loop has been increased by up to 2-fold retain catalytic activity toward ampicillin and/or ceftazidime.

**Expression Levels of Mutated β-Lactamase Proteins**—The levels of variant β-lactamases present during exponential phase growth were assessed by immunoblotting analysis (Fig. 2). Derivatives with pentapeptide insertions in the N-terminal segment of the Ω-loop that no longer hydrolyzed ampicillin were detected reproducibly in lower amounts than the wild-type protein. In contrast, variants with pentapeptide insertions in the C-terminal half of this region that still conferred ampicillin resistance were present at levels indistinguishable from that of the wild-type protein (Fig. 2A). These results agree with previous studies that have demonstrated a correlation between wild-type expression levels and the ability to hydrolyze ampicillin in β-lactamases with Ω-loop substitution mutations (10, 11).

The effect of increasing the size of the Ω-loop insertion on β-lactamase levels was also examined (Fig. 2B). Derivatives containing 11-, 15-, or 19-amino acid insertions at position 170 were present at approximately 20% of the wild-type amounts. A 17-amino acid insertion at this position reduced protein levels to approximately 10% of the wild-type concentration. In contrast, insertions of >11 amino acids at position 164 reduced β-lactamase concentrations to undetectable levels, although these proteins still conferred ceftazidime resistance (Table I). Therefore, insertions at position 164 may induce greater instability of the β-lactamase enzyme than insertions at position 170. This appears to parallel the higher level of ceftazidime resistance conferred by the former (Table I). Alternatively, the folding properties of derivatives with insertions of >11 amino acids may be altered such that the recognition of the protein by the antibody is affected.

**DISCUSSION**

The Ω-loop of TEM-1 β-lactamase contains residues required both for enzyme catalysis and for maintaining the correct topology of the active site (6, 7, 12). Single or multiple substitution mutations in this region expand the resistance spectrum of β-lactamase to include third generation cephalosporin antibiotics that the wild-type enzyme hydrolyzes poorly (5, 8–12, 23). The present study demonstrated that the Ω-loop is even more amenable to mutations that extend β-lactamase substrate specificity than previously foreseen. First, penicillinase insertions into the β-lactamase substrate profile of the Ω-loop substitution mutations (10, 11). Second, larger insertions that increased the length of the Ω-loop 2-fold also retained significant catalytic activity in vivo. These results emphasize further the remarkable
flexibility of the Ω-loop with respect to the primary sequence requirements for ceftazidime hydrolysis, as has been noted previously in substitution mutation studies (10, 11). Interestingly, a tripeptide duplication in the catalytic region of a class C β-lactamase natural variant has recently been shown also to extend substrate specificity to include cephalosporins (25).

Detailed kinetic and structural studies of proteins that harbor Arg-164 and/or Glu-166 mutations have provided insight into the mechanism by which the Ω-loop modifications extend the resistance spectrum of β-lactamase (9, 12, 13). Hydrolysis of β-lactams involves formation of an acyl-enzyme intermediate in which the substrate is ester-linked transiently to the active site Ser-70 residue of the enzyme (26). Glu-166 is required both for activation of the Ser-70 OH group (9) and for deacylation of the acyl-enzyme intermediate (7, 27, 28). The increased activity spectrum of enzymes with mutations of Glu-166 may be due to a combination of effects including displacement of a catalytic water molecule from the active site, easier access by the larger side chain of cephalosporins to the active pocket, and movement of a large segment of the helical domain that contains vital catalytic residues (9, 12). Arg-164 forms salt bridges with Glu-171 and Asp-179 (6, 7). Mutations of Arg-164 that disrupt these interactions alter the stability and conformation of the Ω-loop such that residues both involved in deacylation of the acyl-enzyme intermediate and in the catalytic helical domain are displaced, thereby allowing access of larger substrates (3, 12). The altered resistance spectra associated with the relatively large insertion mutations described in this study may be due to perturbations of the active site topology analogous to those induced by Arg-164 and Glu-166 substitution mutations. How can insertions as large as 19 amino acids have an effect on substrate specificity similar to that of comparably subtle substitution mutations? If the bulk of the inserted amino acids protrude from the main body of the protein, the tertiary organization of the Ω-loop may be disrupted only as severely as in proteins harboring substitution mutations.

Whereas pentapeptide insertions in both the N- and C-terminal segments of the Ω-loop conferred ceftazidime resistance, enzymes containing insertions in the latter region maintained significant levels of hydrolytic activity toward ampicillin, but enzymes containing insertions in the former region did not (Table I). This may reflect the closer proximity of N-terminal insertions to the important Arg-164 and Glu-166 residues. More detailed kinetic and structural studies of selected derivatives with Ω-loop insertions will address these questions further.

DNA rearrangements caused by transposable elements are often regarded as playing an important evolutionary role by promoting, for example, gene duplications and gene fusions. The excision of transposons (which in pentapeptide scanning mutagenesis is artificially mimicked in vitro by the Km1 deletion of Trn4430) may be responsible for amino acid insertions observed when related protein sequences are aligned (29, 30). The excision of transposable bacteriophage Mu has also been suggested as a mechanism by which adaptive mutations occur (4, 31). Pentapeptide scanning mutagenesis may provide an experimental system for reproducing a naturally occurring mechanism of protein variation and for generating proteins with novel catalytic properties. In this regard, a key result is that two of the 23 β-lactamase pentapeptide insertions generated in a recent study (14) were shown here to alter enzyme substrate specificity by conferring increased levels of resistance to ceftazidime even though no selection for this phenotype was applied at any stage of the mutagenesis procedure (Table I). The utility of pentapeptide scanning mutagenesis was emphasized further by adapting the procedure to allow the direct selection of β-lactamase derivatives with expanded substrate specificities. These derivatives contained insertions at a number of positions just within a 19-amino acid region. Because the thermosensitivity (14) and intracellular levels (Fig. 2) of the various mutated β-lactamases also suggest that pentapeptide insertions may affect protein folding and stability, it is tempting to propose that nonspecific destabilization caused by short peptide insertions might modify enzyme function, e.g., substrate specificity, by locally increasing the conformational flexibility of the protein, e.g., in the catalytic site. Secondary mutations might then occur to stabilize the protein in its novel function.

Pentapeptide scanning mutagenesis has also been used to identify regions of the XerD site-specific recombinase implicated in recombination activity in vivo, in contacting the substrate target DNA, and in communicating with the partner recombinase, XerC (15). The utility of pentapeptide scanning mutagenesis as a simple means of manipulating enzyme activity is under further investigation.

Acknowledgments—We thank Jean-Marie Frère for providing β-lactamase antisera and Daniela Barilla, Ronald Chalmers, Jean-Marie Frère, and Andrew Spiers for comments on the manuscript.

REFERENCES
1. Frère, J.-M. (1995) Mol. Microbiol. 16, 385–395
2. Ambler, R. P. (1980) Phil. Trans. R. Soci. Lond. Ser. B 299, 321–331
3. Knox, J. R. (1995) Antimicrob. Agents Chemother. 39, 2593–2601
4. Maenhout-Michel, G., and Shapiro J. A. (1994) EMBO J. 13, 5229–5239
5. Jelsch, C., Mourey, L., Masson, J.-M., and Samama, J.-P. (1993) Proteins 16, 364–383
6. Jelsch, C., Mourey, L., Masson, J.-M., and Samama, J.-P. (1993) Proteins 16, 364–383
7. Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., and James, M. N. G. (1992) Nature 359, 700–705
8. Delaire, M., Lenfant, F., Labia, R., and Masson, J.-M. (1991) Protein Eng. 4, 805–810
9. Guillaume, G., Vanhove, M., Lamotte-Brasseur, J., Ledent, P., Jamin, M., Joris, B., and Frère, J.-M. (1997) J. Biol. Chem. 272, 5438–5444
10. Palzkill, T., Le, Q.-Q., Venkatachalam, K. V., LaRocco, M., and Ocera, H. (1994) Mol. Microbiol. 12, 217–229
11. Petrosino, J. F., and Palzkill, T. (1996) J. Bacterial. 178, 1821–1828
12. Maveyraud, L., Saves, I., Burtle-Schiltz, O., Swarṇ, P., Masson, J.-M., Delaire, M., Mourey, L., Promé, J.-C., and Samama, J.-P. (1996) J. Biol. Chem. 271, 10482–10489
13. Raquet, X., Lamotte-Brasseur, J., Fonze, E., Goussard, S., Courvalin, P., and Frère, J.-M. (1994) J. Mol. Biol. 244, 625–639
14. Hallet, B., Sherratt, D. J., and Hayes, F. (1997) Nucleic Acids Res. 25, 1866–1867
15. Cao, Y., Hallet, B., Sherratt, D. J., and Hayes, F. (1997) J. Mol. Biol. 274, 39–53
16. Mahillon, J., and Leresche, D. (1988) EMBO J. 7, 1515–1526
17. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. C., Boyer, H. W., Crosa, J. H., and Falkow, S. (1977) Gene (Amst.) 1, 95–113
18. Botstein, D., White, R. L., Sklar, L., Davis, R. W., and Boguski, M. S. (1987) Nucleic Acids Res. 15, 6116–6121
19. Summers, D. K., and Sherratt, D. J. (1988) EMBO J. 7, 851–858
20. Woodcock, D. M., Crowther, P. F., Doherty, J., Jefferson, S., Dezu, E., Noyer-Weidner, M., Smith, S. S., Michael, M. Z., and Graham, M. W. (1989) Nucleic Acids Res. 17, 3469–3478
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Ambler, R. P., Coulson, F. W., Frère, J.-M., Ghuysen, J.-M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G., and Waley, S. G. (1991) Biochem. J. 276, 269–272
23. Sowk, J. A., Singer, S. B., Ohringer, S., Mallet, M. F., Dougherty, T. J., Gouguoutsas, J. Z., and Bush, K. (1991) Biochemistry 30, 3179–3188
24. Kralupi, P. (1991) J. Appl. Crystallogr. 24, 946–950
25. Naka, M., Haruta, S., Tanimoto, K., Kusumoto, K., Taniguchi, K., Tsuchiya, K., Takeuchi, T., and Tanaka, M., and Sawad, T. (1995) J. Biol. Chem. 270, 5729–5735
26. Fisher, J., Belasco, J., Khosla, S., and Fink, A. L. (1980) Biochemistry 19, 2895–2901
27. Adachi, H., Ohta, T., and Matsuoka, H. (1981) J. Biol. Chem. 266, 3186–3191
28. Escobar, A. W., Tan, A. K., and Fink, A. L. (1991) Biochemistry 30, 10783–10787
29. Berg, D. E., and Howe, M. M. (eds.) (1989) Mobile DNA, American Society for Microbiology, Washington, D.C.
30. Shapiro, J. A. (1993)Curr. Opin. Genet. Dev. 3, 845–848
31. Foster, P. L., and Cairns, J. (1994) EMBO J. 13, 5240–5244